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Interventional radiology therapies for liver cancer

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ABSTRACT

Hepatocellular carcinoma (HCC) is the fifth most frequently found primary malignant tumor in the world. Hepatic surgery and liver transplantation are considered optimal for the curative treatment of HCC. However, only 15-20% of HCCs may be surgically treated. Most of the surgically-non-eligible patients have to receive locoregional image-guided interventional treatments including intra-arterial and percutaneous ablative therapies. The goal of this paper is to review these interventional oncology approaches. Ablative therapeutic approaches include chemical therapies (such as ethanol or acetic acid injection), and thermal therapies (such as radiofrequency ablation, laser-induced thermotherapy, microwave ablation, cryoablation, and high-intensity focused ultrasound ablation). Catheter-based therapies include embolotherapy/chemotherapy-based treatments (such as transcatheter arterial chemoembolization, bland embolization, transcatheter arterial chemoinfusion, and chemoembolization with drug-eluting beads), and radiotherapy-based treatments (such as radioembolization with yttrium-90 and injection of iodine-131-labeled lipiodol). As a result of the technical development of locoregional approaches for HCC during the recent decades, the range of combined interventional therapies has been continuously extended. In this article, an evidence-based approach will be used to review the current role of interventional radiology therapies in the management of unresectable HCC.

Key words: Hepatocellular carcinoma; local ablative therapy; radioembolization; transarterial chemoembolization

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INTRODUCTION


Hepatocellular carcinoma (HCC) ranks the fifth in overall frequency and fourth in annual tumor mortality.^[1] Surgical treatments including hepatic resection and liver transplantation are considered the most effective treatments of HCC. However, less than 20% of HCC can be treated surgically because of multifocal diseases, proximity of the tumor to key vascular or biliary strictures precluding a margin-negative resection and inadequate functional hepatic reserve with cirrhosis.^[2-4] Usually,

patients with single small HCC (≤ 5 cm) or up to three lesions ≤ 3 cm are indicated for surgery.^[5,6] When surgery is precluded, interventional treatments can be used to improve the prognosis of the patients. Such therapies, which rely on imaging guidance for tumor targeting and response assessment, include various catheter-based and percutaneous ablative techniques. These minimally invasive therapies have been used mainly for palliation but have also increasingly been used with curative intent.

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This review outlines the current status of the most commonly used image-guided therapeutic approaches for the management of patients with HCC.

INTRA-ARTERIAL CATHETER-BASED THERAPIES

Embolotherapy/chemotherapy-based therapies

Transarterial chemoembolization

The radiological technique for tumor devascularization was developed in the 1970s.^[7] Now, it is the most widely used primary treatment for unresectable HCC. It is also the most extensively used therapy for patients on the waiting list for liver transplantation. Embolization agents, like gelatin, may be administered together with selective intra-arterial chemotherapy mixed with lipiodol (iodized oil). Doxorubicin, mitomycin, and cisplatin are commonly used anti-tumor drugs.^[8] The rationale of transarterial chemoembolization (TACE) is as follows: cytotoxic drugs achieve higher intra-tumoral concentrations when injected in the hepatic artery, and lipophilic or amphiphilic anticancer drugs, when mixed with lipiodol, are thought to be liberated progressively inside the tumor. Lipiodol, which destroys capillary beds and induces extensive necrosis in HCC with abundant blood supply, can be transported in a tumor and may remain for weeks or months, for which the absence of Kupffer cells would presumably be responsible.

Usually, lesions that are rich in arterial blood supply can be anticipated to undergo complete necrosis, while those that lack arterial blood supply have less iodine oil deposits and need other combinative therapies. The whole procedure can be repeated monthly or longer to achieve higher degree necrosis and avoid recurrence. However, the injection of cytotoxic drugs mixed with lipiodol but not followed by embolization has not shown any substantial anti-tumor effect, suggesting that ischemia plays a key role in tumor necrosis.^[9] Still, some authors reported that transcatheter arterial infusion chemotherapy had a better anti-tumor effect than TACE.^[10] With respect to the relationship between TACE and pulmonary metastasis, Lin *et al.*^[11] reported that TACE did not significantly increase the risk of pulmonary metastasis. Post-embolization syndrome including abdominal pain and fever is extremely frequent and fades in a few days. Complications related to aberrant arterial embolization, such as acute cholecystitis, stenosis of the biliary tract, acute pancreatitis, or gastroduodenal ulcerations have also been reported. The selection of candidates for TACE is a key point. The benefits of the procedure should not be offset by treatment-reduced liver function failure. Patients with preserved liver function and asymptomatic multinodular tumors without vascular invasion or extra-hepatic spread are indicated for TACE.^[8] Child-Pugh class C is considered a contraindication.^[12]

TACE achieves partial responses in 15-55% of patients and significantly delays tumor progression and vascular invasion.^[8,12-14] For HCC invading the portal venous system, TACE could be an effective treatment with the 1-, 3-, and 5-year survival rates of 42%, 11%, and 3%, respectively.^[15] Although an earlier study showed that TACE could not improve the survival of the patients,^[12] survival benefits were identified by two studies on chemoembolization.^[13,14] Overall, the effect may be considered modest.

Arterial bland embolization

Transcatheter arterial bland embolization, which simulates arterial ligation, induces tumor ischemia by disrupting the blood supply to the tumor. Advocates of this catheter-based therapy claim that bland embolization may be equally effective as TACE for palliative treatment of primary liver cancer.^[16] Despite a trend toward improved survival with TACE, no study to date has demonstrated a difference in survival between the two techniques.^[17] A randomized trial comparing embolization (without chemotherapy) vs. symptomatic treatment in patients with hepatitis C virus-related liver disease and Child-Pugh class A liver function failed to demonstrate a 2-year survival advantage.^[18]

Drug-eluting bead chemoembolization

Drug-eluting bead (DEB)-TACE is a drug delivery system that combines the local embolization of vasculature with the release of chemotherapy into adjacent tissue.^[19,20] It is intended for use in the treatment of hyper-vascular tumors such as HCC. Its administration is similar to that of conventional TACE. Beads are composed of biocompatible polymers such as polyvinyl alcohol (PVA) hydrogel that have been sulfonated to enable the binding of chemotherapy.^[21] The beads occlude vasculature, causing embolization, and the chemotherapy is delivered locally.^[22,23]

Like conventional TACE, DEB-TACE is considered a palliative option for unresectable HCC. DEB-TACE may also use as an adjunctive therapy for liver resection or as a bridge to liver transplantation, as well as before or after radiofrequency ablation (RFA).^[24-28] There are currently two types of microspheres available for drug loading: DC Bead microspheres (Biocompatibles, UK) and the recently introduced superabsorbent polymer (SAP) HepaSphere microspheres (BioSphere Medical, USA). Most of the literature involves the application of DC Bead microspheres. These microspheres are non-biodegradable PVA microspheres that are approved for the treatment of malignant hyper-vascular tumors and loading of doxorubicin. Precision Bead (Biocompatibles, UK) microspheres are the first factory-preloaded (doxorubicin 37.5 mg/vial) microspheres. They can be polymerized to formulate different-sized spheres,

ranging in maximum diameter from 100 to 900 μm . SAP HepaSphere microspheres (BioSphere Medical) are biocompatible, hydrophilic (absorbent), non-resorbable, and acrylic copolymer microspheres designed for hepatic arterial embolization with an ability to absorb fluids at up to 64 times their dry state volume. The expansion rate is dependent on the ionic concentration of its surrounding media. The size of dry particles ranges between 50 and 200 μm , corresponding to an expanded size range of 200 and 800 μm . The SAP microspheres can be loaded with doxorubicin or cisplatin for drug delivery during TACE.^[29] Initial *in vitro* and *in vivo* studies showed encouraging results, and these microspheres now have CE mark approval for TACE of HCC in combination with doxorubicin.

DEB-TACE appears to be a relatively safe procedure, with few long-term serious complications associated with its administration. Although symptoms of post-embolization syndromes, such as fever, nausea, vomiting, and abdominal pain appears to occur in most patients, these symptoms are associated with short hospital stays averaging 2.3 days among publications, which is significantly lower than conventional TACE procedures. The most frequent major complication associated with this procedure is liver abscess, which occurred in approximately 0.75-1.58% of publications. Other complications are infrequent, although some are quite severe. Overall mortality is potentially lower than the reported values (2.06-4.74%) because reported mortality rates include both procedure-related causes of death, such as sepsis and hepatic failure, and death secondary to progressive disease, cardiovascular disease, pulmonary embolism, and other causes. Patients selected for most of these studies are predisposed to comorbidities as a result of their diminished hepatic function and potentially other age or lifestyle-related conditions, which should be taken into consideration.^[30]

The current results show that DEB-TACE produces beneficial tumor response and has exceptionally low complication rates. The technique has the potential to become an effective alternative therapy or palliative measure in the treatment of HCC, but both delivery and data collection must be standardized in order to clarify efficacy. It is a safe alternative for the treatment of unresectable HCC but is unproven as an adjunctive treatment for other standard therapies such as resection and RFA. Further investigation is essential to better define its role as an adjunct in treating HCC.

Transcatheter arterial chemoinfusion

Transcatheter arterial chemoinfusion (TACI) is a catheter-based intra-arterial therapy that traps high concentrations of chemotherapeutic agents in tumor tissues followed by

minimal embolization.^[31] TACI with maximally selective catheterization and highly concentrated chemotherapy preparations minimizes the risk of hepatocellular ischemic and cytotoxic complications and maximizes chemotherapy delivered to tumor tissue. TACI with super selective catheterization, although labor intensive, has been shown to be safe. The eligibility criteria for TACI are similar to those for TACE. Portal venous thrombosis is not a contraindication. Caution should be exercised to avoid injecting large volumes ($> 10 \text{ mL}$) of lipiodol. Moreover, patients with poor hepatic function and tumors with diameters of $> 9 \text{ cm}$ have a high risk of irreversible hepatic failure. A recent retrospective study by Kim *et al.*^[32] compared clinical outcomes of patients treated with TACE ($n = 49$) vs. TACI ($n = 61$) in HCC patients with major portal vein occlusion. The morbidity rate was similar for both TACE (6.1%) and TACI (6.5%) patients, and complications were adequately managed by medical treatment. Median survival for TACE was longer than for TACI (14.9 vs. 4.4 months, respectively, $P < 0.001$).

Radiotherapy-based therapies

Yttrium-90 radioembolization

Transarterial radioembolization (TARE) with intra-arterial injection of yttrium-90 microspheres (Y-90) is another form of hepatic arterial therapy that is available as glass (TheraSpheres; Theragenics Corp., Ottawa, Canada) or resin (Sirtex; Sirtex Medical, Wilmington, MA, USA) and can be delivered to single or multiple segments based on selective arterial cannulation. Its small size (20-60 μm) results in preferential trapping in the tumor capillary bed. These spheres can safely deliver up to 150 Gy of β radiation to induce tumor necrosis by radiation and microscopic embolization once they obstruct the tumor capillary bed. This limits radiation exposure to adjacent healthy tissue, given its half-life of 62 h and radius of action of up to 1 cm.^[33] Patient selection requires pre-treatment procedures, including an angiogram to perform prophylactic embolization in which variant anatomy is identified to avoid non-target delivery of Y-90, and a macro-aggregated albumin scan to confirm that hepatic artery-to-lung shunting is $< 16\%$ to prevent lung injury.^[34] An advantage of this treatment over TACE is its applicability in patients with portal vein thrombosis and potential complications caused by non-target delivery of Y-90 include gastrointestinal ulcerations, pancreatitis, pneumonitis, and cholecystitis.^[35] Salem *et al.*^[36] recently published a comprehensive study on the long-term outcomes after intra-arterial radiotherapy for unresectable HCC. In this study, 291 patients with HCC were treated with Y-90 as part of a single-center, prospective, longitudinal cohort study. Response rate and time to progression were determined by the World Health Organization (WHO) and

the European Association for the Study of the Liver (EASL) guidelines. Survival by stage was assessed. Univariate and multivariate analyses were performed. Toxicities included fatigue (57%), pain (23%), nausea and vomiting (20%), and 19% exhibited grade 3/4 bilirubin toxicity. The 30-day mortality rate was 3%. Response rates were 42% and 57% based on WHO and EASL criteria, respectively. The overall time to progression was 7.9 months. Survival times differed between patients with Child-Pugh class A and B disease (class A, 17.2 months; class B, 7.7 months; $P = 0.002$). Patients with Child-Pugh class B disease who had portal venous thrombosis survived 5.6 months (95% confidence interval, 4.5-6.7). Baseline age, sex, performance status, the presence of portal hypertension, tumor distribution, levels of bilirubin, albumin, and alpha-fetoprotein, and WHO/EASL response rate were important predictors of survival. While Y-90 has anti-tumor activity, controlled data comparing TARE with TACE is lacking, and its impact on survival is not well established.

Intra-arterial injection of radiolabeled lipiodol

Lipiodol is a mixture of iodized ethyl esters from the fatty acids of poppyseed oil, containing 37% iodine by weight. It is selectively taken up by hepatic tumors when administered via the hepatic artery, and it is retained by HCC for many weeks, even up to a year, while it is cleared from normal or cirrhotic liver within 4 weeks. When injected into the hepatic artery, it travels the peribiliary plexus to the portal veins, resulting in a dual embolization.^[37] Early in the course of exploiting lipiodol's unique features, the addition of a radionuclide to this substance gave a new dimension to its clinical use. So far, most clinical research has been performed with 131I-labeled lipiodol, which is commercially available as Lipiocis (CIS Bio International, Gif sur Yvette, France). 131I-lipiodol has been used for the palliative, adjuvant, or neoadjuvant treatment of HCC.^[38] Although most studies have failed to demonstrate any survival benefits, it seems that 131I-lipiodol is much better tolerated (fewer side effects) than chemoembolization. 131I-lipiodol has the theoretical advantage that there is no particle embolization at the end of the procedure and that portal venous thrombosis is thus not a relative or absolute contraindication.

PERCUTANEOUS LOCAL ABLATION THERAPIES

Chemical ablative therapies

Percutaneous ethanol injection

One of the first methods devised to ablate liver tumors involved percutaneous ethanol injection (PEI). Several non-randomized trials in the 1990s confirmed that PEI could safely achieve complete necrosis of small HCCs,^[39-41] with 5-year survival rates of 32-38%. However, the

technique suffered from the need for multiple treatment sessions, the uncertainty of the ablation zone, and a high local progression rate of 17-38%.^[42,43] Several randomized controlled trials compared PEI vs. RFA in the treatment of small HCC.^[44-46] These trials demonstrated an approximately 20% advantage for RFA vs. PEI in overall survival at 3-4 years, mainly as a result of a much lower incidence of local tumor recurrence in the RFA group. In addition, approximately threefold fewer treatment sessions were required for RFA compared to PEI. Two recent meta-analyses comparing RFA vs. PEI echoed these sentiments, declaring RFA superior to PEI in the treatment of small HCC.^[47,48] PEI maintains the advantage of allowing treatment of tumors near sensitive organs and tissues and avoids the problem of the "heat-sink" effect adjacent to vessels. The applicability of PEI in other situations is limited.

Percutaneous acetic acid injection

Ohnishi *et al.*^[49] reported percutaneous acetic acid injection (PAAI) in 1994. Acetic acid is a noxious chemical characterized by better tissue diffusion than ethanol. Usually, it is proposed as an alternative to ethanol, to decrease the number of sessions.^[50] Sequential therapy with TACE and PAAI is superior to repeated PAAI alone for patients with 3-5 cm HCC.^[51] Acetic acid has a higher diffusion capacity; it is easily available and cheap. A smaller volume of acetic acid and fewer treatment sessions can achieve the same degree of tumor ablation as ethanol.^[50] In addition, PAAI, unlike PEI, helps in infiltrating the tumor septae and capsule. There is not much literature about the efficacy of PAAI in ablating HCC.^[49-51] The procedure of PAAI is similar to PEI. This amount is injected in multiple sessions (1-2 mL of acetic acid per tumor per session per week) using a 23 G spinal/Chiba needle. The response to the treatment is assessed by contrast enhanced computed tomography (CECT) of the liver after 4 weeks. CECT characterizes the liver lesion better, and the residual or recurrent disease can be seen well. The ideal lesion for PEI is small HCC < 3 cm in size. The local tumor recurrence rate is 51% at 1 year and 74% at 3 years. The survival rate at 1 and 3 years is 84% and 51%, respectively.^[50] PAAI is a safe technique, with no major complications. The rare side effects include transient hemoglobinuria (but without any renal impairment), fever, right upper abdominal pain and with larger doses, segmental infarction, and metabolic acidosis can occur.^[49-51] Transient hemoglobinuria can occur immediately after tumor ablation, even after using small volumes (5-10 mL) of 50% acetic acid and it usually clears with a few urinary voids. Precautionary alkalization of urine by administering intravenous fluids containing bicarbonates can be helpful.

Thermal ablative therapies

Radiofrequency ablation

Radio frequencies are the part of the electromagnetic spectrum that are bound by a low oscillation of 3 Hz and a high of 300 GHz. RFA refers to the coagulative necrosis of tissue as a result of heat deposition around a probe generating electromagnetic radiation within the radiofrequency spectrum. The probe (energy source) is inserted within the target lesion, and the circuit is closed by placing grounding pads on the patient's body, usually the thighs. A generator modulates the radio frequency amplitude, and the energy is locally deposited as a result of molecular frictional loss resulting in heating of the tissues around the probe tip. The eventual ablated zone geometry is a result of complex interactions that includes the type and shape of the probe, the duration of ablation, the maximum temperature reached, and the proximity of the target lesion to vessels.^[52] Computed tomographic scanning or ultrasound is used for percutaneous probe guidance, although magnetic resonance imaging (MRI) is emerging as a possible alternative. Effective ablation depends on good tissue conductivity, which allows heat transfer farther away from the probe and a larger ablation zone. Counterintuitively, a fast power increase will result in the tissue around the probe being desiccated, which limits heat conduction and the ablation zone. Therefore, slow and methodical ablation with a gradual power increase is desired. RFA of liver lesions usually takes from 10 to 30 min per lesion.

The efficacy of RFA depends on technical aspects and to a lesser extent, on patient selection. Lesion size is the most important determinant of RFA success. Lesions up to 3 cm can be treated effectively with reported complete ablation rates of about 90%.^[53-56] For lesions > 3 cm,^[53,57,58] the efficacy of RFA decreases with increasing lesion size. Complete ablation is possible with favorable anatomy for lesions of 3-5 cm; however, beyond the 5 cm size, a complete response is unlikely. The rate of recurrence is nearly 0% for smaller lesions and > 50% for lesions > 5 cm. Another determinant of success is lesion location. Central (near the hilum) lesions should be avoided because of the risk of the central bile duct and vascular injury. Additionally, the lesions bordering a large (> 3 mm) vessel may not respond because of thermal protection provided by the adjacent blood flow, a phenomenon termed "heat-sink". Survival of patients with unresectable HCC treated with RFA is reportedly 75-92% at 1 year, 80% at 2 years, 37-59% at 3 years, and 28% at 5 years.^[53,55] Even for resectable tumors, RFA appears to offer the same benefit as resection in selected patients. Survival rates for Child-Pugh class A or B patients with lesions up to 3 cm are not different between groups treated with RFA vs. surgical

resection.^[59] Liver transplantation for HCC remains the best treatment option and offers the longest survival for the approximately 10% of patients who are candidates. Treatment with RFA, while a patient is awaiting for liver transplantation, has been shown to be an independent prognostic factor for longer survival.^[56] Although Child-Pugh class C patients may be safely treated with RFA, a survival benefit is unlikely as life expectancy is determined by the progression of cirrhosis. On the other hand, although prospective, randomized trials are lacking, there is strong evidence that Child-Pugh class A and B patients may benefit from RFA of unresectable HCC.

Percutaneous RFA for HCC carries certain unique risks. The mortality of percutaneous liver RFA is extremely low (< 1%). However, this assumes preserved liver function and small ablation volumes. Because most deaths after RFA are attributed to liver failure, this risk increases with larger ablation volumes and diminished liver reserve (resulting from prior hepatectomy, cirrhosis, previous ablations, and other). The overall major risks associated with liver RFA are on the order of 4-5%.^[56-58,60] Most patients treated with RFA for HCC may be discharged home on the day of the procedure after a 3- to 6-h observation unless a complication.

RFA is also known to enhance host immune response. However, the epitopes at which enhanced immune responses occur, the impact on patient prognosis, and the functions and phenotypes of T-cells induced are still unclear. To address these issues, Mizukoshi *et al.*^[61] analyzed immune responses before and after RFA in 69 HCC patients using 11 tumor-associated antigens (TAA)-derived peptides that were identified to be appropriate for analyzing HCC-specific immune responses. The immune responses were analyzed using enzyme-linked immunospot (ELISPOT) assays and tetramer assays using peripheral blood mononuclear cells. An increase in the number of TAA-specific T-cells detected by interferon- γ ELISPOT assays occurred in 62.3% of patients after RFA. The antigens and its epitope at which enhanced T cell responses occur were diverse, and some of them were newly induced. The number of TAA-specific T cells after RFA was associated with the prevention of HCC recurrence, and it was clarified to be predictive of HCC recurrence after RFA by univariate and multivariate analyzes. The number of TAA-specific T cells after RFA was inversely correlated with the frequency of CD14+ HLA-DR(-/low) myeloid-derived suppressor cells (MDSCs). Modification of the T cell phenotype was observed after RFA. The number of TAA-specific T-cells at 24 weeks after RFA was decreased. Although RFA can enhance various TAA-specific T-cell responses and the T-cells induced contribute to

the HCC recurrence-free survival of patients, besides immunosuppression by MDSCs, the memory phenotype and lifetime of TAA-specific T-cells are not sufficient to prevent HCC recurrence completely. Additional treatments by the vaccine or immunomodulatory drugs might be useful to improve the immunological effect of RFA.^[61]

Microwave coagulation therapy

Microwave ablation is the term used for all electromagnetic methods of inducing tumor destruction by using devices with frequencies greater than or equal to 900 kHz. The passage of microwaves into cells or other materials containing water results in the rotation of individual molecules. This rapid molecular rotation generates and uniformly distributes heat, which is instantaneous and continuous until the radiation is stopped. Microwave irradiation creates an ablation area around the needle in a column or round shape, depending on the type of needle used and the generating power.^[62] The local effect of treatment in HCC was assessed by examining the histological changes of the tumor after microwave ablation.^[63,64] In one study, 89% of 18 small tumors were ablated completely.^[63] Coagulative necrosis with faded nuclei and eosinophilic cytoplasm were the predominant findings in the ablated areas. There were also areas in which the tumors maintained their native morphological features as if the area was fixed, but their cellular activity was destroyed as demonstrated by succinic dehydrogenase staining. One study compared microwave ablation and PEI in a retrospective evaluation of 90 patients with small HCC.^[65] The overall 5-year survival rates for patients with well-differentiated HCC treated with microwave ablation and PEI were not significantly different. However, among the patients with moderately or poorly differentiated HCC, overall survival with microwave ablation was significantly better than with PEI. In a large series including 234 patients, the 3- and 5-year survival rates were 73% and 57%, respectively.^[66] At multivariate analysis, tumor size, the number of nodules, and Child-Pugh classification had a significant effect on survival.^[67] Only one randomized trial compared the effectiveness of microwave ablation with that of RFA.^[68] Seventy-two patients with 94 HCC nodules were randomly assigned to RFA and microwave ablation groups. Unfortunately, the data in this study were analyzed with respect to lesions and not to patients. Although no statistically significant differences were observed with respect to the efficacy of the two procedures, a tendency of favoring RFA was recognized with respect to local recurrences and complications rates.^[68]

Laser-induced interstitial thermotherapy

Laser-induced thermotherapy uses optical fibers to deliver high-energy laser radiation to the target lesion. Because of

light absorption, temperatures of up to 150°C are reached within the tumor, leading to substantial coagulative necrosis. The most commonly used device for laser ablation is the Nd-YAG laser. The optical fibers are inserted directly into the lesion under MRI guidance through a percutaneously placed needle, which is removed after localization. A multi-needle approach is essential to treat large lesions successfully (> 5 cm). In such tumors, treatment time can approach 1 h. Thermocoagulation is monitored in real time under MRI, allowing accurate estimation of the actual extent of the thermal damage. The indications and contraindications of laser ablation are the same as those for RFA and microwave ablation.^[69] Laser ablation has been shown to be effective in inducing complete necrosis in HCC. Because with other ablative techniques, long-term success rates are related to tumor size, and an 82% complete response rate has been reported for lesions measuring 3.2 cm in diameter. In a series of 74 patients with small HCCs, survival rates at 1, 3, and 5 years were 99%, 48%, and 15%, respectively.^[70]

Percutaneous cryoablation

Cryotherapy can destroy tumors directly. With different physical and chemical mechanisms of the therapy, cell death depends on the rate of cooling, absolute depth of hypothermia, the rate of thawing, the number of freeze-thaw cycles and delayed effects of post-thaw ischemia. Most tumor cells die at -40 °C; repeated freezing can improve the efficacy. The larger diameter of current cryoprobes and the location of tumors within the liver still limit its application. Guo *et al.*^[71] reported of 26 patients with HCCs of 10-14 cm in diameter receiving argon-helium cryotherapy after TACE. After this therapy, the average neoplasm necrosis rate was 28.7%, significantly higher than that of TACE only.

High-intensity focused ultrasound ablation

High-intensity focused ultrasound ablation (HIFU) as a new modality for the treatment of HCC has been applied clinically. In the treatment area, all tumor cells seem to be irreversibly dead in the forms of nuclear pyknosis, debris, and dissolution. Blood sinusoids were collapsed with endothelial cell damage.^[72] In combination with TACE, HIFU gives a 1-year survival rate of 42.9% for I/IIa stage patients ($P < 0.05$ compared to patients receiving TACE only) and median reduction rates of 28.6%, 35.0%, 50.0%, and 50.0% of tumor sizes at 1, 3, 6, and 12 months, respectively.^[73] However, the need for general anesthesia and high expenses are its disadvantages.

COMBINATION THERAPIES

Both TACE and RFA have well-known limitations in terms

of control of large tumors. The effectiveness of RFA depends on thermal necrosis and blood flow through the tumor promotes heat loss and prevents proper heating of the tumor. A strategy of combining TACE with RFA by performing TACE before RFA treatment to reduce the heat-sink effect and increase the ablation volume of the tumor was recently evaluated in a randomized study.^[74] In this study, patients with tumors larger than 3 cm were randomized to TACE, RFA, and TACE-RFA. The combination modality was superior in median survival (TACE-RFA at 37 months, TACE at 24 months vs. RFA at 22 months) and rate of objective tumor response (TACE-RFA at 54%, TACE at 35% vs. RFA at 36%). The positive findings in this study represent initial evidence in support for the use of combining local regional modalities to improve outcomes in patients with unresectable tumors. Despite aggressive local treatments with this combinational strategy, recurrence, and distant metastasis continue to have a significant effect on the overall survival of patients with HCC. Therefore, studies that combine effective systemic treatment such as sorafenib with either TACE or RFA have the potential of further improving treatment outcomes. Although the combination of RFA and TACE is most commonly used, TACE has also been combined with interstitial laser photocoagulation, microwave coagulation, ethanol injection, or HIFU.^[73,75,76] On the other hand, the combination of TACE and immunotherapy or anti-angiogenesis therapy could also be an attractive field for future clinical application.

CONCLUSION

Image-guided transcatheter and ablative approaches currently play an important role in the management of patients with HCC, a role that is likely to grow even more given the rapid pace of evolution in these technologies. In selected patient populations, these approaches already offer survival rates that are comparable to that of surgery, with the added benefits of reduced morbidity and costs, improved quality of life and shortened recovery time. As the management of patients with HCC continues to evolve toward disease containment rather than a cure and locoregional targeted therapy rather than systemic approaches, image-guided techniques pose as perfectly suited methods for this direction. Results from clinical trials involving such approaches are increasingly promising, and the potential for improvement remains vast. As a result, these therapeutic approaches will undoubtedly positively impact the outcomes of patients with HCC.

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Role of antiviral therapy in patients with chronic hepatitis B or C virus in preventing the development of hepatocellular carcinoma

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ABSTRACT

Patients with chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection are at significant risk for hepatocellular carcinoma (HCC). The most important risk factor associated with HCC is liver cirrhosis, which is again predominantly caused by chronic HBV or HCV infection. The most effective approach to avoid HCC development is to prevent HBV and HCV infection through vaccination. Indeed, HBV vaccine is the first vaccine demonstrated to prevent cancers. However, a vaccine for HCV is not available. Thus, the prevention of HCV-related HCC and to a large extent HBV-related HCC (among persons who are already chronically infected) will rely on antiviral therapy to prevent progressive liver disease. The evidence that these patients can effectively be protected against HCC risk by the treatment with antiviral therapy is rather controversial, due to the lack of randomized controlled trials (RCTs) that are ideally needed to establish the efficacy, but are logistically and ethically challenging. Although the strongest evidence to support that antiviral therapy can prevent HCC should be derived from RCTs with HCC as an endpoint, it should be emphasized that clinical trials showing the efficacy of antiviral therapy on virus suppression or eradication, and/or improvement in liver histology can be considered indirect evidence that antiviral therapy can prevent HCC because high virus levels (in the case of HBV infection) and cirrhosis (in both HBV and HCV infection) are the most important risk factors for HCC.

Key words: Antiviral therapy; cirrhosis; hepatitis B virus; hepatitis C virus; hepatocellular carcinoma; nucleos(t)ide analogs; pegylated interferon; ribavirin

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INTRODUCTION

The World Health Organization estimates that over 350 million persons are infected with hepatitis B virus (HBV) and about 250 million people are chronically infected with


hepatitis C virus (HCV).^[1] This population is constantly exposed to an increased risk of developing cirrhosis, hepatocellular carcinoma (HCC), liver decompensation,

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and esophageal variceal bleeding, ultimately explaining why HBV and HCV infection are currently the leading causes of liver-related death and the main indication for liver transplantation in developed countries.^[2] There is no clear evidence about the role of antiviral therapies in HCC prevention in patients with chronic hepatitis B (CHB) and hepatitis C.^[3]

Reanalysis of studies with antivirals suggested that virus-induced HCC was more likely to be prevented in younger patients with mild liver inflammation rather than in older patients with advanced liver fibrosis or cirrhosis, who in fact, were at higher risk of developing liver cancer.^[4] In this review, we will address the possible role of antiviral therapy in reducing the risk of HCC in patients affected by HBV and HCV.

We reviewed in PubMed database reports published in English language up to January 2015, using the following keywords: “HCC”, “hepatocellular carcinoma”, “hepatitis B”, “HBV”, “hepatitis C”, “HCV”, “antiviral therapy”, and “cirrhosis”. We selected the pivotal randomized controlled trials (RCTs) and meta-analysis on this issue. In addition, a manual search for American Association for the Study of Liver Diseases and European Association for the Study of the Liver 2012-2014 conference abstracts were performed using the same search terms.

HBV

HBV is one of the most etiologic agent of HCC in the world, in particular, in areas prevalent for HBV infection such as Asia, Africa, Southern part of Eastern and Central Europe, and the Middle East.^[5] A report published in 2006 showed that HBV infection accounted for about 60% of the total liver cancer occurrence in developing countries and about 23% in developed countries.^[6]

There are viral and host factors that are associated with an increased risk of HCC among patients with HBV.^[7] Although a majority of liver cancers develop from cirrhotic livers, a significant fraction of HBV-related HCCs occurs in a background of CHB in the absence of liver cirrhosis. The lower rate of underlying cirrhosis in HBV-related HCCs as compared to other etiologies argues for a more direct role of HBV in the oncogenetic process.^[8]

The molecular and genetic features of HBV chronic infection involving cancer development could be summarized into (1) Pre-core and basal core promoter mutations, genotype B and C^[9-13] and (2) integration of HBV DNA into the host genome and the expression of HBV proteins such as surface proteins and the X protein.^[14-16]

Studies on the natural history of chronic HBV infection have shown that active HBV replication contributes to the development of acute hepatitis flare, hepatic decompensation, cirrhosis, and HCC.^[17] A prospective cohort study with 11 years of follow-up observed that there was a significant increase in HCC-related mortality across viral load categories, with a relative risk (RR) for HCC mortality in the low viral load group of 1.7 [95% confidence interval (CI): 0.5-5.7] when compared with 11.2 (3.6-35.0) in the high viral load group.^[18] In the REVEAL-HBV study, serum HBV DNA levels, and HCC risk correlate in a linear relationship, independently of hepatitis B early antigen (HBeAg) status, serum alanine aminotransferase level, and the presence or absence of liver cirrhosis.^[19] In addition to these viral factors, older age, male gender, heavy alcohol consumption, and exposure to carcinogens such as aflatoxin B, a family history of HCC, and more recently, the elevated levels of quantitative hepatitis B surface antigen, as well as metabolic syndrome, associated with obesity and diabetes mellitus have been established as the risk factors for HBV-related HCC.^[17,20-22]

The primary prevention of HBV-related HCC concerns in the prevention of the population exposure to HBV, treatment of HBV infection itself, elimination of those factors which contribute to the progression of liver disease and risk scores have also been established to estimate the risk of developing HCC in < 10 years after presentation. Such scores based on age, gender, HBV DNA levels, core promoter mutations, and cirrhosis, can be used to identify high-risk patients.^[23-25] However, these models were found lacking accuracy for the prediction of HCC in Caucasian patients, for whom different models are, therefore, deemed necessary.^[26] The implementation of universal hepatitis B vaccination program has reduced the incidence rates of childhood HCC in several countries including Taiwan.^[11] Prompt treatment is the only strategy to prevent end-stage liver disease, incidence, and mortality for HCC in unvaccinated adults with chronic HBV infection.

Current therapeutic options for patients with CHB infection are treatment with interferon-alpha (IFN- α), pegylated interferon-alpha (Peg-IFN- α), lamivudine, adefovir, entecavir, telbivudine, and tenofovir. IFN- α has antiviral, immunomodulatory and perhaps antitumoral activities. It has been used in the treatment of CHB for decades and beneficial effects, including HBeAg/HBV-DNA, clearance the reduction of HCC development, and better complication free survival have been documented. However, the effect on the prevention of cirrhosis and HCC development was controversial. Colombo and Iavarone^[3] have recently reviewed the six meta-analysis published to date: The administration of IFN decreased the rate of HCC

development in three meta-analyses, but it appeared to be unchanged in another three. The effect is more evident in Asian than in European studies possibly related to the lower incidence of HCC in European patients.^[27-32] These controversial results can be explained by extrapolating HCC chemoprevention through the retrospective scrutiny of the studies that were originally designed to assess the antiviral efficacy of IFN therapy. The reanalysis of these studies was biased by the lack of a separate analysis of the treatment outcomes between sustained responders and non-responders, who represent a majority of all patients with CHB receiving IFN.^[3] Therefore, proving a direct anti-HCC effect of IFN-based therapy with clinical trial data beyond what is currently available will be difficult if not impossible. However, IFN still has a role as an effective antiviral for HBV, with finite treatment duration and the potential for a durable effect. Theoretically, the promotion of immune control of viral replication by IFN may have a more solid rationale in terms of HCC prevention unless HBV DNA levels have a direct carcinogenic effect, in which case nucleos(t)ide analog therapy is likely more effective.^[33]

The role of nucleos(t)ide analog therapies in preventing HCC has already been widely investigated. The first data date back to the first antiviral agent chronically administered to reduce viral load in patients with the chronic HBV-related liver disease. In 2004, a large RCT conducted in Asia in patients with chronic hepatitis B, who had histologically confirmed cirrhosis or advanced fibrosis, proved that lamivudine was effective in reducing rates of progression of disease and hepatic decompensation as well as the incidence of HCC.^[22] Further studies confirmed these results. Papatheodoridis *et al.*^[31] showed that long-term therapy with nucleos(t)ide analogs (NUCs) starting with lamivudine monotherapy did not eliminate the HCC risk in HBeAg-negative patients with CHB, especially those with pre-existing cirrhosis. A recent meta-analysis reported that lamivudine treatment significantly reduced the incidence of HCC when compared with no treatment. However, HCC still develops at a rate of 1.3 per 100 patient years in CHB patients receiving an oral antiviral agent.^[34] Recent paper on a nationwide study in Greece indicates that the HCC risk remains increased in entecavir-treated HBeAg-negative CHB patients with cirrhosis, in particular, of older age, at least for the first 5 years. The HCC risk does not seem to be significantly reduced with entecavir when compared with antiviral therapy starting with lamivudine.^[31] This finding highlights the need for continued HCC surveillance, particularly in CHB patients with inadequate viral suppression, older age, and cirrhosis.

Maintenance of virological remission is also important for the reduction of HCC risk. Among treated patients, HCC

incidence is significantly higher among those who do not achieve virologic response than in those who do, with a significant treatment effect observed in the subgroup of cirrhotic patients.^[35-38] This observation provides further evidence that older nucleos(t)ide analogs are not an optimal first-line treatment for chronic hepatitis B, as they are associated with very high rates of drug resistance during the long-term treatment, especially in cirrhotic patients. The nucleos(t)ide analogs entecavir and tenofovir, currently recommended as first-line options for the treatment of chronic hepatitis B, maintain long-term viral suppression in over 95% of patients and improve liver histology.^[39-41] Treatment with entecavir and tenofovir can reduce the risk of HCC.^[42-45] The treatment effect was significant in patients with cirrhosis,^[36] whereas a significant HCC risk reduction in non-cirrhotic patients was noticeable only in some reports.^[45,46]

Finally, there is an increasing evidence to suggest that antiviral therapy may reduce recurrence and also improve survival on post-hepatectomy outcome for hepatitis B-related HCC. A registry-based study from Taiwan showed that of 4569 HBV-related HCC patients who received curative liver resections, patients treated with lamivudine, telbivudine, or entecavir had a significantly lower risk of HCC recurrence as compared to those who received no antiviral therapy (hazard ratio 0.67, 95% CI: 0.55-0.81, $P < 0.001$).^[46] Another study by Chan *et al.*^[47] demonstrated that antiviral therapy with lamivudine or entecavir improves the prognosis of HBV-related HCC: The 1-, 3-, and 5-year overall survival rates in the treatment group were 88.1%, 79.1%, and 71.2%, respectively; in the control group, 76.5%, 47.5%, and 43.5%, respectively ($P = 0.005$). Huang *et al.*^[48] in a recent RCT showed that, in patients with hepatitis B-related HCC treated with adefovir, antiviral therapy leads to a reduction of late HCC recurrence and significantly improves overall survival after hepatic resection when compared with no treatment. IFN treatment as tertiary prevention of HBV-HCC-related recurrence remains controversial according to the findings in systematic reviews. Furthermore, the use of IFN is burdened by several side effects, including liver decompensation.

HCV

Increasing incidence of HCC in many countries, especially in the United States, is the result of an increase in the prevalence of HCV infection. HCV has been the dominant viral cause of HCC in North America, some Western countries, and Japan.^[49] The incidence of HCC in HCV-infected patients amounts to 1-3% at 30 years after the infection.^[50]

The molecular mechanism of a malignant transformation of hepatocyte induced by HCV infection is still unclear.^[51] The pathogenesis of HCC is generally accepted as chronic inflammation and injury, which leads to fibrosis with eventual progression to cirrhosis and subsequent development of HCC.^[52] In this setting, the prevention of HCC could be achieved by preventing cirrhosis and chronic liver inflammation and injury. The most effective approach to prevent HCC is averting HCV infection by vaccination. Unfortunately, despite researcher's efforts, HCV vaccine is not yet available.^[53] When infection is acquired the only way to preventing cancer and progression of liver disease depends on antiviral therapy.

Not all patients with chronic hepatitis C (CHC) progress to cirrhosis and not all patients with HCV-related cirrhosis develop HCC, and the risk factors involved are still unknown. Furthermore, the progression from chronic hepatitis to cirrhosis occurs over several decades thus implying that for RCTs to assess efficacy of antiviral therapy to preventing HCC as a primary endpoint, need to enroll large sample size of patients and long-term follow-up. These limitations ensure that evidence to support the role of antiviral therapy to prevent cancer is based mainly on cohort follow-up, retrospective analysis, and meta-analysis.

In the 2000s, the standard therapy of HCV was Peg-IFN and ribavirin; many reports in this period showed a benefit of treatment, even though only a few of these were RCTs, and most of these studies were retrospective or cohort studies.^[54-57] The protective effect of antiviral therapy was seen in most studies when patients achieved sustained virological response (SVR).^[58,59] These data have recently been confirmed by Moon *et al.*^[60] in a retrospective analysis including 494 CHC patients: Among the group of patients who did not achieve SVR, the incidence of HCC was significantly higher (5.5%) vs. the group of patients with SVR (1%, $P = 0.005$). In this study, the clinical factors associated with SVR were non-cirrhosis, age younger than 40 years, HCV genotype 2 or 3, low HCV RNA level, and low body weight, as reported in the previous studies. This suggests that the main chemoprotective effect is achieved for younger patients without cirrhosis and non-advanced liver disease.

The strength of these data are enforced by three meta-analyses suggesting that IFN therapy reduces the incidence of HCC in patients with CHC with an RR among treated patients of 0.43 (95% CI: 0.33-0.56, $P < 0.00001$).^[58,61,62] Some studies report that the risk of HCC is reduced in these patients independent of fibrosis stage, while among cirrhotic patients that achieve SVR incidence of HCC is

reduced by 20%.^[63-65] In the group of patients with chronic hepatitis treated with IFN \pm ribavirin, the incidence rate of HCC is markedly reduced, while in the group of cirrhotic patients data are not sufficient to support the efficacy of therapy to preventing cancer.^[64-66] A meta-analysis in 2010 compared 20 studies with 4,700 patients overall; the risk in treatment group of HCC was reduced (RR: 0.43, 95% CI: 0.33-0.56).^[58] Pinzoni *et al.*^[67] showed that the risk of developing HCC after achieving SVR persisted in patients with HCV-related cirrhosis: among 598 patients with CHC who underwent a complete course of treatment with Peg-IFN and ribavirin, 221 (37%) patients obtained a SVR and throughout the 10-year post-treatment follow-up, 5.8% of these 221 patients developed HCC. Authors conclude that these patients should continue to undergo long-term surveillance for HCC, to ensure the early detection and treatment. Standard therapy can decrease the risk of HCC, but the patients with this benefit are those who achieve SVR and who have not yet progressed to cirrhosis or advanced fibrosis.

The risk of HCC is reduced but not eliminated also in patients with SVR: these patients are older, thus reflecting a long duration of infection or increased prevalence of cirrhosis and other risk factors for HCC in aged population.^[68,69] In addition, non-viral carcinogenic factors such as diabetes, obesity, and alcohol abuse may explain the failure of HCC prevention in SVR patients.^[70] Although this calls for a reassessment of current strategies of patient prioritization to antiviral therapies, which are mostly dictated by cost-utility criteria and, therefore, target the most in need patients with advanced liver disease, we became progressively aware that uncertainty regarding rates and the pattern of HCC chemoprevention by antiviral regimens is mainly the consequence of methodological flaws generated by the retrospective scrutiny of the literature. Because of its chemopreventive and antifibrotic effects, IFN monotherapy has been adopted as a long-term maintenance therapy to prevent HCC development.

Three large RCTs of long-term (3-4 years), low-dose Peg-IFN in patients with advanced fibrosis or cirrhosis showed no benefit of treatment on overall clinical outcomes or HCC.^[71-73] A subsequent report of the HALT-C Trial focusing on HCC development with a slightly longer duration of follow-up also showed no difference in the incidence of HCC between the patients that were randomized to the maintenance IFN or no treatment.^[74] The same results were observed even when the duration of follow-up in these studies was more prolonged.^[75] Even after radical treatment, tumor recurrence of de novo second primary HCC was extremely frequent (70% after 5 years of surgical resection) and treatment options available,

especially for advanced-stage liver disease, including liver transplantation were limited.^[76] In a meta-analysis of ten studies including eight RCTs conducted in 1029 subjects: 528 HCC patients were treated with adjuvant treatment with IFN and 501 patients with placebo. When compared to the control group, the recurrence rates of HCC in IFN group was significantly lower [odds ratio (OR): 0.66, 95% CI: 0.50-0.86, $P = 0.02$], especially after TACE treatment according to subgroup analysis (OR: 0.73, 95% CI: 0.52-1.01, $P = 0.06$ for surgical resection; and OR: 0.54, 95% CI: 0.33-0.86, $P = 0.01$ for TACE).^[77] In another meta-analysis of 10 controlled studies conducted in 655 patients undergoing local ablation or resection of a HCC, the 2-7 years pooled estimated risk reduction of HCC recurrence in SVR patients to IFN based regimens, was 74% and a 60% pooled risk reduction of mortality was observed in parallel. The study showed no correlation between SVR and risk of local recurrence (12.6% vs. 21.3%, $P = 0.22$), whereas the prevalence of recurrent tumors was greater in untreated patients and non-responders (79% and 61.3%) than in responders (35.6%). Finally, these findings support tertiary chemoprevention of hepatitis C-related HCC by IFN, even though applicability of IFN treatment is limited by its toxicity profile in most cirrhotic patients with a previous resection or tumor ablation.^[78]

DISCUSSION

The actual public health measures for preventing HCV/ HBV transmission, including testing blood donors for HBV and HCV, needle exchange programs, lifestyles preventing alcohol abuse, uncontrolled sexual behaviors, and surveillance of high-risk individuals, could allow a significant decline of the disease in future generations.^[79] Successful treatment of HBV and HCV could decrease the risk of HCC, but does not completely eliminate it.

Regarding HBV, the protective effect of IFN- α is likely to be limited to patients with cirrhosis who are sustained responders, which represented a relatively small proportion of all their patients. The effect of IFN- α in patient without cirrhosis is unclear. Treatment with nucleos(t)ide analogs appears more effective in lowering the risk of HCC development, probably through more powerful and long-standing suppression of viral replication, though the effect may be blunted with the occurrence of resistance.^[80] Risk scoring systems for HCC in CHB should be useful to identify the high-risk patients and also to encourage all available prevention measures targeting adjustable HCC risk factors. However, these models need to be applied and validated in worldwide patients setting.

Furthermore, the current therapeutic options do not

eradicate HBV infection and in spite of adequate treatment, the virus remains indefinitely latent in the host genome, representing a continuous threat of reactivation and an oncogenic HCC booster should be mandatory to start viral suppression in patients with active chronic liver disease, in particular with those who have already developed advanced hepatic disease, to avoid future complications, blackout the liver damage and hopefully reducing some degree of inflammation and fibrosis.^[32]

In HCV setting, new direct antiviral therapies seem to be more effective to achieve a complete sustained virological response, and these new results will be compared with those of patients treated with IFN or Peg-IFN and ribavirin. Some patients who achieved an SVR with IFN- α based therapy also develop the complications of cirrhosis including HCC years after they have been cleaned from HCV.^[81] Although nearly all patients will be cured of HCV by the new therapeutic approach, many of these cannot achieve a restorage of the underlying liver damage if yet established. Thus, it is essential that HCV should be identified and eradicated in all patients, despite the presence of symptoms and different severity grades of liver disease.

CONCLUSION

The risk of HCC in patients with chronic HBV or chronic HCV infection is not avoided if the treatment is started after cirrhosis is established. These data indicate that treatment could be useful if administrated earlier in the course of CHB or CHC.

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Conflicts of interest

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Association of serum levels of epidermal growth factor with disease severity in patients with unresectable hepatocellular carcinoma

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ABSTRACT

Aim: Epidermal growth factor (EGF) is a mitogen for hepatocyte grown *in vitro*, and its expression is up-regulated during liver regeneration. EGF also plays an important role in tumor initiation and progression. The goal of this study is to assess whether EGF is associated with advanced hepatocellular carcinoma (HCC) and also whether it is a predictive factor of shortened survival. **Methods:** Serum EGF levels were evaluated in a total of 151 subjects: 51 patients with unresectable HCC, (21 of them were eligible for transarterial chemoembolization (TACE) and serum EGF levels were measured before and 1 week after TACE), 40 patients with chronic hepatitis without cirrhosis, 40 patients with cirrhosis, and 20 healthy controls. Patient demographic and laboratory variables were evaluated as predictive factors of survival in a Cox regression multivariate analysis using SPSS software. **Results:** The mean serum level of EGF in patients with HCC was 784.49 pg/mL, which was significantly higher ($P < 0.05$) than all other groups. Mean EGF level in cirrhotic patients was 144.69 pg/mL; in those with chronic hepatitis C without cirrhosis, it was 338.64 pg/mL; and in healthy controls, it was 297.15 pg/mL. In group Ia patients who underwent TACE, the mean serum level of EGF was 759.76 ± 287.88 pg/mL before TACE, and 801.14 ± 276.12 pg/mL 1 week after treatment ($P = 0.34$). On multivariate Cox regression analysis only age ($P = 0.03$) and higher serum EGF level ($P = 0.005$), were inversely correlated with overall survival. **Conclusion:** EGF levels were found to be significantly higher in HCC patients and together with age were the only predictors of poor survival in these patients. There was an increase in EGF levels 1 week after TACE in response to hypoxia; however, this increase was not statistically significant.

Key words: Cirrhosis; epidermal growth factor; hepatocellular carcinoma; survival; transarterial chemoembolization



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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the second leading cause of cancer deaths all over the world according to the World Health Organization data.^[1,2] In Egypt, incidences of liver cancer have risen dramatically over the last two decades, and now it is the most common cancer in men and the second most common cancer in women,^[3] with an annual rate of HCC development of 1-4%, when hepatitis C virus (HCV)-related cirrhosis is established.^[1] Patients suffering from HCC unlike most solid tumors are confronted with the coexistence of two life-threatening conditions, malignancy and cirrhosis, which makes their prognostic assessments difficult. Despite the usefulness of clinical staging systems for HCC in routine clinical decision making [e.g., Barcelona-Clinic Liver Cancer (BCLC) algorithm], there is still a need to refine and complement outcome predictions.^[4]

There is an obvious lack of minimally invasive, cost-effective, highly sensitive, and specific biomarkers for accurate diagnosis of HCC independent of the cirrhosis status. Alpha-fetoprotein (AFP) is not a reliable marker in early HCC diagnosis due to its low specificity and sensitivity, which renders it unsatisfactory and suggests an urgent need for novel biomarkers for early stage HCC detection.^[5] Measurement of circulating levels of angiogenic factors in patients with cancerous tumors have several advantages over the direct assessment of tumor angiogenesis, it does not require a tumor specimen, thus they are theoretically applicable to every cancer patient for their technical simplicity and the availability of repeated measurements during (i) initial diagnosis, (ii) course of various anticancer treatments, and (iii) long after the treatment is over.^[6,7] Vascular endothelial growth factor (VEGF) is well known to play a crucial role in tumor angiogenesis by inducing new vessel formation and promoting tumor invasion and metastasis, also VEGF levels are higher in HCC patients. VEGF is used as a biomarker of lymph node metastasis in HCC. In addition, the expression of VEGF is closely correlated with tumor recurrence and prognosis. Of note, VEGF receptor expression levels have also been found to correlate with the development of tumor.^[8] Epidermal growth factor (EGF), another key regulator of cell survival and proliferation, is another biomarker identified in the pathogenesis and progression of different types of cancer.^[9] During 1980s, several reports described the overexpression of EGF and EGF receptor (EGFR) in a variety of epithelial tumors, which may have a critical role in the etiology of human cancers.^[10,11]

EGF is also speculated to enhance the transformation of fibroblasts to fibrosarcomas by inducing the development of HCC in transgenic mice.^[12] Additionally, a functional polymorphism in the EGF gene is reported to be associated with the risk of development of HCC.^[13] Kannangai *et al.*^[14] reported overexpression of EGFR associated with late-stage HCC, increased cell proliferation, and degree of tumor differentiation. All these reports support our hypothesis that EGF is a viable candidate for screening for different cirrhotic populations for early detection of HCC. Transarterial chemoembolization (TACE) being the standard of care treatment for patients with intermediate stage HCC, the best candidates are patients with Child A^[1,2] cirrhosis. And, multifocal non-invasive HCC was also included in the study as an arm to determine the EGF levels in response to the treatment. Most importantly, we tried to identify whether circulating EGF levels were altered in cirrhotic livers with and without HCC. The results from the studies showed that EGF was indeed a sensitive biomarker indicative of poor survival outcome, and it was positively correlated with age in the older population.

METHODS

This case-control study was conducted on 151 patients with chronic liver disease, presented to the Hepatology Clinic, from June 2010 to June 2011. Four groups of patients were studied: HCC, chronic hepatitis C with or without liver cirrhosis, in addition to a fourth group of healthy control subjects with well-matched age and sex. Group I comprised 51 patients with unresectable HCC (intermediate, advanced, and terminal stages), lesions were assessed regarding the number, size, vascular invasion, and distant metastasis. Patients in this group were subdivided according to eligibility for TACE into two subgroups. Subgroup Ia comprised 21 patients with an intermediate stage HCC, who were eligible for TACE (BCLC stage B). Their EGF levels were assessed before and 1 week after TACE. Subgroup Ib comprised 30 HCC patients who were not eligible for TACE, in advanced and terminal stages (BCLC stages C and D). Group II comprised 40 chronic hepatitis C patients without cirrhosis. Group III comprised 40 patients with HCV-related cirrhosis with no evidence of HCC. Group IV comprised 20 apparently healthy subjects as a control group with no evidence of liver disease and/or neoplasm. They were all with well-matched age and sex.

All patients were subjected to the following history taking, complete physical examination, and routine laboratory biochemical and hematological tests.

Laboratory investigations

Five milliliter venous blood samples were collected from patients and controls, centrifuged, the serum was separated and divided into two aliquots. The first aliquot was used for routine laboratory investigations including liver function tests (aspartate transaminase, alanine transaminase, bilirubin, and albumin) using fully automated auto analyzer SYNCHRON CX9ALX (Beckman Coulter Inc., CA, USA). Serum AFP concentration was measured using the Automated Chemiluminescence System ACS: 180 provided by Siemens Medical Solutions Diagnostics Corporation, USA. The second aliquot was stored in the deep freezer (-70 °C) for detection of EGF^[15]

Serum EGF enzyme-linked immunosorbent assay

Estimation of serum EGF using Human EGF enzyme-linked immunosorbent assay (ELISA) kit (sandwich ELISA), Anogen, catalogue number EL10010 Mississauga, Ontario, Canada (up to 336 pg/mL) following manufacturer's protocol.

Radiological examination

Abdominal ultrasonography, triphasic computed tomography, and dynamic magnetic resonance imaging was performed on patients when required.

TACE

Chemoembolization was performed percutaneously at the angiography unit of the National Liver Institute with the patient under conscious sedation. After infiltration of local analgesic, the Seldinger technique was used to gain access to the common femoral artery through femoral artery puncture. A 5-french vascular sheath was placed into the common femoral artery over a 0.035-inch guide-wire. Under fluoroscopic guidance, a 5-french glide Cobra catheter (Cordis Corporation, Miami Lakes, Florida, USA) was advanced into the aorta. Angiographic study of the superior mesenteric artery, celiac trunk, and the common hepatic artery was performed to identify all of the vessels feeding the HCC nodule, and to assess patency of the portal vein. In some patients, selective angiography of the phrenic or intercostal arterial branches was required. The arterial branches feeding the tumor were selectively cannulated by microcatheters to proceed with TACE and to ensure better preservation of the surrounding non-tumoral liver tissue. Injection was done using an emulsion of lipiodol-doxorubicin (50 mg of doxorubicin mixed with 6-20 mL of lipiodol according to tumor size, number, and vascularity to form the emulsion); injection was performed far from the origin of the gastroduodenal, right gastric, and cystic arteries; the amount injected into the tumor was adjusted according to the size and uptake of the tumor. Gel foam was the

embolic material injected in all patients.

Follow-up of HCC patients

Follow-up was conducted for a minimum of 1 year to assess their survival and mortality rates.

Statistical analysis

Statistical analysis was conducted using SPSS program version 13 for windows (SPSS Inc., Chicago, Illinois, USA) and for all the analysis. A $P < 0.05$ was considered statistically significant.

RESULTS

Most of our patients were males (92.1% male, 7.9% female in group I; 87.5% male, 12.5% female in group II; and 85% male, 15% female in group III). Mean age for groups I, II, III, and IV was 58.2 ± 8.7 [standard deviation(SD)] years, 48.47 ± 11.51 years, 49.47 ± 6.94 years, and 47.50 ± 6.15 years, respectively, with statistically non-significant difference ($P > 0.05$).

Liver function tests in different patient groups are shown in Table 1. Child-Pugh score and BCLC staging for patients are shown in Tables 2 and 3, respectively.

Radiological criteria of hepatocellular carcinoma

Twenty-three patients (45.1%) had a single focal lesion, 9

Table 1: Liver function tests in the three patient groups

| | Group I (n = 51) | Group II (n = 40) | Group III (n = 40) | P |
|------------------------------|---------------------|----------------------|-----------------------|-------|
| Bilirubin (mg/dL) | 1.76 ± 1.2 | 0.83 ± 0.24 | 1.72 ± 1.59 | <0.05 |
| Albumin (g/dL) | 3.16 ± 0.65 | 4.35 ± 0.53 | 3.06 ± 0.84 | <0.05 |
| ALT (U/L) | 67.34 ± 38.4 | 61.80 ± 41.13 | 55.82 ± 30.17 | 0.31 |
| AST (U/L) | 90.03 ± 55.8 | 48.55 ± 25.20 | 67.30 ± 32.03 | <0.05 |
| Hb (g/dL) | 11.53 ± 1.86 | 14.04 ± 1.78 | 10.76 ± 2.31 | <0.05 |
| Platelet (/mm ³) | 114.58 ± 55.0 | 192.62 ± 47.9 | 104.37 ± 62.9 | <0.05 |

Data shown as mean \pm SD. Group I: HCC patients; Group II: chronic hepatitis; Group III: cirrhotic patients; ALT: alanine transferase; AST: aspartate aminotransferase; Hb: hemoglobin; SD: standard deviation; HCC: hepatocellular carcinoma

Table 2: Study of CPS in groups I and II

| | CPS (A) (%) | CPS (B) (%) | CPS (C) (%) | P |
|----------|-------------|-------------|-------------|-------|
| Group I | 24 (47) | 16 (31.3) | 11 (21.7) | <0.05 |
| Group II | 19 (47.5) | 7 (17.5) | 14 (35) | |

CPS: Child-Pugh score; (A): score 5-6; (B): score 7-9; (C): score 10-15; Group I: HCC patients; Group II: chronic hepatitis; HCC: hepatocellular carcinoma

Table 3: BCLC staging in HCC studied patients

| Groups | BCLC (B) (%) | BCLC (C) (%) | BCLC (D) (%) | Total | P |
|----------|--------------|--------------|--------------|-------|-------|
| Group Ia | 18 (85.8) | 3 (14.3) | 0 | 21 | <0.05 |
| Group Ib | 0 | 13 (44) | 17 (56) | 30 | |
| Total | 18 | 16 | 17 | 51 | |

Group Ia: HCC, underwent TACE; Group Ib: HCC, did not undergo TACE. BCLC: Barcelona-Clinic Liver Cancer; (B): intermediate stage; (C): advanced stage; (D): end stage; HCC: hepatocellular carcinoma; TACE: transarterial chemoembolization

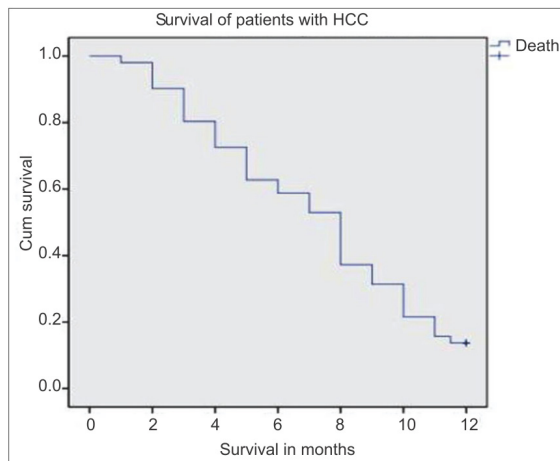


Figure 1: Overall mortality in patients with HCC. HCC: hepatocellular carcinoma

patients (17.6%) had 2 focal lesions, and 19 (37.3%) had ≥ 3 focal lesions. Portal vein thrombosis was present in 12 patients (23.5%), while metastasis was detected in 10 patients (19.6%) [Table 4].

Survival in studied subjects

Overall mortality in patients with HCC was 86% at 12 months. Median survival time was 8 months [Figure 1]. Figure 2 is the Kaplan-Meier cumulative survival curve showing the role of serum EGF in patients' disease-related mortality and cumulative survival. Out of 51 patients with HCC, 44 were deceased after 1 year of follow-up, 9 of them died from upper gastrointestinal bleeding, 15 died from sepsis and spontaneous bacterial peritonitis, 5 died of hepatorenal syndrome, 6 patients died at the intensive care unit after an attack of hepatic encephalopathy, 1 patient died from diabetic hypoglycemic, and the exact cause of death could not be identified in 8 patients.

The majority of patients (85.8%) of group Ia were categorized as BCLC stage B, and 14.3% were in BCLC stage C; while 44% of group Ib were categorized in BCLC stage C and 17% were in BCLC stage D. In Cox regression analysis, age, and serum EGF level were the only factors significantly predicting poor survival in HCC patients ($P < 0.05$) [Table 5].

EGF studies in our subjects

Group I levels were 784.49 ± 313.25 , group II levels were 338.64 ± 224.68 , group III levels were 144.69 ± 124.30 , and for group IV, they were 297.15 ± 175.36 pic/mL. The values are also summarized in Table 6. In pair-wise comparison among individual groups, we found that EGF serum levels were significantly higher in HCC patients compared with the other groups. Statistically significant differences were observed in pair-wise comparison between groups I and II, I and III, I and IV, II and III, and II and IV with $P < 0.05$. Groups III and IV

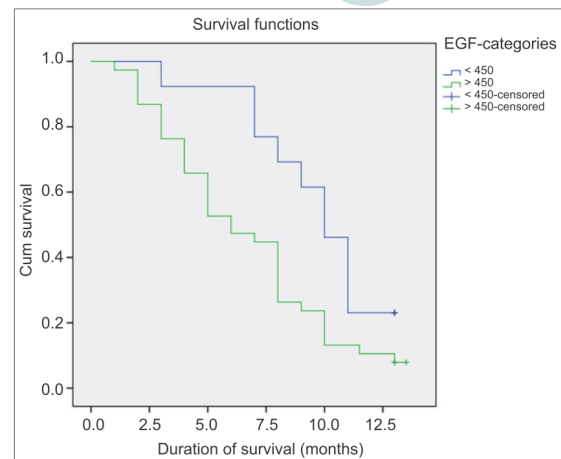


Figure 2: Kaplan-Meier cumulative survival curve showing the role of serum EGF in patients' disease-related mortality and cumulative survival. EGF: epidermal growth factor

Table 4: Radiological criteria of the tumors in HCC patients

| HCC criteria | Patients, n (%) |
|--------------------------------|------------------|
| Lesion | |
| Single lesion | 23 (45.1) |
| 2 lesions | 9 (17.6) |
| > 3 lesions | 19 (37.3) |
| PVT | |
| With PVT | 12 (23.5) |
| Without PVT | 39 (76.5) |
| Metastasis | |
| With metastasis | 10 (19.6) |
| Without metastasis | 41 (80.4) |
| Lesion size | |
| Mean \pm SD in Group Ia (cm) | 6.17 ± 3.055 |
| Mean \pm SD in Group Ib (cm) | 5.67 ± 1.683 |

HCC: hepatocellular carcinoma; PVT: portal vein thrombosis; SD: standard deviation

Table 5: Factors affecting survival in patients with HCC

| | HR (95% CI) | P |
|-------------------|---------------------|-------|
| Age | 1.031 (0.984-1.079) | 0.03 |
| Sex | 1.324 (0.353-4.965) | 0.850 |
| Smoking | 1.014 (0.893-1.151) | 0.490 |
| Pesticide | 1.046 (0.851-1.286) | 0.206 |
| AFP | 1.000 (1.000-1.000) | 0.600 |
| EGF | 1.003 (1.001-1.004) | 0.005 |
| Number of lesions | 0.709 (0.478-1.053) | 0.705 |
| Lesions size | 1.026 (0.890-1.184) | 0.066 |

AFP: alpha-fetoprotein; EGF: epidermal growth factor; HR: hazard ratio; CI: confidential interval; HCC: hepatocellular carcinoma

showed no significant difference in the EGF levels ($P > 0.05$) [Table 7]. EGF levels were 766.05 ± 299.64 pg/mL in BCLC stage B patients, 738.06 ± 320.707 pg/mL in BCLC stage C, and 847.705 ± 328.70 pg/mL in BCLC stage D with no significant difference ($P > 0.05$) [Table 8]. Non-significant difference was found between EGF serum levels in patients with metastatic HCC (mean \pm SD of EGF 847.5 ± 245.4 pic/mL) and in patients with no metastasis (mean \pm SD of EGF 769.1 ± 328.4 pic/mL) ($P > 0.05$) [Table 9]. EGF levels in patients with portal vein thrombosis (mean \pm SD 825.5 ± 318.04 pic/mL) and those without portal vein thrombosis (772.02 ± 314.89 pic/mL) ($P > 0.05$) [Table 9] were similar. Mean

EGF level in HCC patients with one focal lesion was 757.1 ± 327.8 pic/mL, in those with 2 focal lesions was 873.8 ± 334.7 pic/mL, and in those with multiple focal lesions was 775.2 ± 293.9 pic/mL ($P < 0.05$) [Table 9]. Serum EGF levels were strongly correlated to the tumor size and serum AFP levels (using Spearman correlation test, with $P < 0.05$).

Analysis of the receiver operating characteristic (ROC) curve of EGF in HCC prediction [Figure 3] revealed that the area under the curve was 0.93 with 95% confidential interval (CI): 0.89-0.97. Cut-off value of 450 had 74.5% sensitivity, and specificity of 84%, while cut-off value of 700 pg/mL had sensitivity of 60.78% and specificity of 97%, and cut-off value of 900 had sensitivity 39.22% and specificity 98% [Table 10].

Regarding EGF levels in HCC group who underwent TACE (Ia) although EGF levels were higher after TACE than before, no statistically significant difference was found, mean \pm SD 759.76 ± 287.88 pic/mL before TACE, 801.14 ± 276.12 pic/mL after TACE with $P < 0.05$.

DISCUSSION

This case-control study was designed to assess the role of EGF as a predictor factor of progression of HCC in terms of correlation with tumor criteria: size, number, vascular

Table 6: Epidermal growth factor serum levels in the four studied groups

| | Number of subjects | EGF serum levels (mean \pm SD, pg/mL) | P |
|-----------|--------------------|---|--------|
| Group I | 51 | 784.49 ± 313.25 | < 0.05 |
| Group II | 40 | 338.64 ± 224.68 | |
| Group III | 40 | 144.69 ± 124.30 | |
| Group IV | 20 | 297.15 ± 175.36 | |
| Total | 151 | 432.35 ± 350.35 | |

Group I: HCC patients; Group II: chronic hepatitis; Group III: cirrhotic patients; Group IV: healthy control; SD: standard deviation; EGF: epidermal growth factor; HCC: hepatocellular carcinoma

Table 7: Pair-wise comparison of epidermal growth factor between individual groups

| Groups | P |
|------------------|--------|
| Group I vs. II | < 0.05 |
| Group I vs. III | < 0.05 |
| Group I vs. IV | < 0.05 |
| Group II vs. III | < 0.05 |
| Group II vs. IV | < 0.05 |
| Group III vs. IV | 0.65 |

Group I: hepatocellular carcinoma patients; Group II: chronic hepatitis; Group III: cirrhotic patients; Group IV: healthy control

Table 8: Epidermal growth factor levels in HCC patients according to different stages of BCLC classification

| | n | EGF serum levels (mean \pm SD, pg/mL) | P |
|----------|----|---|------|
| BCLC (B) | 18 | 766.05 ± 299.64 | 0.66 |
| BCLC (C) | 16 | 738.06 ± 320.707 | |
| BCLC (D) | 17 | 847.705 ± 328.70 | |
| Total | 51 | 784.49 ± 313.25 | |

BCLC: Barcelona-Clinic Liver Cancer; (B): intermediate stage; (C): advanced stage; (D): end stage; SD: standard deviation; EGF: epidermal growth factor; HCC: hepatocellular carcinoma

invasions, and patient survival. Subjects of our study were selected from the Hepatology Clinics, National Liver Institute, Menoufia University in the period from June 2010 to June 2011. Four groups of patients were studied: Group I comprised 51 patients with unresectable HCC (which were further subdivided according to the eligibility for TACE into subgroups Ia and Ib), group II comprised 40 chronic hepatitis C patients, and group III comprised 40 cirrhosis patients. A fourth group of 20 healthy control subjects (age and sex-matched), was also included in the study. HCC patients were followed up for 1 year for evaluation of their 1-year survival rates.

In this study, 45% of our patients had a single tumor, while 17.6% had 2 lesions, and 37.3% had > 3 lesions. Similar results were presented by Shaker *et al.*^[16] who showed that 38.6% of their cohort had more than one hepatic focal lesion. Vascular invasion was found in 23.5% in our HCC patients. These results are not congruous

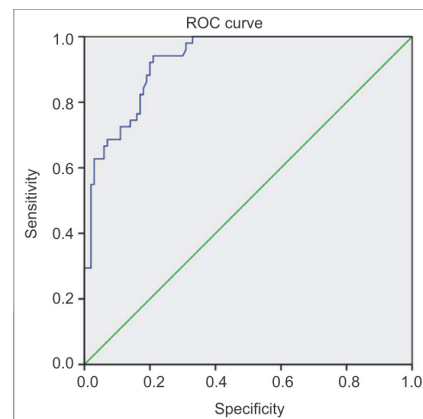


Figure 3: ROC curve of epidermal growth factor value in prediction of hepatocellular carcinoma development. Diagonal segments are produced by ties. ROC: receiver operating characteristic

Table 9: Epidermal growth factor level in HCC patients according to tumor metastases, portal vein thrombosis and number of lesions

| Serum level of EGF | EGF serum levels (mean \pm SD, pg/mL) | P |
|---------------------------|---|------|
| Non-metastatic tumors | 769.1 ± 328.4 | 0.39 |
| Metastatic tumors | 847.5 ± 245.4 | |
| No PVT | 772.02 ± 314.89 | |
| PVT | 825.5 ± 318.04 | 0.64 |
| One lesion (n = 23) | 757.1 ± 327.8 | |
| Two lesions (n = 9) | 873.8 ± 334.7 | |
| ≥ 3 lesions (n = 19) | 775.2 ± 293.9 | |

EGF: epidermal growth factor; PVT: portal vein thrombosis; SD: standard deviation; EGF: epidermal growth factor; HCC: hepatocellular carcinoma

Table 10: Sensitivity and specificity of epidermal growth factor in HCC group

| Studied variable | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) |
|---------------------------------|-----------------|-----------------|---------|---------|
| EGF at the cut-off value of 450 | 74.5 | 84 | 70.37 | 86.60 |
| Cut-off level 700 | 60.78 | 97 | 91.98 | 82.91 |
| Cut-off level 900 | 39.22 | 98 | 90.91 | 75.97 |

EGF: epidermal growth factor; PPV: positive predictive value; NPV: negative predictive value; HCC: hepatocellular carcinoma

with the results of Pirisi *et al.*^[17] which showed that the portal vein thrombosis represented 44% in an autopsied HCC specimen. Another study done by Abdel-Wahab *et al.*^[18] documented that only 15.9% had portal vein thrombosis. This wide range of discrepancy is attributed to the heterogeneity of the studies (some studies evaluated vascular invasion based on histology while others evaluated it based only on imaging). Follow-up of our HCC patients for 1 year revealed that the overall 1-year mortality was 86% with a median survival time of 8 months. Altekruze *et al.*^[19] reported a median survival of < 5 months although a study in Italy found median survival in an untreated group as 10 months,^[20] this could be explained by the fact that the majority of HCC patients had more advanced liver disease.

Evaluation of serum levels of EGF in the four groups revealed significantly higher levels of EGF in HCC patients (784.49 ± 313.25 pg/mL) compared to the other three non-HCC groups. These results signified the role of EGF in tumor growth and progression. Shehata *et al.*^[21] showed higher EGF and transforming growth factor beta 1 levels in patients with HCC compared to the non-HCC counterparts with HCV viral infection and the control subjects. In our study, age and serum EGF levels were the only factors that significantly predicted survival in our HCC patients; higher EGF levels may be associated with tumor aggressiveness and shortened survival. This hypothesis is supported by the in vitro findings of Klocke *et al.*^[22] who demonstrated that the Ig EGF (secreted variant of human EGF) imparts immortality to hepatocyte in vitro. This also was reported by Inoue *et al.*^[23] who studied vandetanib, an inhibitor of VEGF receptor-2 and EGF receptor, in liver cancer in mice and found that it suppressed tumor development and improved prognosis of liver cancer; improved survival, and reduced number of intrahepatic metastases. Yoneda *et al.*^[24] found that higher levels of EGF were associated with activation of EGF-EGFR pathway associated with the development of CK19-positive HCC, and the EGF-induced increase in growth abilities of HCC may account for the poor prognosis of those patients. DeCicco *et al.*^[25] reported overexpression of EGF receptors (EGFR) in hepatoma cells of rats, suggesting that EGFR may be useful as a dynamic marker for the development of hepatoma. This was confirmed by Sung *et al.*^[26] who concluded that serum EGFR level was a potential biomarker of liver cancer. Kannangai *et al.*^[14] added that EGFR can be considered as a marker for predicting the metastasis and recurrence of HCC. Wu *et al.*^[27] found that EGF was a promoting factor for hepatoma cells stressing on the critical role in EGF-induced proliferation. Wu *et al.*^[28] demonstrated that overexpression of epidermal growth factor-like domain

7 was found predominantly in hepatoma cells and closely correlated with poor prognosis.

ROC curve analysis of EGF in HCC showed that the area under the ROC curve of EGF for the prediction of HCC progression was 0.93 with 95% CI: 0.89-0.97. Cut-off value of 450 had 80% sensitivity while cut-off value of 700 had sensitivity 60.78% and specificity 97% while cut-off value of 900 had sensitivity 39.22% and specificity 98%. Shehata *et al.*^[21] showed that significant higher serum levels of EGF in patients with HCC compared to their levels in patients with HCV infection and control subjects with cut-off value of 914 pg/mL, EGF shows 63.3% sensitivity, and 87.5% specificity for HCC patients.

Our results revealed that the EGF serum level increased slightly in chronic hepatitis activity than levels in established cirrhotic group, reflecting potential role of EGF in fibrosis process as described by other reports such as Iagoda *et al.*,^[29] who studied the growth factors and the histological picture of the liver in chronic viral hepatitis and hepatic cirrhosis and found that EGF levels decreased with increase in histological activity and the degree of hepatic fibrosis to cirrhosis. Predictive factors for progressive HCC in our patients were analyzed by binary logistic regression, serum EGF level was found to be a predictive factor of HCC progression. These results agree with the results of a meta-analysis of eight studies concluding that EGF polymorphism is a risk factor in hepatocarcinogenesis.^[30] Tanabe *et al.*^[31] stated that in a dose-dependent fashion EGF measurements in serum and in liver tissue were presumed to be most relevant to hepatocyte transformation in cirrhosis and concluded that the EGF gene polymorphism was associated with development of HCC in liver cirrhosis through modulation of EGF levels. Regarding factors affecting patients' survival using Cox regression analysis, older age and higher serum EGF levels were the only factors significantly affecting survival ($P < 0.05$).

Overall, there was a strong correlation ($P < 0.05$) between EGF level and tumor size, signifying its potential role in tumor proliferation and its use as a predictive factor of HCC progression. A major limitation of our study is the relatively small number of patients who underwent TACE, heterogeneity of the study cohort is a limitation in many of the TACE studies because of the wide spectrum of HCC patients eligible for TACE compared with the other modalities of treatment of HCC, this can be overcome by conducting future prospective studies on larger number of patients with similar disease. Interestingly, serum EGF levels were higher post-TACE, although this difference was not statistically significant. The explanation of

this marginal increase is not yet known, TACE-induced hypoxia (and angiogenesis) might be a contributing factor which needs further studies. The time point to measure serum EGF (1 week after TACE) was chosen at random as an initial evaluation to also address the effect of TACE on EGF, future studies focusing on including 2 additional time points at 1 and 3 months are warranted. Philip *et al.*^[32] tested five EGFR inhibitors: Erlotinib, gefitinib, cetuximab, lapatinib, and vandetanib. Erlotinib showed activity in a phase II study with mixed HCC populations with a median survival of 13 months, and it was being tested in combination with sorafenib in phase III. The other drugs either have not shown meaningful signals of efficacy in phase II, such as gefitinib and lapatinib, or are still in early stages of investigation.^[33] Gefitinib, a selective EGFR tyrosine kinase inhibitor, is reported to successfully treat lung cancer. When investigating the effects of gefitinib on tumor-induced angiogenesis, it was found that production of both VEGF and chemokine factor by EGF-stimulated HCC was more markedly inhibited by gefitinib. Sogawa *et al.*^[34] in their study used a novel human monoclonal antibody against EGFR as an imaging probe for HCC concluded that the radiolabeled human anti-EGFR monoclonal antibody 048-006 has the potential to be a safer imaging probe for predicting tumor uptake of anti-EGFR antibody therapeutic agents in HCC. Studying EGF and its receptors: pathway, therapies, and pipeline concluded that the exploitation of EGFR-directed therapies offered an improvement in survival and quality of life in non-small cell lung cancer and colorectal carcinoma.^[35] Additional efforts should be exerted directing further studies on EGFR-directed therapies to the poorly treated HCC patients.

In conclusion, serum EGF levels were found to be significantly higher in HCC group in comparison with cirrhosis, chronic hepatitis, and control groups. A serum level of EGF is a predictor factor of HCC progression and together with older age were the only two predictive factors for poor survival in patients with HCC after 1 year of follow-up. There was an increase of serum EGF levels in response to TACE without significant difference. Future studies should be conducted to focus on EGFR and their inhibitors as new promising therapeutic agents for HCC with the inclusion of more patients with respectable tumors amenable to resection, ablation, and/or liver transplantation who are expected to survive long enough to study any potential prognostic importance of EGF.

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Liver Institute, Menoufiya University, Egypt.

Conflicts of interest

There are no conflicts of interest.

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Hepatocellular carcinoma and type 2 diabetes mellitus: two cases highlighting changes in tumor glycogen content

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ABSTRACT

This article reports two patients with hepatocellular carcinoma (HCC) and type 2 diabetes mellitus (T2DM), who showed marked changes in hepatocellular glycogen content. Periodic acid-Schiff (PAS)-positive and diastase-PAS-negative (glycogen-storing) hepatocytes were detected in both background liver parenchyma and in HCC tissues. In HCC tissues, the number of glycogen-storing cells resembling hepatocytes was considerably reduced and unevenly distributed as compared with hepatocytes in background liver. To be known, changes in hepatocellular glycogen content in T2DM patients have not been previously described. It is hypothesized that the reduction in glycogen content in both patients was likely associated with the emergence of Warburg type of glycolysis.

Key words: Glycogen; hepatocellular carcinoma; hexokinase II; steatohepatitis; type 2 diabetes mellitus

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INTRODUCTION


Most cases of hepatocellular carcinoma (HCC) occur in patients with chronic liver disease and advanced fibrosis. Well-known causes of chronic liver disease leading to HCC include chronic hepatitis B virus (HBV) and hepatitis C virus infection,^[1] chronic alcohol abuse^[1,2] and more recently, non-alcoholic fatty liver disease (NAFLD).^[3] In addition, type 2 diabetes mellitus (T2DM) has been

associated with HCC.^[4] Patients with T2DM and NAFLD-related non-cirrhotic or cirrhotic livers may develop HCC, suggesting a role for T2DM in hepatocarcinogenesis.^[3]

Glycogen loading of the liver was first documented as a component of Mauriac's syndrome in 1930, and

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enhanced glycogen deposits were observed with increasing frequency in patients with brittle diabetes.^[5,6] Excessive storage of glycogen [glycogen-storing foci (GSF)] has been observed in pre-neoplastic foci of altered hepatocytes (FAH), and in highly differentiated subpopulations of benign and malignant hepatocellular lesions in animal models of hepatocarcinogenesis.^[7-9] Glycogenotic cells (clear cell) have been observed in liver biopsies and explants from the patients harboring foci and nodules of altered hepatocytes.^[10,11] Although clear cell HCCs have been described, their glycogen content was usually not determined.^[12]

To our knowledge, there have been no comparative studies on changes in hepatocellular glycogen content of HCC and background livers in patients with T2DM. This study describes two patients with HCC and T2DM, who showed marked changes in hepatocellular glycogen content.

CASE REPORT

Case 1

A 72-year-old Japanese man with T2DM and alcoholic liver disease was diagnosed with HCC by computed tomography (CT) examination. Laboratory data showed aspartate transaminase (AST) 95 IU/L, alanine transaminase (ALT) 65 IU/L, alpha-fetoprotein (AFP) 8.2 ng/mL, protein-induced by vitamin K absence factor II (PIVKA-II) 26 mAU/mL, fasting blood sugar (FBS) 228 mg/dL, and hemoglobin A1c (HbA1c) 7.9%. CT arterial portography and CT hepatic arteriography revealed 2 minor nodules (3-4 mm) at S5, and a larger nodule (2.5 cm × 2.3 cm) at S8.

A specimen, obtained from needle biopsy of the S8 tumor, was fixed with Carnoy's solution, and formalin for a routine histological diagnosis. Samples were stained with periodic acid-Schiff (PAS) and PAS after diastase pre-treatment (D-PAS). Hexokinase II (HK-II) was detected immunohistochemically using anti-HK II (C64G5) rabbit mAb (Cell Signaling Technology, Inc. Danvers, US). HK-II

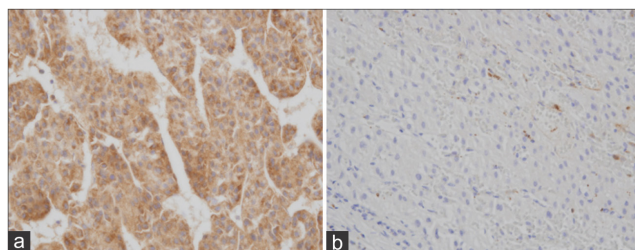


Figure 1: Control - hexokinase II activity in hepatocellular carcinoma tissues (a) and background liver (b) of positive control (65-year-old male, well-differentiated adenocarcinoma in background of chronic hepatitis C) (a and b: hexokinase II, ×400)

activity in positive control was also indicated [Figure 1a and b].

Histologic examination showed a well- to moderately-differentiated HCC [Figure 2a], with the background liver showing steatohepatitis with alcoholic pericellular fibrosis [Figure 2b]. Both PAS-positive [Figure 2c and d] and D-PAS-negative (glycogen-storing) hepatocytes [Figure 2e and f] were detected in the background liver and in HCC tissues. However, the PAS-positive hepatocytes were more abundant in the background liver than in the HCC tissues. No pronounced clear cells were detected. HK-II expression was weak in HCC [Figure 2g] and faint in background liver [Figure 2h]. Clinical and pathological data are summarized in Table 1.

Case 2

A 73-year-old Japanese man with T2DM and non-alcoholic steatohepatitis (NASH) was diagnosed with HCC by CT examination. At the age of 64, he was diagnosed with T2DM and NASH via needle biopsy of the liver. Laboratory examination showed AST 51 IU/L, ALT 22 IU/L, AFP 4.4 ng/mL, PIVKA-II 22 mAU/mL, FBS 140 mg/dL, and HbA1c 6.3%.

Partially, hepatectomized liver tissue was fixed as described in Case 1. Macroscopically, the HCC was revealed as simple nodular type (size, 1.8 cm × 1.5 cm; stage 1, T1N0M0; Child-Pugh grade A). Histological examination showed a well-differentiated HCC [Figure 3a], with the background liver presenting as type B cirrhosis [Figure 3b]. PAS-positive [Figure 3c and d] and D-PAS-negative [Figure 3e and f] hepatocytes were detected in both background liver and in HCC tissues, although the PAS-positive hepatocytes were more abundant in background liver than in the tumors. HK-II was weakly expressed in HCC [Figure 3g] and faintly expressed in background liver [Figure 3h]. No obvious clear cells were detected.

DISCUSSION

This study describes the two patients with T2DM, who developed HCC. Background liver in both patients showed steatohepatitis, suggesting that HCC may have been mainly due to steatohepatitis. The alcohol intake may have been a risk factor for HCC in Case 1,^[2] whereas occult HBV infection with positivity for hepatitis B surface anti-body/hepatitis B core anti-body may have been a risk factor in Case 2.^[13]

Glycogenotic hepatocytes are a common pre-neoplastic liver lesion in human at a high risk of HCC development.^[11,14] FAH, including GSF, was detected in

Table 1: Summary of clinical and pathological data

| | Case 1 | Case 2 |
|--------------------------------------|---|--|
| Age/gender | 72 years/male | 73 years/male |
| DM type/duration | 2/16 years | 2/9 years |
| Insulin level | 3.5 μ U/mL | 20.1 μ U/mL |
| Body mass index (kg/m ²) | 20.8 | 22.1 |
| Alcohol | 63 g/day, 40 years | No |
| HBsAg/cAb/sAb/HCV | -/-/-/- | -/+/-/- |
| Biopsy or resection | Needle biopsy | Partial resection |
| HCC | | |
| Size (location) | 2.5 cm \times 2.3 cm (S8) | 1.8 cm \times 1.5 cm (S8) |
| Histology | (3-4) mm \times (3-4) mm, double (S5) | Well-differentiated adenocarcinoma |
| PAS-positive cells diastase-PAS | Well- to moderately-differentiated adenocarcinoma | Uneven negative |
| HK-II immunostaining | Small numbers negative | Weak positive |
| Background liver | Weak positive | |
| Histology | Steatohepatitis with pericellular brosis (F2-3) | Liver cirrhosis, type B NASH (9 years ago) |
| PAS-positive cells diastase-PAS | Abundant numbers negative | Abundant numbers negative |
| HK-II immunostaining | Faint positive | Faint positive |

DM: diabetes mellitus; HBsAg: hepatitis B surface antigen; cAb: core anti-body; sAb: surface anti-body; HCV: hepatitis C virus; HCC: hepatocellular carcinoma; PAS: periodic acid-Schiff; HK-II: hexokinase II; NASH: non-alcoholic steatohepatitis

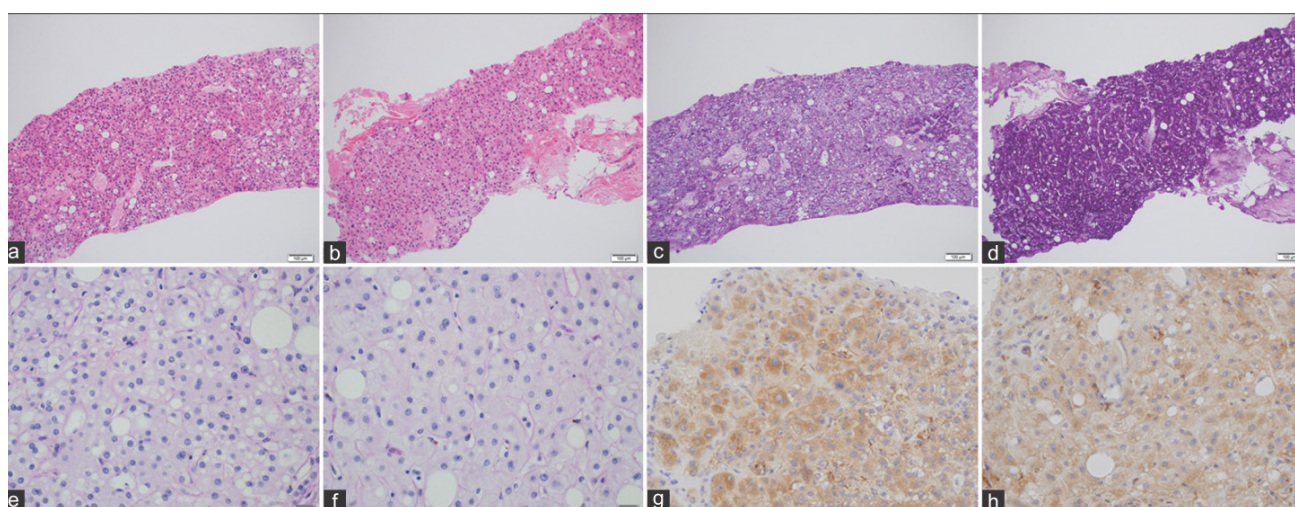


Figure 2: Case 1-histochemical comparison of glycogen content and hexokinase II activity in hepatocellular carcinoma tissues (a, c, e, and g) and background liver (b, d, f, and h) (a and b: HE, \times 100; c and d: periodic acid-Schiff, \times 100; e and f: diastase-periodic acid-Schiff, \times 100; g and h: hexokinase II, \times 400)

84 of 111 (75.7%) patients with cirrhotic liver diseases, with a higher incidence in patients HCC than those without HCC.^[11] GSF were also detected in a significant number of human non-cirrhotic livers (88 of 236; 33.6%).^[15] A combination of enzymatic and molecular biological approaches has shown the striking similarities in metabolic changes in human and rat GSF, including the activation of the AKT/mammalian target of rapamycin (mTOR) and Ras/MAPK signaling cascades.^[15]

Studies in more than 150 human explants showed the evidence for a characteristic sequence of cellular changes, from pre-neoplastic glycogenotic FAH via various intermediate stages [mixed cell foci (MCF)] to glycogen-poor malignant phenotypes, similar to that in animal models.^[9,11] These phenotypic cellular changes are due to a metabolic switch from gluconeogenesis toward the pentose phosphate pathway and the Warburg type of glycolysis.^[9,11] In human HBV-associated tumorigenesis, the mTOR signaling cascade has been shown to play a crucial role

in driving the metabolic alterations toward increased aerobic glycolysis.^[16] When initial excess glycogen stores are reduced, the storage of polysaccharides is often largely replaced by the accumulation of neutral lipids.^[17] In both of our patients, PAS-positive/D-PAS-negative hepatocytes, which store glycogen albeit not the excessive amounts, were detected in background livers and HCC tissues. Hepatocytes rich in glycogen were abundant in background liver parenchyma but were mixed with glycogen-poor cells in HCC tissues. Neither pronounced clear cells nor MCF were detected. Fat deposits were rare in HCC tissues and background livers of both of these patients.

Changes in glycogen content frequently accompany a shift in the expression of isoenzymes during progression, e.g., from low-affinity (glucokinase/HK IV) to high affinity (HK-II) HK,^[17,18] HK-II being characteristic of Warburg type of glycolysis occurring in rapidly growing tumors, including HCC.^[17,19]

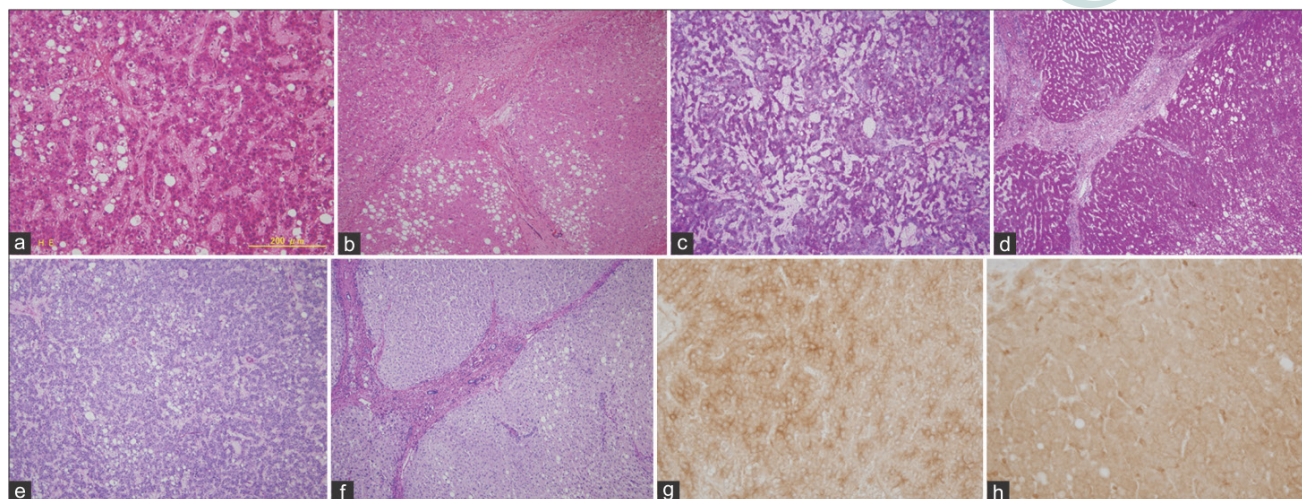


Figure 3: Case 2 - histochemical comparison of glycogen content and hexokinase II activity in hepatocellular carcinoma tissues (a, c, e, and g) and background liver (b, d, f, and h) (a and b: HE, $\times 100$; c and d: periodic acid-Schiff, $\times 100$; e and f: diastase-periodic acid-Schiff, $\times 100$; g and h: hexokinase II, $\times 400$)

Guzman reported that the higher levels of HK-II in HCC were associated with more aggressive histological behavior; however, HK-II expression was not associated with DM.^[20] HK-II was expressed in both the HCC tissues and background liver parenchyma of our patients, but its intensity was inversely related to PAS-positivity, being higher in cells with lower glycogen content. Histological examination showed that our HCC patients have less aggressive phenotypes. It is hypothesized that the reduction of glycogen content in HCC may, therefore, be associated with the appearance of Warburg type of glycolysis. Non-invasive monitoring of the glycogen content of the liver might serve as a basis for predicting the development of HCC. Unfortunately, such an approach is currently not available.

In summary, this study described the two patients with HCC and T2DM, both of whom experienced marked changes in glycogen content in HCC tissues and background liver parenchyma. These studies in larger numbers of patients are needed to clarify a possible relationship between the changes in hepatocellular glycogen content and the development of HCC in diabetic patients with steatohepatitis.

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Conflicts of interest

There are no conflicts of interest.

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Recent updates of genetic and genomic alterations in hepatocellular carcinoma

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ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most common malignant cancers worldwide. However, the molecular mechanisms underlining the development and progression of HCC remain unclear. Genetic and genomic alterations are common events in various types of cancers including HCC. With the development and application of next generation sequencing technology, novel genetic and genomic alterations in HCC have been identified. Here, the article reviews recent updates on the genetic and genomic alterations in HCC.

Key words: Hepatocellular carcinoma; genetics; genomics; next generation sequencing

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant neoplasms worldwide, with a prevalence of more than 50% in China. Hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, ingestion of food contaminated with aflatoxin B1, and alcohol consumption are considered major risk factors for HCC development.^[1] Despite well-established risk factors, the specific molecular mechanisms underlining pathogenesis of HCC remain unclear. Genetic and genomic alterations are common events in various types of cancers including HCC, and may be associated with the development and progression of cancer. With the development of the technology of next generation sequencing, that is, whole-genome sequencing, novel genetic and genomic alterations have been identified. Recent studies on whole-genome sequencing of

HCC confirmed the important roles of previously reported genetic and genomic alterations in the development and progression of HCC.^[2] However, the fact that the most frequently mutated genes were generally previously reported, and that few novel mutated genes with high frequency were identified by the whole-genome sequencing suggests the complexity regarding the role of genetic mutations in the pathogenesis of HCC. In this paper, we review recent updates on genetic mutations and genomic imbalances in HCC.

GENETIC ALTERATIONS: MUTATION AND SINGLE NUCLEOTIDE POLYMORPHISM

Somatic mutation

Previous studies have demonstrated that the most significantly mutated genes in HCC include tumor

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protein p53 (*TP53*), catenin (cadherin-associated protein) beta 1 (β -catenin, *CTNNB1*), and AT-rich interactive domain-containing protein 2 (*ARID2*), with mutation frequency over 15%.^[2-4] Other mutated genes such as *SMAD2/SMAD4* in the transforming growth factor beta ($TGF-\beta$) pathway, caspase 8 (*CASP8*), and Kruppel-like factor 6 were identified with mutation frequency around 10% in HCC,^[5-7] while most other mutated genes were identified with relatively low frequency of < 10% in HCC.^[2] Germline mutations in the *TP53* gene have been identified in patients with Li-Fraumeni syndrome, which is an inherited cancer predisposition syndrome characterized by a wide spectrum of neoplasms.^[8] Somatic *TP53* mutations were identified in virtually any tumor type including HCC, particularly following exposure to aflatoxin.^[9,10] According to the IARC database, 1840 *TP53* somatic mutations have been identified in 31.19% of liver cancer cases (IARC *TP53* Database R17, <http://p53.iarc.fr/>). The mutation of β -catenin gene in *WNT/CTNNB1* pathway has been identified in HCC with a frequency of 15.9%, which can lead to the activation of *CTNNB1* gene with the consequence of overexpression and accumulation of β -catenin.^[3] *ARID2* is a subunit of the PBAF chromatin-remodeling complex, which facilitates ligand-dependent transcriptional activation by nuclear receptors. In the United States and Europe, 18.2% of HCV-associated HCC cases were identified with *ARID2*-inactivating mutations.^[4] However, studies have also reported mutation frequencies of approximately 5-10% for *ARID2* in HCC and truncation of *ARID2* leads to loss of protein function and chromatin dysregulation.^[4,11,12]

With the development of whole-genome sequencing technology, the next generation sequencing of genome DNA provides the possibility that more novel genetic and genomic alterations may be discovered and may provide new insights for understanding the pathogenesis of HCC. However, several recent studies using next generation sequencing for analysis of mutation in HCC showed that the most frequent mutations with mutated rate over 10% were mainly genes reported previously such as *TP53*, β -catenin, and genes of chromatin-remodeling complex such as AT-rich interactive domain 1A (*ARID1A*) (14/110).^[2,13,14] Only a few genes were identified with mutation rates over 10%, for example, the low-density lipoprotein receptor-related protein 1B gene, reported by Kan *et al.*^[2] to have a mutation rate of 11.4% in patients with family hypercholesterolemia. Notably, several components of the chromatin-remodeling complex, such as *ARID1A* and *ARID2*

were mutated in over 10% HCC specimens,^[13,14] similar to previous reports, confirming the important role of chromatin-remodeling in the pathogenesis of HCC. In addition, Janus kinase 1 (*JAK1*) mutation was identified with mutation rate of 9.1% through the whole-genome sequencing of 88 HCC cases, and the *JAK/STAT* pathways were altered in 45.5% of cases,^[2] inconsistent with a previous study which reported low frequency (1/84, 1.2%) of *JAK1* mutation in HCC,^[15] implying that the *JAK/STAT* pathways may act as major oncogenic drivers in HCC. However, the fact that the most frequently mutated genes were generally previously reported, and that few novel mutated genes with high mutation frequencies were identified by whole-genome sequencing suggests the complexity regarding the role of genetic mutations in the pathogenesis of HCC.

It has been reported that genomic instability is a characteristic of most cancers. Genomic instability results from mutations in DNA repair genes and drives cancer development in hereditary cancers. However, in sporadic cancers, previous studies and recent high-throughput sequencing studies suggested that mutations in DNA repair genes were infrequent. Instead, the mutation patterns of the tumor suppressor *TP53*, ataxia telangiectasia mutated (*ATM*), and cyclin-dependent kinase inhibitor 2A (*CDKN2A*) support the oncogene-induced DNA replication stress model, which attributes genomic instability and *TP53* and *ATM* mutations to oncogene-induced DNA damage, that is, high frequency of *TP53* mutations in human cancers could be in response to oncogene-induced DNA damage.^[16] The hypothesis was confirmed by several studies showing that deletion of the *TP53* gene in mouse models and human cells did not lead to aneuploidy, and that in human precancerous lesions, genomic instability was present before the establishment of *TP53* mutations.^[17-19] Consistent with the above studies, previous studies and recent whole-genome sequencing of HCC also showed that mutations in DNA repair genes in HCC were infrequent,^[2,13,14,20] suggesting there may be similar mechanisms of genetic mutations in somatic HCC, that is, high frequency of *TP53* mutations and additional genetic mutations favoring cancer development in somatic HCC could be in response to oncogene-induced DNA damage.

Single nucleotide polymorphism

Single nucleotide polymorphism (SNP) is the most common genetic variation in the human genome.

Genome-wide association study (GWAS) was also applied for SNP analysis of HCC in recent years. In a GWAS of HCC in Japanese population, one intronic SNP (rs1012068) in the DEP domain containing 5 gene was identified to be associated with HCC risk.^[21] In a GWAS of HCC in chronic HBV carriers of Chinese ancestry, one intronic SNP (rs17401966) in kinesin family member 1B was identified to be highly associated with HBV-related HCC.^[22] In addition, SNP (rs9679162) in polypeptide N-acetylgalactosaminyl transferase 14 (*GALNT14*) have been shown to be associated with chemotherapy response in patients with advanced HCC; for advanced HCC patients treated with FMP (fluorouracil oxantrone cisplatin) chemotherapy, *GALNT14* genotype (rs9679162) was an effective predictor of the therapeutic outcome.^[23,24]

GENOMIC ALTERATION: GENOMIC IMBALANCES

Copy number variation-genomic gain or loss

Chromosomal abnormalities in HCC have been well reported, and comparative genomic hybridization (CGH) has revealed a consistent pattern of genomic gains and losses involved in the development and progression of HCC. The most prominent changes are partial or entire gains of chromosome arms 1q, 8q, and 2q; and losses of 1, 4q, 8p, 13q, 16q, and 17p. In one meta-analysis, using conventional CGH analysis with low resolution (approximately 2 Mb) from several studies, it was revealed that the most prominent changes were gains of 1q (57.1%), 8q (46.6%), 6p (23.3%), and 17q (22.2%); and losses of 8p (38%), 16q (35.9%), 4q (34.3%), 17p (32.1%), and 13q (26.2%).^[25] Using array CGH analysis from four studies, it was revealed that loci with genomic gains with a prevalence of more than 25% included 1q, 6p, 8q, 17q, 20p, 5p15.33, and 9q34.2-34.3; and loci with genomic losses with prevalence of more than 25% comprised 4q, 6q, 8p, 9p, 13q, 14q, 16q, and 17p; and were associated with 31 classical molecular pathways, particularly the antiviral immunological pathway.^[25] A series of tumor suppressor genes have been identified in these regions, such as PR domain containing 5 (*PRDM5*, 4q26), *TP53* (17p13.1), retinoblastoma 1 (*RB1*, 13q14), and cadherin 1, type 1 (*CDH1*, 16q22.1).^[26-28] Some clinicopathological associations have been noted with specific abnormalities: Losses of 4q, 13q, and 16q are associated with HBV infection,^[25] loss of 4q has been associated with elevated α -fetoprotein levels, *TP53* mutations,^[29] tumor size, and vascular invasion^[30] while 9p and 6q losses have been reported to be independent predictors of poor outcome of HCC

patients,^[31] and that losses of 4q, 13q, and 16q are associated with HBV infection.

Similar to the finding reported by the previous array CGH based study, a recent whole-genome sequencing study on HCC showed similar patterns of genomic imbalances: The copy number variation in HCC genomes is dominated by large-scale amplifications and deletions of chromosomal arms or entire chromosomes including gain at 1q, 5p, 6p, 8q, 17q, and 20q; and deletion at Xq or loss at 4p/4q, 8p, 13p/13q, 16p/16q, 17p, 21p/21q, and 22q.^[2]

Loss of heterozygosity

Loss of heterozygosity (LOH) refers to one of two polymorphic alleles on a tumor chromosome. Zhang *et al.*^[32] identified a high frequency of LOH 4q (48.1%) in HCC, in which the caspase-6 and ras-related C3 botulinum toxin substrate 1 pseudogene 5 in the region 4q24-26 may be related with tumor growth. Additionally, inhibitor of growth family, member 2 (*ING2*) in the region 4q34.3-4q35 was found to be down-regulated frequently in HCC, and its gene expression was also significantly decreased, suggesting that *ING2* might be a tumor-specific glycoprotein of HCC.^[32] In a variety of human tumors, the most common chromosomal changes were 8p allelic loss, suggesting that there might be one or several tumor suppressor genes on the short arm of chromosome 8. LOH was frequently observed on chromosomes 8p22-23, but the gene closely related with HCC was still unknown. However, Peng *et al.*^[33] identified that LOH of zinc finger, DHHC-type containing 2 (in 8p22-23 was associated with early metastatic recurrence of HCC after liver transplantation.

Gene amplification and deletion

Gene amplification in certain regions of chromosomes plays a crucial role in the development and progression of human malignancies. Recently, researchers found amplification of the ecotropic viral integration site 1 (*EV11*) gene at the chromosomal region 3q26 in the HCC cell line JHH-1.^[34] A copy number gain of *EV11* was observed in 36% (24/66) of primary HCC tumors. *EV11* antagonizes TGF- β -mediated growth inhibition in HCC cells, suggesting the *EV11* may be a potential molecular target for the development of novel therapies to treat HCC.^[34] In another study, granulatin-epithelin precursor, a secretory growth factor, was identified with gene amplification in 20% of HCC cases, and this amplification was correlated with enhanced expression levels in the same HCC

cases.^[35] Human epithelial growth factor receptor-2 (*HER2*) and topoisomerase II alpha (*TOP2A*) have been identified to be co-amplified in breast and some other cancers,^[36] but the *HER2* gene status and *HER2* protein expression in HCC has been controversial.^[37] However, no correlation was shown between *TOP2A* amplification and *TOP2A* overexpression in HCC.^[38]

Gene deletion of tumor suppressor genes in certain regions of chromosomes also plays a crucial role in the pathogenesis of cancer. *CDKN2A* is a tumor suppressor gene that encodes for p16 and p14ARF. In a recent whole-genome sequencing study, *CDKN2A* deletion was identified in 10.2% HCC cases.^[2] Protein tyrosine phosphatase, receptor type D, a tumor suppressor gene, which was previously identified to be frequently deleted in several cancers,^[39-41] was also identified with homozygous deletion in human HCCs.^[42]

PROSPECTS

In summary, multiple lines of evidence have shown that the genetic and genomic alterations play important roles in the development and progression of HCC. The next generation sequencing of genomic DNA provides the possibility that more novel genetic and genomic alterations may be discovered and may provide new insights for understanding of the pathogenesis of HCC. However, further studies on the role of genetic mutation and genomic imbalances in the pathogenesis of HCC, as well as related functional and mechanistic studies are also urgently needed.

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Conflicts of interest

There are no conflicts of interest.

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Portal vein thrombosis in liver transplantation: radiologic evaluation, risk factors, and occult diagnosis

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ABSTRACT

Aim: Portal vein thrombosis (PVT) in the liver transplant recipient poses many challenges. Unfortunately, the risk factors and effects on outcomes of PVT are not well-defined. **Methods:** This study analyzed the experience with PVT in liver transplant program from 2007 to 2013. This included the effectiveness of PVT diagnostics and its risk factors using logistical regression. The primary endpoints were Kaplan-Meier patient and graft survival. The secondary endpoints were the length of stay (LOS), transfusion rate, and overall morbidity. Independent predictors of survival were identified using a Cox's proportional hazards model. **Results:** Two hundred and sixteen consecutive liver transplant recipients were examined, and 30 (13.8%) had either a total or partial PVT. Two hundred and five patients had imaging within 1 year of liver transplantation with only 7 (23.3%) of the 30 PVTs identified pre-operatively. Calculated sensitivity (4.8-50%) and negative predictive values (10.5-22.2%) were poor. Only, age significantly predicted PVT [$P = 0.037$ /hazard ratio (HR) = 0.95]. Ninety-day-patient and graft survival for PVT was similar at 6 months, although 1-year survival was significantly lower. "Occult" PVT was not associated with inferior survival. Model for end-stage liver disease score > 25 ($P = 0.001$, HR = 0.49/ $P = 0.004$, HR = 0.52) and age > 60 years ($P = 0.017$, HR = 0.64/ $P = 0.013$, HR = 0.67) were significant predictors of patient and graft survival. Although the transfusion rate was significantly greater with PVT, LOS, and morbidity were not. **Conclusion:** Older recipients had a greater likelihood of PVT. Diagnostic studies were not effective at excluding PVT, and occult diagnosis did not affect the outcome. PVT was not an independent predictor of mortality or graft loss, but was associated with greater blood loss but not increased LOS or morbidity.

Key words: Advanced age; graft survival; patient survival; post-transplant outcomes; resource utilization

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INTRODUCTION

In 1985, Shaw *et al.*^[1] reported the first successful cases of liver transplantation (LT) in the setting of recipient portal vein thrombosis (PVT). PVT was once considered an absolute contraindication to LT due to the considerable risk of intraoperative mortality and the technical difficulty of the operation.^[2] Advancements in operative technique, greater experience with the operation, and improved intra-operative anesthesia management have now made LT in patients with PVT increasingly common.^[3]

It is estimated that the prevalence of PVT in cirrhotic patients who are the candidates for LT ranges from 5% to 26%.^[4] Despite its prevalence, the understanding of PVT in the context of LT remains incomplete. Furthermore, the impact of PVT on the natural history and progression of cirrhosis is uncertain.^[5] Although there is no clear evidence that PVT leads to further deterioration of liver function in advanced cirrhosis, this is often a common assumption or observation. Furthermore, PVT may be a source of technical difficulties in the particular setting of transplantation leading to a negative impact on outcomes.^[4] To date, the evidence regarding the effect of PVT on LT outcomes is mixed.

The mixed evidence exists regarding the risk factors for PVT, as well as the utility of preoperative imaging protocols in identifying the patients with, or at risk for PVT.^[6-9] As a result, it is estimated that more than 50% of patients with PVT remain undiagnosed until the time of surgery, even when a rigorous pre-operative screening protocol is utilized.^[10,11] In addition, the full extent of PVT is not evident until the LT operation.^[7] Since surgeons are unable to rely on imaging, pre-operative planning according to the severity of thrombosis remains difficult. However, as is the case with known PVT, it is still undetermined whether or how the occult, or incorrectly graded PVT, discovered at LT, impacts outcome.

Regarding the resource utilization in LT, it has been shown that longer length of stay (LOS) and higher cost of care are associated with increased severity of illness, increased number of procedures performed, and younger age.^[12] Resource utilization data specific to LT with PVT is limited. However, PVT has been associated with longer operative times and increased use of blood products.^[13]

Herein, an analysis of the risk factors for PVT and independent predictors of survival were undertaken. We review the commonly used modalities for detection of PVT, and the effects of an uncertain pre-operative diagnosis of PVT on survival and resource utilization as determined by blood utilization/transfusion rate [packed red blood cell (PRBC)], LOS, and post-operative morbidity at our institution.

METHODS

A retrospective analysis of 216 consecutive adult patients undergoing cadaveric LT from January 2007 to December 2013

at a single institution was undertaken. Patients with complete mesenteric venous thrombosis were excluded from LT, and all other patients were included in the analysis. Pre-operative patient demographics and clinical status were evaluated to identify any potential risk factors for PVT. Routine imaging at our center consists of liver Doppler ultrasound (US) and a cross-sectional imaging either a triple phase computed tomography (CT) or an Eovist magnetic resonance imaging (MRI). All imaging was reviewed by a multidisciplinary conference held weekly with all surgeons, hepatologists, body imaging radiologist, and interventional radiologists present. Interval imaging after listing a patient for transplantation consists of the US every 6 months. In patients with malignancy, contrasted CT, or MRI is done every 3 months until LT. The effectiveness of diagnosing PVT pre-LT, when PVT was later identified at LT, was evaluated for US, CT, MRI, and retrograde portal venography (RPV). Patient and graft survival were considered as primary endpoints. Blood utilization, LOS, and overall morbidity (Clavien grade II or greater) were used as surrogates of resource utilization.^[14] These were our secondary endpoints.

Statistical analysis

Continuous variables were compared between the groups using Student's *t*-test, categorical variables were compared using Chi-square test, and the serial values were compared using analysis of variance (ANOVA). Kaplan-Meier with log-rank analysis of actuarial patient and graft survival were calculated. LOS and PRBC were analyzed using ANOVA. Overall morbidity (Clavien grade II or greater) was compared between the groups by Chi-square analysis. Pre-operative characteristics that were significant on univariate analysis were evaluated by logistic regression to identify any potential risk factors for PVT. Multivariate survival analysis was done with a Cox proportional hazards model, and independent predictors of LOS and PRBC were analyzed by multivariate analysis of covariance.

RESULTS

Of 216 patients undergoing cadaveric LT, 30 (13.8%) patients had PVT at the time of operation. Two hundred and five patients had at least one diagnostic imaging study within 1-year of LT. Only, 7 of 30 patients with PVT (23.3%) had at least one positive imaging study suggestive of PVT pre-LT. The sensitivity of imaging techniques ranged 4.8-50%, and the negative predictive value ranged 10.5-22.2% [Table 1].

Analysis of perioperative variables for those patients with and without PVT revealed that there was a significantly higher model for end-stage liver disease (MELD) score (25.0 vs. 21.4, $P = 0.049$) and age (57.8 vs. 53.8, $P = 0.041$) in those with PVT, although intensive care unit (ICU) status approached statistical significance (30% vs. 15.6%, $P = 0.07$) [Table 2]. However, in our small series, the only factor by logistic regression that significantly predicted PVT was age [$P = 0.037$; hazard ratio (HR) = 0.95].

Overall 90-day, the patient and graft survivals were 90.7% and

Table 1: Pre-operative imaging studies

| Diagnostic study | Number of studies | Median days pre-LT | Sensitivity (%) | NPV (%) | Specificity (%) | PPV (%) |
|--|-------------------|--------------------|-----------------|---------|-----------------|---------|
| US (no flow = PVT) | 149 | 26 | 4.8 | 13.8 | 97.7 | 25.0 |
| US (no, diminished, or reversal of flow = PVT) | 149 | 26 | 31.6 | 10.5 | 85.4 | 24.0 |
| CT | 158 | 56.5 | 19.0 | 11.2 | 98.5 | 66.7 |
| MRI | 51 | 66 | 12.5 | 14.3 | 97.7 | 50.0 |
| RPV | 11 | 45 | 50.0 | 22.2 | 100.0 | 100.0 |

The efficacy of pre-operative diagnostic studies has long been questioned. Our data support this as well. Even when we set criteria for ultrasound diagnosis liberally (2nd US row), the sensitivity and NPV were wholly inadequate. Though the number is small, in our series even RPV, a direct and invasive technique only detected PVT pre-LT in half the cases. LT: liver transplant; PVT: portal vein thrombosis; NPV: negative predictive value; PPV: positive predictive value; RPV: retrograde portal venography; US: ultrasound

Table 2: Variables related to PVT

| Perioperative variables | PVT | No PVT | P |
|-------------------------------------|-------|--------|--------------|
| Pre-operative variables | | | |
| Age | 57.8 | 53.8 | 0.041 |
| Gender: female | 23.3% | 33.3% | NS |
| Non-Caucasian race | 30.0% | 29.0% | NS |
| Medicare or medicaid | 20.0% | 43.0% | NS |
| Hepatocellular carcinoma diagnosis | 2.8% | 13.6% | NS |
| Hepatitis C virus diagnosis | 50.0% | 52.7% | NS |
| MELD | 25.0 | 21.4 | 0.049 |
| Cr | 1.6 | 1.8 | NS |
| Total bilirubin | 7.2 | 5.0 | NS |
| INR | 2.0 | 1.8 | NS |
| Pre-LT ICU status | 30.0% | 15.6% | 0.07 |
| Pre-LT hemodialysis | 26.7% | 15.6% | NS |
| Previous upper abdominal surgery | 30.0% | 25.8% | NS |
| Intra- and post-operative variables | | | |
| Cold ischemic time | 367.7 | 350.2 | NS |
| Warm ischemic time | 35.9 | 34.4 | NS |
| PRBC | 28.9 | 17.5 | 0.001 |
| Reentry | 40.0% | 36.0% | NS |
| Morbidity (\geq Clavien II) | 43.3% | 37.6% | NS |
| LOS, total (days) | 19.8 | 16.6 | NS |

Age, MELD score, and the amount of blood loss were greater in patients who had PVT (bold print signifies significant values). The proportion of patients in the ICU with PVT was greater but only approached statistical significance. With logistic regression, the only pre-operative factor independently associated with PVT was age ($P = 0.037/\text{HR} = 0.95$). Pre-LT: pre-liver transplant; LOS: length of stay; PVT: portal vein thrombosis; ICU: intensive care unit; MELD: model for end-stage liver disease; PRBC: packed red blood cell; HR: hazard ratio; NS: not significant

90.3%, and 1-year were 83.7% and 83.3%. The patient and graft survival were inferior in those with PVT [Figure 1a]. The divergence of both patient and graft survival occurred at approximately 6 months post-operatively. The patients with PVT at LT without a pre-LT diagnosis ("occult" PVT) did not have inferior patient or graft survival as compared to those with a definite pre-LT diagnosis ($P = 0.79$) [Figure 1b]. On multivariate analysis of patient survival, only MELD > 25 ($P = 0.001$, $\text{HR} = 0.45$) and age > 60 years ($P = 0.017$, $\text{HR} = 0.64$) were independent risk factors for patient death. Similarly for graft survival, MELD > 25 ($P = 0.004$, $\text{HR} = 0.52$) and age > 60 years ($P = 0.013$, $\text{HR} = 0.67$) predicted graft loss independently [Table 3]. The presence of PVT diagnosed pre-LT or as an occult finding was not an independent predictor of either patient or graft survival on multivariate analysis.

LOS and PRBC requirements were also assessed. Although PRBC requirements were greater with PVT (28.9 vs. 17.5,

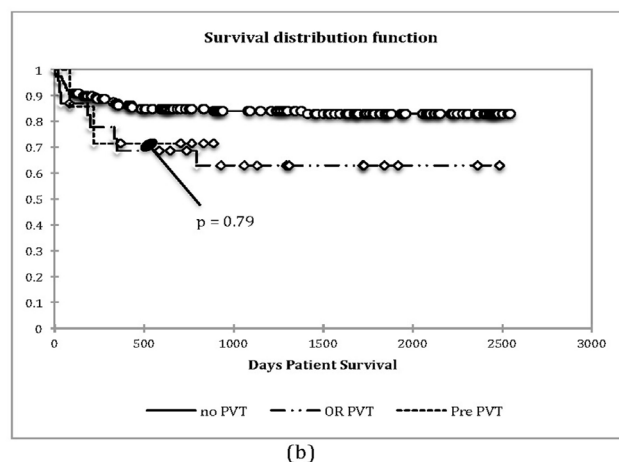
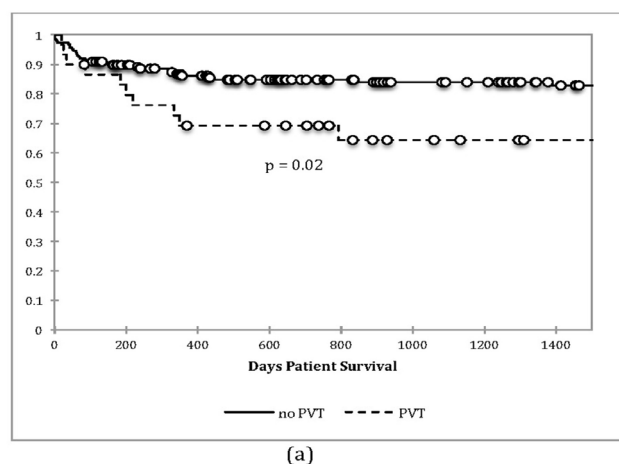


Figure 1: Patient survival and portal vein thrombosis (PVT). (a) PVT was associated with significantly reduced survival at approximately 6 months. Early perioperative survival (i.e., 90-day) was not significantly different, but divergence in survival occurred at 6 months; (b) there was no difference in survival between those with pre-liver transplantation diagnosis (pre-PVT) and those with "occult" PVT (OR PVT). Results for graft survival demonstrated the similar findings. On multivariate analysis [Table 3], PVT was not an independent predictor of survival

$P = 0.001$), patients with PVT did not have a longer LOS (19.8 vs. 16.6, $P = 0.36$) or greater morbidity (43.3% vs. 37.6%, $P > 0.05$) [Table 2]. Only PVT ($P = 0.002$) and pre-LT hemodialysis (HD) ($P = 0.013$) were significant covariates associated with increased PRBC [Figure 2]. When examining LOS, only female gender ($P = 0.008$), pre-LT HD ($P = 0.012$), and re-laparotomy ($P < 0.0001$) were significant at predicting the longer LOS [Figure 3].

Table 3: Analysis of patient and graft survival

| Independent variable | Patient survival | | HR | Graft survival | | HR |
|------------------------------------|-------------------|--------------|-------------|----------------|--------------|-------------|
| | Univariate | Multivariate | | Univariate | Multivariate | |
| Age > 60 | 0.046 | 0.017 | 0.64 | 0.027 | 0.013 | 0.67 |
| Gender: male | 0.144 | | | 0.102 | | |
| Hepatitis C virus diagnosis | 0.52 | | | 0.76 | | |
| Hepatocellular carcinoma diagnosis | 0.26 | | | 0.28 | | |
| Private insurance | 0.138 | | | 0.244 | | |
| Socioeconomically disadvantaged | 0.284 | | | 0.135 | | |
| Pre-LT ICU stay | 0.015 | NS | | 0.023 | NS | |
| Pre-LT hemodialysis | < 0.001 | NS | | 0.001 | NS | |
| Simultaneous kidney transplant | 0.04 | NS | | 0.045 | NS | |
| Lab MELD > 25 | 0.02 | 0.001 | 0.49 | 0.03 | 0.004 | 0.52 |
| PVT | 0.02 | NS | | 0.031 | NS | |
| Occult PVT | 0.062 | | | 0.092 | | |
| Complete PVT | 0.04 | NS | | 0.045 | NS | |
| Past upper abdominal surgery | 0.236 | | | 0.331 | | |
| PRBC > 20 | 0.002 | NS | | 0.001 | NS | |
| Reentry | 0.001 | NS | | 0.001 | NS | |

All univariates were analyzed by Kaplan-Meier method with a log-rank test for significance. The significant univariates (bold print signifies significant values, $P < 0.05$) were analyzed by a multivariate Cox's proportional hazards model to determine which independent predictors of survival. The only factors that appear to independently predict patient and graft survivals are advanced age (> 60) and advanced liver disease (MELD > 25). Interestingly, PVT was not an independent predictor of survival. Pre-LT: pre-liver transplant; MELD: model for end-stage liver disease; PRBC: packed red blood cell; PVT: portal vein thrombosis; ICU: intensive care unit; HR: hazard ratio; NS: not significant

DISCUSSION

The risk factors for PVT are often conflicting and not well established. Previously identified risk factors in historical patient series have included: Male gender, Child-Pugh class C disease, treatment for portal hypertension, variceal bleeding, abdominal surgery, as well as various etiologies of liver disease.^[7-10] Conversely, age, sex, MELD score, treatment for portal hypertension, abdominal surgery, and etiology of liver disease have been identified as non-contributory factors in overlapping patient series.^[7,9,10] Such contradictory results highlight the need for further investigation to identify the independent risk factors associated with PVT.

Of 216 patients undergoing cadaveric LT, the prevalence of PVT in this center (13.8%) fell within the expected range predicted by most historical series. Advanced age and perhaps higher MELD and ICU status were the risk factors for PVT in our series. It is possible that the duration and/or severity of portal hypertension seen in older patients with higher MELD scores contribute to PVT risk. The lack of statistical significance of higher MELD score and ICU status in predicting PVT on multivariate analysis may represent a type II statistical error. Furthermore, other factors such as a patient's sex, race, insurance status, diagnosis of hepatocellular carcinoma, hepatitis C virus, need for pre-LT HD, or surgical history did not contribute significantly to PVT risk in this study [Table 2]. These data, while relevant to this institution, do little to clear up the mixed picture of PVT risk factors overall, especially given the relatively small number of patients in this study. Further multicenter studies are clearly warranted.

In addition to examining the risk factors associated with PVT, we also attempted to examine the diagnostic capabilities

for detecting PVT at our institution. Results from our series demonstrated that imaging was not effective at excluding PVT. The sensitivity and negative predictive values of various imaging modalities (US, CT, MRI, and RPV) in detecting PVT were poor [Table 1]. This is congruent with the results from the previous series, which have been demonstrated that the degree of PVT may be overestimated or underestimated, or it may be missed entirely by pre-operative imaging.^[6,7] It is estimated that more than 50% of patients with PVT remain undiagnosed until the time of surgery even with rigorous screening protocols in place.^[10,11] These high false negative rates are often attributed to the variability in the skill and experience of a US technicians and radiologists.^[7] Experience and preference of the radiologist greatly impact the quality of information obtained from any imaging study.

Missed diagnoses are most common in patients with partial PVT,^[6,15] although they have been described in those with complete thrombosis as well.^[8] In other patients, PVT is graded incorrectly, such that the full extent of thrombosis is not evident until the time of operation.^[6]

The evidence regarding the impact of PVT on LT outcome is mixed, and whether an occult diagnosis has any additional effect on outcome is also uncertain. Using Kaplan-Meier survival curves, we found that the patient and graft survival were inferior in those with PVT and that the divergence of both patient and graft survival occurred at approximately 6 months following LT [Figure 1]. On multivariate analysis, MELD > 25 and age were significant independent predictors of patient and graft survival, while the presence of PVT was not. Age thus appears to be an independent predictor of PVT, as well as survival, and that survival is not predicated on the presence of PVT in this study. Furthermore, the discovery of PVT at

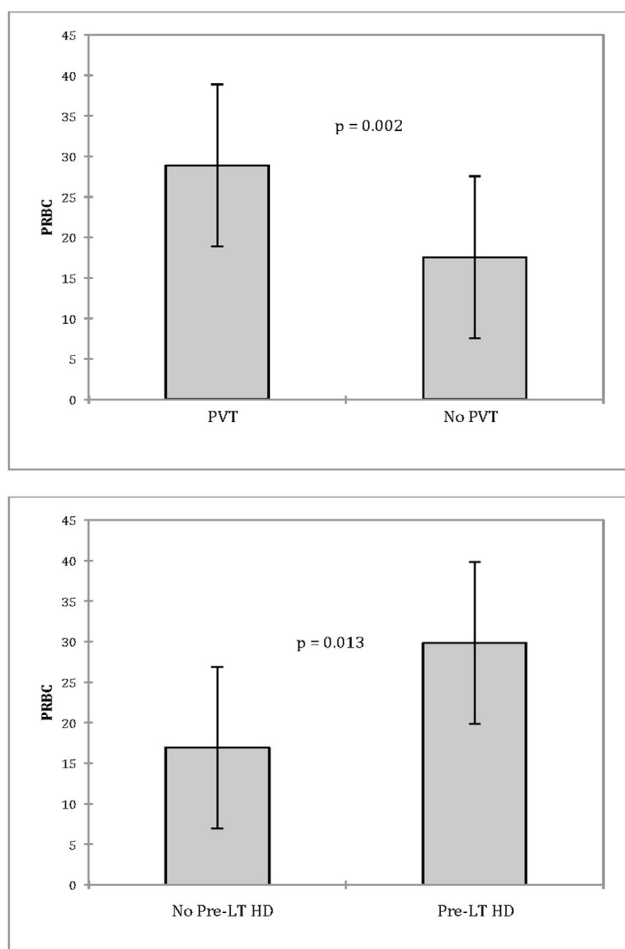


Figure 2: Predictors of PRBC utilization. Two independent predictors of blood utilization (PRBC) were identified by multivariate analysis of covariance: PVT and pre-LT HD. PRBC: packed red blood cell; PVT: portal vein thrombosis; Pre-LT: pre-liver transplant; HD: hemodialysis

the time of LT, without a pre-LT diagnosis (“occult” PVT), did not correlate with inferior patient or graft survival. Previous studies have also demonstrated that PVT does not have a significant effect on survival.^[6,11,16,17] The largest patient series to date, however, found that independent of MELD score; pre-transplant PVT was associated with up to a 50% increase in 1-year mortality post-transplant.^[4,18] Once again, the data in the literature is conflicting.

Survival in patients undergoing LT with PVT varies with the severity of thrombosis and the type of revascularization performed.^[4,6] When conventional end-to-end portal anastomosis can be achieved, whether PVT is partial or complete, results are comparable to LT recipients without PVT, with 1- and 5-year survival ranging from 84% to 86% and 65% to 80%, respectively.^[4,6,11,16,19,20] When alternative, non-anatomical revascularization techniques are necessary, such as renoportal anastomosis or cavoportal hemitransposition, survival is inferior with 1- and 5-year survival rates of only 60% and 38%, as well as early post-operative mortality risks of 25%. These techniques are typically reserved for the cases with extensive thrombosis involving the splenic or superior mesenteric veins and are only performed at a handful of centers.^[21-23] In our

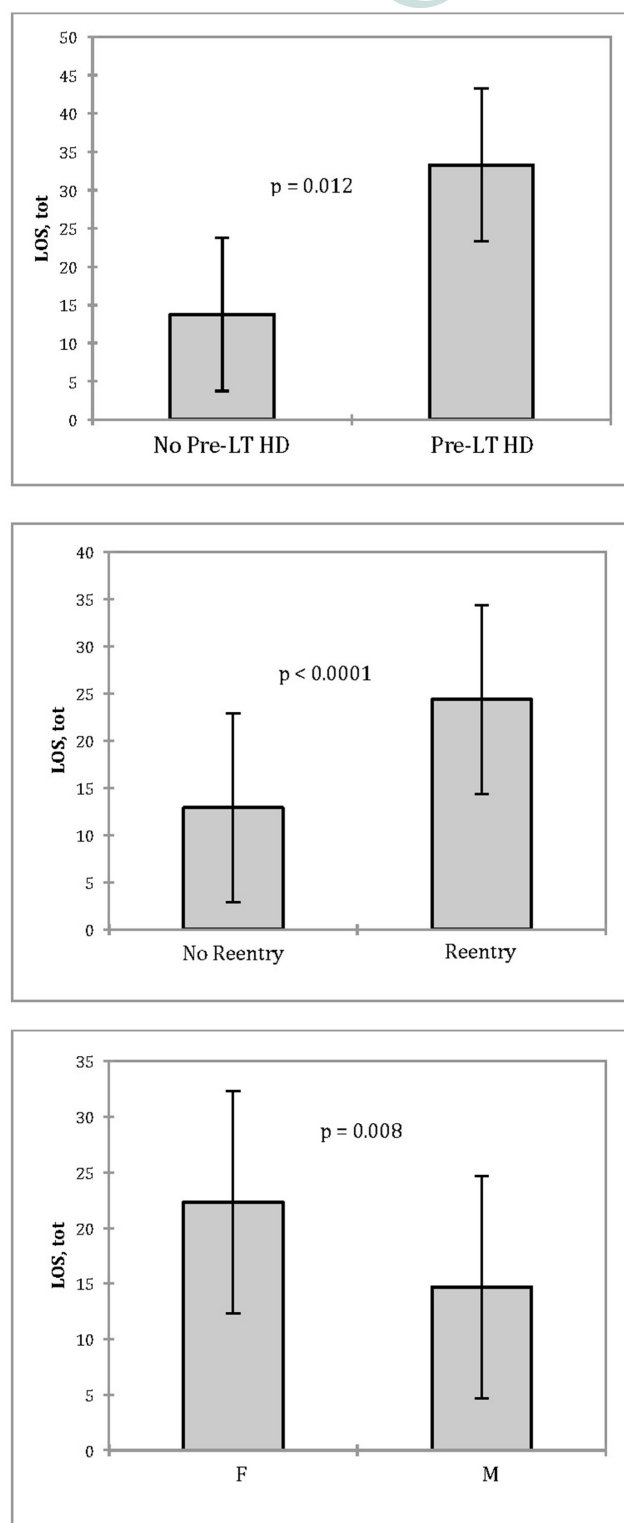


Figure 3: Predictors of LOS. Three independent predictors of prolonged LOS were identified by multivariate analysis of covariance: pre-LT HD, need for reentry, and female gender. LOS: length of stay; Pre-LT: pre-liver transplant; HD: hemodialysis

series, all patients with pre-operatively recognized extensive PVT of the entire portal venous system were excluded. Given the relatively small size of this patient series, and the conflicting data within the literature, the true effect of PVT on survival after LT remains incompletely understood. Further

studies, along with a multicenter pooling and analysis of data would be a key to providing insight into this area.

Not only is PVT thought to increase post-transplant mortality, but morbidity has been argued by some to be affected as well. PVT has been associated with increased risk of sepsis,^[6,22-25] gastrointestinal bleeding due to persistent portal hypertension,^[4,21,22,24,25] ascites, renal dysfunction,^[4,24] and thrombotic events such as thrombosis at the anastomosis, hepatic artery, and pulmonary embolism.^[24,25] This may reflect the greater technical difficulty in the operation, the advanced disease state of patients with PVT, or a combination of the two. In our series, PVT was not an independent determinant of survival. Our data suggest that it is the advanced age and more advanced liver disease in patients with PVT that contributes to reduced patient and graft survival. Furthermore, we found that PVT was only associated with greater blood loss. Aside from blood loss, PVT did not contribute significantly to resource utilization, as measured by LOS or post-operative morbidity.^[14] These data are encouraging but need to be combined with larger patient series to establish more generalizable data.

In conclusion, neither patient or graft survival nor resource utilization in the form of LOS or overall post-operative morbidity was adversely affected by a known diagnosis or an occult finding of PVT in LT at our center. However, PVT at the time of LT did result in increased blood loss. Although this probably affected cost, it did not affect LOS or morbidity in our series. These results are reassuring given the poor sensitivity and negative predictive value of current imaging. The data from our study indicate that an extensive search for PVT may not be warranted, and imaging should be ordered only when otherwise indicated (e.g., malignancy surveillance and known malignancy). Clinical suspicion for PVT should be high in older patients, especially with a high MELD score and/ or ICU status. By optimization of the patient and anticipatory anesthesia care, the patient may benefit by a reduction in blood loss.

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Conflicts of interest

There are no conflicts of interest.

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Fascin-1 depletion from hepatocellular carcinoma cells inhibits migfilin and vasodilator-stimulated phosphoprotein expression and enhances adhesions

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ABSTRACT

Aim: Extracellular matrix (ECM)-adhesions and their interaction with actin cytoskeleton are fundamental for hepatocellular carcinoma (HCC). Fascin-1, an actin-bundling protein, is correlated with poor HCC prognosis, and is known regarding the molecular mechanism of its action. In this study, the authors investigated Fascin-1 basic molecular mechanism and cellular properties in HCC cells. **Methods:** Fascin-1 was silenced by small interfering RNA and the expression of actin. The ECM-adhesion-related proteins were assessed along with the cells' adhesion capacity in two cell lines that differ in terms of aggressiveness; the hepatoma cell line PLC/PRF/5 (Alexander) and the highly invasive HCC cell line HepG2. **Results:** This study shows that Fascin-1 is upregulated in HepG2 cells compared to Alexander cells and when silenced leads to increased cell adhesion only in HepG2, while at the same time is associated with reduced migfilin and vasodilator-stimulated phosphoprotein (VASP) expression. **Conclusion:** This is the first study to show that Fascin-1 contributes to a more aggressive phenotype in HCC cells and acts through migfilin and VASP.

Key words: Adhesion; Fascin-1; hepatocellular carcinoma; migfilin; vasodilator-stimulated phosphoprotein

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INTRODUCTION

Extracellular matrix (ECM), focal adhesions, and their interaction with actin cytoskeleton are fundamental for a number of vital cellular processes such as cell survival, differentiation, development, and tissue homeostasis. This is particularly true for the organ liver as the main parenchymal cells of the liver, hepatocytes, interact to a great extent with ECM, and express a number of focal adhesions and actin-related proteins. Moreover, the fact that ECM and ECM-related proteins are fundamental for liver biology is evident by a number of studies showing how they affect hepatocyte differentiation,^[1] survival,^[2] and normal liver function.^[3,4] More specifically, matrix overlay on primary hepatocytes cultured *in vitro* inhibits the dedifferentiation that normally occurs due to culturing and maintains hepatocytes in a fully differentiated state.^[5] Along the same line, loss of a cell adhesion protein related to integrins (namely, integrin-linked kinase) from mouse hepatocytes *in vitro* and *in vivo* leads to increased apoptosis and hepatitis,^[2] showing clearly that cell-ECM interactions are critical for hepatocyte survival. Finally, liver-specific ablation of integrin-linked kinase leads to abnormal liver histology^[4] and increased liver regeneration capacity,^[3] indicating the crucial role played by cell-ECM adhesion proteins in liver structure and function.

Moreover, ECM plays central role during carcinogenesis in the liver, as hepatocellular carcinoma (HCC) is known to result from a series of events which include fibrosis, cirrhosis, generation of phenotypically-altered hepatocytes, and dysplasia ultimately leading to HCC.^[6] Thus, cell adhesion proteins that connect cells to the ECM and/or to neighboring cells are evidently fundamental for HCC development and progression. Moreover, cell adhesion proteins are often directly or indirectly connected to actin cytoskeleton creating a network of interacting proteins that is crucial for tissue homeostasis. Interestingly, most of these proteins are found to be deregulated in cancer and cancer metastasis.^[7,8] This deregulation destabilizes cell attachment to the surrounding ECM and neighboring cells, thus facilitating cancer cells dissociation from the original tumor mass and invasion of surrounding tissues, and ultimately leads to metastases formation.

Fascin-1 is an actin-bundling protein that is found in membrane ruffles and stress fibers, and its expression has been shown to be greatly increased in many transformed cells^[9] and a spectrum of cancers such as breast cancer, colon cancer, pancreatic cancer and prostate cancer. In HCC in particular, Fascin-1 has been correlated with poor prognosis.^[10]

Moreover, Fascin-1 has been suggested as a novel marker of HCC progression and a significant indicator of poor prognosis for HCC patients.^[11] However, little is known regarding the molecular mechanism of its action. In a recent work, we have shown that Fascin-1 is negatively regulated by migfilin,^[12] a novel cell-matrix adhesion protein known to interact with

vasodilator-stimulated phosphoprotein (VASP),^[13] and is localized both at cell-matrix and cell-cell adhesions.^[14]

In the present study, we tested the expression and molecular mechanism of the action of Fascin-1 in two liver cell lines that differ in terms of aggressiveness; the hepatoma cell line PLC/PRF/5 (Alexander) and the highly invasive HCC cell line HepG2.

METHODS

Liver cell lines

Two liver cell lines of different invasive capacity were used in the present study; the hepatoma cell line PLC/PRF/5 (Alexander) and HCC cell line HepG2. Both cell lines were purchased from American Type Culture Collection.

Transfection with small interfering RNAs

Both Alexander and HepG2 cells were treated for 48 h with 100 nmol/L small interfering RNA (siRNA), non-specific control (NSC) siRNA, or siRNA against Fascin-1 using the Lipofectamine 2,000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the company's guidelines. The siRNA sequence used to silence Fascin was as purchased from Santa Cruz, while the sequence 5'AAA CUC UAU CUG CAC GCU GAC3' was used as NSC. Silencing efficiency prior to every experiment performed was tested by western blot.

Antibodies

Anti- β -actin antibody (Sigma-Aldrich) was used as loading control. Antibodies VASP and Fascin-1 were purchased from Cell Signaling. The monoclonal antibody against migfilin was kindly provided by Dr. Chuanyue Wu (Professor at the University of Pittsburgh Medical School, Pittsburgh, PA, USA).

Protein extraction and western blot analysis

Total cell lysates were obtained using 1% sodium dodecyl sulfate in radioimmunoprecipitation assay buffer (20 mmol/L Tris/Cl pH 7.5, 150 mmol/L NaCl, 0.5% NP-40, 1% TX-100, 0.25% sodium deoxycholate, 0.6-2 μ g/mL aprotinin, 10 μ mol/L leupeptin, and 1 μ mol/L pepstatin). Protein concentrations in the samples were determined by the BCA protein assay kit (Pierce) using bovine serum albumin as standard. An equal amount of protein was loaded on each lane of a 10-12% acrylamide gel and transferred to a PVDF membrane (Millipore) using the Bio-Rad Semi-dry transfer system (Bio-Rad, Hercules, CA, USA). Signals were detected using suitable secondary immunoglobulin G, conjugated with horseradish peroxidase (Invitrogen). Antibody detection was performed using super-signal ECL detection system (Pierce).

Cell adhesion assay

Cell adhesion assay was performed as described previously.^[13] Briefly, cells were transfected with a control NSC siRNA or siRNA against Fascin-1. Forty-eight hours post-transfection, 10⁴ cells/well were seeded in 6 wells of a 96-well plate pre-coated with 0.1% gelatin. After a 60-min incubation at 37 °C,

three of the wells were washed three times with phosphate-buffered saline while the remaining three were fixed with 4% paraformaldehyde (PFA). Washed wells were also fixed with PFA and then cells in all wells were quantified using crystal violet.^[13] Crystal violet was washed using ddH₂O and cells were solubilized using acetic acid. Absorbance was measured at 570 nm using Perkin Elmer EnSpire plate reader (Waltham, MA, USA). Adhesion was presented as the ratio of the absorbance at 570 nm of adhered cells (washed) divided by the absorbance at 570 nm of the total seeded cells (not washed). The data from two independent experiments were analyzed using the Student's *t*-test. $P < 0.05$ was considered statistically significant.

Statistical analysis

Comparison of means using Statgraphics software (Statgraphics Company, Warrenton, VA, USA) was used for the statistical analysis. *t*-test was performed, and $P < 0.05$ was considered statistically significant.

RESULTS

Fascin-1 protein expression is dramatically elevated in HepG2 compared to Alexander cells

We first tested Fascin-1 protein expression in Alexander and HepG2 cells using western blotting. As shown in Figure 1a, Fascin-1 protein expression was found to be dramatically elevated in the highly invasive HepG2 cells compared to the

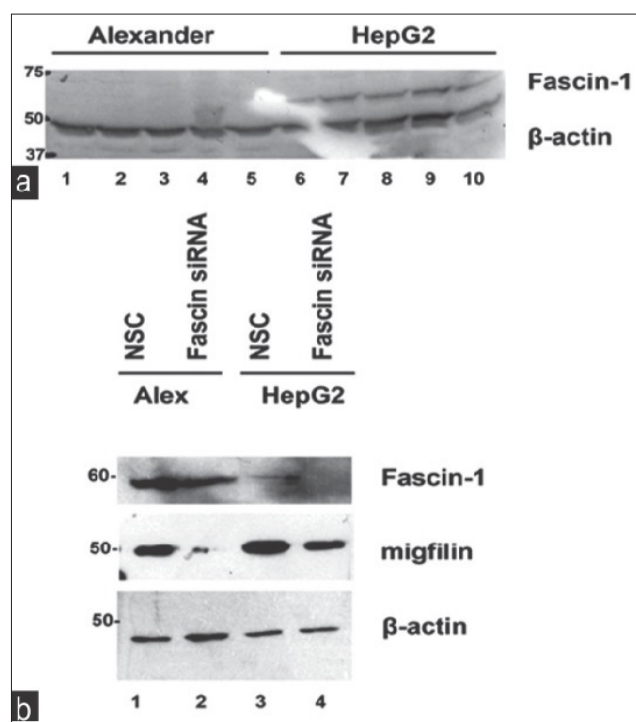


Figure 1: Fascin-1 is upregulated in HepG2 cells compared to Alexander while its depletion leads to a reduction in migfilin expression. (a) Representative western blot showing Fascin-1 protein expression in the two hepatocellular carcinoma cell lines tested; the low invasiveness Alexander and the highly invasive HepG2 cells; (b) the effect of Fascin-1 silencing on migfilin protein expression. β -actin is used as loading control. NSC: non-specific control

nonaggressive Alexander cells [Figure 1a]. This piece of data further confirms a critical role played by Fascin-1 in HCC and cancer cell aggressiveness.

Fascin-1 gene silencing leads to downregulation of both migfilin and VASP

We then proceeded with knocking down Fascin-1 gene in both HCC cell lines to better understand its function as well as its effect on known ECM-related proteins. As shown in Figure 1b, Fascin-1 was successfully silenced in both cell lines transfected with Fascin-1 siRNA compared to the cells transfected with an NSC siRNA (compare lanes 2 and 4 with lanes 1 and 3).

As ECM and actin cytoskeleton are fundamental for HCC progression and aggressiveness, we tested the expression of focal adhesion proteins migfilin (also known as Filamin Binding LIM-protein-1) a novel LIM domain-containing protein present both at cell-ECM,^[15] cell-cell adhesions,^[14] and VASP, a focal adhesion phosphoprotein known to regulate actin polymerization.^[16-18] Interestingly, migfilin and VASP interact with each other and are implicated in cellular adhesion to ECM as well as migration.^[13]

As shown in Figure 1b, migfilin was significantly downregulated upon Fascin-1 silencing indicating a connection between the two molecules. Interestingly, in addition to migfilin, VASP was also found to be downregulated [Figure 2a] following Fascin-1 knock-down, engaging both proteins in Fascin-mediated effects.

Fascin-1 silencing leads to increased cell adhesion in HepG2 cells

Since both migfilin and VASP played significant roles in cell adhesion, we next investigated whether Fascin-1 silencing affected the property of cells to adhere to ECM. Thus, we performed a series of adhesion assays on 1% gelatin in both cell lines using cells that were transfected with NSC or Fascin-1 siRNA. As shown in Figure 2b, inhibition of Fascin-1 expression by siRNA induces an increase in cell adhesion ability of HepG2 cells, whereas this is not the case for the less

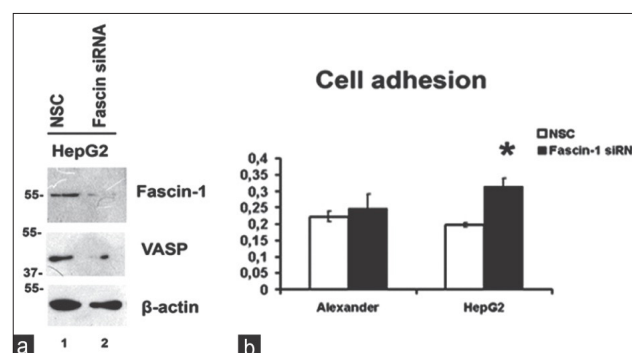


Figure 2: Fascin-1 silencing leads to VASP downregulation and promotion of cell adhesion. (a) The effect of Fascin-1 silencing in HepG2 cells on VASP protein expression assessed by western blotting. β -actin is used as loading control; (b) cell adhesion on 1% gelatin-coated 96-well plates following Fascin-1 silencing in both cell lines. VASP: vasodilator-stimulated phosphoprotein; NSC: non-specific control

invasive Alexander cells, which remain unaffected.

DISCUSSION

Cell adhesion proteins connecting cells to the ECM and/or to the neighboring cells are often interconnected to the actin-cytoskeleton and this network of interacting proteins is fundamental for tissue homeostasis while at the same time being deregulated in cancer and cancer metastasis.^[7,8] Fascin-1 is an actin-bundling protein that is found in membrane ruffles and stress fibers.^[19] The expression of Fascin-1 is greatly increased in many transformed cells, as well as in specialized normal cells including neuronal cells and antigen-presenting dendritic cells. A morphological characteristic common to these cells expressing high levels of Fascin-1 is the development of many membrane protrusions in which Fascin-1 is predominantly present.^[9] Recent studies show that Fascin-1 also localizes to invadopodia, membrane protrusions formed at the adherent cell surface that facilitate ECM invasion, thus providing a potential molecular mechanism for how Fascin-1 increases the invasiveness of cancer cells since Fascin-1 expression is upregulated in a spectrum of cancers such as breast cancer, colon cancer, pancreatic cancer, and prostate cancer.^[20,21] In HCC, in particular, Fascin-1 has been correlated with poor prognosis.^[10]

In fact, Fascin-1 has been recently introduced as a migration factor associated with epithelial to mesenchymal transition in HCC cells facilitating their invasiveness in combination with matrix metalloproteinases.^[22] Moreover, it has been suggested to be a novel marker of progression in HCC and a significant indicator of poor prognosis for HCC patients.^[11] However, little is known regarding the molecular mechanism of its action.

In this study, we tested the expression and molecular mechanism of action of Fascin-1 in two HCC cell lines that differ in terms of aggressiveness; the hepatoma cell line PLC/PRF/5 (Alexander) and the highly invasive HCC cell line HepG2. Interestingly, we show that Fascin-1 is dramatically upregulated in HepG2 cells compared to more benign Alexander cells [Figure 1a].

We then utilized a siRNA-mediated silencing approach to knock-down the Fascin-1 gene. Fascin-1 silencing led to a reduction in the expression level of two important focal adhesion proteins related to the cytoskeleton, namely, migfilin^[14,15] [Figure 1b], and its interactor VASP [Figure 2a].^[13]

More importantly, Fascin-1 silencing led to significantly increased cell adhesion in the highly invasive and aggressive HepG2 cells [Figure 2b] but had no effect on the less invasive Alexander cells, indicating that Fascin-1 silencing has, indeed, a great impact on more aggressive cells. Furthermore, the fact that it results in elevated cell adhesion in HepG2 cells shows that Fascin-1 depletion stabilizes cell-ECM adhesions leading to a less aggressive cancer phenotype. These findings are evidence confirming previous studies showing the potential of Fascin-1 as a therapeutic target for metastasis.^[9]

The fact that Fascin-1 silencing leads to migfilin and VASP downregulation and increased adhesion indicates that Fascin-1 may regulate migfilin and VASP and/or be physically associated with them. This evidence further complements recent work from our laboratory showing that migfilin silencing, among other things, reduces VASP expression, and leads to Fascin-1 upregulation, and promotion of cell adhesion in HepG2 cells.^[12] Therefore, the evidence clearly indicates a molecular interplay between the three proteins, migfilin, Fascin-1, and VASP, and the potential existence of a regulatory feedback loop in HCC cells.

Although our study was performed in cancer cell lines, which have their limitations in terms of modeling the physiological complexity of human cancer, it still offers significant insight into the molecular mechanism by which Fascin-1 is implicated in HCC pathogenesis. Of course, more studies are needed to decipher the exact sequence of molecular events taking place and the importance for HCC progression.

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Conflicts of interest

There are no conflicts of interest.

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Predictive factors for the success of “one-off” ablation in single hepatocellular carcinoma patients who underwent percutaneous radiofrequency ablation

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ABSTRACT

Aim: To investigate the technique's effectiveness and evaluate the risk factors affecting the success of “one-off” percutaneous ultrasound-guided radiofrequency ablation (RFA) for single hepatocellular carcinoma (HCC). **Methods:** A total of 462 consecutive patients who received RFA from February 2010 to December 2013 at a single center (Eastern Hepatobiliary Surgery Hospital, Shanghai, China) were enrolled in the study. The patients were followed up for at least 6 months. Herein, this study adopted a new terminology named “one-off” ablation which is defined as achieving complete necrosis and no local residual or recurrent tumor within 6 months after single-session RFA. The incidence of “one-off” RFA was observed and the attributing risk factors were analyzed. A multivariate analysis was conducted to determine the independent predictive factors for the success of “one-off” ablation. **Results:** The technique's effectiveness was 90.0% (416/462). After 6 months, 281 patients achieved “one-off” ablation, while 181 patients failed. On univariate analysis, tumor size ≤ 3 cm and tumor further from organs were found to be significantly correlated with “one-off” complete ablation ($P = 0.003$, and $P = 0.010$, respectively). On multivariate analysis using a logistic regression, tumor size ≤ 3 cm [odds ratio (OR), 0.534; 95% confidence interval (CI): 0.346-0.825, $P = 0.005$] and tumor further from organs (OR, 0.593; 95% CI: 0.387-0.909, $P = 0.017$) remained predictive. **Conclusion:** Tumor size and tumor location are the predictive factors for the success of “one-off” ablation in patients with single HCC.

Key words: Hepatocellular carcinoma; radiofrequency ablation; tumor location; tumor size

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INTRODUCTION

Liver cancer, one of the most fatal cancers, is the second most common cancer in China. Each year, nearly 383,000 people died from liver cancer in China, which accounts for 51% of the deaths from liver cancer worldwide.^[1] Hepatocellular carcinoma (HCC) has the highest incidence

in all the hepatic malignancies. Liver transplantation (LT) and partial hepatectomy are considered as the main curative treatments for HCC.^[2] However, LT for patients who meet the Milan criteria is limited due to the insufficient availability of donors.^[2] In addition, anatomic location, multicentric tumor occurrence, and

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poor liver function status also preclude liver resection in majority of patients, with only 9-29% of HCC patients being suitable for partial hepatectomy.^[3]

Over the years, local ablation including percutaneous ethanol injection, radiofrequency ablation (RFA), and microwave ablation have gained more interests. Among these techniques, RFA was the most widely applied due to its low mortality, minimal invasiveness, high effectiveness, outpatient-use, and repeatability for recurrence.^[3] It was reported that RFA was the most effective treatment for unresectable liver cancer.^[4] Some lines of evidence also indicated that RFA can be used as a bridge to LT.^[5] The therapeutic goal of RFA is complete necrosis. For patients who had incomplete necrosis, RFA can be repeated.^[6] However, a series of studies showed that multiple-session RFA would increase the incidence of complications such as bleeding, hollow organ injury, and tumor diffusion.^[7] Meanwhile, the cost-effectiveness of a standardized percutaneous RFA treatment was \$20,424.^[8] In China, about 75% of the population has no insurance to guarantee their basic health care and nearly 30% of poor families suffered financially due to illness. Therefore, most patients in China cannot afford to take many sessions of RFA.

Herein, we adopted a new terminology named “one-off” ablation, which was proposed by Jiang *et al.*^[9-11] and defined as achieving complete necrosis after a single-session of RFA with no local residual or recurrent tumor within 6 months. The present retrospective study tried to investigate the predictive factors related to the success of “one-off” ablation.

METHODS

Patients

The Healthcare Ethics Committee and Institutional Review Board of our hospital have approved that we could use the data of patients for this retrospective study. We reviewed the data of a single center database (Eastern Hepatobiliary Surgery Hospital, Shanghai, China) and screened all patients with single HCC from February 2010 to December 2013. HCC was diagnosed according to the guidelines of American Association for the Study of the Liver Disease (AASLD), that is, a positive result in biopsy or concordant results of at least two imaging techniques or positive finding on one imaging study together with alpha fetal protein (AFP) > 400 ng/mL.^[12] Clinical data were collected including demographic characteristics, imaging examinations, intra-RFA parameters, and laboratory tests results.

Inclusion and exclusion criteria

The inclusion criteria were as follows: (1) single HCC nodule measuring 5.0 cm or less in diameter; (2) liver function of Child-Pugh Class A or B; (3) no macrovascular

thrombosis and extra-hepatic metastasis; (4) performance status Eastern Cooperative Oncology Group 0 or 1; and (5) platelet count > 50,000/mL. Exclusion criteria were: (1) poor or absent visualization of nodules on ultrasound (US); (2) any previous treatments aimed at HCC nodules.

RFA procedures and techniques

All RFA sessions were performed by the same team who had more than 30 years of experience in interventional radiology. The Cool-Tip Radiofrequency System (Radionics, Burlington, Massachusetts, USA) contains a generator, a monopolar-array needle electrode (LeVeen, RadioTherapeutics), which has a 2 or 3 cm exposed tip and a dispersive electrode pad. The radiofrequency electrode is 17-gauge which contains internal channels and the five hook-shaped expandable electrode tines with a diameter of 2.0-, 3.0- or 3.5-expansion. For nodules < 1.5 cm in diameter, an electrode with 2.0-cm expanded tines; for nodules 1.5-2.5 cm in diameter, an electrode with 3.0-cm expanded tines; and for nodules larger than 2.5 cm in diameter, and an electrode with 3.5-cm expanded tines were used.

Prior to the operation, pethidine 100 mg and anisodamine hydrochloride (654-2) 10 mg were given through intra-muscular injection as a basal anesthesia. Tumor localization detection was under real-time US. Patient's posture would be changed according to the tumor location. The insertion site of the skin depends on the biggest cross-section of tumors in US. Local anesthesia with 1% lidocaine was given from the insertion site down to the peritoneum along the planned puncture track, and conscious analgesia-sedation was induced by intravenous administration of 0.1 mg of Tramadol (SanJiu Pharmaceutical Ltd., Zhejiang, China). During the puncture procedure, damage to the visceral organs, such as gallbladder, bowels, and stomach, was avoided by keeping 1 cm away from adjacent organ so that we can place the needle into nodules easily. After the electrode was placed into the center of the nodule under the guidance of US, the hooks then expanded. The initial output was 30-50 W with an increase of 10 W every 60 s till the power of about 60-90 W, which was maintained for 5 min, and then, increasing the power again to the maximum level (90-130 W) step by step. The selection of the power level depended on the size of tumor. Ablation was maintained for at least 15 min.^[8] During ablation, water was administered at a base rate of 20 mL/10 min by the syringe pump to cool the electrode tip to reduce injury to the surrounding tissue. For larger tumors (≥ 3.0 cm), the RF probe with 3.5-cm expanded tines was introduced into a 0.5-1.0 cm deep position from the center of the nodule to create overlapping coagulation zones with adequate ablation margin of 0.5-2.0 cm. At the end of the procedure, the needle track was cauterized for 15 s to prevent possible tumor seeding or bleeding.

Follow-up and endpoint

Two days after RFA, contrast-enhanced computer tomography (CT) or magnetic resonance imaging (MRI) was performed. If any irregular contrast enhancement was found inside or beside the ablation zone, additional RFA would be performed in 1 week. Thirty days after the first RFA, contrast-enhanced CT or MRI was carried out again. If the enhancing tissue at the tumor site disappeared, it was classified as “complete necrosis”.^[6] Laboratory test of AFP was also used to evaluate the efficiency of RFA in patients with high pre-operative AFP levels. Then, patients were regularly followed up in the outpatient clinic every 3 months for the first 2 years. In our study, the endpoint was “one-off” ablation, which was assessed at the 6th month after RFA.

Statistical analysis

Data were analyzed with the SPSS statistical software (SPSS version 20.0, Chicago, IL, USA). Homogeneity of continuous data was performed by the Gaussianity test, and described as means \pm standard deviations or median (range) and compared using the unpaired *t*-test. Categorical variables were compared using Chi-square test or the Fisher's exact test, where appropriate. Variables with a *P* < 0.05 in the univariate analysis would be added to the multivariate model. In the multivariate analysis, a multiple logistic regression was used to determine the predictors of the success of “one-off” ablation.

RESULTS

Baseline data

A total of 983 patients were screened while 735 patients were included in the study, 273 patients were excluded based on our study exclusion criteria and failure to follow-up. Therefore, a total of 462 patients were enrolled for the analysis. Clinical and demographic characteristics were summarized in Table 1. There were 373 male patients and 89 females, with a mean age of 56.6 ± 11.0 years. Most patients (85.7%) had a background of viral hepatitis (hepatitis B and/or hepatitis C). Tumor diameter ≤ 3.0 cm and > 3 cm diameter were present in 362 (70.6%) and 136 (29.4%) patients, respectively. Tumor location included deep-parenchyma (307 patients, 66.5%) and sub-capsular (155 patients, 43.5%). Among them, 109 (23.6%) tumors were close to organs (space between tumor and organ < 1 cm)^[13] (22 nodules close to stomach, 48 close to gallbladder, 23 close to jejunum, 8 close to pericardium, and 17 close to kidney), and 40 tumors (3.9%) were close to the main blood vessels (between tumor and vessels < 5 mm)^[11] such as post-hepatic vena cava, hepatic vein, and the portal vein.

Complications of RFA

Most patients experienced mild pain or discomfort during ablation. Twenty patients (4.3%) had one or more complications. One patient died in the hospital due to

liver failure. Other complications were listed on Table 2. Further analyses showed that there was no significant difference between the “one-off” group and other treatment groups.

“One-off” ablation and predictive factors for its success

During the CT evaluation 2 days after RFA, there were 416 (90.0%) patients who had achieved “complete necrosis”, while 46 (10.0%) patients had not. When evaluated at 6 months after the treatment, 281 (60.8%) patients achieved “one-off” ablation, while 181 (39.2%) patients failed. Clinical data were compared between patients who achieved “one-off” ablation and those who failed

Table 1: Baseline characteristics of all 462 patients

| Variables | n = 462 |
|---------------------------------------|----------------------|
| Gender (male/female) (%) | 373 (80.7)/89 (19.3) |
| Age (years) | 56.6 ± 11.0 |
| PLT ($\times 10^9$ /L) | 131.1 ± 57.1 |
| PT (s) | 12.3 ± 0.95 |
| Total bilirubin ($\mu\text{mol/L}$) | 17.2 ± 10.9 |
| ALT (IU/L) | $86.5 (9.4, 546.8)$ |
| Albumin (g/L) | 41.3 ± 4.0 |
| Prealbumin (mg/dL) | 186.6 ± 52.1 |
| AFP (ng/mL) | $26.5 (0.6, 584.0)$ |
| Child-Pugh classification | |
| Class A | 442 |
| Class B | 20 |
| Hepatitis background | |
| HBV | 333 |
| HCV | 7 |
| HBV-HCV# | 4 |
| HBsAg | |
| Present | 333 |
| Absent | 129 |
| HBeAg | |
| Present | 117 |
| Absent | 345 |
| Tumor size (cm) | 2.6 ± 1.1 |
| Tumor location | |
| Parenchyma | 307 |
| Sub-capsular | 155 |
| Close to organs | |
| Gallbladder | 48 |
| Stomach | 22 |
| Jejunum | 23 |
| Pericardium | 8 |
| Kidney | 17 |
| Close to main blood vessels | |
| Yes | 40 |
| No | 422 |

#Co-occurrence of HBV and HCV. PLT: platelet; PT: prothrombin time; ALT: alanine aminotransferase; AFP: alpha fetal protein; HBV: hepatitis B virus; HCV: hepatitis C virus; HBsAg: hepatitis B surface antigen; HBeAg: hepatitis B e antigen

Table 2: Complications of radiofrequency ablation

| Complications | Number |
|--------------------|--------|
| Severe pain | 3 |
| Cholecystitis | 6 |
| Bile leakage | 2 |
| Intestinal leakage | 1 |
| Abdominal bleeding | 2 |
| Liver abscess | 2 |
| Pleural effusion | 3 |

[Table 3]. On univariate analysis, patients with tumor size ≤ 3 cm had a higher rate of achieving “one-off” ablation than those with tumor size > 3 cm (92.0% vs. 85.3%, $P = 0.003$), while tumor close to the organs had a lower rate of achieving “one-off” ablation than those further from organs (50.8% vs. 64.2%, $P = 0.010$). On multivariate analysis using a logistic regression, tumor size ≤ 3 cm [odds ratio (OR), 0.534; 95% confidence interval (CI): 0.346-0.825, $P = 0.005$] and tumor further from organs (OR, 0.593; 95% CI: 0.387-0.909, $P = 0.017$) remained predictive for the success of “one-off” RFA [Table 4].

DISCUSSION

RFA, a newly developed local ablative technique,^[14] is suggested by AASLD and the European Association for the Study of the Liver (EASL) as the first-line treatment for HCC due to its safety, lower mortality and morbidity, and shorter hospitalization.^[15] “One-off” ablation, first proposed by Jiang *et al.*,^[9-11] defined as (1) the diameter of post-RFA zone demonstrated by contrast-enhanced CT is more than the maximal length of the tumor, and (2) no tumor recurrence within 6 months after RFA. However, not all tumors can achieve “one-off” ablation after a single-session RFA. So far, numerous investigators have described prognostic factors for survival after RFA. However, no large study has illustrated the predictive factors for the success of “one-off” ablation after a single-session RFA. In the study, we focused on the analyses of the effectiveness of single-session RFA in single HCC, and investigated the risk factors influencing the success of “one-off” ablation to provide clinicians a guideline for their routine medical treatments.

Our study showed that tumors measuring 3 cm in greatest dimension and which are further to organs were most suitable for a single-session, single application of percutaneous RFA [Table 3]. As reported, when RFA was performed on small HCC nodules (≤ 3 cm), complete necrosis can be achieved in more than 90% patients.^[16] As the tumor size increased, the therapeutic effect of RFA decreased. For tumors 3.0-5.0 cm and tumors larger than 5.0 cm, complete tumor necrosis rates was 71% and 45%, respectively.^[17] In this study, the mean tumor size is 2.6 ± 1.1 cm. The primary effectiveness was 90.0% and the rate of “one-off” ablation in our study was 60.8%. Patients with tumor size ≤ 3 cm had a higher rate to achieve “one-off” ablation than those with tumor size > 3 cm, similar to observations by Komorizono *et al.*^[18] Komorizono’s study showed that tumors measuring ≤ 2 cm in greatest dimension were indicated for an optimal ablation.^[18] Tumor size may influence the success of “one-off” RFA due to three possible reasons: first, RFA induced tumor coagulative necrosis by putting high-frequency alternating electrodes within the tumor tissue. The temperature inside the ablated tissue must be $> 60^\circ\text{C}$ to achieve coagulation necrosis. Some authors suggested

Table 3: Univariate analysis of factors related to “one-off” radiofrequency ablation

| Variables | Achieved (n = 281) (%) | Failed (n = 181) (%) | P |
|---------------------------------|---------------------------|-------------------------|-------|
| Sex | | | |
| Male | 221 (59.2) | 152 (40.8) | 0.156 |
| Female | 60 (67.4) | 29 (32.6) | |
| Age | | | |
| ≤ 60 | 180 (59.2) | 125 (40.8) | 0.268 |
| > 60 | 101 (64.3) | 56 (35.7) | |
| PLT ($\times 10^9/\text{L}$) | 143.0 ± 57.9 | 119.2 ± 54.6 | 0.119 |
| PT (s) | 12.2 ± 0.98 | 12.4 ± 0.93 | 0.533 |
| Bilirubin ($\mu\text{mol/L}$) | 17.8 ± 14.3 | 16.7 ± 6.1 | 0.713 |
| Albumin (g/L) | 41.2 ± 4.2 | 41.4 ± 4.0 | 0.857 |
| Prealbumin (mg/dL) | 189.5 ± 54.9 | 183.8 ± 50.1 | 0.687 |
| ALT (IU/L) | 94.8 (9.40, 546.80) | 70.2 (18.10, 154.80) | 0.710 |
| AFP (ng/dL) | | | |
| ≤ 400 | 225 (60.3) | 148 (39.7) | 0.652 |
| > 400 | 56 (62.9) | 33 (37.1) | |
| Child-Pugh classification | | | |
| Class A | 267 (60.4) | 175 (39.6) | 0.390 |
| Class B | 14 (70.0) | 6 (30.0) | |
| Hepatitis background | | | |
| HBV and/or HCV | 204 (59.3) | 140 (40.7) | 0.253 |
| None | 77 (65.3) | 41 (34.7) | |
| HBsAg | | | |
| Present | 197 (59.2) | 136 (40.8) | 0.239 |
| Absent | 84 (65.1) | 45 (34.9) | |
| HBeAg | | | |
| Present | 67 (57.2) | 50 (42.7) | 0.362 |
| Absent | 214 (62.0) | 131 (38.0) | |
| Tumor size (cm) | | | |
| ≤ 3.0 | 184 (92.0) | 142 (8.0) | 0.003 |
| > 3.0 | 97 (85.3) | 39 (14.7) | |
| Tumor location | | | |
| Parenchyma | 181 (59.0) | 126 (41.0) | 0.248 |
| Sub-capsular | 100 (64.5) | 55 (35.5) | |
| Close to organs | | | |
| Yes | 60 (50.8) | 58 (49.2) | 0.010 |
| No | 221 (64.2) | 123 (37.8) | |
| Close to blood vessels | | | |
| Yes | 25 (62.5) | 15 (37.5) | 0.820 |
| No | 256 (60.1) | 166 (39.3) | |

PLT: platelet; PT: prothrombin time; ALT: alanine aminotransferase; AFP: alpha fetal protein; HBV: hepatitis B virus; HCV: hepatitis C virus; HBsAg: hepatitis B surface antigen; HBeAg: hepatitis B e antigen

Table 4: Multivariate analysis of factors related to “one-off” radiofrequency ablation

| Variables | OR | 95% CI | P |
|--|-------|-------------|-------|
| Tumor size (≤ 3 cm vs. > 3 cm) | 0.534 | 0.346-0.825 | 0.005 |
| Tumor close to organs (no vs. yes) | 0.593 | 0.387-0.909 | 0.017 |

OR: odds ratio; CI: confidence interval

that the cirrhotic tissue around small HCC behaved like a thermal insulator, increasing the heat retention within the tumor and preventing heating outside the tumor. However, when the tumor is > 3 cm, heat may be lost in the periphery. Meanwhile, Ahmed *et al.*^[19] used an established computer simulation model of RFA to characterize the combined effects of varying perfusion, electrical, and thermal conductivity on radiofrequency (RF) heating. They observed that electrical and thermal

conductivity had greatest differences in effect seen in tumor range. Therefore, some researchers suggested that when tumor size > 2 cm, repeated RFA or combination treatment may be beneficial. Second, as reported by Kim *et al.*,^[20] a margin of 3 mm or more is associated with a lower rate of local tumor recurrence after percutaneous RFA of HCC. Some clinicians have reported difficulty in obtaining adequate circumferential ablative margin for large tumors after a single-session of RFA. Overlapping treatment or combining with transcatheter arterial chemoembolization were needed.^[21] Third, the effectiveness of RFA may be related with the perfusion of the tumor, although it is still debated. Some researchers found that RFA with occlusion of tumor blood supply in tumors measuring 3.5 cm was beneficial.^[22] Documented pathology showed that blood supplies changed as tumors grow larger. As the perfusion of tumors aggravated, the “heat-sink effect” (HSE) may be induced which will influence the effectiveness of the RFA.^[23]

In addition to tumor size, proximity of the tumor to organs is also one of the most important factors influencing the success of “one-off” ablation. In the clinic, tumors adjacent to gallbladder, kidney, diaphragm, and so on were thought to be high-risk.^[24] Local ablation for tumors in “high-risk” location is technically challenging because of the poor visibility of the tumor and for fear of collateral thermal injury to the adjacent organs and causing serious post-operative complications.^[25,26] The complication rate of our study is 4.3%, similar to the report of Lau and Lai,^[15] which indicated a complication rate of RFA ranging from 3% to 7%. Most patients experienced mild pain or discomfort during the ablation. Six patients had bile leakage on the 3rd or 4th post-operative day. One patient died from liver failure. These tumors were all located in “high-risk” areas. To achieve better ablation effects, some clinicians suggest departing the vulnerable structures from the area of ablation^[27] or using laparoscopic ablation (LA).^[28] LA was proved to be a safe and effective technique for high-risk lesions not manageable by percutaneous approach and not suitable for surgical resection.^[28]

Surprisingly, our study indicated that tumor close to vascular and capsular sites did not influence the success of “one-off” RFA. Tumor located near the capsular has no influence on the success of “one-off” ablation, which is contrary to Komorizono’s retrospective study that showed patients who had sub-capsular tumors had significantly shorter recurrence free intervals compared with patients who had non-sub-capsular tumors.^[18] Further prospective study is needed to clarify this inconsistency. In addition, whether tumor close to vascular will influence the effectiveness of ablation is also unclear. Our result is similar to the study of Komorizono *et al.*^[18] which also showed that proximity of a tumor to vessel did not influence the local effect

of ablation, which was contrary to previous reports.^[29,30] In the current study, one patient whose tumor was seen adjacent to the portal vein, hepatic artery, and bile duct by enhanced CT died due to liver failure. Using a pig model, Lu *et al.*^[31] found that when vessel size was > 3 cm, HSE and river-flow effect occurred. Heat could be carried away by the blood flow, infusing into regional hepatic segments or lobes along the blood flow, causing thermal lesion to liver cells and finally impairing liver function with sustained high heat.^[9] Hence, to achieve “one-off” ablation and decrease these complications, laparoscopic approaches or pringle maneuver seem to be appropriate for tumors close to vasculature.^[31,32]

This study has several limitations. First, most patients did not have pathological examination. The diagnosis of HCC relied on their hepatitis history and imaging examination. Therefore, it is possible that benign liver diseases were included, which may influence the judgment of “one-off” ablation. Second, all RFA procedures were performed by the same team, which may introduce bias to our results. Third, our study was a retrospective study, and limited to single-center (Eastern Hepatobiliary Surgery Hospital). Further analyses including randomized controlled trials in multi-center sites are needed.

In conclusion, for single HCC with diameters smaller than 3 cm and which are further from organs, “one-off” percutaneous RFA was beneficial. Our study also elucidated the scientific rationale of RFA treatment criteria (AASLD and EASL) for HCC regarding tumor size. For tumors located at specific sites of the liver, open or laparoscopic RFA or combination with other techniques may be a better choice.

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Conflicts of interest

There are no conflicts of interest.

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Beneficial and detrimental effects of natural dietary products on the risk of hepatocellular carcinoma, and their roles in its management

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ABSTRACT

Hepatocellular carcinoma (HCC) is a common solid malignancy and a leading cause of cancer-related death worldwide. The mechanisms underlying the pathogenesis and development of HCC are complex and heterogeneous. Although mainly related to hepatitis B and C chronic infection; HCC may also arise from diet-associated conditions such as non-alcoholic fatty liver disease and non-alcoholic steatohepatitis. Furthermore, toxins and nutrients such as mycotoxins and alcohol have an established role in the pathogenesis of chronic liver diseases, whereas specific diet patterns or foods have been associated with a reduction in HCC risk. The aim of this review is to provide a thorough overview of the clinically relevant effects - either beneficial or detrimental - of natural products consumed by humans on HCC risk and management.

Key words: Hepatocellular carcinoma; natural products; diet; dietary supplements



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INTRODUCTION

The risk of hepatocellular carcinoma (HCC), associated with nutritional and metabolic factors, has been underestimated until recently. HCC may represent a late complication of non-alcoholic steatohepatitis-related cirrhosis,^[1] which in turn is strongly related to diet-associated conditions such as obesity, type 2 diabetes mellitus and dyslipidemia.^[2] Furthermore, several foods, beverages and food

contaminants are known to affect the risk of developing HCC. Nutritional compounds that display anti-inflammatory and antioxidant effects may have specific applications in preventing oxidative stress-induced injury, which characterizes the pathogenesis of cirrhosis and steatosis.^[3] The pivotal role of diet is highlighted by the results of two large case-control

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studies conducted in Italy and Greece, indicating that strong adherence to the Mediterranean diet may be protective against HCC (approximately a 50% reduction in risk), with potential benefits also in patients with chronic viral hepatitis.^[4] As for patients with established chronic liver disease, nutritional interventions to support sufficient energy intake significantly improve patient survival.^[5-8] A better knowledge of the detrimental or beneficial effects of foods is therefore important in the prevention and management of HCC, and the evaluation of dietary supplements potentially able to reduce the risk and/or the progression of cirrhosis and steatosis is of the highest interest.

The potential protective and therapeutic mechanisms of natural compounds in the prevention and treatment of hepatotoxicity and HCC have been recently reviewed.^[9] The aim of the present review is to provide an insight on the clinically relevant effects—either beneficial or detrimental—of natural products consumed by humans on HCC risk and management.

DETRIMENTAL NATURAL PRODUCTS

Foods and beverages

Alcohol

The detrimental effects of alcohol on the liver are well known; ethanol exerts toxic effects that can cause cell injury and a reactive response culminating in alcohol-induced hepatic cirrhosis. More in detail, reactions catalyzed by the main enzymes involved in alcohol metabolism, namely alcohol dehydrogenase and aldehyde dehydrogenase, lead to the production of reactive oxygen species (ROS) that can exert toxic effects such as lipid peroxidation, enzymes inactivation, DNA mutations, and destruction of cell membranes; in addition, in conditions of chronic alcohol abuse there is an increased production of acetaldehyde from ethanol, due to induction of the microsomal system and in particular of the Cytochrome P450 enzyme Cytochrome P450 2E1.^[10] Acetaldehyde is one of the main mediators of alcohol-induced fibrogenesis in the liver, as it can stimulate synthesis of fibrillar-forming collagens and structural glycoproteins of extracellular matrix in hepatic stellate cells, and increase the secretion of transforming growth factor- β . Eventually, these events may lead to hepatic cirrhosis, which is associated with a 5-year cumulative risk for HCC of 8%.^[10] The immunosuppressive effects of alcohol^[11,12] and alcohol-induced epigenetic modifications^[13] may also contribute to the development of HCC in

patients with alcoholic liver disease.

Red meat

Red meat consumption has been reported to be associated with an increased risk of HCC.^[14] Meat processing, e.g. curing and smoking, can in fact result in the formation of carcinogenic chemicals, including *N*-nitroso-compounds and polycyclic aromatic hydrocarbons. cooking, especially if high-temperature, can also produce known or suspected carcinogens, including heterocyclic aromatic amines and polycyclic aromatic hydrocarbons.^[15] The International Agency for Research on Cancer has recently classified red meat and processed meat as “probably carcinogenic to humans” (Group 2A) and “carcinogenic to humans” (Group 1), respectively.^[15] However, the strongest association appears to be with colorectal cancer, pancreatic cancer and prostate cancer,^[15] and currently available evidence supporting a causative role for red meat in HCC is inconsistent.^[16,17]

Pickled foods

A possible carcinogenic effect of pickled vegetables was first reported in 1992.^[18] Traditionally, pickled vegetables are prepared by packing moist vegetables in a jar for weeks to months, allowing fermentation and growth of fungi and yeasts. This process can potentially yield carcinogenic compounds such as the *N*-nitroso compound Roussin’s red (dimethylthiot etranitrosodiiron).^[19] Consistently, a large systematic review and meta-analysis revealed that those who consume pickled vegetables/foods have an about 50% increase in risk of gastric cancer vs. those who consume little or no pickled vegetables/foods.^[20] An association between pickled food and HCC has also been reported.^[21]

Sugar

Non-alcoholic fatty liver disease (NAFLD) is considered as the hepatic manifestation of the metabolic syndrome. It is characterized by an increase in intrahepatic triglyceride content (i.e. steatosis), with or without inflammation and fibrosis [i.e. non-alcoholic steatohepatitis (NASH)]. Hepatic *de novo* lipogenesis (DNL) has been suggested to be abnormally increased in NAFLD, and to contribute to its development.^[22] As glycolysis and the metabolism of carbohydrates are the main providers of substrates for DNL, a high-carbohydrate diet can prime the DNL pathway with a large substrate load and increase rates of DNL.^[23] Dietary fructose may contribute to NAFLD by promoting DNL, insulin resistance, oxidative

stress, bacterial overgrowth, and inflammation.^[24] Both NAFLD and NASH can further progress to hepatic fibrosis and eventually to cirrhosis,^[25] older age and deterioration of metabolic status being major risk factors for fibrosis progression.^[26] NAFLD/NASH that progresses to cirrhosis carries the highest risk for HCC, due to the erratic liver remodeling with repeated cycles of hepatocellular destruction and compensatory regeneration that characterizes cirrhosis. However, there is increasing concern that NAFLD-associated HCC may also occur in non-cirrhotic liver, due factors specifically associated with NAFLD (e.g. lipotoxicity associated with DNL and increased levels of proinflammatory adipokines/cytokines).^[27] Recent findings indicate that the incidence rate of HCC in NAFLD and NASH is 0.44 and 5.29 cases per 1,000 person-years, respectively.^[28] Although these rates are lower than those reported for patients with hepatitis B virus (HBV) or hepatitis C virus (HCV), the number of patients with NAFLD and NASH-related HCC is projected to increase, given the increasing prevalence of these conditions. Epidemiological evidence linking dietary sugar, and specifically, fructose consumption, with cancer derives from case-control studies that found an association between high dietary glycemic load and increased risk for HCC, especially in patients with chronic viral hepatitis.^[16,17] However, a recent analysis of the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort revealed an association between higher total sugar intake and risk of HCC, but not between glycemic index/glycemic load and HCC.^[18]

Overnutrition

Hepatic cirrhosis and an associated increased risk of developing HCC independent of viral hepatitis frequently occurs secondary to NASH and NAFLD,^[29] which often has a nutritional basis. NAFLD is very common in obesity and is present in 60-75% of obese persons and 85-95% of morbidly obese persons.^[30] Furthermore, it has been proposed that obesity, diabetes, and insulin resistance may predispose to HCC in patients with cirrhosis.^[31-33] Thus, in the case of HCC arising from NAFLD, it appears that overnutrition is leading to obesity and its complications may increase the risk of developing HCC, rather than specific nutrients in the diet.

Food contaminants

Mycotoxins

Aflatoxin B1 (AFB1) is a mycotoxin (i.e. toxic compounds produced by fungal secondary

metabolism) produced by *Aspergillus flavus* and *Aspergillus parasiticus*, widely represented in nature. The mycotoxin is found in many foods such as corn, rice, oil seeds, dried fruits, and peanuts that have been improperly stored in hot, humid, and unsanitary conditions.^[34] Metabolism of aflatoxins by hepatic enzymes may generate reactive epoxide species with the potential of forming a covalent bond with guanine,^[35] generating adducts that can promote cellular and macromolecule damage, including mutations in the *p53* tumor-suppressor gene.^[36] Exposure to AFB1 has been associated with HCC in several cohort studies, supporting a role of AFB1 in liver cancerogenesis-particularly among subjects who are carriers of hepatitis B surface antigen.^[37] It has been estimated that aflatoxin exposure may account for 5-28% of total HCC cases worldwide.^[38]

Fumonisin are ubiquitous mycotoxins that contaminate cereal grains, primarily maize. More than 10 compounds have been isolated and characterized; fumonisin B1 is believed to be the most toxic among them, as it has been shown to be hepatocarcinogenic in rodents.^[39-41] Fumonisin are thought to impair the de novo synthesis of ceramide and sphingolipid metabolism due to a structural resemblance with ceramide; this may lead to disruption of signal transduction pathways in the target cells.^[42] A pathogenic role of exposure to fumonisins through consumption of moldy corn in human HCC has been suggested by studies carried out in China.^[43-45]

Ochratoxin A is another mycotoxin that may have a role in the development of HCC.^[46] It may be found in cacao and derived products, dried fruits, wine, cereals, green coffee, and spices (mainly nutmeg, paprika, coriander, and pepper powder).^[47] The carcinogenic effect of ochratoxin A is the result of both direct genotoxic (covalent DNA adduct formation and mutagenicity)^[48] and epigenetic mechanisms leading to protein synthesis inhibition, oxidative stress and the activation of specific cell signaling pathways.^[49] In a recent case-control study, high performance liquid chromatography analysis of serum samples from HCC patients and controls indicated that the incidence of elevated ochratoxin A was highest in the HCC group, being 5-fold higher than in the control group.^[50] These findings support a strong association between the presence of ochratoxin A and HCC. Ochratoxin A is a stable compound that is not destroyed by common food preparation procedures.

Available data on the presence of mycotoxins in grains and foods indicate that there may be a continuous low-level exposure to these toxic metabolites.^[47] Foods mainly contributing to the intake of mycotoxins with diet are cereals, maize being the most risky commodity due to the potential co-occurrence of more than one mycotoxin. It has been postulated that individuals with increased maize-based products consumption such as celiac patients could be particularly at risk of mycotoxin exposure. However, studies have shown that the intake of mycotoxins in these potentially vulnerable populations is generally below the tolerable daily intake.^[51,52]

Pyrrolizidine alkaloids

Pyrrolizidine alkaloids such as riddelline, which is found in *Senecio riddellii* (Riddell groundsel) and *Senecio longilobus* (also known as woolly groundsel and thread-leaf groundsel),^[37] can be found as a contaminant in foods such as meat, grains, seeds, milk, herbal tea and honey.^[53] In hepatocytes, Cytochrome P450s convert dehydropyrrolizidine alkaloids to 6,7-dehydropyrrolizine esters, i.e. the toxic metabolites. Dehydroretronecine and dehydroheliotridine that are produced from the initial toxic metabolites via ROS react rapidly with the SH, OH, NH groups on nucleotides, as well as with proteins to form adducts, eventually leading to DNA damage and carcinogenesis.^[54] There is a large body of evidence from studies in animals supporting the carcinogenicity of pyrrolizidine alkaloids.^[37] Of note, there are published reports of primary liver tumors in natives of Central and South Africa associated with the consumption of traditional medicinal plants containing of pyrrolizidine alkaloids.^[55-58] Honey and tea have been reported to be a significant source of pyrrolizidine alkaloids in Western countries.^[59] Although health impairment due to chronic intake of pyrrolizidine alkaloids is improbable for adult consumers with average amounts of consumption of honey and tea,^[60,61] longer-term regular consumption of products with containing high amounts of pyrrolizidine alkaloids could be associated with a risk of health impairment.

BENEFICIAL NATURAL PRODUCTS

Foods and beverages

Coffee

A protective effect of coffee against HCC was first suggested by Gallus *et al.*^[62] in 2002. Since then, several other studies have confirmed this hypothesis.

Meta-analyses of epidemiological studies found that an increased consumption of coffee may reduce the risk of liver cancer.^[63-65] In a recent analysis of EPIC, a large epidemiological study designed to investigate the association between diet, lifestyle and environmental factors and the incidence of various types of cancer and other chronic diseases, coffee consumers in the highest compared to the lowest quintile had 72% lower risk of developing HCC.^[66] Consistently, high levels of coffee consumption were associated with reduced risk of incident HCC and chronic liver disease mortality in a population-based prospective cohort study of more than 215,000 men and women from Hawaii and California.^[67] Coffee has been shown to exert beneficial effects on body weight, development of diabetes, the prevention of hepatic fibrosis in NAFLD, and other chronic liver diseases, including chronic hepatitis C.^[68] There are approximately 1,000 substances in coffee, including caffeine, diterphenolic alcohols and chlorogenic acid [(CGA), a polyphenol].^[68] It is uncertain which are the exact substances and mechanisms responsible for the beneficial effects of coffee on the liver. Several substances as well as the method of preparation are thought to be of importance. As an example, filtered coffee may provide the most benefit due to a reduction in cafestol and kahweol, which can raise serum cholesterol, while maintaining CGA and caffeine content.

Fish

By virtue of its high content in omega-3 fatty acids, which may have anti-carcinogenic and anti-inflammatory effects,^[69] fish might be protective against HCC. Evidence supporting a protective role of fish comes from the EPIC study. In EPIC, total fish intake was inversely associated with HCC risk (20% reduction in risk per 20 g/day of fish, after calibration).^[70] Lean/white fish (cod, haddock, and plaice), fatty fish (salmon, tuna, trout, herring, kippers, and mackerel, and crustaceans and mollusks) were independently associated with lower HCC risk, even after adjusting for HBV/HCV status and liver function biomarkers.^[70]

Olive (Olea europaea)

Epidemiological studies have shown that intake of virgin olive oil is associated with low incidences of several types of cancer,^[71] likely due to its high content in phenolic antioxidants. These include hydroxytyrosol and oleuropein.^[72] Hydroxytyrosol (HT) is a natural polyphenolic compound with significant antioxidant properties.^[73] It has been recently demonstrated

that HT inhibited the proliferation and induced apoptosis in HCC *in vitro* and in a tumor model of HCC,^[74] and exerted antiproliferative, antioxidant and anti-inflammatory effects on human hepatoma HepG2 and Hep3B cell lines.^[75,76] Oleuropein, a major constituent of *O. europaea*, was shown to effectively inhibit cell viability and to induce apoptosis in HepG2 human hepatoma cells in a dose -dependent manner, through activation of the caspase pathway.^[77]

Oleanolic acid (3 β -hydroxyolean-12-en-28-oic acid) is a pentacyclic triterpenoid found in olive leaves. Antitumor effects of oleanolic acid have been investigated recently both *in vitro* and *in vivo*. It exhibited inhibitory effect on HCC through induction of apoptosis and cell cycle arrest both in transplanted tumors in mice and in HepG2 cells, indicating that oleanolic acid has significant antitumor activities on HCC both *in vitro* and *in vivo* models.^[78] Olive fruit pulp is a rich source of antioxidants and possesses very good hepatoprotective activity against CCl₄-induced hepatic damage in mice.^[79]

Other foods and beverages

Several other foods and beverages have been reported to have a protective role against HCC, including tea polyphenols (i.e. green and black tea),^[80] tomatoes and tomato-based products^[81] (a rich source of lycopene, an antioxidant carotenoid that has even been shown to prevent HCC metastases in animal studies^[82]), dietary fiber,^[18] green-yellow vegetables and fruit.^[83]

Nutraceuticals and dietary supplements

A large proportion of HCC patients use dietary supplements.^[3] However, only in few cases their use is supported by clinical evidence.

Branched chain amino acids

Branched chain amino acids (BCAA) may suppress hepatocarcinogenesis by several mechanisms, including improvement of immune function, reduction of oxidative stress and improvement of insulin resistance.^[84] Supplementation of BCAA for 2 years in patients with cirrhosis (Child-Pugh class A) has been associated with increases albumin synthesis in a multicenter, randomized controlled trial.^[85] However, another randomized controlled trial did not find an improvement in serum albumin levels with BCAA supplementation, possibly due to different patient characteristics (patients were Child-Pugh class B or C).^[86] Clinical trials have reported that long-term oral supplementation with BCAAs is associated

with decreased frequency of development of HCC in obese patients with cirrhosis and hepatitis C virus infection,^[87] significant reduction in HCC incidence rate and improvement of event-free survival rate in patients with cirrhosis,^[88] and reduced HCC recurrence after treatment with radiofrequency ablation in patients with cirrhosis.^[84,89] Finally, BCAAs have been also shown to improve health-related quality of life^[85,86] and sleep disturbances in patients with cirrhosis.^[90]

Milk thistle (Silybum marianum)

Milk thistle is an herbal agent that has been used to treat liver diseases for centuries. Silymarin, the main active constituent of milk thistle, is a mixture of polyphenols, including flavonolignans and flavonoids. Despite a strong anticancer activity against human HCC cells are demonstrated *in vitro*,^[91] clinical studies supporting the use of silymarin as a hepatoprotective agent have yielded conflicting results.

A Cochrane systematic review revealed that the evidence supporting a role of milk thistle for the treatment of patients with alcoholic and/or hepatitis B or C virus liver diseases is scanty, and that milk thistle vs. placebo or no intervention had no significant effect on mortality, complications of liver disease or liver histology.^[92] Milk thistle was not associated with increased risk of adverse events.^[92] Silymarin use in 1,049 patients with advanced fibrosis or cirrhosis unsuccessfully treated with peginterferon plus ribavirin has been associated with reduced progression from fibrosis to cirrhosis.^[93] In a 24-week multicenter, double-blind, placebo-controlled trial that included 154 patients with chronic HCV infection and elevated serum alanine aminotransferase (ALT) unsuccessfully treated with interferon-based therapy, silymarin did not significantly reduce serum ALT levels.^[94] Silymarin has also been used as an adjuvant therapy in conjunction with chemotherapy and other supplements (α -tocopheryl acetate and a product containing stem cell differentiation stage factors) in a case report of a patient with locally advanced HCC, with encouraging results.^[95]

Omega-3 fatty acids

Preclinical data indicate that omega-3 polyunsaturated fatty acids (PUFAs) inhibit HCC cell growth and might therefore be useful for the chemoprevention and treatment of human HCC.^[96] This hypothesis is supported by the results of a population-based prospective cohort study of 90,296 Japanese subjects, in which consumption of omega-3 PUFAs, particularly

eicosapentaenoic acid, docosapentaenoic acid, and docosahexaenoic acid, protected against the development of HCC, irrespective of HCV or HBV status.^[97] The effect of treatment with high dose purified long chain omega-3 fatty acids on liver fat percentage and scores for liver fibrosis in patients with NAFLD is currently being investigated in a randomized double blind placebo controlled trial.^[98]

Spirulina platensis

Spirulina is a blue-green alga (cyanobacterium) available as a dietary supplement. *In vitro* studies have demonstrated that spirulina may exert hepatoprotective effects.^[99] In patients with chronic hepatitis C infection, viral load and ALT levels tended to improve after 6 months of treatment with spirulina in a small, active-controlled trial.^[100] Another small, uncontrolled trial reported significant improvements in aspartate aminotransferase, alanin aminotransferase, gamma-glutamyltransferase, triglycerides, low-density lipoprotein-cholesterol, total cholesterol, and the ratio of total cholesterol to high-density lipoprotein cholesterol after 6 months of treatment in patients with NAFLD. According to the authors, spirulina supplementation resulted also in a significant reduction in weight and insulin resistance, and a significant improvement in health-related quality of life was observed. However, no changes in sonographic findings were observed.^[101]

Antioxidants

Reduced glutathione (GSH) is a potent antioxidant naturally occurring in the body, and is available for parenteral administration. Very few studies have assessed the therapeutic role of GSH in liver diseases. In an Italian study that compared the effects of reduced GSH and vitamin K in patients with alcoholic liver disease, those treated with reduced GSH showed a greater improvement of hepatic function vs. patients treated with vitamin K.^[102] A study published several years ago assessed the effect of GSH treatment on HCC in 8 patients with biopsy-proven HCC not amenable to surgery, but results were inconclusive.^[103] Besides direct GSH supplementation, hepatic GSH deposits can be restored by administering compounds such as *S*-adenosyl-*L*-methionine and *N*-acetylcysteine. Both compounds are generally very well tolerated, although of limited clinical value in improving liver function in chronic liver diseases.^[104-106]

Finally, it has been observed that patients with HCC have low levels of serum vitamin B^[107] and vitamin D,^[108] which suggests that these patients might benefit from supplementation with these vitamins.

Traditional Chinese medicines

It has been suggested that use of Chinese herbal medicines might result in the protection of liver function during chemotherapy.^[109] The herbal formulation PHY906 consists of four commonly used herbs, i.e. *Scutellaria baicalensis* Georgi, *Paeonia lactiflora* Pall, *Glycyrrhiza uralensis* Fisch, and *Ziziphus jujube* Mill, at a ratio of 3:2:2:2. Studies have shown that PHY906 not only reduces gastrointestinal toxicity and enhances the antitumor efficacy of some anticancer drugs but also alleviates chemotherapy-induced side effects, such as diarrhea.^[109] Preliminary clinical data indicate that PHY906 can serve as an adjuvant to chemotherapy in the treatment of advanced HCC.^[110] Other traditional Chinese medicines that may have a role in the treatment of HCC are bufotoxin (toad skin secretion), astragalus and products containing ginseng (*Panax ginseng*), astragalus and mylabris (dried body of the Chinese blister beetle).^[111]

CONCLUSION

Identifying modifiable risk factors such as diet is important to counteract HCC. Dietary patterns are complex to assess, and are entangled with other aspects of lifestyle. To date, conclusive evidence supporting a detrimental or beneficial role in the prevention of chronic liver diseases is available only for few products. Available information on coffee, fish and BCAAs supplementation is of acceptable quality and supports a beneficial role for these products in the prevention and management of HCC. On the other hand, the detrimental effects of alcohol and aflatoxins are widely recognized. Excessive sugar and calorie consumption should also be avoided.

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Conflicts of interest

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Curcumin: an adjuvant therapeutic remedy for liver cancer

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ABSTRACT

The molecular signalling pathways for hepatocellular carcinoma and hepatoblastoma have been extensively studied. The treatment of these highly vascular tumors mainly revolves around chemotherapy and surgery. Yet there is a high associated morbidity and mortality due to advanced stages, adverse effects owing to chemotherapy and recurrence. The role of Curcumin as an adjuvant remedy is explored in this article. Curcumin stimulates apoptosis of cancer cells, acts as anti-proliferative agent, has anti-angiogenic action, prevents tumor invasiveness and metastasis and prevents recurrence. It also has been proven to decrease the adverse effects of chemotherapeutic agents and has a synergistic anticancer action. It acts at the molecular level and affects the various metabolic pathways involved in tumorigenesis. It also promotes healing and has anti-inflammatory, anti-oxidant and anti-infective action. This natural phytochemicals has immense anti-cancer potential and holds future promise as an adjuvant remedy to treat liver cancer.

Key words: Curcumin; hepatoblastoma; hepatocellular carcinoma; diferuloylmethane

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INTRODUCTION

Primary liver cancer characterised by active neovascularization is among the most common lethal cancers worldwide and can occur at any age. Hepatocellular carcinoma (HCC) occurs in older children and adults and has a high prevalence in developing Asian


and African countries. In children under five years of age, hepatoblastoma (HB) accounts for more than 90% of primary hepatic malignant tumors and HCC for 12.5%.^[1]

With recent advances in diagnostic technology, the incidence of HCC and HB has been increasing in the past decades, especially in Europe and North America.^[2] Risk

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factors for HCC include cirrhosis, hepatocarcinogenic like aflatoxins and nitrosamines, dietary and environmental carcinogens by generation of reactive oxygen species (ROS) and infections like hepatitis B and C viruses.^[2]

The current management of liver tumors is not satisfactory. Chemotherapy, surgery, and radiofrequency ablation are all directed at reducing the tumor bulk. However, in the majority of cases, tumor recurrence and relapse occurs on completion of therapy. Also, liver cancer is diagnosed at an advanced stage quite frequently; hence the available chemotherapy regimens fail to offer a complete cure. Even if chemotherapy has been instituted timely, the available chemotherapeutic agents are reported to show severe adverse effects. Angiogenesis plays a significant role in human HCC tumor progression and recent studies are focussing on anti-angiogenic agents targeting specific tumor vasculature.^[3]

In this regard, discovery of natural phytochemicals having anti-tumor and anti-angiogenic activities could have greater clinical significance as they do not affect physiology and survival of normal cells. Many phytochemicals have proven anti-tumor action including catechins, quercetin in apples and onions, resveratrol in grapes, red wine, peanuts, and ellagic acid in pomegranates.^[4-7] This review describes firstly the molecular pathology of liver cancers and then summarizes the evidence based literature that describes the various proven mechanism demonstrating the anti-tumor potential of curcumin in turmeric (*Curcuma longa*) and thus exploring its role as an adjuvant therapeutic remedy for liver cancer.

CURCUMIN

Curcumin is the active phytoconstituent of turmeric. It has been widely used as a therapeutic medicine in Indian traditional medicine. Of late, scientists all over the world have recognized its therapeutic potential as an anti-inflammatory, anti-oxidant and anti-cancer agent.^[8-11] Curcumin inhibits lipid peroxidation and maintains the normal concentration of intracellular antioxidant enzymes like catalase, glutathione peroxidase and superoxide dismutase and scavenges reactive oxygen species effectively.^[12,13]

TUMORIGENESIS AND MOLECULAR BIOLOGY OF LIVER CANCER

Tumorigenesis of liver cancer is a complex process. The recognition of tumor stem cells and their molecular signaling has opened new pathways for therapeutic strategies. The liver has great potential to regenerate after the loss of hepatic tissue which depends on proliferation of existing mature hepatocytes.

Growth factors like hepatocyte growth factor, epidermal growth factor and transforming growth factor (TGF)-alpha

control normal hepatic regeneration via DNA synthesis stimulation. TGF- β and activin serve as negative feedback mechanisms and regulate the end point of the hepatocyte proliferation. This termination is regulated by the ratio of liver to body mass thus providing a check on the extent of liver regeneration.^[14]

Liver stem cells are proposed to be from dual origins, intrahepatic with short-term proliferative capacity present within the canals of Herring and interlobular bile ducts and extrahepatic derived from bone marrow and peripheral blood cells with long-term proliferation capacity.^[15]

MOLECULAR SIGNALING PATHWAYS IN LIVER CANCER

Liver cancer stem cells have many signals to maintain self-renewal and pluripotency including EpCAM, Wnt/ β -catenin pathway, Sonic Hedgehog pathway, and Notch pathway, which play a decisive role in the regulation and maintenance of stemness and in tumor formation. Tumorigenesis results from uncontrolled activation of these pathways. Wnt pathway proteins regulate the cellular fate and self-renewal of stem cells.^[16] The Notch pathway is involved in cellular differentiation, fate of the cell, cellular proliferation, apoptosis, and cell adhesion. Notch signaling in the liver is involved in cholangiocyte differentiation.^[17]

HEPATOCELLULAR CARCINOMA

EpCAM signaling pathway

EpCAM consists of a large extracellular, a single transmembrane and a short intracellular domain. There is a cross-talk between EpCAM signaling and the Wnt pathway.^[18,19]

Wnt/ β -catenin signaling pathway

The Wnt/ β -catenin pathway is essential for development, growth, survival, regeneration, and self-renewal.^[20] Disruption of Wnt/ β -catenin signaling by mutational and non-mutational events is associated with many cancers, including HCC. Disrupted Wnt/ β -catenin signaling pathway has been reported in around one third of all HCCs.^[21] However, the point at which cross-talk occurs in the signaling cascades of Wnt/Frizzled and EpCAM remains unknown.

SALL4 signaling pathway

As an oncofetal gene, SALL4 is expressed at high levels in fetal-liver progenitor cells but not in adult hepatocytes, and it has an important role in hepatic cell lineage commitment.^[22,23]

TGF- β family

The TGF- β family controls cellular differentiation and proliferation in both cancer stem cells and cancer cells. Impaired TGF- β signaling through the activation of

interleukin-6 in hepatic stem/progenitor cells can cause HCC.^[24] TGF- β inhibits cell proliferation and promotes tumor cell invasion. Many studies have reported a reduction of TGF- β receptors in up to 70% of HCCs that also correlated with metastasis within the liver. On the other hand, high TGF- β levels have been correlated with advanced clinical stages of HCC. This twofold role of TGF- β signaling in HCC is explained by the tumor microenvironment and selective loss of TGF- β -induced antiproliferative pathway. Tumor cells that have selectively lost their growth-inhibitory response to TGF- β , but retain a functional TGF- β signaling pathway may exhibit increased migration and invasive behaviour on TGF- β stimulation. Cells with dysfunctional TGF- β signaling have been reported to be cancer progenitor cells giving rise to HCC.^[25]

The Notch signaling pathway

This plays an important role in stem cell self-renewal and differentiation. Notch signaling is important in liver embryogenesis, bile duct formation; angiogenesis and endothelial sprouting. However, other signaling pathways have a control on whether Notch functions as a tumor suppressor or oncogene.^[26] The increased expression of genes involved in this pathway has been shown in CD133-positive liver cancer cells vs. CD133-negative cells. The activated intracellular form of Notch-3, and the Notch ligand Jagged, is highly expressed in HCC. Activation of

Notch-1 signaling increases the death receptor 5 (DR5) expression with augmentation of tumor necrosis factor (TNF)-related apoptosis-inducing ligand induced apoptosis *in vitro* and *in vivo*.^[27]

Sonic Hedgehog pathway

Activation of Hedgehog signalling is related to liver cancer.^[28] Up to 60% of human HCCs express Sonic Hedgehog. After specific blockade of the sonic Hedgehog pathway, concomitant down regulation of Gli-related target genes is observed. Furthermore, tumorigenic activation of SMO can mediate over expression of *c-myc*, a gene having an important pathogenic role in liver carcinogenesis.

miRNAs

miRNAs directly interact with specific messenger RNAs (mRNAs) through base pairing and inhibiting the expression of target genes. MiRNAs can undergo anomalous regulation during carcinogenesis, and can act as oncogenes or tumor suppressor genes. MiR-181 also regulates the Wnt/ β -catenin signaling pathway with a positive feedback loop within stem cells. This is used by cancer cells to self-propagate continuously, metastasize and develop drug resistance.

HEPATOBLASTOMA

The best characterized pathways in pathogenesis of HB

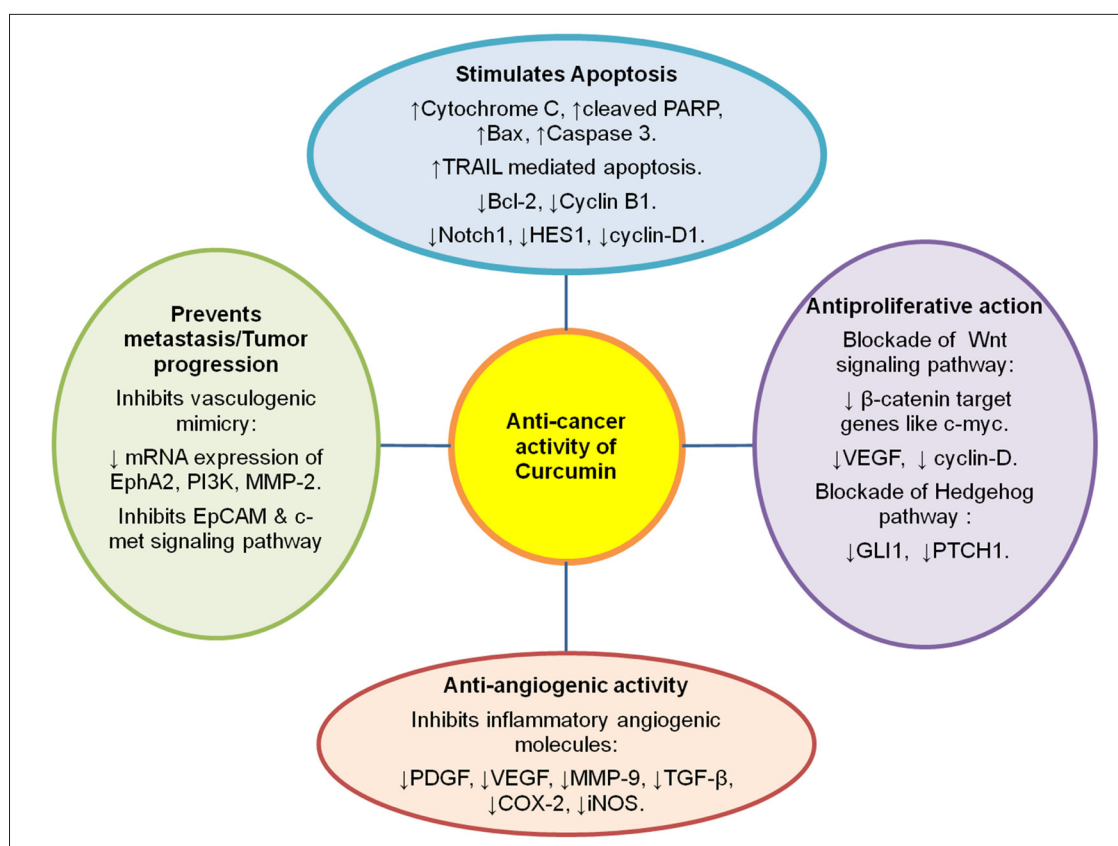


Figure 1: Flow chart depicting the various anti-cancer properties of curcumin. VEGF: vascular endothelial growth factor; MMP: matrix metalloproteinase; PDGF: platelet derived growth factor; TGF: transforming growth factor; COX: cyclooxygenase; iNOS: inducible nitric oxide synthase; EpCAM: epithelial cell adhesion molecule

include the following.

Canonical Wnt/beta-catenin signaling pathway

Multiple Wnt/beta-catenin target genes are key regulators of cellular proliferation, anti-apoptosis and angiogenesis. These include c-myc, cyclin D1, FRA-1, matrix metalloproteinase-7, c-Jun, urokinase plasminogen activator receptor, immunoglobulin transcription factor 2, endothelial growth factor receptor and vascular endothelial growth factor (VEGF) receptor.^[29-31] In the absence of Wnt ligand, the Wnt/beta-catenin signaling pathway is turned off and beta-catenin undergoes ubiquitin-mediated degradation.^[32]

Majority of HBs contain beta-catenin gene mutations that prevent beta-catenin from being degraded.^[33] As a result, beta-catenin accumulates aberrantly in the cytoplasm, and then translocates to the nucleus. Most HBs have cytoplasm and nuclear beta-catenin levels.^[34] Nuclear localization of beta-catenin leads to uncontrolled hepatoblast proliferation.^[35] Beta-catenin has been considered as a highly sensitive tumor marker for HB.

Some HBs without beta-catenin mutations may also display beta-catenin accumulation due to other aberrant components. About 65% of sporadic HBs possess adenomatous polyposis coli APC gene alterations.^[36] In absence of beta-catenin mutations, HBs with over expression of a catalytic subunit of the enzyme telomerase, telomerase reverse transcriptase also demonstrate beta-catenin accumulation.^[37] The Wnt/catenin signaling in HB is dependent on the liver and may contribute more to the genesis of the embryonal than the fetal component of HB.^[38]

Hepatocyte growth factor/c-met signaling pathway

Hepatocyte growth factor (HGF), the natural ligand for c-met receptors HGF/c-met signaling also leads to aberrant beta-catenin accumulation in hepatoblasts.^[34,39] After binding to HGF, c-met undergoes autophosphorylation on tyrosine residues and further downstream signaling. Beta-catenin is a substrate for tyrosine kinase. Tyrosine phosphorylation of beta-catenin shields beta-catenin from serine/threonine phosphorylation and subsequent degradation, and leads to beta-catenin accumulation in the tumor cells. Though this process is independent of Wnt but the result is the same.

Notch signaling pathway

The Notch signaling plays a critical role in stem cell renewal, differentiation, angiogenesis and endothelial sprouting. It is relevant for both hepatocyte embryogenesis and cholangiocyte differentiation.

Deregulation of Notch signaling in HB has been documented.^[40] Notch activation is associated more with the subtype pure fetal HB. The role of Notch signaling in tumorigenesis is dependent on the cellular context. The

crossstalk between Notch and Ras, a cell survival pathway, or the death receptor 5, an apoptotic pathway, may decide whether Notch functions as an oncogene or a tumor suppressor, respectively.

Hedgehog signaling pathway

Activation of Hedgehog signaling induces hepatic malignancies.^[41] Many signaling molecules of the Hedgehog pathway, Sonic Hedgehog, PTC, SMO and GLI-1, are over expressed in HB. Specific blockade of Hedgehog signal transduction inhibits the growth of HB.^[42]

ANTI-TUMOR PROPERTIES OF CURCUMIN IN LIVER CANCER

As an anti-tumor agent, curcumin has been reported to exhibit direct action by inhibiting proliferation of tumor cells as well as an indirect action by inhibiting angiogenesis [Figure1].

Curcumin stimulates apoptosis of cancer cells

Apoptosis or programmed cell death can be triggered by extrinsic and intrinsic pathways.^[43] Intrinsic pathway is stimulated by internal stimuli such as DNA abnormality, hypoxia, viral infection, cellular distress, *etc.* Extrinsic (receptor mediated) pathway is initiated by extracellular messenger proteins such as TNF. Intrinsic pathway is regulated by the members of the Bcl-2 family of proteins, which can be divided into three groups: (1) pro-apoptotic members that promote apoptosis, e.g. Bax, Bak; (2) anti-apoptotic members that protect cell from apoptosis, e.g. Bcl-2, Bcl-w; (3) BH-3, only protein that promote apoptosis through indirect mechanism. Extrinsic pathway of apoptosis is mediated by several caspases which are proteases with specific cellular targets, caspase-8 followed by caspase 3, 6 and 7. Cancer cells are resistant to apoptosis and this leads to their uncontrolled growth.

Curcumin affects the following pathways and promotes apoptosis of cancer cells.

EF24 is a synthetic compound and a potent curcumin analogue with enhanced bioavailability. Liu *et al.*^[44] demonstrated that EF24 significantly suppressed HCC and induced apoptosis in mouse liver cancer cell line. The levels of cytochrome c, cleaved-PARP, Bax and activated caspase-3 were increased whereas the levels of PARP and Bcl-2 were down-regulated as compared to control (non-EF24 treated) groups. Incubation of human hepatoma SMMC-7721 cells with curcumin for 24 h resulted in decreased expression of bcl-2 protein whereas expression of bax protein increased significantly and a higher curcumin concentration showed potent cytotoxicity.^[45]

EF24 induces cell cycle arrest at G2/M phase in mouse liver cancer cells. Passage from G2 to M-phase requires the activation of cdc2 by cyclin B1. With the use of curcumin, the levels of cyclin B1 and cdc2 in the cells

were significantly reduced.^[44] Wang *et al.*^[46] showed that treatment with curcumin resulted in the activation of Chk1 mediated G2 checkpoint which caused the induction of G2/M arrest and resistance of cancerous cells to curcumin-induced apoptosis. In hepatoma cell lines Chk1-mediated activation of G2 checkpoint was required for curcumin induced G2/M arrest. Chk1 inhibition reversed this arrest significantly and sensitizes curcumin resistant cells to apoptosis. Single knockdown of Chk1 in Hep3B cells caused the abrogation of curcumin-induced G2/M arrest and decreased phosphorylation of Cdk1. Thus G2/M arrest is Chk1-mediated and may be responsible for the resistance of cancer cells to curcumin-induced apoptosis.^[46]

Caspase-3 is the key member of caspase family proteins that are crucial in apoptosis. The pro-apoptotic effect of curcumin was assessed by measurement of caspase-3 activity. Dai *et al.*^[47] demonstrated that curcumin significantly elevated the activity of caspase-3.

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) can induce apoptosis in cancer cells by binding to four types of membrane bound death receptors (DR4, DR5, DcR1 and DcR2). Jung *et al.*^[48] established that curcumin sensitizes human renal cancer cells to TRAIL mediated apoptosis. Membrane bound death receptors DR4 and DR5 have a conserved cytoplasmic region called the death domain which is necessary for TRAIL-induced apoptosis.^[48] TRAIL induces apoptosis only in the cancer cells without any toxicity to normal cells because normal cells have decoy receptors on their surface.^[49]

Notch signaling can either behave as an oncogene or as a tumor suppressor. When the pathway is unregulated, it behaves as an oncogene and hence it results in increased cell proliferation, prevention of differentiation and inhibition of apoptosis.^[50] Aziz *et al.*^[51] proved that curcumin has inhibitory effects on Notch1 signaling and its target genes (Hes1 and cyclin D1).

Cytotoxic/anti-proliferation activity of curcumin

Curcumin has been demonstrated to inhibit the proliferation of HepG2 cells (Hepatoma cell line) in a dose and time dependent manner in *in vitro* studies.^[47] Curcumin demonstrates anti-proliferative action by blocking two important pathways; the Wnt signaling pathway and the Hedgehog pathway. Both these pathways affect the cancer stem cells.

Blockade of the Wnt signaling pathway

Wnt signaling pathways have important role in carcinogenesis as well as embryonic development. Wnt proteins can activate different pathways but canonical wnt/ β -catenin pathway is the most studied. In the absence of wnt proteins, β -catenin is targeted to the destruction complex for its phosphorylation at specific sites, β -catenin accumulates and recruited to the nucleus by Bcl-9 adaptor

proteins. In the nucleus, β -catenin binds to the T-cell factor/lymphocyte enhancer factor, transcription factors and activates the expression of target genes like c-myc, VEGF, cyclin-D1, that results in cell proliferation.^[52] Curcumin has been shown to interrupt this pathway and thus suppress the expression of β -catenin target genes like c-myc, VEGF, cyclin-D. Curcumin has been reported to suppress cell proliferation and induced apoptosis by interrupting wnt signaling via decreasing β -catenin activity.^[53] Curcumin and its reduced analogue tetrahydrocurcumin showed anti-proliferative effects on HepG2 cell lines.^[54] HepG2 cells (hepatoma cell line) when treated with novel curcumin derivative and mesenchymal stem cells showed a significantly decrease of proliferation rate as compared to the control group.^[51] Xu *et al.*^[53] found that curcumin significantly suppressed the cell proliferation, decreased the β -catenin accumulation and induced apoptosis in human HCC cell lines BEL-7402 and QGY-7703 in a dose dependent manner. A dose dependent decrease in the expressions of c-myc and VEGF was also reported. Thus curcumin attenuated wnt signals in HCC cells.

Blockade of the Hedgehog pathway

The Hedgehog pathway is another potential target for cancer stem cell eradication. In liver cells, the suppression of the Sonic Hedgehog pathway by small interfering RNA decreased HCC cell proliferation also chemosensitized the cells to 5-fluorouracil and induction of cell apoptosis.^[55] In HB, blocking the Hh Hedgehog signaling pathway with an antagonist cyclopamine strongly inhibited cell proliferation of HB cell lines.^[56] A significant decrease in expression of Notch1, Hes1 and cyclin D1 was observed in HepG2 cells upon treatment of hepatoma cell lines (HepG2) with mesenchymal stem cells conditioned medium (MSCs CM) and novel curcumin derivative (NCD).^[51] Pre-treatment of MSCs with NCD resulted in a more significant decrease in the expression of these genes. Thus NCD and MSCs had synergistic effect in suppression of Notch1 signaling.^[51]

Induce differentiation of cancer stem cell

Cancer stem cells comprising a small proportion of cancer cells sustain tumor growth and are more resistant to conventional chemotherapy than other more differentiated cancer cells. Malignancy may thus be treated by inducing the differentiation of cancer stem cells and thus making them lose their self-renewal property. Curcumin has been shown to induce differentiation of embryonic stem cells through possible modulation of nitric oxide-cyclic GMP pathway.^[57]

Anti-angiogenic effects of curcumin

Active neovascularisation is a predominant feature in HCC and supports tumor growth. Angiogenesis starts when tumor cells start sending signals to the nearby surrounding normal host tissue and encourage the release of signaling molecules that initiate and promote angiogenesis. This angiogenesis provides the tumor cells with oxygen and

nutrients and also a route to enter general circulation. HCC cells secrete various angiogenesis activators like VEGF, platelet derived growth factor, TGF- β . Among these, VEGF is most critical antigenic factor.^[3] Cancer cells grow in hypoxic conditions that lead to expression of several hypoxia response genes which are involved in metabolic dysregulation.^[58] These include inflammatory angiogenic molecules secreted by tumor cells like cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase. Angiogenesis requires the expressions of COX-2, VEGF and matrix metalloproteinase-9 (MMP-9). Anti angiogenic effects of curcumin have been demonstrated.^[54] COX-2 and VEGF are associated with angiogenesis in HCC.^[59] ROS generated as a result of oxidative stress in the cells also causes up regulation of MMPs that causes angiogenesis and invasiveness.^[60] Cao *et al.*^[61] found that curcumin treatment inhibited the cell proliferation and induce apoptosis in cancer cells. Curcumin also exhibited inhibitory action on cancer metastasis by inhibiting the secretion of MMP-9.^[62]

Vasculogenic mimicry (VM) refers to the functional plasticity of the aggressive and metastatic tumor cells forming the non-endothelial tumor cell-lined microvascular channels which contribute to the tumor progression. VM is detected in gliomas.^[63] Liang *et al.*^[64] demonstrated that curcumin inhibits vasculogenic mimicry through down regulation of protein and mRNA expression of erythropoietin producing hepatocellular carcinoma-A2, phosphoinositide 3-kinase and MMP-2. The same authors reported that curcumin was found to inhibit the VM formation of glioma U251 cells which they were unable to form network structures, inhibit the migration and invasion in a dose dependent manner and reduced the mRNA expression of EphA2, PI3K and MMP-2 as detected by quantitative polymerase chain reaction (QPCR).

Prevents metastasis and tumor progression

TNF- α inhibition

TNF- α has a very important role in tumor cell survival and metastasis. Curcumin inhibits TNF- α expression. However, the hydrophobicity and low bioavailability of curcumin are the major barriers. Thus, scientists have encapsulated curcumin in microcells to make it a sustained release preparation in order to increase its solubility and bioavailability.^[65] Moreover curcumin bearing microcells significantly reduced the levels of the liver enzymes in HCC induced animal group as compared to the free form curcumin. In addition, curcumin bearing microcells induced expression of proapoptotic molecules like p53 and Bax.

DNA damage induced by curcumin

Mitochondrial DNA (mtDNA), being in closer contact to ROS produced in mitochondria, is more prone to oxidative damage. Cao *et al.*^[66] reported mitochondrial and nuclear DNA damage induced by curcumin in human hepatoma (HepG2) cells, a cell line that retains many characteristics

of hepatocytes. Furthermore, QPCR assay revealed that curcumin led to dose dependent damage in nuclear as well as mitochondrial genomes.

EpCAM as a target in cancer therapy

EpCAM is potentially a promising target as it is highly expressed in most cancer cells as well as on cancer stem cells. In normal tissue, EpCAM is localized to basolateral membranes. Thus, the ease of access for EpCAM-binding antibodies is lower for normal cells than for cancer cells. EpCAM is strongly over expressed in cancer cells and thus might be partly unbound and more accessible for targeting antibodies and curcumin-loaded lipid-polymer-lecithin hybrid nanoparticles have been used against EpCAM for targeted delivery to colorectal adenocarcinoma cells.^[67]

ROLE OF CURCUMIN IN DECREASING ADVERSE EFFECTS OF CHEMOTHERAPY

Neuroprotective effect of curcumin

Cisplatin is potent chemotherapeutic agent with adverse effects like nephrotoxicity and peripheral neuropathy. Mendonca *et al.*^[68] reported the neuroprotective effect of curcumin against cisplatin induced cytotoxicity without any interference of curcumin with the cytotoxic activity of cisplatin.

Anti-inflammatory action

Curcumin has proven anti-inflammatory, antioxidant, antimicrobial, hepatoprotective, immunostimulant, antiseptic, and antimutagenic properties.^[69] This anti-inflammatory action of turmeric helps to decrease the side effects like gastro intestinal inflammation due to chemotherapy or radiotherapy.

Anti-infective action

Patients who receive chemotherapy are immuno-compromised and prone to multiple infections. Curcumin with its beneficial anti-infective action would help to prevent infections and take care of minor infections.^[70-72]

ROLE OF CURCUMIN IN WOUND HEALING

After liver resection of the tumor, liver regeneration takes place. Patients with cancer have poor nutrition and poor healing following chemotherapy. The catabolic phase following surgery is enhanced and hence healing takes a long time. Curcumin would be beneficial to expedite the liver regeneration.^[73]

POTENTIAL SIDE EFFECTS OF CURCUMIN

Curcumin is generally considered safe and has been used since ages in Asian countries as a condiment. The low incidence of colorectal carcinoma in India has been linked to the consumption of curcumin in all meals. There have been no side effects in the daily consumption in cooked food. However, when consumed raw in larger doses, it may

cause gastric irritation, stomach upset, nausea, diarrhoea, allergic skin reaction, and antithrombosis activity. The Food and drug administration has declared curcumin as: generally regarded as safe.^[70] Curcumin exhibits both antioxidant and prooxidant activities.^[73] These opposing actions of curcumin might be regulated by its concentration that might switch roles. Thus research studies are needed to study the effects of curcumin in different conditions and the doses need to be titrated to get the maximum benefit. Till date, there have not been any long-term studies with curcumin, which show its toxic or adverse effects. Such studies are necessary in both animal models and human subjects to determine the long term safety of curcumin. Currently, there are no carcinogenic effects of consuming curcumin in doses of around 100-200 mg/day over long periods of time.^[70]

CONCLUSION

Liver cancer is a leading cause of death in children and adults. The treatment revolves around chemotherapy, radiotherapy and surgery. Recent advances include transcatheter arterial chemoembolization, radioembolization, anti-angiogenic drugs like sorafenib and liver transplantation in advanced stages. Despite improving diagnostic methods, the results have been far from satisfactory mainly due to advanced stage at diagnosis and the side effects of chemotherapy. However, the successful cure of liver cancer mandates destruction of both the differentiated neoplastic cells and the potential cancer stem cells. The conventional anticancer therapies reduce the tumor mass, but potentially leave behind cancer-initiating cells. Thus, new combinations of therapies may be needed to overcome the complex network of signaling pathways, and ultimately inhibit the signaling that controls tumor growth and survival. Adjuvant curcumin along with the current modalities of treatment may help to overcome the side effects and also have synergistic action as an anti-cancer agent.

Curcumin has been reported to inhibit telomerase activity in human cancer cell lines.^[74] Synergistic anti-cancer effects of curcumin has also been demonstrated in conjunction with chemotherapeutic drugs such as doxorubicin and paclitaxel by *in vivo* animal models, and with cisplatin, 5-FU, and adriamycin by *in vitro* studies.^[75-79]

Synergistic effects of curcumin have also been demonstrated in combined treatment with anti-angiogenic agents such as leflunomide and perindopril in *in vivo* mice models.^[80]

Thus, to conclude, curcumin has a lot of potential to act as an adjuvant remedy in liver cancer. As far as toxicity issue is concerned, herbal medicines are much safer, have less adverse effects and relatively cheaper than conventional medicines. Curcumin as an adjunct would have a synergistic

anti-cancer action and would also protect against the side effects of the current chemotherapeutic agents. Previous studies have also claimed its antitumor effects against various types of cancers due to its inhibitory effects on many types of pathways. In this article we have discussed various pharmacological activities of curcumin along with its various antitumor mechanisms.

As we have discussed, oxidative stress is a risk factor cancer. Curcumin, being a strong antioxidant has been proved to scavenge reactive species and can control tumor cell proliferation. Although preclinical results are promising but its clinical use in the treatment of HCC and HB remains to be elucidated.

Curcumin has the ability to modify many signaling pathways demonstrating its anti-tumor potential. Also, we noticed that curcumin has been proved to possess strong anti-oxidant and anti-inflammatory properties. Curcumin also targets principal anti-angiogenic molecules like VEGF and COX-2. All these properties of curcumin are essential for its use as a therapeutic anti-tumor agent. It provides a future perspective for the development of a novel adjuvant anticancer agent for humans.

Poor bioavailability and hydrophobicity of curcumin are the main obstacles in its path to be used clinically as an anti-tumor agent. However this issue can be resolved with the advancements in the drug delivery like formation of nanoparticles and microcells of curcumin via polymerization and these can be used to target cancerous cells without affecting other normal cells. Thus we can conclude that curcumin might be a promising candidate as an adjuvant therapy for liver cancer in the future but further research is needed to elucidate its various mechanisms of action, to reveal its therapeutic strategy and to titrate the dose required to reap maximum benefit.

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Conflicts of interest

There are no conflicts of interest.

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Coffee, Traditional Chinese Medicine and cannabinoids as potential tools for prevention and treatment of hepatocellular carcinoma

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ABSTRACT

In the last decade, the incidence of hepatocellular carcinoma (HCC) is growing in both Europe and United States. Conventional therapies such as liver resection, transplantation, ablation, chemoembolization and sorafenib are not enough to avoid a significant mortality. Many studies suggested the positive effect of caffeine for prevention of HCC. Nevertheless, the amount of therapeutic caffeine and the high-dose safety are unknown. Many authors proposed Traditional Chinese Medicine as preventive and/or curative approach. Although it reveals limits such as the uncertain safety profile and the lack of evidence about a unique product, it shows interesting results in terms of survival and quality of life if given in combination with standard loco-regional therapy. Among the future promises, cannabinoids show interesting background mechanisms of blocking cell proliferation and neoangiogenesis. It is conceivable that in the next years, some natural products may have a role in improving the standards of care of HCC.

Key words: Hepatocellular carcinoma; caffeine; Traditional Chinese Medicine; cannabinoids

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INTRODUCTION

Liver neoplasm represents the sixth most common cancer and the third cause of cancer-related mortality worldwide.^[1] Hepatocellular carcinoma (HCC) is the main liver cancer,

accounting for more than 90% of cases of liver tumors. In the last decades, the HCC incidence and HCC-related mortality are increasing in both United States and Northern Europe.^[2] Cirrhosis due to chronic hepatitis B and C, is the major risk factor for the HCC development. However, also other

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potentially risky conditions such as alcohol intake, tobacco habit, overweight, diabetes, aflatoxin consumption and oral contraceptives use, should be considered.^[3]

Barcelona-Clinic Liver Cancer (BCLC) staging system is a widely used set of criteria to guide management of patients with HCC. It takes into account tumor stage, liver functional status, physical status and cancer-related symptoms.^[4] Surgical treatment of HCC is a potentially curative approach, including liver transplantation (LT) and liver resection. LT is the best treatment option for patients fitting the “Milan criteria” since it removes both neoplasm and underlying liver disease. For patients with single tumor < 2 cm, with a Child-Turcotte-Pugh class A, without clinically significant portal hypertension and with normal bilirubin, liver resection represents a feasible strategy.^[4] Ablation with ethanol or acetic acid or thermal, is another potentially curative option. It is practicable in patients with single, small tumors not candidates for surgery.^[4] Many HCC cases are diagnosed in stage B of BCLC algorithm, for which the standard of care is the transcatheter arterial chemoembolization (TACE).^[4] Lastly, sorafenib is the unique universally approved systemic palliative drug for BCLC C patients.^[4]

In the European^[5] and in the North-American^[6] guidelines, no natural product is mentioned neither for the prophylaxis nor for the treatment of HCC. On the contrary, in the Asian-Pacific ones,^[7] natural products are cited for both primary and secondary prophylaxis.

Literature data are available regarding the coffee-derived substances as prevention tools in high-risk populations, the possible prevention or adjuvant effect of many kinds of Traditional Chinese Medicine (TCM), and possible utility of cannabinoids as antineoplastic drugs.

Hereby, we sought to review the current knowledge on the role of some natural products in the prevention and treatment of HCC. The research included published articles (peer reviewed original articles, review articles and meta-analyses). The search terms included “natural products and hepatocellular carcinoma”, “natural products and liver”, “hepatocellular carcinoma treatment options”, “coffee and HCC”, “Traditional Chinese Medicine and HCC”, and “cannabinoids and HCC”.

COFFEE

Many data are available about the dose-dependent protective effect of coffee respect to the development of liver disease and HCC.^[8] Both *in vitro* and *in vivo* studies showed that several coffee compounds such as diterpenes, cafestol and kahweol, may act on some enzymes involved in carcinogenesis.^[9,10] Diterpenes, cafestol and kahweol seem to modify the xenotoxic metabolism via induction of glutathione-S-transferase and inhibition of N-acetyltransferase.^[11] Caffeine and antioxidant substances from coffee beans, may improve some liver enzymes, such as γ -glutamyltransferase and

aminotransferase. Interestingly, this positive effect of caffeine is mainly relevant in heavy drinkers.^[12,13] Notably, coffee consumption would be inversely related to the hazard of cirrhosis, which is the main risk factor of HCC.^[14,15]

Although some authors^[16,17] suggested a not statistically significant association between coffee consumption and risk of HCC, many other studies reported positive results.

In an Italian case-control study (including 250 HCC),^[18] coffee intake showed a significant protective role against HCC. In all patients, ten-year coffee intake was associated with a decreased risk of HCC with a dose-effect relation (double with 3-4 cups/day respect to 1-2 cups/day).

In a further Italian study (185 HCC),^[19] patients drinking ≥ 4 cups/day (no decaffeinated) had a lesser risk of HCC respect to the others.

Tanaka *et al.*^[20] developed a Japanese case-control study (209 HCC) showing that coffee consumption during the last 1-2 years, was associated with a decreased risk of HCC. Another Japanese case-control study including 73 HCC, analyzed the role of coffee in patients with hepatitis C.^[21] Coffee drinking ≥ 1 cup/day significantly reduced the risk of HCC compared to the abstinence. The same data were found for hepatitis B chronic carriers^[22] with a risk reduction of 30-80%.

Two large Japanese prospective studies^[23,24] including hepatitis B, C and sieronegative subjects, reported that drinkers of ≥ 5 cups/day had a lower dose-dependent HCC risk respect to abstinent patients.

The relationship between coffee and risk of HCC was studied also by Johnson *et al.*^[25] through a large prospective study including 63,257 patients. The authors reported that subjects consuming ≥ 3 cups/day experienced a 44% of HCC risk reduction.

Hu *et al.*^[26] firstly analyzed the possible association between coffee consumption, serum gamma-glutamyltransferase and HCC. The study cohorts included 60,323 patients without cancer. During a median follow-up period of 19.3 years, 128 participants developed HCC. According to the author's data, a combination of very low coffee consumption and high level of serum GGT was associated with nearly nine-fold increased risk of HCC.

In 2007, Bravi *et al.*^[27] performed a meta-analysis based on 10 studies (both European and Asian) and a total of 2,260 HCC cases. Authors reported a 41% of reduction in HCC risk among coffee drinkers compared to non-drinkers. In the same year, Larsson *et al.*^[28] published another meta-analysis with similar conclusions. In 2013, Bravi *et al.*^[29] conducted a further meta-analysis including more recent studies. According to the authors, coffee drinkers had a decrease of 40% in the risk of HCC compared to abstinent patients. Moreover, high coffee drinkers showed more than 50% of risk drop. Notably,

the protective effect of coffee was reliable across different subgroups at increased HCC risk.

After the publication of these meta-analyses, other studies regarding the protective role of coffee in the HCC setting have been published. The first one, was a multicentre study by Bamia *et al.*^[30] including 201 HCC cases. Authors demonstrated that coffee intake was associated with a decrease of 72% in HCC risk. Setiawan *et al.*^[31] conducted a large population-based prospective cohort study (451 HCC) showing that drinkers of 2-3 cups/day respect to abstinent subjects, had a 38% of HCC risk reduction. In addition, patients drinking 4 or more cups/day had a 41% of risk drop. Feld *et al.*^[32] again suggested that regular ingestion of coffee in patients with chronic liver disease can make slower the progression of liver fibrosis, preventing both cirrhosis and HCC. Petrick *et al.*^[33] developed the Liver Cancer Pooling Project based on North-American data and including 1,212,893 patients (with 860 HCC cases). A high caffeinated coffee consumption (≥ 4 cups/day) was associated with a lower risk of HCC in comparison to a lesser intake. In a Japanese cohort-study^[34] including 258 cases, an inverse association was reported between coffee and mortality associated to HCC. Interestingly, the hazard of HCC-related death for abstinent patients was two-fold higher compared to coffee drinkers, and this was true also for few consumption (≥ 1 cup/day).

TCM

Many authors proposed TCM-based therapy alone or in combination with standard loco-regional therapies for prevention or treatment of HCC [Table 1]. The main TCM products include combinations of different herbal medicines or animal/insect extracts. Astragalus shows immunomodulatory properties and anti-tumor activity. It seems to reinforce Lymphokine Activated Killer cell activity restoring the T-cell function suppressed in cancer patients.^[35,36] The Panax Ginseng has inhibitory effects on cell proliferation and angiogenesis^[37] restraining tumor cell

invasion and defeating sister chromatid interactions in human lymphocytes.^[38] Toad skin secretion bufalin (Bufotoxin) could induce apoptosis in human-leukaemia cells modifying the expression of some apoptotic genes.^[39] Other toad skin secretions such as 3-formyloxyresibufogenin, 19-oxobufalin, 19-oxodesacetylcinobufagin, 6-hydroxycinobufagin and 1-hydroxybufalin seem to have inhibitory properties on KB, human promyelocytic leukemia cells (HL-60) and MH-60 cancer cell lines.^[40] *Mytilus phalerata* (Mytilus) can lead to the apoptosis of tumor cells^[41] while *Atractylodes* might bring apoptosis and have cytotoxic effects against tumor cells.^[42] *Bupleurum falcatum* shows a significant anti-cell adhesive activity on solid tumor cells^[43] and *Curcuma longa* has a relevant immunostimulatory activity.^[44]

Concerning the prevention ability of herbal products, a Japanese herb called Sho-saiko-to has to be cited since it is reported in the Asian-Pacific guidelines.^[7] In a randomized controlled trial (RCT),^[45] Sho-saiko-to was shown to improve liver function in patients with chronic hepatitis. Also Oka *et al.*^[46] reported that Sho-saiko-to may prevent the development of HCC in cirrhotic subjects. Successive studies with liver cell lines confirmed the above-cited suggestions.^[47,48]

In 2013, Zhai *et al.*^[49] compared in a RCT the efficacy of TCM and TACE in preventing recurrence of small HCC after resection. Authors tested TACE or TCM as adjuvant therapy for patients who underwent surgery without evidence of recidivism. One hundred and eighty-eight patients received Cinobufacini injection (extract from *Bufo bufo gargarizans* Cantor) and Jiedu Granule (a compound herbal medicine). The other patients (191 cases) were assigned to the TACE subgroup. TCM was associated with decreased HCC recurrence after resection in comparison to TACE, with similar adverse events.

Regarding the use of TCM alone as therapeutic tool, Tian *et al.*^[50] demonstrated that it may be effective in subjects affected by middle/late stage HCC. In this RCT, 97 patients were treated with *Oleum fructus bruceas*, Ganji Decoction and external application of Ailitong, and 48 patients received

Table 1. The main natural products from Traditional Chinese Medicine

| Product | Type | Main property/ies | Studies in humans | RCTs | Meta-analysis | Ref. |
|--------------------|---------------------|-------------------------------------|-------------------|------|---------------|---------------|
| Astragalus | Herb | Restores T-cell | Yes | Yes | Yes | [35,36,53,55] |
| Panax | Herb | Anti-proliferation and angiogenesis | Yes | Yes | Yes | [37,38,55] |
| Ginseng | | | | | | |
| Bufotoxin | Toad skin secretion | Induces apoptosis | Yes | Yes | Yes | [39,55] |
| Atractylodes | Herb | Induces apoptosis | No | No | No | [42] |
| Bupleurum falcatum | Herb | Anti-adhesive activity | No | No | No | [43] |
| Curcuma longa | Herb | Immunostimulatory activity | No | No | No | [44] |
| Cinobufacini | Bufo skin extract | Induces apoptosis | Yes | Yes | No | [49] |
| Jiedu | Herb | Unreported | Yes | Yes | No | [49] |
| Sho-saiko-to* | Herb | Decreases collagen type 1 | Yes | Yes | No | [7,45-48] |
| Bruceas | Fruit extract | Unreported | Yes | Yes | No | [50] |
| Ganji | Herb | Unreported | Yes | Yes | No | [50] |
| Ailitong | Herb | Unreported | Yes | Yes | No | [50] |
| Kanglaite | Herb | Immunomodulation | Yes | Yes | Yes | [56] |

*It comes from the Japanese Tradition

chemotherapy. The HCC progression was similar between the two groups, but the TCM approach showed less adverse reactions. Moreover, survival rate at three months was comparable, while the test group had a better half- and 1-year survival.

Man *et al.*^[51] studied 94 patients with unresectable HCC. Authors compared three subgroups: (1) patients receiving TCM with non-curative antitumor treatments of Western Medicine; (2) patients receiving TCM alone; and (3) patients treated with non-curative antitumor treatments of Western Medicine or supportive treatment alone. They showed that patients treated with the combination schedule respect to patients in Western therapy alone, showed a significantly better 1- and 2-year survival (76.0% and 55.5% vs. 55.8% and 30.8%, respectively).

In 2005, Shu *et al.*^[52] analysed 26 RCTs reporting that TCM might determine an advantage in terms of both neoplasm response and long-term patient survival. Notably, authors did not specify the kind of used natural product. McCulloch *et al.*^[53] compared 34 RCTs, including 2,815 subjects, demonstrating that *Astragalus*-based TCM increased the efficacy of platinum chemotherapy. In 2009, two meta-analyses reported data concerning the possible role of TCM in association with TACE. Cho and Chen^[54] analyzed 30 studies showing an improved long-term survival in patients treated with the association between TACE and TCM respect to the subjects who did not receive TCM. According to this study, TCM determined a relevant increase in white blood cell count, a substantially lower nausea and vomiting, and a significant rise in the body weight. Wu *et al.*^[55] systematically reviewed and meta-analyzed a series of Chinese RCTs concerning the efficacy of TCM for the treatment of HCC. Authors reported some criticisms of the analyzed trials suggesting that the methodological issues were poor. Nevertheless, the studies with bufotoxin, *astragalus* (with or without *mylabris*) and ginseng associated to TACE, showed lower HCC recurrence and better patient survival in comparison to TACE alone. However, authors suggested that these data should be confirmed in further well-conducted Western RCTs.

Kanglaite (KLT) is a TCM coming from the seeds of a tropical Asian grass called Coix. It exhibits antitumor and immunomodulatory activity. Fu *et al.*^[56] performed a meta-analysis including nine clinical trials to evaluate the efficacy of KLT injection combined with hepatic arterial intervention for the treatment of unresectable HCC. KLT injection combined with hepatic arterial intervention respect to arterial therapy alone, seemed to improve both short-term clinical efficacy and pain's control.

CANNABINOIDS

Cannabinoids are lipid mediators isolated from the hemp

plant *Cannabis sativa* that can activate two G-protein-coupled receptors.^[57] The active ingredients of Cannabis, as well as their synthetic analogues, are bioactive lipids that seem to block cell proliferation, reduce cell migration and inhibit angiogenesis.^[58] The molecular mechanisms involved in the antineoplastic and anti-HCC action are debated. G protein-coupled receptor type 1 and 2 are typically considered the cannabinoid receptors. However, these substances may impact on other targets such as nuclear receptors peroxisome proliferator-activated receptor (PPAR)s.^[59] PPARs are ligand-activated transcription factors, which belong to the nuclear receptor superfamily and mediate lipid metabolism, energy balance and anti-inflammatory cascade.^[60] Several PPAR ligands have been shown to decrease HCC cell proliferation and migration through PPAR activation.^[61] Moreover, utilizing a PPARγ knockout mice model, it was suggested that PPAR decreases HCC carcinogenesis acting as tumor-suppressor gene in the liver.^[62] Notably, the synthetic cannabinoid WIN 55,212-2 seemed to increase PPAR expression leading to apoptosis in the HCC HepG2 cell line.^[63] Vara *et al.*^[57] demonstrated that D9-tetrahydrocannabinol and JWH-015 (two kind of cannabinoids), might induce autophagy in HCC cells stimulating the AMP-activated protein kinase pathway. Jiang *et al.*^[64] studying the PPAR-deficient mice, demonstrated the accumulation of autophagic vacuoles and up-regulation of autophagic marker LC3 protein expression. These results are in agreement with the above reported observations by Vara *et al.*^[57] These authors suggested a connection between PPAR and autophagy-essential proteins in mammalian HCC. Also Vara *et al.*^[65] reported the involvement of PPAR activation in the anti-cancer effect of cannabinoids. The authors showed that THC and JWH-015 might increase mRNA and protein levels of PPAR inducing PPAR activation *in vitro*. Moreover, the authors showed that, when endoplasmic reticulum stress-related protein tribbles homolog 3 (TRIB 3) is genetically inhibited, the expression of both PPAR mRNA and protein decreased. Indeed, TRIB 3 seemed to have a significant role in regulating cannabinoid-induced PPAR overexpression. Cannabinoid treatment could improve phosphorylated-eIF2α (an endoplasmic reticulum stress marker) and the endoplasmic reticulum stress-related pseudokinase TRIB 3. Notably, this latter is necessary for cannabinoid-induced cell death and the consequent anti-tumor effect.^[66] Regarding the role of TRIB 3, Takahashi *et al.*^[67] demonstrated that it can downregulate PPAR transcriptional activities by protein-protein interaction in 3T3-L1 adipocytes.

CONCLUSIONS AND FUTURE PERSPECTIVES

HCC represents one of the most common cancers worldwide and is the third cause of neoplasm-related death. Since chronic viral hepatitis are the main risk factors for HCC, the vaccination against hepatitis B and the treatment of both hepatitis B and C, represent the main preventive therapies. Today, the potentially curative (LT, resection, ablation) and palliative (arterial chemoembolization, sorafenib) standards of care still do not protect from a relevant rate of mortality.

Cohort studies and meta-analyses suggest that high coffee intake might prevent the HCC in subgroups of patients at increased risk. Nevertheless, the mechanisms involved and the specific components of coffee beverages that may determine this sort of protection are unknown. The available studies often report different cut-offs of coffee intake, besides not taking into account many potential confounders. Moreover, registration of coffee consumption depends mainly on the self-reporting questionnaires with intrinsic relevant statistical limits. Consequently, it is difficult to establish the temporal relationship between coffee use, liver disease and HCC onset. Indeed, the open questions are the following: how much coffee is necessary and for how long time? Which is the long-term safety profile of high-dose caffeine?

Concerning TCM, many authors proposed it, alone or in association with standard therapy. Notably, the studies proposing TCM approach alone for the treatment of HCC show no strong data. The TCM treatment obtains some interesting results if administered together with TACE. In particular, RCTs and meta-analyses demonstrate an advantage in terms of both patients' survival and quality of life in comparison with the Western approach alone. However, there are many unclear aspects: which single product of the TCM large family is the best? Which is the impact of TCM on the liver function? Which is the safety profile of each TCM product?

Many basic studies suggest that cannabis could block cell proliferation, reduce cell migration and inhibit angiogenesis thus showing an anticancer attitude. Several data show a relationship between PPAR receptor and autophagy-essential proteins in HCC but the mechanisms involved in the antineoplastic action of cannabinoids are still debated. Furthermore, the lack of data on humans makes difficult to consider these substances as therapeutic choices.

It may be that the described natural products could have a future in the prevention of HCC, in the strengthening of the standard therapy and in the palliative phase. Still, further RCTs with strong results are mandatory for their effective broad application.

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Conflicts of interest

There are no conflicts of interest.

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Dietary incorporation of jojoba extract eliminates oxidative damage in livers of rats fed fumonisin-contaminated diet

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ABSTRACT

Aim: This study aimed to determine the composition of ethanol extract of jojoba seeds, and to evaluate its hepatoprotective effects in rats fed fumonisin B₁ (FB₁)-contaminated diet. **Methods:** Jojoba seeds were extracted in 95% ethanol, and the chemical composition was determined. Male rats were divided into six groups and treated for 8 weeks as follows: (1) Untreated control; (2) FB₁-contaminated diet (80 mg/kg diet); (3) low dose (0.5 mg/kg b.w.) jojoba extract; (4) high dose (1.0 mg/kg b.w.) jojoba extract; (5) low dose jojoba extract plus FB₁; and (6) high dose jojoba extract plus FB₁. Blood and liver samples were collected for different biochemical analyses and histological examinations. **Results:** The results indicated that the ethanolic extract of jojoba is rich in protein, phenolic compounds, phytic acid, and considerable amounts of simmondsin. Animals fed FB₁-contaminated diet showed severe biochemical and histological changes typical to those reported in literature. Treatment with jojoba seed extract alone at the two tested doses did not induce significant alterations in all parameters tested. Combined treatment of jojoba seed extract with FB₁ eliminated hepatotoxicity induced by FB₁, especially at low dose of jojoba seed extract. **Conclusion:** The authors concluded that jojoba seed extract can be incorporated in FB₁-contaminated feed to eliminate FB₁-induced hepatotoxicity.

Key words: Fumonisin B₁; health hazards; jojoba seed; liver; mycotoxins; oxidative stress

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INTRODUCTION

Fumonisin B (FBs) are mycotoxins produced by the fungal species *Fusarium*, including *Fusarium verticillioides* and *Fusarium proliferatum*.^[1,2] This mycotoxin is mainly produced on corn and possibly sorghum, which remain the primary sources of human exposure.^[3,4] At least 28 FBs have been isolated and characterized.^[5]

Fumonisin B₁ (FB₁) is the most common toxin, which has been classified by the International Agency for Research on Cancer as a Group 2B carcinogen (possibly carcinogenic in humans).^[6] Long-term studies indicated that FB₁ was hepatocarcinogenic in rats^[7-9] while another study reported on the nephrocarcinogenicity and cancer promoting activity in rats.^[10,11] Epidemiological

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evidence suggests that it may be an etiological agent in human esophageal cancer.^[12,13] Several studies in rodents have shown that FB₁ promotes pre-neoplastic lesions in the liver, suggesting a role for FB₁-induced genotoxicity.^[14] Recently, Chuturgoon *et al.*^[15] reported that FB₁ induces global DNA hypomethylation and histone demethylation in human hepatoma cells that causes chromatin instability and may lead to liver tumorigenesis. FB₁ is resistant to conditions normally used in food processing and, therefore, poses a significant hazard to human and animal health.^[16] The cytotoxic mechanism of FB₁ is attributed to its disruption of sphingolipid metabolism; the underlying mechanisms of its cancer initiating/promoting properties are unknown. This disturbance of sphingolipid metabolism plays a role in membrane and lipoprotein structure and in cell regulation as secondary messengers for growth factors, differentiation factors, and cytokines.^[8]

Jojoba (*Simmondsia chinensis* L) is a perennial woody shrub native to semi-arid regions all over the world.^[17] Currently, it is cultivated in the Ismailia Desert in Egypt.^[18] The jojoba plant produces seeds that contain up to 50% liquid wax used as a lubricant additive and in cosmetics.^[19] It has been reported that jojoba seeds possess anti-inflammatory activity.^[20] Moreover, jojoba liquid wax was used in folk remedies for renal colic, sunburn, chaffed skin, hair loss, headache, wounds, and sore throat.^[21] Jojoba meal is the protein residue remaining after oil extraction, and it has potential as dietary supplements for animal feeds, as well as for the treatment of overweight animals and humans.^[22] This protein meal consists mainly of 79% albumins and 21% globulins.^[23] Previous reports indicated that jojoba meal contained anti-nutritional compounds known as simmondsins (5-demethylsimmondsin, 4,5-didemethylsimmondsin, simmondsin, and simmondsin 2'-ferulate),^[24] which have been identified as the component in jojoba that is most responsible for the inhibition of food intake and for appetite suppression in rodents, rats, dogs, and chickens.^[25] However, the meal also contains several beneficial compounds, such as phytic acid and polyphenols, which shows antioxidant and anti-cancer activity.^[26] The aim of this study was to evaluate the effect of ethanol extract of jojoba seed in rats fed FB₁-contaminated diet.

METHODS

Chemicals and kits

FB₁ standard was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Kits for analysis of aspartate

aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), triglycerides, and cholesterol were obtained from Quimica Clinica Aplicada (SA, Spain). Interleukin-1 α (IL-1 α), procollagen III, and tumor necrosis factor- α (TNF- α) kits were purchased from Origenium (Helsinki, Finland). Kits for measuring nitric oxide (NO), malondialdehyde (MDA), total antioxidant capacity (TAC), carcinoembryonic antigen (CEA), and superoxide dismutase (SOD) were obtained from Biodiagnostic (Giza, Egypt).

Preparation of jojoba seed extract

Jojoba (*S. chinensis*) seeds were obtained from the Crops Department, National Research Center, Dokki, Cairo, Egypt. The seeds (200 g) were ground to a powder and immersed in 95% ethanol overnight. The extract was filtered and evaporated under reduced pressure of nitrogen to obtain a semisolid residue.

Determination of chemical composition in jojoba seed extract

Crude protein (N X 6.25) was determined according to AOAC^[27] and phytic acid content in jojoba seed extract was determined according to the method described by Mohamed *et al.*^[28] Total phenolics were determined according to the modified method described by Chandler and Dodds^[29] and simmondsin content was determined according to Abbott *et al.*^[30]

FB₁ production

FB₁ was produced through the fermentation of corn by *Fusarium moniliforme* (obtained from Plant Pathology Department, National Research Center, Dokki, Cairo, Egypt) as described by Voss *et al.*^[31] The fermented corn was autoclaved; ground to a powder and the FB₁ content was measured by high-performance liquid chromatography (HPLC) according to Shaphard *et al.*^[32] The corn powder was incorporated into the basal diet to provide the desired level of 80 mg FB₁/kg diet. The diet containing FB₁ was analyzed, and the presence of FB₁ was confirmed by HPLC.

Experimental animals

Three months old male Sprague-Dawley rats (100-120 g) were purchased from the Animal House Colony, Giza, Egypt and were maintained on standard laboratory diet (protein: 160.4; fat: 36.3; fiber: 41 g/kg and metabolizable energy: 12.08 MJ) in artificial illuminated and temperature controlled room free from any other sources of chemical contamination at the Animal House Lab., National Research Center, Dokki, Cairo, Egypt. All animals received humane care in compliance with the guidelines of the Animal Care and Use Committee of the National Research Center, Dokki, Cairo, Egypt.

Experimental design

After an acclimatization period of 1 week, the animals were divided into six groups (10 rats/group) and housed in filter-top polycarbonate cages. The rats were maintained on their respective diet for 8 weeks as follows: (1) Untreated control; (2) FB₁-contaminated diet (80 mg/kg diet); (3) low dose of jojoba seed extract (JELD) (0.5 mg/kg b.w.); (4) high dose of jojoba seed extract (JEHD) (1.0 mg/kg b.w.); (5) FB₁-contaminated diet and treated with JELD; and (6) FB₁-contaminated diet and treated with JEHD. Body weight and food intake were recorded daily throughout the treatment period. At the end of the treatment period, blood samples were collected from the retro-orbital venous plexus of all animals after fasting for 12 h. The blood sample from each animal was left to clot and centrifuged at 5,000 g under cooling for 10 min to separate the serum for the determination of ALT, AST, ALP, triglycerides, cholesterol, NO, IL-1 α , TNF- α , and CEA according to the respective kit instructions. After collection of blood samples, all animals were sacrificed and liver samples of each animal were dissected, weighed, and homogenized in phosphate buffer (pH 7.4) to give 20% w/v homogenate. This homogenate was centrifuged at 1,700 g and 4 °C for 10 min and the supernatant was stored at -70 °C for the determination of lipid peroxidation by measuring the formed MDA using thiobarbituric acid reactive substances. The level of lipid peroxidation was expressed as nmol MDA per gram tissue. The liver homogenate was further diluted to give 5% homogenate (w/v), centrifuged at 3,000 g for 5 min at 0 °C and used for the determination of SOD and TAC. Another liver sample of each animal was dissected, excised, and fixed in 10% neutral formalin; dehydrated in ascending grades of ethanol; cleaned in xylene; and embedded in paraffin. Five micrometer thick sections were prepared and stained with hematoxylin and eosin according to Drury *et al.*^[33]

Statistical analysis

All data were statistically analyzed using the General Linear Models Procedure of the Statistical Analysis System.^[34] The significance of the differences among treatment groups was determined by Waller-Duncan k-ratio.^[35] All statements of significance were based on probability of $P \leq 0.05$.

RESULTS

Composition of ethanol extract of jojoba seeds

The results of this study revealed that ethanol extract of jojoba seeds is rich in crude protein (27.32 g/100 g seeds) and total phenolic content (65.53 mg/100 g). Phytic

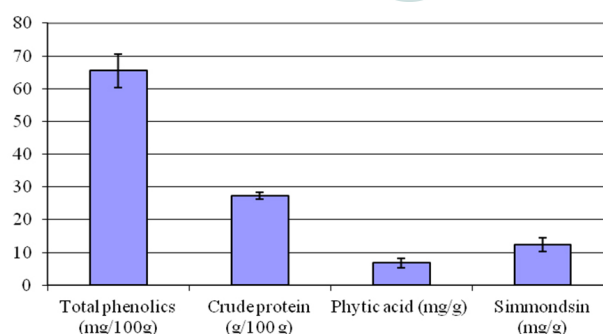


Figure 1: Chemical composition of the ethanol extract of jojoba seeds

acid content was 6.83 mg/g, and simmondsin content reached 12.43 mg/g [Figure 1].

Effect of jojoba seed extract on food intake and body weight

The effect of different treatments on food intake indicated that the acute toxicity of FB₁ first appeared as a significant decrease in food intake [Figure 2a]. Animals fed FB₁-contaminated diet showed a significant decrease in food intake throughout the treatment period compared to the control group. Animals treated with jojoba seed extract at both the low and high doses also showed a gradual decrease in food intake which became severe by the 7th week of treatment and was pronounced in the JEHD group. The combined treatment of FB₁ and jojoba seed extract induced a significant improvement in food intake, although the food consumption was still lower than in the control group. It is of interest to mention that the improvement in food intake was pronounced in the group fed FB₁-contaminated diet and treated with JELD [Figure 2a].

The effect of different treatments on body weight gain of rats is depicted in Figure 2b. Animals fed FB₁-contaminated diet failed to gain weight; however, animals treated with jojoba seed extract showed slight weight gain, although there was a significant difference between these groups and the control. Moreover, animals in the groups treated with the FB₁ and jojoba seed extract did not show any significant increase in body weight, and they were below the normal weight of the control group. Animals receiving combined treatment of FB₁ and jojoba seed extract showed slightly higher weight gain than those receiving FB₁ alone.

Biochemical effects of treatment with FB₁ and jojoba seed extract

The biochemical results [Table 1] revealed that FB₁ alone induced a significant increase in all biochemical parameters tested. The jojoba seed extract alone at both low and high doses did not induce any significant changes in ALT, AST, and triglycerides. However, ALP

showed a significant increase accompanied by a significant decrease in cholesterol level, especially in the high dose group. Animals fed FB₁-contaminated diet and treated with jojoba seed extract showed a significant improvement in all biochemical parameters; although all levels tested were still higher than in the control group. The observed improvement in all biochemical parameters was more pronounced in the group fed FB₁ and treated with JELD.

The data presented in Table 2 showed that treatment with FB₁ resulted in a significant increase in serum CEA, TNF- α , IL-1 α , and NO. Animals treated with JELD or JEHD alone were comparable to the control group in terms of the levels of CEA, TNF- α , and NO, however, the level of IL-1 α showed a significant increase. Treatment with FB₁ plus JELD or JEHD resulted in a significant improvement in all the tested parameters toward the control values; in the JEHD group, CEA and TNF- α levels were normalized [Table 2].

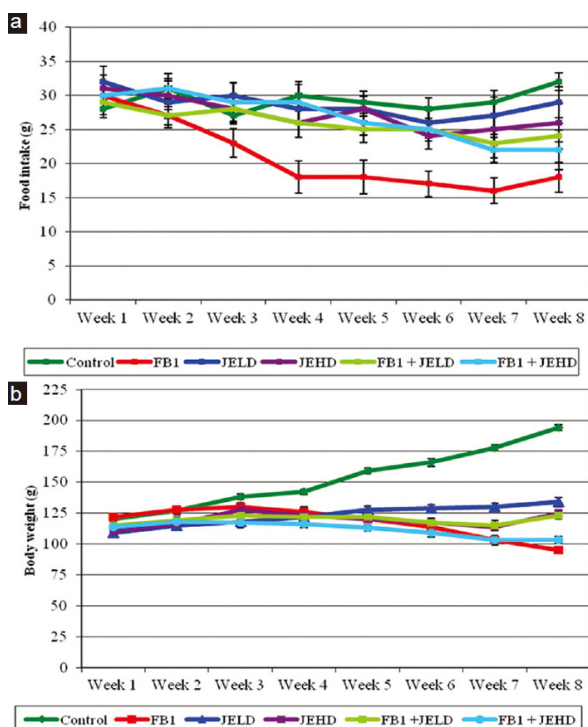


Figure 2: Effect of jojoba extract on (a) food intake and (b) body weight in rats fed FB₁-contaminated diet. FB₁: fumonisin B₁; JELD: low dose of jojoba seed extract; JEHD: high dose of jojoba seed extract

The effect of different treatments on MDA level, glutathione (GSH), and TAC in liver tissue [Table 3] revealed that animal fed FB₁-contaminated diet showed a significant increase in MDA accompanied by a significant decrease in GSH and TAC. Treatment with JELD or JEHD did not affect MDA significantly; however, it resulted in a significant increase in GSH and TAC levels. The combined treatment of FB₁ with jojoba seed extract resulted in a significant improvement in the activity of antioxidant enzymes and decreased lipid peroxidation in the liver tissues although they were still significantly different from the control. Of note, treatment with JEHD showed the best results at improving antioxidant enzymes activity and at decreasing lipid peroxidation.

Histological changes induced by treatment with FB₁ and jojoba seed extract

The above biochemical findings were further confirmed by histological examinations in the liver tissues. The microscopic examination of the liver section of the control rats showed the normal histological structure of liver lobule and hepatocytes which form cords radiating from the central vein [Figure 3a]. The liver sections of rats fed FB₁-contaminated diet showed vacuolar degeneration, hepatocellular necrosis, and congestion of blood sinusoids which were surrounded by an aggregation of inflammatory cells, proliferation and dilation of bile duct, as well as signs of fibrosis [Figure 3b]. The liver sections of rats treated with JELD showed normal hepatocytes architecture, and dilation of sinusoid [Figure 3c]; however, liver sections of rats treated with JEHD did not show any pathological changes [Figure 3d].

The liver of rat fed FB₁-contaminated diet and treated with JELD showed marked improvement in the histological features of the hepatic tissue although minimal vacuolar degeneration was still present [Figure 3e]. However, the liver sections of rats fed FB₁-contaminated diet and treated with JEHD showed histological features resembling normal hepatocytes [Figure 3f].

DISCUSSION

Previous reports indicated that jojoba meal contained 25-30% crude protein, was high in dietary fiber, and could

Table 1: Effect of jojoba extract on biochemical parameters in rats fed FB₁-contaminated diet

| Groups parameter | Control | FB ₁ | JELD | JEHD | JELD + FB ₁ | JEHD + FB ₁ |
|-----------------------|----------------------------|----------------------------|----------------------------|----------------------------|----------------------------|----------------------------|
| ALT (IU/L) | 25.43 ± 2.73 ^a | 76.21 ± 5.18 ^b | 26.72 ± 1.33 ^a | 27.44 ± 2.28 ^a | 34.93 ± 2.28 ^c | 38.21 ± 2.93 ^c |
| AST (IU/L) | 85.16 ± 3.27 ^a | 112.73 ± 4.22 ^b | 88.25 ± 2.53 ^a | 88.76 ± 4.73 ^a | 96.23 ± 4.92 ^c | 102.32 ± 2.83 ^c |
| ALP (IU/L) | 82.25 ± 4.28 ^a | 123.32 ± 6.43 ^b | 92.28 ± 4.34 ^c | 97.28 ± 6.22 ^c | 95.28 ± 7.22 ^c | 115.24 ± 2.93 ^d |
| Triglycerides (mg/dL) | 122.21 ± 3.74 ^a | 243.24 ± 6.43 ^b | 122.34 ± 3.37 ^a | 125.74 ± 2.56 ^a | 142.32 ± 4.89 ^c | 142.73 ± 3.82 ^c |
| Cholesterol (mg/dL) | 87.23 ± 3.26 ^a | 287.82 ± 7.78 ^b | 79.83 ± 5.38 ^c | 72.34 ± 5.64 ^c | 111.96 ± 3.88 ^d | 117.26 ± 3.95 ^d |

Within each row means superscript with different letters are significantly different at $P \leq 0.05$. FB₁: fumonisin B₁; JELD: low dose of jojoba seed extract; JEHD: high dose of jojoba seed extract; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase

serve as a feed supplement.^[19] It represents one of the non-traditional plant protein sources. However, several trials have demonstrated growth retardation in animals consuming diets supplemented with jojoba meal^[36] due to the presence of simmondsin and simmondsin-

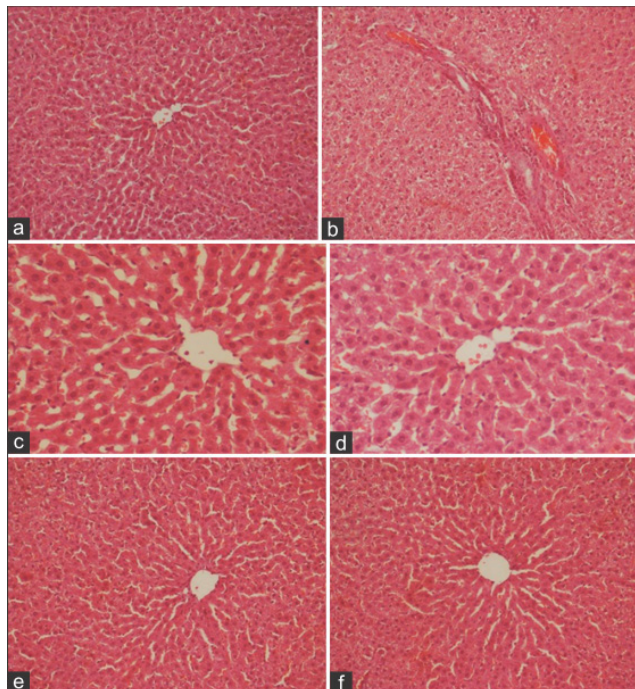


Figure 3: A photomicrograph of liver section of (a) control rat showing normal structure of hepatic lobule, central vein, and blood sinusoid(s); (b) rat fed FB₁-contaminated diet showing vacuolar degeneration, hepatocellular necrosis, and congestion of blood sinusoids which are surrounded by aggregation of inflammatory cells, proliferation and dilation of bile duct, and signs of fibrosis; (c) rat treated with JELD showing normal hepatocytes architecture and dilation of sinusoid; (d) rat treated with JEHD showing no pathological changes; (e) rat fed FB₁-contaminated diet and treated with JELD showing marked improvement in histological features of hepatocyte tissue with minimal vacuolar degeneration still present; and (f) rat fed FB₁-contaminated diet and treated with JEHD showing histological features resembling that of normal hepatocytes (a, e, f: HE, ×200; b, c, d: HE, ×400). FB₁: fumonisin B₁; JELD: low dose of jojoba seed extract; JEHD: high dose of jojoba seed extract

Table 2: Effect of jojoba extract on serum cytokines and NO in rats fed FB₁-contaminated diet

| Groups parameter | CEA (ng/mL) | TNF-α (ng/L) | IL-1α (ng/mL) | NO (μmol/L) |
|------------------------|--------------------------|---------------------------|--------------------------|---------------------------|
| Control | 1.99 ± 0.42 ^a | 43.2 ± 3.53 ^a | 0.68 ± 0.02 ^a | 23.72 ± 2.11 ^a |
| FB ₁ | 8.66 ± 1.43 ^b | 87.32 ± 3.21 ^b | 5.12 ± 0.87 ^b | 57.28 ± 3.21 ^b |
| JELD | 1.92 ± 0.72 ^a | 43.32 ± 1.98 ^a | 0.81 ± 0.04 ^c | 26.72 ± 1.73 ^c |
| JEHD | 1.98 ± 0.62 ^a | 45.37 ± 3.25 ^a | 0.81 ± 0.06 ^c | 29.83 ± 1.83 ^d |
| FB ₁ + JELD | 3.53 ± 0.43 ^c | 62.11 ± 3.47 ^c | 1.55 ± 0.12 ^d | 28.94 ± 1.29 ^d |
| FB ₁ + JEHD | 2.14 ± 0.22 ^a | 42.18 ± 2.33 ^a | 1.22 ± 0.07 ^d | 32.93 ± 2.94 ^e |

Within each row means superscript with different letters are significantly different at $P \leq 0.05$. FB₁: fumonisin B₁; JELD: low dose of jojoba seed extract; JEHD: high dose of jojoba seed extract; CEA: carcinoembryonic antigen; TNF-α: tumor necrosis factor-alpha; IL-1α: interleukin 1 alpha; NO: nitric oxide

Table 3: Effect of jojoba extract on antioxidants and lipid peroxidation in liver of rats fed FB₁-contaminated diet

| Groups parameter | Control | FB ₁ | JELD | JEHD | JELD + FB ₁ | JEHD + FB ₁ |
|----------------------|----------------------------|----------------------------|----------------------------|----------------------------|----------------------------|----------------------------|
| MDA (mol/mg protein) | 66.85 ± 2.37 ^a | 115.36 ± 3.44 ^b | 67.26 ± 2.73 ^a | 68.23 ± 3.16 ^a | 87.74 ± 3.19 ^c | 85.91 ± 3.02 ^c |
| SOD (u/mg protein) | 331.43 ± 8.65 ^a | 166.74 ± 7.34 ^b | 352.33 ± 3.46 ^c | 348.93 ± 5.88 ^c | 258.33 ± 6.72 ^d | 277.76 ± 4.27 ^d |
| TAC (mol/g protein) | 82.25 ± 4.28 ^a | 123.32 ± 6.43 ^b | 92.28 ± 4.34 ^c | 97.28 ± 6.22 ^c | 95.28 ± 7.22 ^c | 104.33 ± 2.75 ^d |

Within each row means superscript with different letters are significantly different at $P \leq 0.05$. FB₁: fumonisin B₁; JELD: low dose of jojoba seed extract; JEHD: high dose of jojoba seed extract; MDA: malondialdehyde; SOD: super oxide dismutase; TAC: total antioxidant capacity

2-ferulate.^[37] These compounds were considered toxic probably after metabolism by gut microorganisms.^[38] However, elimination of jojoba seed meal anti-nutritional factors could be done by different methods, including solvent extraction, heat, chemical treatment, and microbial fermentation.^[39]

In this study, we evaluated the ability of ethanol extract of jojoba seeds to protect the liver of laboratory animals from the toxic effects of FB₁. The tested animals were given an extreme FB₁ challenge to ensure induction of severe response. The selected doses of FB₁ and jojoba seed extract were based on our previous work and others,^[8,40] respectively. The current results indicated that the ethanol extract of jojoba seeds is rich in total phenolics, crude protein, phytic acid, and simmondsin. These results were in accordance with those reported previously.^[19,41-43] Moreover, Shrestha *et al.*^[23] reported that jojoba protein consisted mainly of albumins and globulins. The decrease in body weight gain and food intake reported in this study in the group fed FB₁-contaminated diet indicated the presence of adverse effects and toxicity in rats caused by ingestion of FB₁. This decrease may indicate protein catabolism, thereby contributing to the observed kidney injury.^[8,9,44,45] Similar decrease in body weight gain and food intake had been reported in rats,^[9,44] swine,^[45] horses,^[46] broiler,^[47] and Turkey poult^[48,49] fed fumonisin. Previously, Abdel-Wahhab *et al.*^[8] and El-Nekeety *et al.*^[9] stated that administration of FB₁ to rats enhanced lipid peroxidation which presumably resulted from free-radical-mediated toxicity. Stockmann-Juvala *et al.*^[50] found that FB₁ evoked oxidative stress, which may contribute at least in part to FB₁-induced toxicity and carcinogenicity.

The elevation of ALT, AST, ALP, triglycerides, and cholesterol in the group fed FB₁-contaminated diet indicated necrosis or hepatocellular injury.^[9] The results of this study also revealed that treatment with FB₁ resulted in a significant increase in serum CEA, TNF-α, IL-1α, and NO suggesting that FB₁ can induce hepatotoxicity in rats. Similar results suggested earlier indicated that TNF-α, IL-1α, and NO were produced by macrophages, and they played a vital role in tumor conditions.^[51] Moreover, TNF-α is an essential factor in tumor promotion^[52] and is a key factor that regulates the production of other

cytokines involved in chronic inflammation and tumor development via the nuclear factor kappa B pathway.^[53] Moreover, the increase of NO and MDA and the decreased level of SOD and TAC in rats fed with FB₁ suggested that FB₁ administration enhanced the generation of free radicals which directly led to free radical-mediated toxicity.^[8,9,54,55] The generation of free radicals is one of the main manifestations of oxidative damage and has been found to play an important role in the toxicity and carcinogenesis induced by many carcinogens.^[56,57] In this respect, Hassan *et al.*^[58] reported that liver damage was directly related to free radical mediated toxicity which was known to attack the highly unsaturated fatty acids of the cell membrane and considered a key process in many pathological events induced by oxidative stress.^[59] Another mechanism of FB₁-induced injury was suggested by Pinelli *et al.*^[60] who stated that FB₁-induced a down-regulation of cytoplasmic phospholipase A2 activity and arachidonic acid metabolism by a mechanism involving prostaglandin production, cyclic adenosine monophosphate synthesis, and protein kinase activation, as well as global DNA hypomethylation and histone demethylation that causes chromatin instability and may lead to liver tumorigenesis.^[61]

The histological findings of the liver strongly confirmed the biochemical results. We demonstrated that jojoba seed extract had a protective role against FB₁-induced liver damage, as indicated by improvements in the histological structure of the liver tissues. Similar histological changes in the liver tissues were reported previously.^[9] Moreover, Abdel-Wahhab *et al.*^[8] and Voss *et al.*^[61] stated that FB₁ specifically disrupt cellular sphingolipid metabolism causing, among other things, increased levels of the sphingoid base sphinganine and an increased sphinganine/sphingosine ratio. Such disruption was associated with a diversity of animal diseases. These include liver and kidney lesions in rats,^[8] liver and brain lesions in horses,^[62] liver and lung lesions in pigs,^[63] and liver lesions in chickens.^[64] FB₁ was reported to induce liver lesions in rats which consisted of one or more of the following features: single cell necrosis, hepatocellular cytoplasmic vacuolation, variation in nuclear size and staining properties, pyknosis, fibrosis and bile duct proliferation, mild to marked hepatocellular hyperplasia, mitotic figures and foci of cellular alteration were found in the more severely affected livers.^[9,54]

In this study, animals treated with the ethanol extract of jojoba seeds at both the low and high doses did not show an acute decrease in body weight and food intake which may be due to the low levels of simmondsin due to the ethanol extraction.^[65,66] The slight decrease in food intake

and body weight gain in these groups may be due to the presence of simmondsin residue which was reported to induce food restriction and growth retardation.^[36,67,68] Treatment with jojoba seed extract to rats fed FB₁-contaminated diet improved food consumption and body weight gain which may be due to the withdrawal of the effect of simmondsin.^[69] Similar growth retardation was observed in male rats fed defatted jojoba meal which, therefore, concluded that the growth retardation seen with defatted jojoba meal was due to its simmondsin activity through its role in food intake reduction.^[70]

The results of this study also revealed that treatment with jojoba seed extract at both low and high doses did not affect the activity of ALS, AST, triglycerides level, or serum cytokines suggesting that the treatment did not cause liver toxicity. However, jojoba seed extract induced a slight increase in NO. According to Kampf *et al.*,^[70] jojoba contains a natural antioxidant postulated to be an allylic derivative of hydroxytoluene. Van Boven *et al.*^[71] isolated eight glucoside compounds from jojoba seeds and Bouali *et al.*^[40] reported that jojoba is rich in phytic acid and omega-3 fatty acid. Phytic acid is well known to have anti-radical effects by chelating iron required for the MPP-enhanced •OH generation *via* the Fenton-type reaction.^[72,73] Phytic acid was also shown to have anti-cancer property,^[74] and to improve serum and hepatic lipid levels in aged mice fed a high-cholesterol diet by increasing their fecal lipid content. Moreover, Pacheco *et al.*^[75] reported that phytic acid protected the membranes of the Intestinal Porcine Epithelial cell line (IPEC-1) against cell damage induced by the mycotoxin deoxynivalenol.

The antioxidant activity of glucoside was reported by Mehta *et al.*^[76] Abdel-Wahhab *et al.*^[77] concluded that glucoside decreased DNA damage and hepatocarcinogenesis induced by aflatoxin B₁ by activating the phase II enzymes GSH S-transferase and GSH peroxidase. These results suggest that glucoside is capable of counteracting FB₁ toxicity by suppressing cytochrome P450 mediated bioactivation of FB₁. Jojobenoic acid in jojoba seed extract also has antioxidant activity and has the ability to bind metal ions, representing an additional mechanism underlying their pharmacological effects.^[40] More importantly, jojoba seed extract itself was not toxic and did not exert any significant changes in the biochemical parameters tested or the histological structure of the liver.

Previous reports showed that jojoba extract did not show any toxic manifestation on the general body metabolism and the blood serum parameters were within the normal range.^[20,21] Moreover, jojoba oil supplement resulted in a 40% reduction of blood cholesterol and altered

lipoprotein pattern which may be attributed to the higher omega-3 fatty acid content.^[78] Moreover, Vermauti *et al.*^[79] reported that jojoba was rich in saponin which was well known to stimulate the cell-mediated immune system, as well as to enhance antibody production.^[80] It was reported to inhibit the growth of cancer cells *in vitro*,^[81,82] to exert an anti-cancer effect at the intestinal level, to reduce the formation of carcinogenic substances in the colon, and to have antioxidant properties.^[83] The higher total phenolic content in the extract reported in this study suggested another mechanism for its antioxidant activity.^[84] In this respect, Zheng and Wang^[85] reported that active polyphenol components such as flavonoids and phenolic acids possess antioxidant activities. Consequently, the protective effects of jojoba seed extract against FB₁-induced biochemical and histological changes in the liver reported herein may be due to the direct mechanism as free radical scavenger, and the indirect mechanism by which the extract may induce its protective effect through the enhancement of the synthesis of antioxidant enzymes in the liver.^[86]

It could be concluded that the ethanolic extract of jojoba seeds is rich in protein, phenolic compounds, and phytic acid. The extract has antioxidant effects and can protect against FB₁-induced hepatotoxicity. This action may be due to its content of several antioxidant compounds that have the ability to scavenge free radicals generated by FB₁ and consequently prevent lipid peroxidation, and/or the enhancement of antioxidant enzyme activities in the cell.

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Conflicts of interest

There are no conflicts of interest.

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Arterial blood supply of hepatocellular carcinoma is associated with efficacy of sorafenib therapy

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ABSTRACT

Aim: There are some previous reports concerning the relationship between prognosis of patients treated with sorafenib and parameters of computed tomography (CT) and magnetic resonance imaging (MRI). This study presents monocentric experience with sorafenib in the treatment of hepatocellular carcinoma (HCC) patients and will try to identify predictive factors for survival based on the correlation of results from imaging and survival. **Methods:** A total of 38 HCC patients treated from April 2009 to December 2010 with sorafenib were included in this study. HCCs were classified as good arterial supply and poor arterial supply according to the enhancement intensity on CT scan or MRI. Clinical data were collected and survival time was analyzed by Kaplan-Meier method. A Cox's regression model was performed to reveal predictive factors for survival. **Results:** Among the 38 patients treated with sorafenib, mean age was 53.3 ± 11.1 years and 35 (92.1%) were males. Tumors in 17 patients were classified as good arterial supply, while the remaining 21 patients belonged to poor arterial supply. The median survival time (MST) was 10.7 months [95% confidence interval (CI), 8.7-12.7] and the 1-year overall survival (OS) was 41.0%. The MST and 1-year OS in patients with a good arterial supply of tumors were 12 months (range: 4-20 months) and 52.9%, compared with that of 7 months (range: 1-16 months) and 23.8% in patients with a poor arterial supply of tumors ($P = 0.002$). Patients who had tumors at Barcelona Clinic Liver Cancer (BCLC) stage B had longer MST and higher OS than those who had tumors at BCLC stage C, but there was no statistical difference between these two stages. On multivariate analysis, only arterial supply of the tumors remained statistically predictive for OS (hazard ratios 0.22, 95% CI, 0.07-0.67, $P = 0.008$). **Conclusion:** Arterial blood supply is an independent predictor for survival in patients treated with sorafenib, and patients with a good arterial supply of tumors benefit more than those with a poor arterial supply of tumors.

Key words: Arterial blood supply; hepatocellular carcinoma; sorafenib

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INTRODUCTION


Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third most frequent cause of cancer-related death.^[1] Only about 15% patients with HCC are suitable for curative treatment, such as

surgical therapy (resection and liver transplantation) and locoregional therapy (radiofrequency ablation). For patients with advanced HCC, curative therapies cannot be applied, and only systemic therapy is

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available.^[2]

Sorafenib is a multikinase inhibitor which inhibits angiogenesis by targeting the vascular endothelial growth factor (VEGF) receptor 2 and platelet-derived growth factor receptor pathway and blocks cell proliferation by targeting the Ras/mitogen-activated protein kinase signaling pathway. Two global phase III trials (SHARP^[3] and Asia-Pacific trial)^[4] showed that sorafenib prolonged the survival of patients with advanced HCC. Following that, multiple studies have been conducted to determine the predictor for survival in patients treated with sorafenib. There are some previous reports concerning the relationship between prognosis of patients treated with sorafenib and parameters of computed tomography (CT) scan and magnetic resonance imaging (MRI).^[5-8] Hahn *et al.*^[6] showed that the area under the contrast concentration vs. time curve 90 s after contrast injection (IAUC90) and volume transfer constant of contrast agent [K (trans)] measured by MRI were prognostic pharmacodynamic biomarkers for metastatic renal carcinoma treated with sorafenib. In addition, Hsu *et al.*^[7] found K (trans) correlated well with tumor response and survival in HCC patients who received sorafenib plus metronomic tegafur/uracil therapy. Sorafenib significantly suppressed tumor perfusion, tumor vascularity, and endothelial permeability-surface area product quantified by CT scan in experimental prostate carcinoma in rats.^[5,9,10] It seems that CT scan or MRI may be applicable for imaging biomarkers of therapy response to antiangiogenic therapy.

We present our monocentric experience with sorafenib in the treatment of HCC patients and will attempt to identify predictive factors for survival, by placing emphasis on the correlation of the results from imaging and survival.

METHODS

Patients

A total of 38 HCC patients treated from April 2009 to December 2010 with sorafenib were included in this study. Hypervascular HCCs were diagnosed by at least 2 radiologic imaging showing characteristic features of HCC (contrast enhancement on the arterial phase with venous washout), or 1 radiologic imaging showing characteristic features of HCC associated with alpha-fetoprotein (AFP) ≥ 400 ng/mL, while hypovascular HCCs were diagnosed by biopsy with cytological or histological confirmation. Eligibility

criteria also included Eastern Cooperative Oncology Group Performance Status of 0 or 1; Child-Pugh liver function class A. All eligible patients received continuous oral treatment with 400 mg of sorafenib (consisting of two 200-mg tablets, provided by Bayer HealthCare Pharmaceuticals) twice daily. HCC is staged according to the Barcelona Clinic Liver Cancer (BCLC) classification.^[11] HCCs were divided into good arterial supply and poor arterial supply according to the enhancement intensity on CT scan or MRI and were assessed by an experienced radiologist who was blind to clinical information. Good arterial supply is defined as enhancement in $\geq 60\%$ lesions while poor arterial supply is defined as enhancement in $\leq 40\%$ lesions.

Study design

Our null hypothesis was that patients with a good arterial supply of tumors and those with a poor arterial supply of tumors benefitted similar outcomes. The primary endpoint of the trial was the 12-month overall survival (OS) rate. The secondary endpoints were the recurrence-free survival rate and the overall recurrence rate. Data were collected and stored in the liver cancer database management system by a designated clinical study center assistant chosen by the Research Ethics Committee.

This study met the requirements of the Declaration of Helsinki and was approved by the Research Ethics Committee of the Eastern Hepatobiliary Surgery Hospital, which is affiliated with the Second Military Medical University. Informed consent was obtained from all recruited patients.

Follow-up

Clinical examinations were performed for each patient, with laboratory assessment (routine tests of liver and kidney function and AFP) every month and imaging exams (chest X-ray and abdominal CT scan or MRI) every other month. A systemic nuclide scan was carried out when metastasis was suspected. Additional treatments, such as transarterial chemoembolization (TACE), were applied when necessary. Adverse events were under surveillance, and proper managements were provided when necessary.

Statistical analysis

Quantitative data were expressed as a mean \pm standard deviation (SD) or median (range) where appropriate and compared using the independent sample *t*-test. For quantitative data, the gaussianity

test was performed to test for homogeneity of variances. Homogeneous variances were indicated as a mean plus or minus SD (mean \pm SD) and the Student's *t*-test was used for statistical analysis. If the variances were not homogeneous, they were presented as median in combination with the range. Categorical variables were compared using the Chi-square test with Yates correction or the Fisher exact test where appropriate. $P < 0.05$ was considered significantly. Hazard ratios (HRs) and their corresponding 95% confidence interval (CI) were calculated using simple logistic-regression analysis.

Survival rates were obtained by the Kaplan-Meier method and were compared using the log-rank test. Cox regression model was used to analyze the prognostic predictors for survival. Survival time started from the date of treatment with sorafenib until death or the closing date. The closing date of this study was August 31, 2011.

RESULTS

Baseline characteristics

Among the 38 patients treated with sorafenib, mean age was 53.3 ± 11.1 years and 35 (92.1%) were males. All the patients had viral hepatitis background, with a hepatitis B prevalence of 94.7%. The baseline characteristics of the 38 patients are shown in Table 1. Tumors in 17 patients were classified as good arterial supply while the other patients belonged to poor arterial supply according to the judgment of the radiologist. A total of 30 patients received 1 time additional therapy of TACE during the period of follow-up, of which 13 patients with a good arterial supply of the tumors and 17 with poor arterial supply.

Safety and adverse events

Each patient experienced at least one adverse event in the duration of sorafenib administration. Hand-foot skin reaction and diarrhea were the most common discomforts complained by the patients. Less common adverse effects included fatigue, alopecia, hypertension, and diabetes. A total of 6 patients had dose reduction due to severe adverse events, of which 3 for diarrhea and 3 for hand-foot skin reaction. None of the patients had drug discontinuation.

Survival analysis

At the closing date of this study, 29 (76.3%) patients died and 9 patients were still alive. The median survival time (MST) was 10.7 months (95% CI, 8.7-

Table 1: Baseline characteristics of 38 patients included in the study

| Variable | n = 38 |
|------------------------------|---------------------|
| Sex (male/female) | 35/3 |
| Age (years) | 53.3 ± 11.1 |
| ECOG PS | |
| 0 | 32 |
| 1 | 6 |
| BCLC stage | |
| B | 18 |
| C | 20 |
| Arterial supply of the tumor | |
| Good | 17 |
| Poor | 21 |
| Portal invasion | |
| Yes | 14 |
| No | 24 |
| Extrahepatic metastasis | |
| Yes | 9 |
| No | 29 |
| Collaborative treatment | |
| TACE | 30 |
| None | 8 |
| Hepatitis background | |
| Hepatitis B | 36 |
| Hepatitis C | 2 |
| Vascular thrombus | |
| Presence | 12 |
| Absence | 26 |
| Tumor size | 8.1 ± 3.1 |
| AFP (ng/mL) | 205.1 (2-2,483,000) |
| Total bilirubin (umol/L) | 15.0 ± 7.6 |
| Albumin (g/L) | 39.3 ± 4.6 |
| Pre-albumin (mg/L) | 144.0 ± 46.0 |
| ALT (IU/L) | 48.3 ± 65.9 |
| AST (IU/L) | 55.3 ± 49.3 |
| PT (s) | 12.5 ± 1.1 |
| BUN (mmol/L) | 5.43 ± 0.69 |
| Cr (umol/L) | 69.18 ± 11.61 |

ECOG PS: Eastern Cooperative Oncology Group Performance Status; BCLC: Barcelona Clinic Liver Cancer; TACE: transarterial chemoembolization; AFP: alpha-fetoprotein; ALT: alanine aminotransferase; AST: aspartate aminotransferase; PT: prothrombin time; BUN: blood urea nitrogen; Cr: creatinine

12.7) and the 1-year OS was 41.0%. On univariate analysis [Table 2], the MST and 1-year OS in patients with good arterial supply of tumors were 12 months (range: 4-20 months) and 52.9%, compared with that of 7 months (range: 1-16 months) and 23.8% in patients with poor arterial supply of tumors ($P = 0.002$). Similarly, patients who had tumors at BCLC stage B had longer MST and higher OS than those who had tumors at BCLC stage C. However, there was no statistically significant difference between these two stages.

Eight variables were selected on multivariate analysis to determine the prognostic predictors for survival in patients treated with sorafenib [Table 3]. Only arterial supply of the tumors remained statistically predictive for OS (HR: 0.22, 95% CI, 0.07-0.67, $P = 0.008$).

DISCUSSION

As a highly vascularized neoplasm, most HCCs exert imaging characteristics of intense contrast uptake in the arterial phase, followed by contrast washout in the delayed venous phase at dynamic imaging by

Table 2: Univariate analysis of factors associated with survival of patients included in the study

| | <i>n</i> | Median survival time (months) | 1-year survival rate (%) | Log-rank test <i>P</i> |
|-------------------------------|----------|-------------------------------|--------------------------|------------------------|
| BCLC stage | | | | |
| B | 18 | 12.5 (2-18) | 61.1 | 0.067 |
| C | 20 | 7.5 (1-20) | 15.0 | |
| Arterial supply of the tumors | | | | |
| Good | 17 | 12 (4-20) | 52.9 | 0.002 |
| Poor | 21 | 7 (1-16) | 23.8 | |
| Portal invasion | | | | |
| Yes | 14 | 8.5 (1-19) | 21.4 | 0.206 |
| No | 24 | 11.5 (2-20) | 50.0 | |
| Extrahepatic metastasis | | | | |
| Yes | 9 | 9 (2-20) | 22.2 | 0.591 |
| No | 29 | 10 (1-18) | 41.4 | |
| Collaborative treatment | | | | |
| TACE | 30 | 10 (1-19) | 40.0 | 0.504 |
| None | 8 | 8 (2-20) | 25.0 | |
| AFP | | | | |
| ≥ 400 ng/mL | 15 | 8.5 (2-18) | 20.0 | 0.347 |
| < 400 ng/mL | 23 | 11 (1-20) | 47.8 | |
| Albumin | | | | |
| > ULN | 12 | 9.5 (4-19) | 41.7 | 0.159 |
| ≤ ULN | 26 | 9 (1-20) | 34.6 | |
| ALT | | | | |
| > ULN | 12 | 13 (1-20) | 58.3 | 0.063 |
| ≤ ULN | 26 | 9 (2-19) | 35.0 | |
| AST | | | | |
| > ULN | 18 | 9 (2-18) | 38.9 | 0.881 |
| ≤ ULN | 20 | 10 (1-20) | 35.0 | |

BCLC: Barcelona Clinic Liver Cancer; TACE: transarterial chemoembolization; AFP: alpha-fetoprotein; ULN: upper limit of normal; ALT: alanine aminotransferase; AST: aspartate aminotransferase

Table 3: Multivariate Cox's model for factors associated with survival of patients included in the study

| Variable | HR | 95% CI for HR | <i>P</i> |
|--|------|---------------|----------|
| BCLC stage (B vs. C) | 0.33 | 1.29-10.53 | 0.335 |
| Portal invasion (yes vs. no) | 1.15 | 0.19-7.03 | 0.881 |
| Extrahepatic metastasis (yes vs. no) | 0.88 | 0.13-5.94 | 0.893 |
| Arterial supply of the tumor (good vs. poor) | 0.21 | 0.07-0.67 | 0.008 |
| Collaborative treatment (TACE vs. none) | 1.54 | 0.48-4.91 | 0.470 |
| AFP (≥ 400 ng/mL vs. < 400 ng/mL) | 1.33 | 0.50-3.49 | 0.568 |
| Albumin (> ULN vs. ≤ ULN) | 2.13 | 1.00-6.50 | 0.064 |
| ALT (> ULN vs. ≤ ULN) | 0.35 | 0.11-1.08 | 0.068 |
| AST (> ULN vs. ≤ ULN) | 1.05 | 0.37-2.98 | 0.925 |

HR: hazard ratio; CI: confidence interval; BCLC: Barcelona Clinic Liver Cancer; TACE: transarterial chemoembolization; AFP: alpha-fetoprotein; ULN: upper limit of normal; ALT: alanine aminotransferase; AST: aspartate aminotransferase

contrast-enhanced CT scan or gadolinium-enhanced MRI.^[11] However, there are also many HCCs, which display poor contrast enhancement on CT scan or MRI on the arterial phase.

In this study, when we concentrated on the relationship between the degree of enhancement on the arterial phase of CT scan/MRI and the prognosis of HCC patients treated with sorafenib, the results showed that patients with good arterial supply benefitted more than those with poor arterial supply. Previously, Li *et al.*^[12] and Ippolito *et al.*^[13] found that CT scan could provide quantitative

information about tumor-related angiogenesis, which could be used to assess tumor vascularization. During hepatocarcinogenesis, arterial and portal blood flow would decrease, and then new arterial vessels formed because of the reduced arterial blood flow. And this caused hypervascular lesions to occur.^[14,15] The degree of tumor enhancement on the arterial phase could be an important symbol of vascularization. Neovascularization played a critical role during growth of solid neoplasms,^[16] and VEGF played an important role in regulating angiogenesis and endothelial cell proliferation.^[17] In the past few years, several studies had shown that the VEGF expression in HCC was correlated with imaging findings.^[18-21] Kwak *et al.*^[21] found that the strong arterial enhancement of HCC resulted from a strong VEGF expression which was responsible for an increased vascular permeability and increased proliferation of the endothelial cells. In contrast, sorafenib inhibited the activity of VEGF receptors and other proangiogenic signaling pathways. In mouse xenograft models of HCC, sorafenib significantly reduced tumor microvessel density. These observations, combined with the relatively short half-life of sorafenib, suggest that sorafenib administered during and after TACE treatment may counteract hypoxia-induced angiogenesis and potentially yield synergistic efficacy in decreasing tumor burden. However, these hypothesis generated findings remain speculative until sufficient clinical trial data can be accumulated.

It is reported that there is a significant correlation between efficacy of sorafenib administered combined with TACE treatment and arterial blood supply of HCC. According to our study, the stronger the enhancement intensity of HCCs on the arterial phase, the longer the HCC patients treated with sorafenib survived. Maybe the level of VEGF could indicate the treatment effect of sorafenib, and further research needs to be done to reveal the correlation between the VEGF activity and efficacy of sorafenib.

The major limitations of this study are the non-comparative design and a limited number of patients. A prospective study should be done to investigate the correlation between enhancement intensity of HCCs in the arterial phase and survival of HCC patients treated with sorafenib.

In conclusion, arterial blood supply is an independent predictor for survival in patients treated with sorafenib, and patients with a good arterial supply of

tumors benefit more than those with a poor arterial supply of tumors. Further prospective studies need to be conducted to reveal the relationship between the degree of tumor enhancement in the arterial phase and the prognosis of HCC patients treated with sorafenib therapy.

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Conflicts of interest

There are no conflicts of interest.

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Surgical resection or radiofrequency ablation in the management of hepatocellular carcinoma: single center experience

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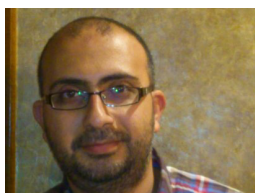
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ABSTRACT

Aim: The aim of this study is to prove or disprove the superiority of surgical resections over radiofrequency ablation (RFA) with respect to efficacy and safety. **Methods:** The study was conducted in Zagazig University Hospitals, which included 40 patients with hepatocellular carcinoma (HCC) during the period from November 2011 to December 2014, using either liver resection or RFA. **Results:** Hepatic resection was done in 20 patients (13 males, 7 females). Interventional RFA was done in 20 patients (12 males, 8 females). There was no in-hospital mortality after resection. One- and two-year survival rates were 85% and 70% respectively. There was no in-hospital mortality after RFA. One- and two-year survival rates were 80% and 65% respectively. **Conclusion:** Surgical resection is preferred over RFA in HCC-liver cirrhosis Child A patients with tumor sizes ≥ 3 cm. HCC-liver cirrhosis Child A patients with masses < 3 cm have almost the same results with both surgery and RFA. But in special cases such as central position lesions, RFA is preferred over resection. Also the decision for management may be changed according to patients well. Surgical resection 1- and 2-year survival rates were better than those treated with RFA.

Key words: Hepatocellular carcinoma; liver resection; radiofrequency ablation



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INTRODUCTION

Hepatic resection (HR) forms part of the conventional treatment for patients with hepatocellular carcinoma (HCC).^[1] Size, site, number of tumors, vascular and extra-hepatic involvement as well as liver function represent some aspects that prompt surgical resection difficulties. Accordingly, the majority of primary liver cancers are not suitable for curative resection at the time of diagnosis.^[2,3]

Radiofrequency ablation (RFA) is recommended for HCC nodules with a maximum diameter of 3 cm in patients with no more than three tumors that are contraindicated for surgery.^[4]


METHODS

The patients were diagnosed through history taking,

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complete physical examination, laboratory investigations [complete blood count, coagulation profile, liver function test, kidney function test and alpha-fetoprotein (AFP)], and radiological investigations [abdominal ultrasonography and triphasic computerized tomography (CT)]. They were categorized into two groups. Group A: 20 patients for whom HR was done (according to the size, site and number of tumors); Group B: 20 patients for whom RFA was done using percutaneous ultrasonography.

Inclusion criteria

Patients with or without liver cirrhosis. Patients with Child A and B (Child-Pugh classification). Patients with or without hepatitis B or C infection. Patients who have HCCs diagnosed by triphasic CT \pm elevated AFP.

Exclusion criteria

Patients with Child C liver disease. Patients with HCC tumors outside of the Milan criteria and are not candidates for RFA (central lesion near common bile duct, lesion adherent to bowel loop, lesion not accessible and lesion exophytic). Patients with HCC metastasis.

Follow-up

The patients in both groups were followed up for 2 years and we then compared the two groups with regards to operative mortality, morbidity, hospital stay, and 1- and 2-year overall states. The results and the recurrence were measured by the changes in AFP levels, abdominal ultrasound, and triphasic CT scan after 1 month then every 3 months in the 1st year and subsequently every 6 months for the 2nd year.

Surgical resection

Group A: From November 2011 to December 2014, 20 consecutive patients with HCC (13 males, 7 females; average age: 53.4 years; range: 45-62 years) underwent HR at Zagazig University Hospitals, Surgical Department. All resections were considered radical (tumor-free resection margins confirmed by pathology) [Figures 1-3].

Patients prepared preoperatively by using central line and epidural catheters a day before surgery. Packed red blood cells and fresh frozen plasma were prepared according to patient labs.

Incision used was usually L-shaped, rarely we needed to conduct bilateral subcostal with midline incisions. Before we started, we usually assessed the operability via feeling of the mass, searching for other masses and searching for enlarged lymph nodes. Complete mobilization was the first step. Identification of the hilar structures is the second step. Even if we were not going to do typical hepatectomies and this for control of possible bleeding. During operation

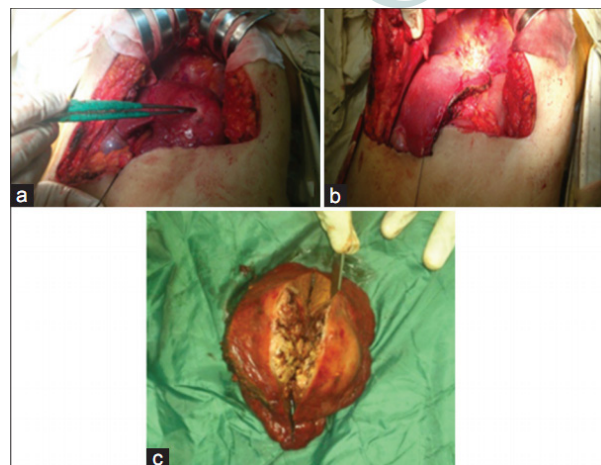


Figure 1: Right liver lobe hepatocellular carcinoma resection. (a) Intraoperative identification of the mass; (b) liver bed after resection of the mass; (c) opening of the mass after excision

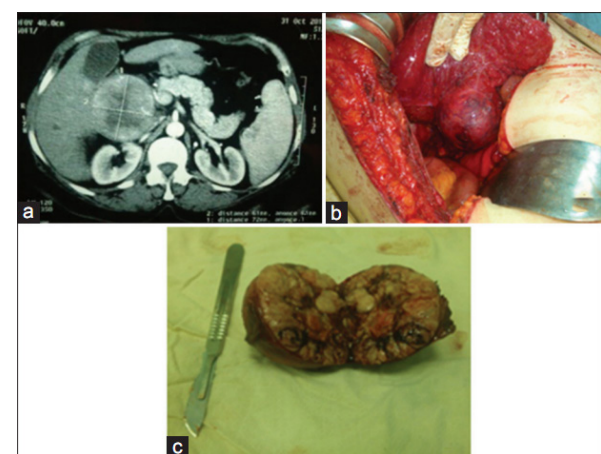


Figure 2: Caudate lobe liver resection. (a) Triphasic computerized tomography identification of caudate lobe mass; (b) intraoperative identification of caudate lobe mass; (c) opening of the mass after excision

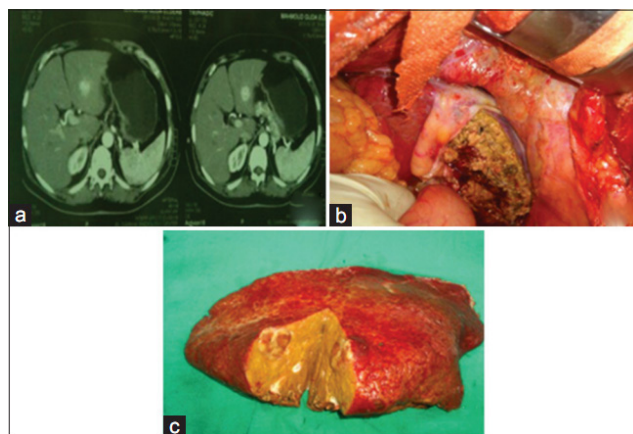


Figure 3: Left liver lobe hepatocellular carcinoma resection. (a) Triphasic computerized tomography identification of left lobe mass (left lateral segment); (b) liver bed after resection of the mass; (c) opening of the mass after excision

we used a harmonic scalpel for parenchyma dissection. We were ready to conduct the Pringle maneuver, but only used it when needed. Meticulous haemostasis was maintained as usual and bile leakage was avoided. Tube drains were only inserted in susceptible patients.

Post-operative management

Five patients were transferred to the Intensive Care Unit and were under observation until patients became stable. A naso-gastric tube was left for 24 h. Patients started oral fluids when intestinal sounds became audible, and gradually returned to a normal diet. Ambulance was started as early as possible. Drains were removed when below 100 mL (usually the 4th day). Hospital stay keep as short as possible to avoid hospital acquired infection, ranging from 5 days to 7 days.

In the same period, 20 consecutive patients with HCC (12 males, 8 females; average age: 54.3 years; range: 48-66 years) underwent percutaneous RFA at Zagazig University Hospitals, Interventional Radiology Department [Figure 4].

Thirteen of them were treated using the Radionics cool tip needle (4 ablated by the single probe and 8 by the cluster probe). Seven patients were treated using the Rita needle with expandable hooks. Fifteen patients were treated with a single electrode insertion, 4 with double insertions and in one case, by three insertions. Only 1 patient received a second session of RFA due to a residual tumor detected by the 1-month follow-up triphasic CT study.

Local anesthesia was performed on the entry site of the skin to the liver capsule along the needle track with 10 mL of 2% xylocaine. Most of the patients undergoing RFA were treated under general intravenous (IV) anesthesia.

The objective in treating the tumors was to ablate the entire tumor and an at least 1 cm tumor-free margin

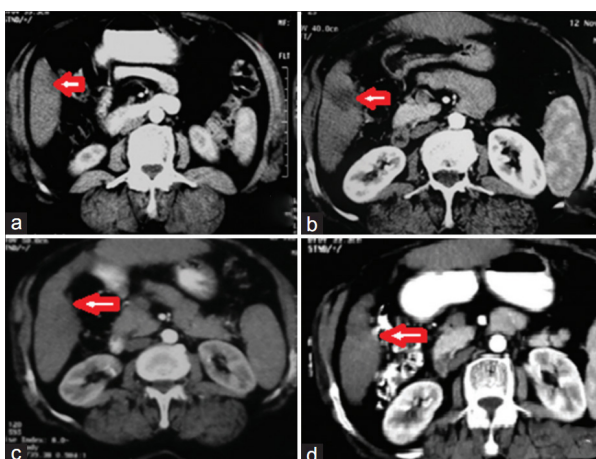


Figure 4: (a) Arterial contrast enhanced triphasic computerized tomography shows right lobe (segment 6) hepatocellular carcinoma about 16 mm × 14 mm; (b) arterial phase 1 month after RFA; (c) arterial phase 3 months after RFA; (d) arterial phase 9 months after RFA. In b, c and d, no enhancement of the ablated right lobe. Significant decrease in mass size is noted. RFA: radiofrequency ablation

of normal liver. The deepest ablations were performed before the superficial ones to minimize the possibility of micro bubbles that might obscure visualization of the deepest portions of the tumor and thus prevent complete ablation. In our cases, we ablated the tract before removal of the needle.

Post-ablation care

IV antiemetic was given. Strong IV analgesics were given to control pain. All patients were observed clinically for 2-3 h in the Radiology Department to detect any acute complications (like bleeding, shock and injury to other organs) and to start IV fluid. Prophylactic antibiotics were started and continued for 3 days.

RESULTS

Sociodemographic characteristics of patients

We compared tumor characteristics in the two different treatment groups (Child-Pugh score, tumor number, tumor diameter and AFP levels), as shown in Table 1.

Group A: Resection

A total of 20 consecutive patients with HCC (13 males, 7 females; average age: 53.4 years; range: 45-62 years) underwent HR. The etiology of the patients' underlying liver disease were characterized by 20 patients with chronic hepatitis (hepatitis B: 3; hepatitis C: 14; hepatitis B + C: 3). On the other hand, 17 had Child A and 3 had Child B, according to the Child-Pugh scoring system.

Group B: Radiofrequency ablation

A total of 20 consecutive patients with HCC (12 males, 8 females; average age: 54.3 years; range: 48-66 years) underwent RFA interventional in the Radiology Department. The etiology of the patients' underlying liver disease was characterized by 20 patients with chronic hepatitis (hepatitis B: 4; hepatitis C: 14; hepatitis B + C: 2). Of these patients, 12 had Child A and 8 Child B.

Table 1: Tumors characteristics in the two different treatment groups

| Underlying cirrhosis | Group A | Group B |
|----------------------|-----------------|------------------|
| | HR (n = 20) (%) | RFA (n = 20) (%) |
| Child-Pugh score | | |
| A | 17 (85) | 12 (60) |
| B | 3 (15) | 8 (40) |
| Number of tumors | | |
| Single | 18 (90) | 13 (65) |
| Multinodular | 2 (10) | 7 (35) |
| Tumor diameter | | |
| maximum 7.5 cm | | |
| ≤ 3 cm | 5 (25) | 4 (20) |
| > 3 cm | 15 (75) | 16 (80) |
| AFP levels (ng/mL) | | |
| ≤ 20 | 3 (15) | 2 (10) |
| > 20 | 17 (85) | 18 (90) |

RFA: radiofrequency ablation; AFP: alpha-fetoprotein; HR: hepatic resection

Treatment mortality and morbidity

We found the difference in overall survival in the two different treatment groups regarding child type as shown in Table 2.

Group A: Resection

There was no operative mortality (within 30 days of surgery) after resection; mean hospital stay was 6 days. One- and two-year survivals were 85% (17) and 70% (14) respectively.

Post-resection complications varied greatly. Wound infection (seroma) occurred in 4 patients and were managed conservatively via repeated dressing and antibiotic administration according to the culture obtained from the wound. Incisional hernia occurred in 2 patients. Hernioplasty was performed in one of them while the other one refused. Chest complications were the most common complications, big incision and severe pain limits respiration, leading to retained secretions and chest infections. Chest complications occurred in 8 patients. Ascitis occurred in 3 patients and were managed medically. One patient developed recurrence after 18 months (this patient was managed by RFA but was excluded from our results, as RFA was done after finishing the study).

Group B: Percutaneous radiofrequency ablation

There was no in-hospital mortality after RFA; the mean hospital stay ranged from 4 h to 24 h with a mean of 7 h. One- and two-year survival was respectively, 80% (16) and 65% (13).

Pain after procedures was present in all patients (mild to moderate pain presented in 16 patients which was managed using analgesia. Severe pain presented in 4 patients and was managed using sedation). Pain lasted for 24-72 h in most patients. Delayed pain occurred in 2 patients lasting for 1 week. This was attributed to the proximity of the ablated lesions to the diaphragm. Pain occurred either isolated or as a part of the post-ablation syndrome that occurred in 12 patients with flu-like manifestations including low-grade fever, pain, malaise, myalgia, nausea, and vomiting.

Table 2: Overall survival by patient and child type in the two different treatment groups

| | 1 year (%) | 2 years (%) |
|----------------|------------|-------------|
| Total patients | | |
| HR (n = 20) | 17 (85) | 14 (70) |
| RFA (n = 20) | 16 (80) | 13 (65) |
| Child A | | |
| HR (n = 17) | 15 (75) | 13 (65) |
| RFA (n = 12) | 10 (50) | 9 (45) |
| Child B | | |
| HR (n = 3) | 2 (10) | 1 (0.5) |
| RFA (n = 8) | 6 (30) | 4 (20) |

RFA: radiofrequency ablation; HR: hepatic resection

One case developed a new lesion detected 4 months post-procedure at the follow-up triphasic CT study managed by a second session.

Cholecystitis developed in 1 patient with a segment 5 nodule adjacent to the gall bladder wall. Bile duct injury developed in another patient 1 month post-procedure.

DISCUSSION

HCC accounts for more than 90% of primary liver cancer, the third most common cause of cancer-related death. It is the fifth most prevalent cancer in men and the seventh in women.^[5,6] The prognosis for untreated HCC is generally poor. Curative treatment consists of surgical resection, RFA, and liver transplantation.^[7]

Management of cirrhotic HCC involves several specialties.^[8] To correctly select candidates for resection, it is essential to consider not only the tumor characteristics, but also the accurate estimate of liver function with the aid of imaging. The risk of incorrect staging of associated cirrhosis may result in post-operative liver failure, followed by chronic decompensated cirrhosis.^[9]

The high mortality and morbidity associated with chronic liver disease limits liver resection in cirrhotic patients.^[10] Liver transplantation is the choice of treatment, with the best results in terms of long-term survival, but this option is feasible in a small number of patients because of the shortage of donors.^[11] However, current progresses in liver resection techniques and in post-operative follow-up have improved the resection results in terms of operative risk and long-term survival.^[9,12]

Indications for resection depend on the size, number and location of lesions as well as the estimation of remnant liver volume (RLV). The best candidates are patients with a single peripheral lesion, which permits the preservation of more than 50% of RLV.^[13]

Tumor location is an essential assessment parameter. With regard to peripheral lesions, no matter how bulky the mass is, resection may be performed with a curative intent and anatomically, without compromising a large parenchymal volume.^[14] In contrast, a small central lesion (< 3 cm) may require the sacrifice of a significantly great parenchymal volume, with risk of post-operative liver failure, so RFA is preferable if possible.^[15]

Surgical resection of HCC remains the gold standard. Unfortunately, its usefulness has been limited by many factors, including tumor multiplicity and poor hepatic reserve to tolerate surgery. Other techniques

(e.g. percutaneous ethanol injection, microwave, RFA, and brachytherapy) may be effective and feasible in the treatment of HCC patients who are not suitable for resection.^[16] Among these, RFA may be beneficial to more patients than the others because of its large coagulated necrosis, fewer treatment sessions, and higher survival rates.^[17-20] Rare studies have evaluated the results of treatment with RFA, by comparing it to liver resection.^[21-23]

There was no in-hospital mortality after resection. One- and two-year survivals were 85% and 70% respectively in our series. There was no in-hospital mortality after RFA. One- and two-year survival was 80% and 65%, respectively. This finding agreed with Parisi *et al.*^[16] who concluded that surgical resection improved the overall survival and recurrence-free survival in comparison with RFA.

Our results regarding masses < 3 cm matched with other results of Nishikawa *et al.*^[24] who found that in patients with HCCs < 3 cm, there was no significant difference between the two treatment groups in terms of overall survival. They concluded that RFA was as effective as resection in the treatment of single and small HCC, and was less invasive than surgery. Chen *et al.*^[25] suggests that RFA and surgery have similar results in terms of overall survival and RFS for single HCCs < 5 cm. Abu-Hilal *et al.*^[21] showed that RFA should be considered as an acceptable alternative when surgery was not possible in small unifocal HCCs. Therefore, RFA could be the first choice of treatment for single and small HCC.

However, regarding masses more than 3 cm, our results agree with Huang *et al.*^[22] who reported that in treating Child-Pugh A cirrhotic patients with a solitary HCC larger than 3 cm but < 5 cm, or with two or three lesions each < 5 cm, surgical resection provided a better survival than RFA.

RFA has some advantages compared with resection such as: Being less invasive, having a relatively rapid recovery period, and short hospital stay. But it also has shortcomings, such as more frequent local recurrence after treatment than resection.^[26,27]

Furthermore, the resection group indicated higher incidences of complications compared with RFA. In addition, resection has weaknesses such as a longer hospital stay and a longer recovery period after operation. Our finding was in agreement with the study of Park *et al.*^[28] and Bruix *et al.*^[29]

The study is based on a limited number of patients, however, our number are near other studies.^[30,31]

Follow-ups were extremely difficult. Usually, when the patient feels improvement; he/she stops visiting our outpatient clinic for follow-ups.

In conclusion, surgical resection is preferred over RFA for HCC-liver cirrhosis Child A patients with tumor sizes ≥ 3 cm. HCC-liver cirrhosis Child A patients with masses < 3 cm have almost the same results as both surgery and RFA. But in special cases such as central position lesions, RFA is preferred over resection. Also the decision for management may be changed according to patients well. Surgical resection 1- and 2-year survival rates were better than those treated with RFA.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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Nutrition profile of a liver transplant recipient

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ABSTRACT

Malnutrition is almost universally present in patients undergoing liver transplantation. In this report, a male adult patient was followed from his pre-liver transplant phase until chronic post-transplant phase (3 months after the transplant). Improvement in nutrition status, quality of life, and performance status was seen from the pre-transplant to chronic post-transplant phase. Day to day nutrition monitoring and gradual increase in calorie and protein intake was seen in the acute post-transplant phase, but during pre- and chronic post-transplant phase, lack of nutrition support was observed in the patient.

Key words: Liver transplant; malnutrition; nutrition profile

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INTRODUCTION

Liver transplantation (LT) is the only treatment for the end-stage liver disease (ESLD).^[1] It is estimated that malnutrition occurs in 65-100% of patients with ESLD.^[2,3] Medical nutrition therapy provided by a registered dietitian is necessary during all phases of LT for improved surgical outcomes.^[4]

CASE REPORT

Nutrition therapy for LT is divided into three phases: (1) pre-transplant - provision of adequate nutrients without aggravating ESLD symptoms; (2) acute post-transplant - high protein feeds through various routes to achieve adequate intakes; and (3) chronic post-transplant - aggressive nutrition therapy for improved survival.^[4]

Pre-transplant phase

A 54-year-old Indian male patient diagnosed with

ethanol and hepatitis C virus-related chronic liver disease underwent living donor LT (Child-Turcotte-Pugh score^[5] = 8, Model for ESLD score^[6] = 14). Medical history showed the patient suffered from jaundice (for 2 years), ascites (for 3 months) and excessive fatigue (for 15 days). The patient was admitted 12 days before LT. Biochemical parameters before LT depicted deranged results [Table 1].


Nutrition status assessment by anthropometry depicted mild malnutrition by mid-arm muscle circumference (MAMC) and severe malnutrition by triceps measurement.^[7] Subjective global assessment (SGA) showed moderate malnutrition.^[8] Hand grip strength (both hands) showed severe malnutrition.^[9]

Body composition analysis depicted standard physique of the patient with normal levels of fat percentage, fat-free mass (FFM), and muscle mass [Table 2].^[10]

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Diet history depicted no gastrointestinal (GI) symptoms, dental or oral problem, or food allergies. The simplified nutritional appetite questionnaire (SNAQ) score was 16 hence there was no significant risk of at least 5% weight loss within 6 months.^[11] The patient was alcoholic (CAGE score > 2).^[12] He was recommended an oral normal diet with supplements providing 2700 kcal, 115 g of proteins with salt (2 g) and fluid restriction (1.5 L/day).^[4] Patients' intake was 1100 kcal and 40 g protein, indicating consumption of 57.6% of the recommended calories.

Table 1: Biochemical parameters of the patient before the transplant

| Biochemical parameter | Value | Range | Biochemical parameter | Value | Range |
|---------------------------------|-------|------------|-----------------------|-------|----------|
| Hb (mg/dL) | 8.5 | 13-17 | Na (mmol/L) | 134 | 137-145 |
| WBC (10 ³ /UL) | 8.31 | 4.00-10.00 | K (mmol/L) | 3.7 | 3.5-5.1 |
| Platelets (10 ³ /UL) | 100 | 150-410 | Ca (mg/dL) | 8.9 | 8.4-10.2 |
| Alb (g/L) | 3 | 3.5-5.0 | Mg (mg/dL) | 1.5 | 1.6-2.3 |
| Bili (D) (mg/dL) | 0.1 | 0.2-1.3 | P (mg/dL) | 4.3 | 2.5-4.5 |
| Bili (T) (mg/dL) | 1.5 | 0.2-1.3 | Cl (mmol/L) | 106 | 98-107 |
| Total protein (g/L) | 6.4 | 6.3-8.2 | PT | 15.6 | 8.8-12.3 |
| ALT/SGPT (U/L) | 23 | 21-72 | INR | 1.51 | |
| AST/SGOT (U/L) | 34 | 17-51 | CR protein (mg/dL) | 11.6 | 0.0-10.0 |
| γ glutamyl transferase (U/L) | 28 | 15-73 | | | |
| Alkaline phosphates (U/L) | 63 | 30-120 | | | |
| Urea (mg/dL) | 61 | 10-50 | | | |
| Cr (mg/dL) | 1.6 | 0.80-1.50 | | | |

Hb: hemoglobin; WBC: white blood cell; Alb: albumin; Bili: bilirubin; ALT: alanine aminotransferase; AST: aspartate aminotransferase; Cr: creatinine; PT: prothrombin time; INR: international normalized ratio; CR protein: C-reactive protein; SGPT: serum glutamic pyruvic transaminase; SGOT: serum glutamic oxaloacetic transaminase

Table 2: Nutrition assessment of the patient

| Parameter | Observation | Evaluation |
|--|-------------|-----------------------|
| Anthropometric evaluation | | |
| Weight (kg) | 73.9 | |
| Height (cm) | 176 | |
| Ideal body weight (kg) | 76 | |
| Triceps ^[7] (cm) | 0.56 | Severe malnutrition |
| MAMC ^[7] (cm) | 22 | Mild malnutrition |
| SGA ^[8] | | |
| SGA ^[8] | 6 | Moderate malnutrition |
| Body composition analysis by bioelectrical impedance analysis ^[9] | | |
| Weight (kg) | 72.55 | Normal |
| Fat (%) | 22.5 | Normal |
| Fat mass | 16.3 | Normal |
| FFM (kg) | 56.25 | Normal |
| Muscle mass (kg) | 53.35 | Normal |
| BMI | 23.2 | Normal |

MAMC: mid-arm muscle circumference; SGA: subjective global assessment; FFM: fat-free mass; BMI: body mass index

Eastern Cooperative Oncology Group (ECOG) performance status score of 3 indicated that the patient was capable of only limited self-care and unable to carry out any work activities that was ≥ 50% of working hours.^[13] Quality of life (QOL) assessment by short form-36 before LT depicted low level in its eight dimensions [Figure 1].^[14]

Acute post-transplant phase

The altered blood parameters are important for implementing the nutrition therapy plan. Deranged biochemical parameters in this phase are presented in Figure 2a-h. The patient had been in intensive care unit for 3 days. At post-operation day (POD), 1 patient was extubated within 24 h and was provided propofol 45 mL (1 kcal/mL) and dextrose normal saline 440 mL (17 kcal/100 mL), KCl 45 mL intravenously. On POD 2 propofol, 120 mL and KCl 120 mL was given. On POD 3 KCl 40 mL along with oral liquids (250 kcal) was given. On POD 4, he was transferred to the LT unit and was given oral high protein normal diet with supplements providing 2,700 kcal and 115 g protein. The patient was not able to complete meals (especially lunch and dinner), because of nausea and lack of appetite. An increasing trend of energy and protein consumption after LT during the hospital stay is indicated in Figure 3. The patient met 76.4% and 103% of the recommended calorie and protein intake, respectively. The patient was discharged on POD 15, on 2,700 kcal and 115 g of proteins (high protein, low potassium normal diet) out of which 375 kcal and 36 g of protein were from low potassium nutrition supplements and about 352 kcal, and 24 g protein was from high calorie-protein biscuits.^[4] He was recommended to take multivitamins and potassium binding medications, to monitor glucose regularly, and to avoid the outer

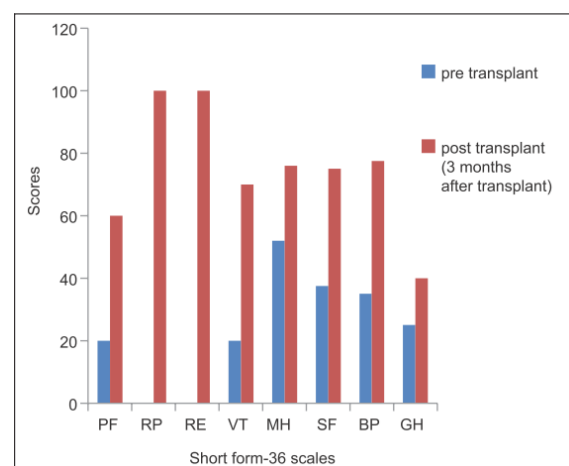


Figure 1: Comparison of quality of life by short form-36 questionnaire pre- and post-transplant. PF: physical functioning; RP: role limitation due to physical health; RE: role limitation due to emotional problem; VT: vitality; MH: mental health; SF: social function; BP: body pain; GH: general health

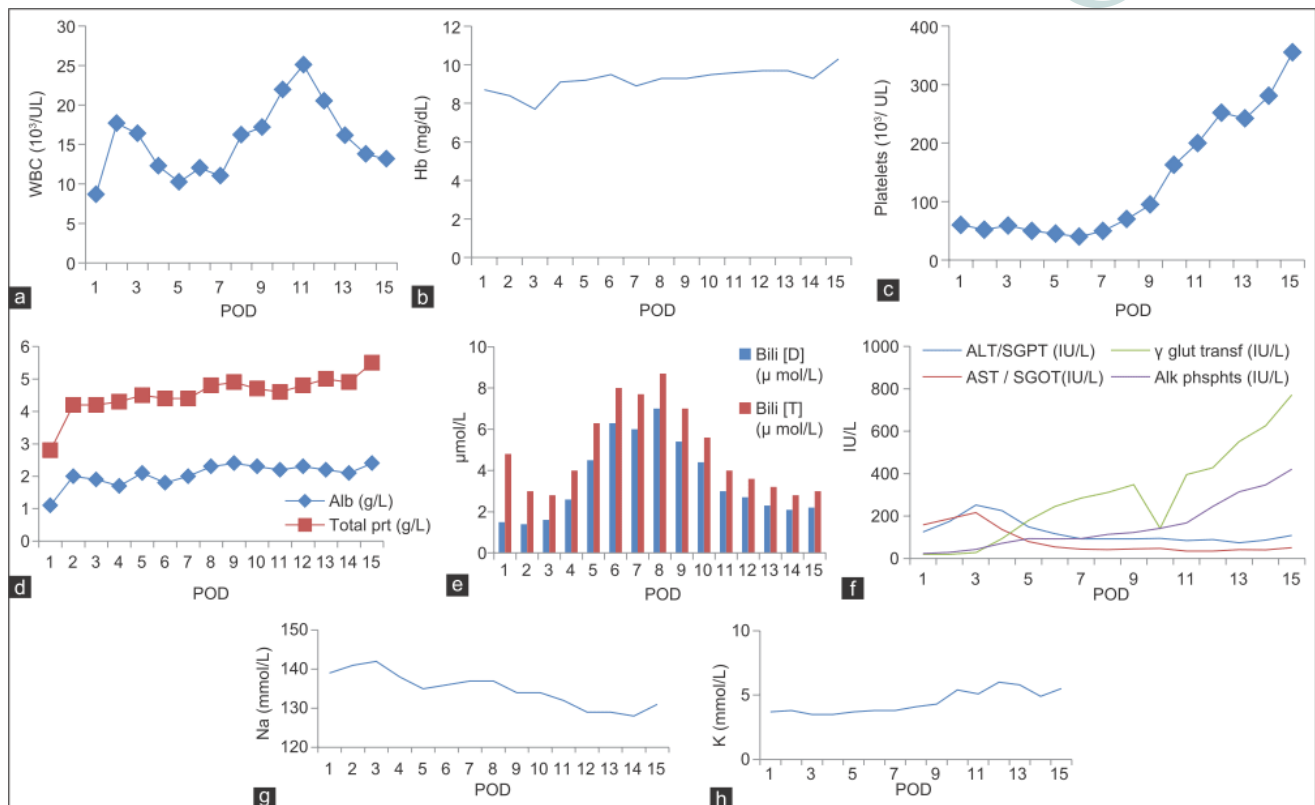


Figure 2: Each panel depicts acute post-operative patient profile of WBC (a), hemoglobin (b), platelets (c), albumin and total protein (d), bilirubin (D and T) (e), AST, ALT, γ glutamyl transpeptidase and alkaline phosphates (f), sodium (g), and potassium (h), respectively. Hb: hemoglobin; WBC: white blood cell; Alb: albumin; Bili: bilirubin; AST: aspartate aminotransferase; ALT: alanine aminotransferase; POD: post-operation day

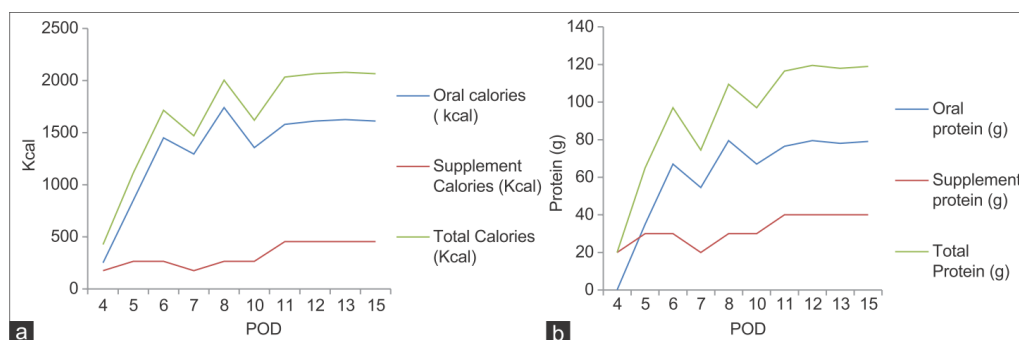


Figure 3: Energy (a) and protein (b) intake of the patient during the hospital stay after the transplant. POD: post-operation day

source of infection.

Chronic post-transplant phase

Gradual improvement in all the biochemical parameters was seen after 3 months of LT [Table 3]. The patient regularly visited the hepatologist after the surgery but never visited the dietician. The patient's intake was 1983 kcal and 78.9 g protein from the oral diet without any nutritional supplement. The recommended intake amounts to 2,280 kcal and 76 g of protein.^[4] Hence, patient met 83.9% of calorie requirements.

The patient was not having any GI problem; he was able to perform daily routine functions. The SNAQ score was 16 which showed no significant risk of at least

5% weight loss within 6 months.^[11] QOL assessment depicted improvement of all the eight dimensions 3 months after LT [Figure 1].^[14] The performance status assessment by ECOG improved from a score of 3 to 1 which indicated that the patient was restricted in physically strenuous activity but was ambulatory and able to carry out work of a light or sedentary nature.^[13] Nutrition status assessment is depicted in Table 4. Anthropometric examination through, MAMC^[7] showed similar results as in pre-transplant phase, which is mild malnutrition. Triceps measurement improved from severe malnutrition to normal range.^[7] SGA scores improved from moderate malnutrition to normal.^[8] Body composition analysis depicted higher levels of fat percentage and FFM after 3 months of LT.^[10] Hand grip

Table 3: Patients' biochemical profile after discharge

| Days after discharge | Hb (mg/dL) | WBC (10 ³ /UL) | Platelets (10 ³ /UL) | Bil (T) (mg/dL) | Bil (D) (mg/dL) | AST (IU/L) | ALT (IU/L) | Alkaline phosphates | γ glutamyl transferase (IU/L) | Alb (g/dL) | Na (mmol/L) | K (mmol/L) | Cr (mg) |
|----------------------|------------|---------------------------|---------------------------------|-----------------|-----------------|------------|------------|---------------------|-------------------------------|------------|-------------|------------|---------|
| 1 | 9.5 | 12.02 | 40 | 8 | 6.3 | 54 | 117 | 92 | 245 | 1.8 | 136 | 3.8 | 0.8 |
| 2 | 8.9 | 11.02 | 50 | 7.7 | 6 | 44 | 92 | 94 | 284 | 2 | 137 | 3.8 | |
| 3 | 9.3 | 16.2 | 70 | 8.7 | 7 | 41 | 92 | 113 | 311 | 2.3 | 137 | 4.1 | 0.8 |
| 4 | 9.3 | 17.18 | 95 | 7 | 5.4 | 45 | 92 | 122 | 348 | 2.4 | 134 | 4.3 | 0.8 |
| 5 | 9.5 | 21.93 | 163 | 5.6 | 4.4 | 47 | 95 | | 362 | 2.4 | 134 | 5.4 | 0.8 |
| 6 | 9.6 | 25.6 | 200 | 4 | 3 | 34 | 84 | 167 | 396 | 2.2 | 132 | 5.1 | 0.9 |
| 7 | 9.7 | 20.51 | 252 | 3.6 | 2.7 | 35 | 89 | 245 | 428 | 2.3 | 129 | 6 | 1 |
| 8 | 9.6 | 16.13 | 242 | 3.2 | 2.3 | 41 | 74 | 314 | 552 | 2.2 | 129 | 5.8 | 1 |
| 9 | 9.2 | 8.09 | 185 | 1.5 | 0.9 | 30 | 117 | 82 | 195 | 1.9 | 131 | 4.6 | 0.8 |
| 10 | 10.3 | 10.17 | 355 | 3 | 2.2 | 51 | 109 | 421 | 772 | 2.4 | 131 | 5.5 | 0.9 |
| 12 | 9 | 13.14 | 305 | 2.1 | 1.6 | 52 | 78 | 287 | 733 | 2.2 | 133 | 4.1 | 1 |
| 15 | 9 | 13.19 | 300 | 2.3 | 2 | 105 | 196 | 294 | 737 | 2.3 | 137 | 3.3 | 0.9 |
| 19 | 9.8 | 17.86 | 373 | 2 | 1.7 | 67 | 221 | 325 | 828 | 2.6 | 138 | 3.7 | 0.9 |
| 26 | 11.20 | 15.48 | 301 | 1.0 | 0.8 | 57 | 119 | 213 | 623 | 2.50 | | | 1.0 |
| 33 | 11.30 | 17.37 | 312 | 0.7 | 0.7 | 42 | 86 | 178 | 474 | 2.50 | | 4.0 | 0.8 |
| 34 | 11.70 | 13.27 | 311 | 0.7 | 0.5 | 39 | 83 | 162 | 449 | 2.60 | | | |
| 41 | 12.40 | 14.80 | 326 | 0.6 | | 44 | 91 | 169 | 382 | 2.90 | 135 | 5.3 | 0.9 |
| 53 | 11.30 | 13.05 | 328 | 0.3 | 0.2 | 38 | 69 | | | | | | |
| 54 | 12.20 | 13.22 | 308 | 0.5 | 0.4 | 55 | 102 | 160 | 283 | 2.70 | | | |
| 72 | 10.90 | 22.63 | | 0.6 | 0.2 | 29 | 42 | 220 | | 4.90 | 146 | 4.2 | 1.3 |
| 88 | | | | 0.4 | 0.3 | 23 | 32 | 116 | 107 | 3.10 | 140 | 4.8 | |

Hb: haemoglobin; WBC: white blood cell; Alb: albumin; Bil: bilirubin; AST: aspartate aminotransferase; ALT: alanine aminotransferase; Cr: creatinine

Table 4: Comparison of nutritional status in pre-transplant and chronic post-transplant phase (3 months after LT)

| | Pre-transplant | Post-transplant (3 months after transplant) |
|--|----------------|---|
| Anthropometric evaluation | | |
| Weight (kg) | 73.9 | 78.6 |
| Height (cm) | 176 | 176 |
| Triceps ^[7] (cm) | 0.56 | 1.5 |
| MAMC ^[7] (cm) | 22 | 21.2 |
| SGA ^[8] | | |
| SGA ^[8] (score) | 6 | 2 |
| Body composition analysis by bioelectrical impedance analysis ^[9] | | |
| Weight (kg) | 72.55 | 76.6 |
| Fat (%) | 22.5 | 28 |
| Fat mass (kg) | 16.3 | 21.45 |
| FFM (kg) | 56.25 | 55.15 |
| Muscle mass (kg) | 53.35 | 52.3 |
| TBW (%) | 53.5 | 47.6 |
| BMI | 23.2 | 24.5 |
| Bone mass (kg) | 2.90 | 2.85 |

MAMC: mid-arm muscle circumference; SGA: subjective global assessment; FFM: fat-free mass; TBW: total body water; BMI: body mass index; LT: liver transplantation

strength (both hands) showed severe malnutrition similar to pre-transplant phase.^[9]

DISCUSSION

A high incidence of malnutrition has been seen in LT recipients.^[5,14,15] Accurate estimation of the nutritional status of patients with ESLD presents a major challenge due to fluid retention found in patients and the effect of liver function on protein synthesis.^[16] Malnutrition

has also been associated with poor surgery outcome and increased morbidity and mortality. In India, LT is a relatively new area, and there is a lack of data about the general and nutritional profile of patients undergoing LT. It is essential to identify and correct nutritional deficiencies in LT recipients. Hence, this case report provides information on the day to day nutrition profile and the medical nutrition therapy of a LT recipient with the aim of improving outcomes.

A gradual improvement in the nutrition, biochemical, and functional parameters was seen after 3 months of transplant. Nutrition assessment by SGA, triceps, and body composition analysis showed better nutrition status 3 months after LT. During the acute post-transplant phase, continuous observation by medical and nutrition experts helped to fulfill nutritional needs through various feeding routes. However, the difference in calorie and protein intake in chronic post-transplant phase is due to lack of counseling from nutrition experts. Hence, proper nutrition monitoring is required during all phases of transplant to maintain the overall health of the patient.

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Conflicts of interest

There are no conflicts of interest.

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Spontaneous rupture of hepatocellular carcinoma

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ABSTRACT

This is a very interesting case of a 64-year-old female with a history of chronic hepatitis C infection, with abdominal pain and was found to have ruptured hepatocellular carcinoma (HCC). She was managed with the two-stage therapeutic approach first using transarterial embolization to provide adequate hemostasis and then surgical resection with an excellent outcome. This case report exemplifies the importance of early diagnosis and treatment of ruptured HCC.

Key words: Hepatocellular cancer; rupture; transarterial embolization

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignant tumor of the liver, and one of the leading causes of death in patients with cirrhosis. Spontaneous rupture is a fatal complication of HCC that occurs in 3-15% of cases and is associated with worse short- and long-term prognosis.^[1-3] In this case report, we are presenting a case of a 64-year-old female with a history of chronic hepatitis C infection who presented with abdominal pain and was found to have a ruptured HCC. She was managed with the two-stage therapeutic approach first using transarterial embolization (TAE) to provide adequate hemostasis and then surgical resection with an excellent outcome. This case report exemplifies the importance of early diagnosis and treatment of ruptured HCC.

CASE REPORT

We are reporting a case of a 64-year-old female with past medical history of hypertension and hepatitis C, who was diagnosed and treated in the year 2000 with interferon and ribavirin. She presented to our emergency department


with worsening right upper abdominal pain for the last few months; the symptoms continued to progressively get worse until her presentation to the emergency department. Initial vital signs showed blood pressure of 140/76 mmHg, pulse rate of 74 beats/min, respiratory rate of 18 breaths/min, and oxygen saturation of 98% on room air. Physical examination showed significant right upper quadrant tenderness, soft abdomen with no guarding or rigidity and active bowel sounds, normal heart sounds with no murmurs or added sounds, and normal breath sounds. Initial lab results showed a white blood cell count of 7.4 k/ μ L, hemoglobin of 14.2 g/dL, hematocrit of 42.8%, platelets of 177 k/ μ L, creatinine of 0.89 mg/dL, aspartate aminotransferase of 30 IU/L, alanine aminotransferase of 29 IU/L, alkaline phosphatase of 63, total bilirubin of 0.8, prothrombin time of 13.6 s, partial thromboplastin time of 25.7 s, international normalized ratio of 1.3, albumin of 3.9 g/dL, alpha-fetoprotein (AFP) of 1380 ng/mL, and hepatitis C antibodies were positive, but with an undetectable viral load, hepatitis B antibodies and surface antigen were negative.

An abdominal ultrasound showed a 7 cm \times 6 cm mass in the right hepatic lobe, abdominal computed tomography (CT)

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scan with contrast and triple phase abdomen CT scan were done for better visualization of the mass which showed a 7.2 cm \times 5.8 cm heterogeneous enhancing mass in the sixth segment of the liver, with a pseudocapsule bulges on the liver capsule, which has an adjacent small 6 cm \times 3 cm accumulation of complex fluid that likely represents a ruptured HCC and less likely a benign liver tumor with regional hematoma, the liver was abnormal in appearance with nodular contour suggestive of underlying cirrhosis/fibrosis [Figures 1 and 2].

Gastroenterology, hepatobiliary surgery, and interventional radiology (IR) were consulted, after that and while the patient was being evaluated, her hemoglobin level dropped to 12.2 g/dL and she became more tachycardic. At that time, the impression was that the patient has a ruptured HCC, and the decision was to do an IR-guided bland embolization of the tumor. The embolization was done using polyvinyl alcohol particles. After that, the patient remained hemodynamically stable. Later, she underwent resection of the tumor and the sixth segment of the liver, during surgery there was no evidence of spread of the tumor outside the liver.

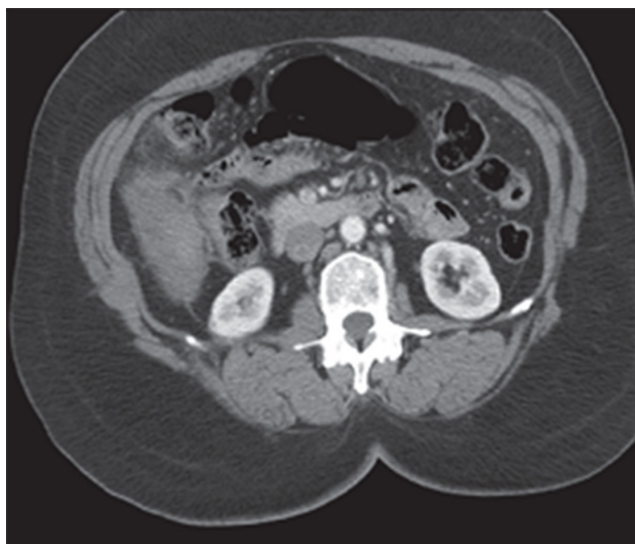


Figure 1: Computed tomography scan revealing evidence of hepatocellular cancer

The pathology report showed a 6.5 cm \times 6.1 cm \times 6.0 cm moderately differentiated HCC with a trabecular and pseudoglandular growth pattern with foci of necrosis and hemorrhage and negative surgical margins, it also showed vascular invasion of the portal triad and diffuse macronodular cirrhosis [Figures 3 and 4]. At this point, the decision was made to follow-up the patient closely with AFP, liver function test, and imaging studies every 3 months for the first 2 years. Follow-up AFP about 1 month after surgery was 54.4 ng/mL and 4.8 ng/mL after 3 months.

DISCUSSION

HCC is the most common primary malignant tumor of the liver; it is also known to be the fifth most common cancer and the third most common cause of cancer-related death worldwide.^[1,2] HCC is a hypervascular tumor that mostly occurs in the settings of liver cirrhosis, and it is one of the leading causes of death in patients with cirrhosis.

Spontaneous rupture is a major life-threatening complication of HCC that occurs in 3-15% of cases with geographical differences among Western, Asian, and African countries, where HCC is more frequent.^[3] The incidence of HCC is on decline due to early detection and screening.

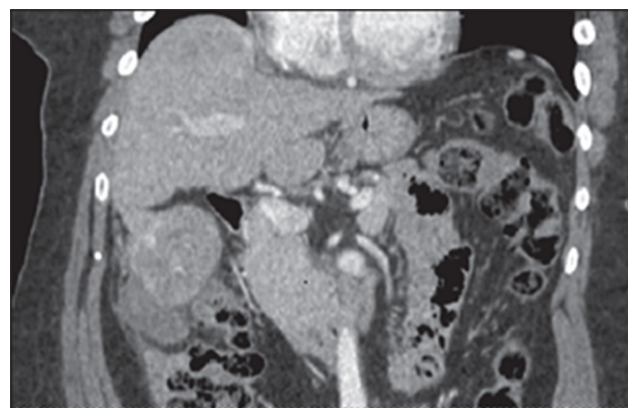


Figure 2: Computed tomography scan confirming evidence of hepatocellular cancer with regional hematoma

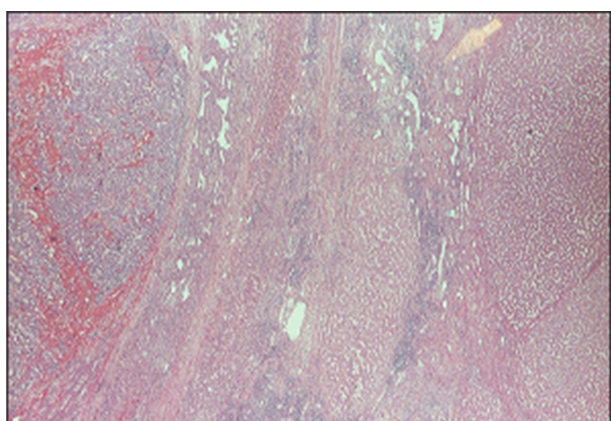


Figure 3: Histopathology of liver tissue revealing a trabecular and pseudoglandular growth pattern with foci of necrosis and hemorrhage

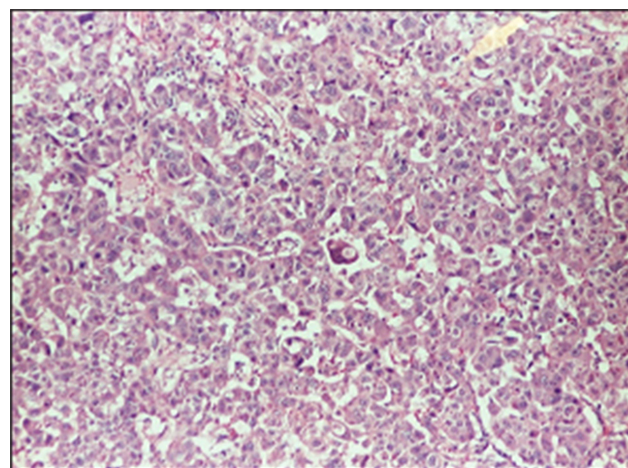


Figure 4: Histopathology of resected hepatic tumor

Generally, short- and long-term survival rates after ruptured HCC are worse compared to non-ruptured HCC patients. Spontaneous rupture is considered the third leading cause of HCC-related death after tumor progression and liver failure, with an associated mortality that is even higher than ruptured esophageal varices.^[4]

The exact mechanism of spontaneous rupture of HCC is not clearly known at this time, but it is believed to be related to a tear in the tumor surface or rupture of a feeding artery.^[5] Risk factors that could be responsible for HCC rupture include subcapsular location, rapid growth of the tumor with necrosis, and erosion of vessels and blunt abdominal trauma, especially with superficial tumors.^[6,7]

The usual symptoms of spontaneous rupture are right upper quadrant or epigastric pain, and when the lesion is more peripheral and located on the free surface of the liver, it might be associated with signs of shock and peritoneal irritation due to hemoperitoneum. Peritoneal irritation due to bleeding is not as common in cases of rupture of a deeper lesion, which does not interrupt the liver capsule. In addition to pain and hemorrhagic shock, there is also a risk of peritoneal seeding of cancer cells, which worsen the prognosis. The diagnosis can be confirmed by the presence of hemoperitoneum on abdominal paracentesis. Ultrasonography may demonstrate a hepatic tumor and ascites, the rupture site appears as a hyperechoic area around the tumor, CT is valuable in showing the tumor with a high attenuation close to it, which represents acute blood clotting. Conventional angiography may reveal extravasations of contrast from the tumor. Zhu *et al.*^[8] reported that the positive rate of correct diagnosis was 86% with paracentesis, 66% by ultrasonography, 100% by CT, and 20% by angiography.

Treatment of spontaneous rupture of HCC is dependent on the pre-ruptured liver function and severity of bleeding, liver resection is the only curative option for ruptured HCC and the first step of treatment is resuscitation and stabilization of the patients.^[9-11]

The open surgical method was the mainstay of treatment for hemostasis in the period from the 1960s to the 1980s. Various surgical procedures, including perihepatic packing, suture plication of bleeding tumors, hepatic artery ligation, and liver resection, were reported to be effective in hemostasis.^[10-13] Open surgical procedures achieved a high rate of hemostasis but were associated with a high in-hospital mortality rate. With the introduction of TAE and transarterial chemoembolization (TACE), TAE has been increasingly used for hemostasis in ruptured HCC. Now, open surgical hemostasis becomes a second-line treatment when TAE fails or it is not available. However, it is still regarded as a reliable method for hemostasis, and permits consideration for resection of the tumor at the same time.^[4]

The two-stage therapeutic approach to manage ruptured HCC consists of initial management by conservative method, hemostasis by TAE or surgical means, and followed by second-stage hepatic resection or TACE.^[7,9] Previous studies suggested that multidisciplinary management with TAE and postponed surgery in selected patients improve the short-term mortality.^[7] If the patient's conditions allow, a two-staged approach involving TAE for hemostasis followed by staged hepatectomy is preferred over emergency hepatectomy. This approach permits to stabilize the patient, assess the liver function, and stage cancer to better plan the surgical resection. Emergency liver resection can achieve hemostasis and provide a definitive treatment in a single operation. However, one-stage hepatectomy is only recommended for patients with preserved liver function (Child-Pugh Classes A and B) and resectable tumors.^[12-14]

Conservative treatment is recommended for patients who are hemodynamically stable at initial presentation. TAE is the first choice of treatment for unstable patients with continuous intra-abdominal hemorrhage, TAE is thought to be the ideal treatment because it is simple and effective with a success rate of about 90%.^[10] Definitive treatment of HCC should follow the initial recovery from ruptured HCC. Patients with preserved liver function and resectable tumors should be considered for curative hepatic resection if a low-risk curative resection is possible for patients with Child-Pugh Classes A and B.^[10-13] TAE as a palliative procedure is indicated when the liver function is compromised or in the case of multifocal bilobar HCC. Long-term survival is correlated with the stage of the disease, its local spread after rupture, and the residual hepatic function.^[9-12]

In summary, HCC has a tendency to rupture spontaneously, which may lead to a life-threatening condition. Though recently TAE followed by a second-stage resection has been the first choice of treatment, laparotomy is still a reliable method for hemostasis and permits consideration for resection of the tumor at the same time. In the presented case, the two-stage therapeutic approach was used, utilizing a multidisciplinary team approach consisting of gastroenterology, hepatobiliary surgery, and IR consultants. Our patient was first managed with TAE to achieve hemodynamic stability and after that she underwent resection of the tumor with excellent outcomes.

To our knowledge, until now, there has been no prospective randomized controlled trial or well-designed comparative study to find out which is the best method of hemostasis. Most evidence comes from cohort series; therefore, more research is needed in this field.

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Conflicts of interest

There are no conflicts of interest.

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Microwave coagulation therapy: the future is quite rosy

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TO THE EDITOR

We read with great interest the review of Guan^[1] on microwave coagulation therapy (MCT) of hepatocellular carcinoma (HCC), and we strongly agree with his conclusion that MCT has a great promise for future use, especially with further technical improvements.

In this regard, one of the main limits of MCT [which discouraged its clinical application in many western countries in favor of radiofrequency ablation (RFA)] was the back heating effect, due to reflected waves along the coaxial line. Such a drawback imposed to use large antennas and to deliver energy for a short time, achieving small ablation areas and requiring multiple insertions even in the presence of small tumors.^[2,3] Internally-cooled MCT partially reduced this problem, allowing for the increase of the ablation time and the amount of power that could be safely delivered.^[4] The introduction into the distal portion of the antenna of a choke coil was proposed to reduce back heating effects. However, this remedy caused remarkable thickening of the antenna (9-10 gauge), making the

device not suitable for percutaneous applications.^[5] In the very last years, a miniaturized device for MW confinement has been developed (Mini Choke®), that enables to minimize back heating effects using slender MW antennas and allowing for percutaneous applications (AMICA MWA System, HS Hospital Service, Aprilia, Italy). In an experimental study, this system produced thermal lesions of 6.5 cm × 4.5 cm in *ex vivo* bovine liver by delivering 60 W for 10 min.^[6] A randomized prospective comparison of MCT and RFA reported significantly larger coagulation areas *in vivo* with MCT than with internally-cooled RFA, using a 16-gauge internally-cooled, minichoked MCT antenna with a power output of 60-70 W and ablation time of 10 min.^[7] Although energy delivery was underpowered with respect to the maximum power output of the system, MCT yielded ablation areas comparable to those previously reported by other authors who performed MCT using a power output of 100 W and a 14-gauge cooled shaft antenna without choke device.^[8] As the minichoked MWA system can also use a 14-gauge antenna with a power output of 100 W, it is hypothesizable that ablation areas even larger than those obtained in the above-mentioned *in vivo* comparison between MCT and RFA could be achieved using the maximum

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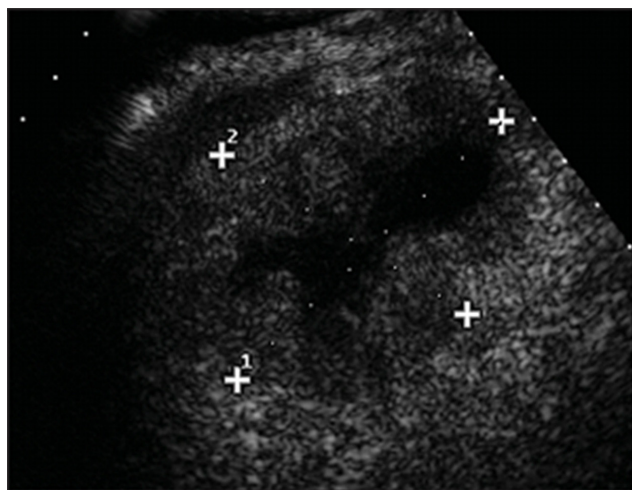


Figure 1: Right intercostal contrast-enhanced sonogram showing a 7 cm × 6 cm colorectal metastasis of the right liver lobe

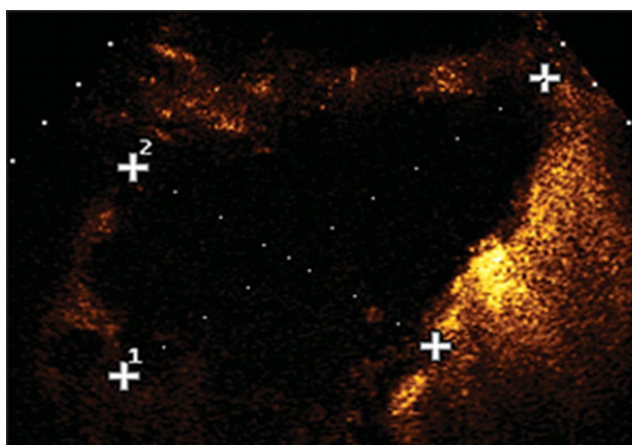


Figure 2: Right intercostal contrast-enhanced sonogram showing a 10 cm × 7 cm ablation area produced after two insertions of a 14-gauge internally-cooled, minichoked MW antenna using a power output of 90 W for a total ablation time of 20 min

power output. Indeed, we treated some HCCs and liver metastases measuring up to 7 cm × 6 cm, performing two insertions of a 14-gauge minichoked antenna and using a power output of 90 W for a total ablation time of 20 min, achieving coagulation areas measuring up to 10 cm × 7 cm [Figures 1 and 2].

Further randomized trials enrolling large series of patients are obviously needed to verify whether the superiority of MCT in obtaining larger ablation areas than RFA can

translate into better long-term outcome and longer survival of patients with primary and secondary liver tumors, but it is indubitable that the technical improvement of MCT systems is already ongoing. According to the conclusion of the interesting review of Guan,^[1] we believe that MCT is leaving its infancy and is running up to a quite rosy future.

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Nil.

Conflict of interest

There is no conflict of interest.

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Review

Human telomerase disease mutants and its relation with hepatocarcinoma

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ABSTRACT

Telomerase is a special reverse transcriptase, which adds telomeric DNA repeats to the ends of chromosome to offset loss. A vast majority of cancer cells have been shown that their telomerase was up-regulated and sustain proliferation and growth. Hepatocellular carcinoma (HCC) is one of the most commonly occurring cancers worldwide. It is also one of the leading causes of cancer death, and is connected with abnormal telomerase function. However, reports about the telomerase mutations and HCC are still insufficient. In this review, the structure and mechanism of action of telomerase, inherited disorders caused by its mutations, hepatocarcinoma, and drug development targeting telomerase are reviewed. However, further investigations are needed to elucidate human telomerase RNA gene regulation for initiation and progression of the liver cancer.

Key words: Telomerase; mutants; hepatocarcinoma; target

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INTRODUCTION

Following genome duplication, eukaryotic chromosomes shrink due to the incomplete replication.^[1] The end of the chromosomes is capped by DNA-protein complex known as telomere. The progressive loss of telomeric DNA threatens genome stability and limits cell division.^[2] Telomerase is a special reverse transcriptase which adds telomeric DNA repeats to the chromosome ends to offset loss.^[3] In human, telomerase is inactive in most of the somatic cells but not stem cells and germlines. So far it has been found that a vast majority of cancer cells, their telomerase is up-regulated in order to sustain proliferation and growth.^[4] Additionally, telomere mediated disorders such as dyskeratosis congenital, aplastic anemia and idiopathic pulmonary fibrosis have been demonstrated to have telomerase mutations.^[5-7]


Cancer is one of the world's greatest disease burdens and hepatocellular carcinoma (HCC) is one of the leading

causes of cancer death especially in Asia and Africa.^[8] HCC is induced by the well known risk factors such as hepatitis B, hepatitis C virus infection as well as cirrhosis.^[8,9] In general, it is widely accepted that telomeric shortening is responsible for limiting the life of human somatic cells and the expression of telomerase in the cells is sufficient to overcome both replication as well as senescence.^[10] Although the mechanism involved in telomerase regulation has not been completely understood, most types of cancer cells reveal a telomere length maintenance, which is responsible for their immortality.^[11,12] Intact telomere signaling has been demonstrated to be essential in the development of HCC. Similar to other types of cancers, it has been shown that around 85% of human HCC specimens exhibit reactivation of telomerase activity.^[13] Transcriptional regulation of the hTERT gene with frequent somatic mutations has been described in several tumor cells including HCC.^[14,15] Additionally, weak activation of telomerase has been reported during chronic viral hepatitis or cirrhosis, which could be potential factors

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for development of HCC.^[16] Thus, telomerase has been recognized as a relevant factor in distinguishing cancer from normal cells and is a very promising target for anticancer therapy.^[17]

STRUCTURE AND WORKING MECHANISM OF TELOMERASE

Telomerase is a unique reverse transcriptase, the core of which is composed of the telomerase reverse transcriptase (TERT) protein and integral telomerase RNA (TR).^[18,19] As a ribonucleoprotein, the TR of telomerase provides the template which specifies the telomere repeat sequence and motifs necessary for the activity; the protein is the catalytic component of the enzyme which comprises four conserved structural domains.^[20] Unlike TRs which varies in length and secondary structure among different species, TERT proteins are conserved and comprise four structural domains: the telomerase essential N-terminal domain (TEN), the TR binding domain (TRBD), the reverse transcriptase domain (RT) and the C-terminal extension domain (CTE).^[20,21]

To date, the only complete crystal structure of the TERT protein is from a flour beetle *Tribolium castaneum*, and subsequent biochemical work showed a DNA/RNA duplex bound to *T. castaneum*. Interestingly, the TERT as a model of TR bound to substrate DNA resemble those observed in human immunodeficiency virus RT.^[22] The recently reported crystal structure of TRBD of TERT and conserved region 4 and 5 of TR from teleost fish *Oryzias latipes* provides useful information for further investigation into the structure and function of telomerase ribonucleoproteins complex.^[23] Unfortunately, the whole structure of the human telomerase remains unsolved mainly because of the requirement for highly purified concentrated protein.

Compared with traditional RT, telomerase extend DNA substrate by using its own short RNA as a template. Therefore besides nucleotide addition, telomerase requires a process called template translocation to recycle its template. Furthermore, there are several working models of human telomerase that have been proposed in last few years by biochemical functional assay or single molecular FRET.^[24,25] However the detail of this process remains unknown.

MUTANTS AFFECT ENZYME FUNCTION

Numerous unique mutations within the hTR gene have been found to reduce the levels of active telomerase. Changes in the primary sequence can disrupt RNA base-pairing and local structure, which will affect telomerase function by: (1) reducing the assembly of hTERT and hTR; (2) mis-positioning of the template region; and (3) dissociation of hTR with accessory proteins.^[26-28] The reduction in telomerase activity or RNA accumulation is experimentally confirmed and is associated with diseases. Similar to hTR mutations, various unique mutations have been identified within the TERT gene, which are linked to human telomere mediated disorders

| hTERT | 5'-UTR | TEN | TRBD | RT | | | CTE | | 3'-UTR |
|-----------|--------|-------------|---------------|--------------------|-------|--------|--------|--|--------|
| Mutations | | P33S V144M | S368F R486C | R631Q L725F | R865H | R951Y | K1050E | | |
| | | L55Q V170M | R361P R522K | R671Y T726M | V867M | S957R | A1062T | | |
| | | V56L A202T | H412Y P524A | A678D V747A | H876Q | R972H | G1063S | | |
| | | P65A G260P | E441del K570N | G682D Y772C | R889X | R979W | R1084P | | |
| | | R83P A279T | | V694M R811C | R901W | C1015R | V1090M | | |
| | | R112P V299M | | P702L L841F | K902N | L1019F | T1110M | | |
| | | | | P704S Y846C | K902R | V1025F | F1127L | | |
| | | | | A716V G861R | P923L | N1028H | I1130V | | |
| | | | | D718N L862_L884del | H925Q | | | | |
| | | | P721R R865C | | | | | | |

Figure 1. The structural scheme for the four domains of the telomerase reverse transcriptase (TERT) protein with mutations. TERT is composed of four structural domains: telomerase essential N-terminal (TEN), telomerase RNA binding domain (TRBD), reverse transcriptase (RT), and C-terminal extension (CTE). The above structure has indicated the locations of mutations known to cause human diseases.

[Figure 1]. When mapped onto the amino acid sequence, the hTERT mutations are located almost exclusively in the conserved functional domains, especially concentrated within the RT motifs.^[29,30] While mutations that disrupt nucleotide addition are well characterized, only those with reduced repeat addition processivity have been discovered recently.^[30]

Table 1: Human telomerase related disease mutants

| Diseases | Mutations | | |
|--------------------------------------|-----------|----|--------------------|
| | TERT | TR | Accessory proteins |
| Aplastic anemia | ✓ | ✓ | ✓ |
| Acute myeloid leukemia | ✓ | | |
| Dyskeratosis congenita | ✓ | ✓ | ✓ |
| Pulmonary fibrosis | ✓ | ✓ | ✓ |
| Pancytopenia | ✓ | | |
| Hoyeraal Hreidarsson syndrome | | ✓ | ✓ |
| Thrombocytopenia | | ✓ | |
| Paroxysmal nocturnal hemoglobinuria | | ✓ | |
| Bone marrow failure | | | ✓ |
| Myelodysplastic syndrome | ✓ | | ✓ |
| Nail dystrophy | | | ✓ |
| Polymorphism | ✓ | ✓ | ✓ |
| Hypoplastic myelodysplastic syndrome | | ✓ | |
| Revesz syndrome | | | ✓ |
| Mucocutaneous features | | | ✓ |
| Intrauterine growth retardation | | | ✓ |
| Menorrhagia | | ✓ | |

TERT: telomerase reverse transcriptase; TR: telomerase RNA

THE INHERITED DISORDERS CAUSED BY THE TELOMERASE MUTATIONS

The hTERT and hTR genes are considered the common cause of inherited human telomerase mediated disease. Numerous mutations within hTERT and hTR including substitution, additions and deletions have been shown connected to inherited disorders that lead to diseases. Congenital dyskeratosis, aplastic anemia and idiopathic pulmonary fibrosis have been demonstrated linking to mutations within the genes that encode for two telomerase core components hTERT and hTR as well as telomerase associated proteins [Table 1].^[31-33] The maintenance of telomere length in highly

proliferative cells, stem cells and germline, is crucial for the preservation of high populations and human health.^[34] Generally, point mutations that lead to single substitution of amino acid are more likely tolerated than frame shift and splicing junction mutations, limiting but not abolishing the enzyme activity. The toleration of reduction and loss of telomerase function decreases with several subsequent generations. The telomeres of the parental generation erode when passed to the offspring with shorter telomeres. The increase in severity of symptoms is linked with the progressive decrease to telomere length.^[35]

HEPATOCARCINOMA WITH EXPRESSION OF ACTIVE TELOMERASE

The relationship between telomerase mutation and development of hepatocellular carcinoma is controversial and inconclusive so far.^[13] Telomeres within HCC were shorter compared to normal liver cells suggesting that it could escape the DNA damage response and subsequent cell cycle arrest signal generated from short telomeres. It has been suggested that telomere shortening may represent a genetic risk factor for the development of cirrhosis.^[36] The beneficial effects of the telomere and telomerase system plays a role for suppression of the development of liver cirrhosis and HCC in gene knock out mouse model which was performed by Wiemann *et al.*^[37] and Kitada *et al.*^[38]

However, some studies of HBV-associated HCC have demonstrated that longer telomeres and higher telomerase activity correlates with a worse prognosis. The expression of dyskerin, the accessory component of telomerase complex, showed a correlation with tumorigenic process, which might be a prognostic factor in patients with HCC.^[39] A nuclear ribonucleoprotein A2/B1, an hTERT-associated protein was proposed as a marker and prognosis factor of HCC.^[40] The study by Lechel *et al.*^[41] provides direct evidence that telomerase is a critical component for *in vivo* progression HCC with short telomeres in the chronically damaged liver and telomerase limits the accumulation of telomere dysfunction thus suppressing hepatocarcinogenesis. Taken together, short telomeres or telomere dysfunction appears permissive for the development of early stage neoplasia, but inhibitory to later stage and more anaplastic lesions.^[42]

Transcriptional regulation of the TERT gene is a cause of cancer specific increase in telomerase activity.^[43] Quaas *et al.*^[44] and other researchers have shown the mutations on promoter region of hTERT in hepatocellular carcinoma. Meanwhile, several reports have shown that increase in telomerase activity was detected in nearly 90% of HCC as compared to only 21% of non-tumor tissue which resulted in increased levels of TERT mRNA implying that TERT mRNA expression could predict or be a marker of HCC.^[45,46] Recent study from Cevik *et al.*^[47] hTERT promoter is one of most frequent mutational targets in liver cancer regardless of the geographical location and two site mutation (C228T AND

C250T) showed very high frequency in HCC. Furthermore, large scale studies by Huang *et al.*^[43] identified TERT promoter mutations to be 31.4% in HCC which shows high frequency similar like other primary cancers.

Cirrhosis is a disease in which liver cells become damaged and is replaced by scar tissues. People with cirrhosis have an increased risk of liver cancer. Most people who develop liver cancer already have some evidence of cirrhosis. Evidence supporting the role of genetic risk factors has been accumulating during the past years and recently it has been also suggested that telomere shortening may represent a genetic risk factor.^[12] Valenti *et al.*^[48] found that HCC arising from cirrhosis contained a TERT mutation in the neoplastic tissue. Furthermore, studies from Hartmann *et al.*^[16] provides experimental evidence that telomerase gene mutations are present in patients who develop cirrhosis as a consequence of chronic liver disease.

DRUG RESEARCH AND DEVELOPMENT FOR CANCER WITH TELOMERASE AS TARGET

A fundamental property of the cancer cells is to replicate without limitation, which is achieved by telomerase-regulated telomere maintenance in most types of cancer cells. Since somatic cells do not utilize activated telomerase to keep the integrity of the telomere length, the telomerase inhibitors have the potential to be a selective anti-cancer agents to disrupt the proliferation of the telomerase-positive cancer cells.^[11] Oligonucleotide can interact with both telomerase RNA and mRNA of telomerase proteins, therefore native or modified oligonucleotides are considered to be potential telomerase inhibitors that can influence the biogenesis of telomerase core components. A promising oligonucleotide, GRN163L, has been developed as telomerase inhibitor, which acts as competitive inhibitor for the template region of the hTR.^[49,50] The compound has already completed phase I trials in patients and now being conducted for phase II trials.^[51] To trigger cancer cells death, it requires a period of treatment of telomerase inhibitor to produce enough short telomeres. However, the therapy may be more effective when combined with conventional chemotherapies.

Some of the telomerase inhibitors have been found in microbes, which target either telomerase holoenzyme activity or regulatory pathways of telomerase expression. Among anticancer compounds, the inhibitors are promising for the chemotherapy by virtue of differential expression of telomerase in cancer cells. Synthetic preparation or modification of already screened natural telomerase inhibitor will become useful weapons in the war against cancer e.g. BIBR 1532.^[52] Most recently the co-crystal structure of telomerase inhibitor BIBR 1532 with *Tribolium castaneum* telomerase catalytic subunit showed a novel motif on the thumb domain could be a target for inhibiting telomerase function.^[53] Kellermann *et al.*^[54] identified a compound that prevent the assembling of the core enzyme and revealed a

target for screening small molecules capable to interfere with telomerase assembly. Indeed, for macromolecular complex, the interfacial drugs have a remarkable potential application.

G-quadruplex stabilizers are potent ligands that indirectly target telomerase resulting in inhibition of its activity. BRACO-19, RHPS4 and Telomestatin are commonly studied G-quadruplex binding ligands. Recently there are several studies showed anticancer drug candidates with G-quadruplex as targets.^[55,56]

Immunotherapy approach which induces T lymphocytes to respond to hTERT antigens in malignant tumor has shown good inhibitory effect. Preclinical studies with hTERT peptides have led to successful progress in the telomerase-targeting immunotherapies. Some telomerase vaccination such as Vx-001, GV1001 showed promising clinical outcome for different types of tumor.^[57,58] Recently an hTERT-derived peptide [hTERT(461)] have shown clinical benefits in HCC patients.^[59]

CONCLUSION AND PERSPECTIVE

Telomere shortening plays an important role in cell senescence. Telomerase which maintains the length of telomere connects with aging, chronic diseases as well as cancer promotion and progression.^[17,34] By looking into the telomerase gene mutations, the relation between the mutants and liver disease including HCC probably is due to the reduced activity. Meanwhile, the mutations at noncoding sequence of the telomerase also involved in the development of the HCC by regulating the expression level of active enzyme. It is commonly believed that the expression of hTERT may be a definitive factor in the activation of telomerase in hepatocarcinogenesis,^[46] however according to the recent paper from Xi *et al.*^[60] overexpression of either hTR or hTERT could increase telomerase activity which indicates that the two core components assemble into active telomerase is an equilibrium process. Further investigation is required to elucidate the regulation of hTR gene with initiation and progression of the liver cancer.

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Conflicts of interest

There are no conflicts of interest.

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Review

Murine double minute 2, a potential p53-independent regulator of liver cancer metastasis

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ABSTRACT

Hepatocellular carcinoma (HCC) has emerged as one of the most commonly diagnosed forms of human cancer; yet, the mechanisms underlying HCC progression remain unclear. Unlike other cancers, systematic chemotherapy is not effective for HCC patients, while surgical resection and liver transplantation are the most viable treatment options. Thus, identifying factors or pathways that suppress HCC progression would be crucial for advancing treatment strategies for HCC. The murine double minute 2 (MDM2)-p53 pathway is impaired in most of the cancer types, including HCC, and MDM2 is overexpressed in approximately 30% of HCC. Overexpression of MDM2 is reported to be well correlated with metastasis, drug resistance, and poor prognosis of multiple cancer types, including HCC. Importantly, these correlations are observed even when p53 is mutated. Indeed, p53-independent functions of overexpressed MDM2 in cancer progression have been suitably demonstrated. In this review article, we summarize potential effectors of MDM2 that promote or suppress cancer metastasis and specifically discuss the p53-independent roles of MDM2 in liver cancer metastasis from clinical as well as biological perspectives.

Key words: Murine double minute 2; metastasis; effectors; hepatocellular carcinoma; p53 independent

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INTRODUCTION

Liver cancer is the 5th most frequently diagnosed cancer worldwide in males (9th in females) and is the 2nd leading cause of cancer-related death in males (6th in females).^[1] Around 80% of hepatocellular carcinoma (HCC) cases occur in developing countries, mainly due to the incidence of hepatitis B and hepatitis C infections.^[2] HCC is often diagnosed at late stages, and the 5-year survival rate for metastatic HCC is less than 10% (<http://www.cancer.org/acs/groups/cid/documents/webcontent/003114.pdf.pdf>).^[3-5] Understanding the mechanisms involved in the regulation of HCC metastasis and discovering methods or compounds to suppress metastasis would be highly beneficial for HCC patients.^[6]

Metastasis is a cellular process which involves multiple

cascades including detachment of cancer cells from primary tumors, migration, intravasation, survival in the vasculature, extravasation, and colonization at a secondary site.^[7] Multiple factors play a role in each metastatic step and the inhibition of any of these steps would be helpful in blocking the cancer spread. Although distant metastasis is not a common event in HCC, HCC often shows vascular invasion, intrahepatic colonization, and lymph node metastasis. This is most likely due to the dense hepatic vasculature which supports the intrahepatic metastasis of HCC.^[8]

The murine double minute 2 (MDM2) was originally identified as a gene which was overexpressed in a spontaneously transformed mouse cell line (3T3-DM),^[9] and the gene product was found to transform normal cells.^[10] The primary function of MDM2 is to ubiquitinate the tumor suppressor p53 for inducing its degradation. Hence, MDM2 overexpression

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Table 1: Metastasis promoters interacting with MDM2

| Gene | Roles in liver cancer metastasis | Binding to MDM2 | Functional association with MDM2 | References |
|----------------|---|--------------------|--|---------------|
| HIF-1 α | Overexpression of HIF-1 α is correlated with vascular invasion and poor survival in human HCC. | Endogenous binding | MDM2 positively regulates HIF-1 α expression in MEFs, colon cancer, and osteosarcoma cell lines independent of p53. Conversely, MDM2 is reported to destabilize HIF-1 α by promoting its ubiquitination. | [32-39] |
| Slug | Overexpression of Slug is associated with invasion and metastasis of HCC by repressing E-cadherin. | Endogenous binding | MDM2 stabilizes Slug mRNA in human non-small cell lung carcinoma and colon cancer cell lines. | [41-44] |
| MMP-9 | Overexpression of MMP-9 is well correlated with invasion, metastasis, and poor prognosis in liver cancer. | Unknown | MDM2 increases the MMP-9 promoter activity in breast cancer cell lines. | [46-49,51,52] |
| HuR/ELAV1 | HuR expression is positively correlated with advanced stages in HCC and poor outcomes in HCC patients. | Endogenous binding | MDM2 neddylates HuR, protects it from degradation, and induces its nuclear localization in MEFs, mouse liver progenitor MLP29, colon cancer RKO, and HCC HepG2 cell lines. | [58,60] |

HCC: hepatocellular carcinoma; MDM2: murine double minute 2; MEFs: mouse embryonic fibroblasts; HuR: Hu antigen R; HIF-1 α : hypoxia-inducible factor-1-alpha; MMP-9: matrix metalloproteinase 9

Table 2: Metastasis suppressors interacting with MDM2

| Gene | Roles in liver cancer metastasis | Binding to MDM2 | Functional association with MDM2 | References |
|-------------|--|--------------------|---|---------------|
| E-cadherin | Reduced E-cadherin expression is associated with high tumor grade, vascular invasion, intrahepatic metastasis, disease progression, and poor outcomes. | Endogenous binding | MDM2 promotes E-cadherin degradation in breast cancer cell lines. | [68-72] |
| NME2 | NME2 expression is increased in HCC. | Endogenous binding | MDM2 suppresses the ability of NME2 to negatively regulate cell motility in renal cell carcinoma and lung cancer cell lines. | [77-79] |
| Tap63 | Role of Tap63 in HCC metastasis is not explored. | Endogenous binding | MDM2 suppresses Tap63 activity by inhibiting its nuclear localization in MEFs and osteosarcoma cell lines. Conversely, MDM2 increases Tap63 levels and its transcriptional activity in osteosarcoma and monkey kidney fibroblast-like cell lines. | [91,92,94] |
| FOXO family | Direct association of FOXO proteins with HCC metastasis remains unknown. | Endogenous binding | MDM2 degrades FOXO 1, 3, and 4 in MEFs, breast cancer, and lung cancer cell lines. | [110-112] |
| MTBP | MTBP inhibits HCC migration and metastasis in ACTN4-dependent and -independent manners. Controversially, MTBP may increase HCC metastasis by stabilizing MDM2. | Exogenous | The roles of MTBP in cancer metastasis, the underlying mechanisms, and functional association between MDM2 and MTBP remain to be further investigated. | [114-117,122] |

MDM2: murine double minute 2; FOXO: forkhead box O; NME2: non-metastatic cells 2; MTBP: MDM2 binding protein; HCC: hepatocellular carcinoma

greatly contributes to tumor development through inhibition of p53 activity. MDM2 is also a transcriptional target of p53, hence forming autoregulatory negative feedback loop.^[11]

Increasing evidence, however, indicates that MDM2 also has p53-independent functions toward malignant progression when overexpressed. Approximately 10% of human cancers have both MDM2 overexpression and mutant p53.^[12] Mice carrying a MDM2 transgene develop a higher percentage of sarcomas regardless of p53 status, as compared with p53-null mice.^[13] Ectopic expression of MDM2 in mammary epithelial cells of mice, as well as in mouse embryonic fibroblasts (MEFs), increases aneuploidy and chromosome/chromatid breaks regardless of p53 status.^[14,15] MDM2 interacts with different proteins and alters their activities, leading to malignant progression independent of p53.^[11] Specifically, MDM2 inhibits Nijmegen breakage syndrome 1, leading to inhibition of double-strand break repair.^[16] MDM2 also promotes p21 degradation.^[17,18] Additionally, MDM2 promotes cell cycle progression through activation of S-phase, via interaction with the retinoblastoma tumor suppressor protein and the transcriptional factor E2F.^[19,20] MDM2 furthermore enhances doxorubicin resistance in acute lymphoblastic leukemia cells through its binding to the Sp1-binding site in the p65 promoter.^[21] MDM2 is shown to bind to Sp1 and inhibit Sp1-dependent transcription.^[22] Thus, numerous MDM2 binding partners and effectors contribute to its p53-independent functions.^[23]

MDM2 overexpression is clinically correlated with metastasis of multiple cancer types including liver cancer,^[24-27] but the underlying mechanisms remain unclear. In this review, we focus on p53-independent roles of MDM2 in cancer metastasis, specifically in liver cancer. We categorize effectors of MDM2 into metastasis promoters [Table 1] and suppressors [Table 2].

METASTASIS PROMOTERS

Hypoxia-inducible factor-1-alpha

Hypoxia-inducible factor-1-alpha (HIF-1 α) and HIF-1 β are a class of transcription factors that play a key role in regulating cellular response against hypoxia.^[28] While HIF-1 β is constitutively expressed, expression of HIF-1 α is dependent on oxygen tension. In normoxic conditions, it is rapidly degraded, whereas in hypoxic states, HIF-1 α heterodimerizes with HIF-1 β on hypoxia response elements in the promoter regions of numerous downstream target genes, thus promoting tumor invasion, angiogenesis, and metastasis.^[29] For example, HIF-1 α transactivates Snail1 and vascular endothelial growth factor (VEGF) that accelerate epithelial-mesenchymal transition (EMT), a crucial biologic process for epithelial tumors to gain metastatic potential, and angiogenesis, respectively, thereby enhancing invasion and metastasis.^[30] HIF-1 α is overexpressed in multiple types of human cancer including HCC.^[31,32] Overexpression of HIF-1 α is correlated with vascular invasion and poor survival in

human HCC.^[32-35]

MDM2 directly binds to HIF-1 α , and overexpression of MDM2 results in accumulation of HIF-1 α in hypoxic cells and increase in hypoxia-induced VEGF transcription.^[36,37] Conversely, MDM2 is shown to degrade HIF-1 α under hypoxic conditions, which is inhibited by phosphorylation of MDM2 at serine 166 by AKT.^[38,39] Thus, the roles of MDM2 in regulating HIF-1 α function need to be further investigated. Although both MDM2 and HIF-1 α play roles in HCC progression, there is no existing study that directly shows MDM2 enhancing liver cancer metastasis through upregulation of HIF-1 α .

Slug

Slug (also known as Snail family zinc finger 2: Snail2) is a member of the Snail family of transcription factors that induce EMT crucial for embryogenesis and cancer metastasis by repressing E-cadherin.^[40] Slug is upregulated in many cancer types, including HCC, and its overexpression is associated with invasion and metastasis of HCC.^[41-43]

MDM2 is shown to stabilize Slug mRNA in a p53-independent manner, while knockdown of Slug nullifies invasion of HCT116 p53-null colon cancer cells induced by MDM2 overexpression.^[44] However, direct evidence demonstrating that MDM2's involvement in promoting HCC metastasis via upregulation of Slug has not yet been demonstrated.

Matrix metalloproteinase-9

Matrix metalloproteinase 9 (MMP-9), is a type IV collagenase which is a group of zinc-containing endopeptidases to degrade structural proteins of extracellular matrix, thus playing a pivotal role in the metastatic process.^[45] Overexpression of MMP-9 is well correlated with invasion, metastasis, and poor prognosis in liver cancer.^[46-49] Correlation between the expression of MMP-9 and MDM2 is shown in benzopyrene-induced lung cancer in rats, where both protein expression is higher in stage III and IV lung cancer tissues as compared with stage I and II tissues.^[50] Also, in human breast cancer, MDM2 expression is positively correlated with that of MMP-9, and is also negatively correlated with disease-free survival.^[51] Moreover, knockdown of MDM2 in pancreatic carcinoma SW1990HM cells results in reduced MMP-9 protein expression,^[52] and MDM2 promotes invasion of both MCF7 and MDA-MB-231 cell lines by increasing the MMP-9 promoter activity.^[51] Although there is definite clinical and functional correlation between MMP-9 and MDM2, it remains unclear whether MDM2 induces invasion and metastasis in liver cancer through upregulation of MMP-9.

Hu antigen R

Hu antigen R (HuR, also known as ELAV-like protein 1) was first identified in drosophila as a member of the embryonic lethal abnormal vision (ELAV) family RNA-binding proteins.^[53,54] HuR binds to AU-rich elements in the 3' untranslated region of target mRNAs and stabilizes them, resulting in regulation of cell proliferation, survival, immune response, and

differentiation.^[55] Elevated expression of HuR is reported in many types of cancer.^[56,57] Specifically, HuR is upregulated in HCC, and its expression is positively correlated with advanced stages of HCC, as well as poor outcomes in HCC patients.^[58] HuR promotes proliferation and differentiation of hepatocytes, as well as HCC transformation.^[59] Importantly, MDM2 neddylation of HuR, protects it from degradation, and induces its nuclear localization in mouse liver progenitor, colon cancer, and HCC cell lines.^[60] Although all the cell lines contain wild-type p53, neddylation of HuR by MDM2 is likely to be p53-independent, which needs to be clarified in the future. Importantly, it also remains unknown whether neddylation of HuR by MDM2 enhances HCC metastasis.

METASTASIS SUPPRESSORS

E-cadherin

E-cadherin is a single transmembrane glycoprotein involved in Ca^{2+} -mediated cell adhesion, mobility, and proliferation of epithelial cells and functions as a metastasis suppressor.^[61,62] Reduced expression of E-cadherin is correlated with high potential of invasion and metastasis, as well as poor prognosis, in many cancer types including breast,^[63] gastric,^[64] lung,^[65] colorectal,^[66] and pancreatic cancer.^[67] Also in HCC, reduced E-cadherin expression is associated with high tumor grade, vascular invasion, intrahepatic metastasis, disease progression, and poor outcomes.^[68-71]

MDM2 is found to directly interact with E-cadherin and facilitate its degradation in a p53-independent manner.^[72] Expression of MDM2 and E-cadherin is inversely correlated in breast cancer having lymph node metastasis.^[72] However, it remains unclear whether or not MDM2 promotes HCC metastasis by degrading E-cadherin.

Non-metastatic cells 2

Non-metastatic cells 2 (NME2, also known as NDPK-B, NM23B, NM23-H2) belongs to the nonmetastatic family and functions as a metastasis suppressor.^[73] Reduced NME2 expression is associated with increased metastatic potential of oral squamous cell carcinoma, lung, ovarian, colon, and breast cancer.^[74-76] However, NME2 expression is found to be increased in HCC.^[77,78]

MDM2 interacts with NME2 in H1299 lung cancer and HEK293 embryonic kidney cell lines and also suppresses the ability of NME2 to negatively regulate cell motility in renal cell carcinoma (UOK117 and its derivative 1.27) and H1299 cell lines.^[79] However, the role of NME2 in metastasis suppression of HCC and its functional association with MDM2 in HCC remain to be investigated.

Tap63

Tap63, along with Tap73, are tumor suppressor proteins that belong to the p53 family with high homology in the DNA binding domain and recognize the same p53 responsive elements.^[80] Tap63 suppresses migration and metastasis in many human cancer types including liver cancer, thus

functioning as a metastasis suppressor.^[81-86] On the other hand, isoforms of p63 lacking N-terminal domain show oncogenic function and are overexpressed in multiple cancer types.^[85,87,88] Mice with deletion of the p63 gene spontaneously develop tumors, while compound knockout mice for p53 and p63 show high frequency of metastasis as compared with p53 or p63 knockout mice.^[89,90]

Tap63 weakly binds to MDM2,^[91] and MDM2 is shown to attenuate apoptotic function of Tap63 by inhibiting its nuclear localization.^[92] However, it is unknown whether or not MDM2 inhibits metastasis suppressor function of Tap63. Conversely, it is also reported that MDM2 competes with Tap63 for binding to p53^{R175H} mutant to restore p63 activity,^[93] and overexpression of MDM2 increases the steady-state level of intracellular Tap63 and enhances its transcriptional activity.^[94] Thus, the functional relationship of MDM2 with Tap63 is controversial.

Forkhead box O family

Forkhead box O (FOXO) proteins (FOXO1, 3, 4, and 6) are members of the forkhead family of transcription factors.^[95] FOXO proteins have been implicated in suppression of tumor progression in multiple cancer types.^[96-100] Expression of FOXO proteins is negatively correlated with migration, invasion, and metastasis of renal cell carcinoma,^[101] lung cancer,^[102] prostate cancer,^[103] and urothelial cancer.^[104] Importantly, FOXO3 inhibits EMT by suppressing activities of β -catenin in prostate cancer^[103] and Twist1 in urothelial cancer,^[104] while FOXO4 functions as a metastasis-suppressor through counteracting the PI3K/AKT signal pathway in prostate cancer^[105] and inhibiting EMT in lung cancer.^[106] Although reduced expression of FOXO proteins is correlated with hepatocarcinogenesis and poor survival of HCC patients, direct association of FOXO proteins with HCC metastasis remains unknown.^[107-109] MDM2 functions as an E3 ubiquitin ligase for FOXO1, FOXO3, and FOXO4 to promote their degradation.^[110-112] However, it remains unsolved whether degradation of FOXO proteins by MDM2 accelerates cancer metastasis.

MDM2 binding protein

MDM2 binding protein (MTBP) was originally identified as a protein that binds to MDM2.^[113] Although these two proteins interact exogenously, their endogenous interactions have not yet been demonstrated. Overexpression of MTBP is shown to suppress cell migration and metastasis of osteosarcoma and HCC in alpha-actinin 4 (ACTN4)-dependent and -independent manners.^[114-116] Also, in MTBP knockout mice, MTBP haploinsufficiency increases metastasis of tumors induced in the p53 heterozygous background.^[117] Clinically, reduced MTBP expression is associated with reduced patient survival with head and neck carcinoma, as well as capsular/vascular invasion and lymph node metastasis in HCC.^[116,118] On the other hand, increased MTBP expression is observed in B-cell lymphoma and triple negative breast cancer where it contributes to tumor progression through its interaction

with Myc.^[119-121] In another study on human HCC, increased MTBP expression is shown to be associated with increase in MDM2 levels and metastasis, as well as poor survival of HCC patients, which is contrary to previously published studies.^[122] Thus, the roles of MTBP in cancer metastasis, the underlying mechanisms, and functional association between MDM2 and MTBP need to be further clarified in the future.

CONCLUSION

Approximately 30% of human cancers have MDM2 overexpression. Specifically, in well differentiated liposarcomas, MDM2 overexpression is detected in over 90% of the cases.^[123] These observations indicate significance of MDM2 overexpression in cancer progression. The mechanisms of MDM2 overexpression or hyper-activation include MDM2 gene amplification,^[124] single nucleotide polymorphisms in the MDM2 promoter,^[125] silencing/inhibition of MDM2 negative regulators,^[126] phosphorylation of MDM2,^[127] enhanced translation,^[128] or other mechanisms.^[129] Although the best characterized function of MDM2 is to inhibit p53 activity, an increasing body of evidence suggests that MDM2 has a p53-independent function. Such function is found specifically when MDM2 is overexpressed. MDM2 mainly exerts its p53-independent function by interacting with its downstream effectors.^[11] These effectors frequently play integral roles in cancer progression including cancer metastasis and drug resistance. Indeed, MDM2 overexpression is implicated in cancer metastasis through enhancing EMT, activation/upregulation of other oncoproteins, and suppression of tumor suppressors or metastasis suppressors. However, there is scarce evidence showing direct involvement of MDM2 in invasion and metastasis of HCC. It is thus imperative to have future studies that could appropriately demonstrate the direct role of overexpressed MDM2 in promoting HCC metastasis.

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Conflicts of interest

There are no conflicts of interest.

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Can gender predict virological response to standard antiviral therapy for chronic hepatitis C? A retrospective study

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ABSTRACT

Aim: The liver is a sexually dimorphic organ presenting gender differences in its metabolism, functions, enzyme activity, membrane lipid composition and immune response. This paper aimed to assess whether gender may predict virological response to standard antiviral therapy in subjects with chronic hepatitis C (CHC). **Methods:** The authors retrospectively analyzed 100 patients with genotype 1 CHC (55 men, 45 women), who performed standard antiviral therapy (interferon and ribavirin for 12 months) in the period 2002-2012, evaluated with blood tests and abdominal ultrasound to compare different virological and biochemical response in both gender. **Results:** Rate of sustained virological response (SVR) was higher, but not significant, in women than men (46.7% vs. 34.5%, $P = 0.05$); difference became significant after stratification by age (< 50 and ≥ 50 years). Specifically in the group aged under 50 years, rate of SVR was significantly higher in women than in men (66.7% vs. 38.2%, $P < 0.05$). **Conclusion:** Female gender may predict virological response to standard antiviral therapy in subjects with CHC aged below 50 years. Considering new potent and more expensive antiviral drugs actually available for HCV treatment, it could be useful to identify candidates firstly eligible to therapy.

Key words: Liver; gender; antiviral therapy

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INTRODUCTION

The liver is a sexually dimorphic organ with gender differences in gene expression, mitochondrial function, microsomal enzyme activity, membrane lipid composition, immunological response. Many studies found gender differences in hepatic response to different stressors, postulating as pattern of secretion and expression of receptors of growth hormones and sex hormone levels may underlie sexual dimorphism. The hepatic circulation depends by a balance between vasoconstrictor and vasodilator

substances; in stress conditions, the liver produces prevalent vasodilating substances in females than in males, probably due to estrogens, contributing to protect microcirculation.^[1]

Clinical studies also showed how females are more susceptible to the alcohol detrimental effects, as they develop liver disease following alcohol exposure, although reduced in quantity and time. Thus chronic alcohol assumption may modify the

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hormonal balance in both sexes, suggesting a role for sex hormones in the pathogenesis of alcohol-induced liver disease. Furthermore, compared to men, women have a lower volume of distribution and gastric alcohol dehydrogenase activity, so being more prone to liver injury.^[2] Furthermore, gender differences have been reported in both incidence and progression of specific liver diseases, such as autoimmune hepatitis, genetic hemochromatosis, non-alcoholic hepatic steatosis and chronic hepatitis C (CHC).

In this study, we aimed to assess whether gender may predict virological response to standard antiviral therapy in subjects with CHC. The identification of predictive factors for response to treatment may allow personalize therapy and improve the cost-effectiveness profile.

METHODS

Patients

We retrospectively evaluated 100 subjects (55 men, 45 women) with genotype 1 CHC who performed standard antiviral therapy [interferon (IFN) and ribavirin for 12 months] in the period 2002-2012, followed by the Department of Medical Science of "San Giovanni Battista" hospital-Turin (Italy). Criteria to start therapy were: serum alanine aminotransferase (ALT) levels 1.2 times the upper limit of the normal range in at least two assessments during the previous 6 months; anti-hepatitis C virus (HCV) antibody positivity; positive polymerase chain reaction for HCV-RNA; hemoglobin values >13 g/dL in males and > 12 g/dL in females, leukocytes count > 3,000 cells/mm³, platelets (PLTs) count > 100,000 cells/mm³, normal serum bilirubin, international normalized ratio (INR) and thyroid function tests.

Exclusion criteria included previous antiviral treatments for CHC; co-infections with hepatitis B virus (HBV) or human immunodeficiency virus; immunosuppression state; autoimmune hepatitis; primary biliary cirrhosis; chronic alcohol abuse; uncontrolled psychiatric illness; decompensated cirrhosis; chronic kidney failure; heart disease; hepatocellular carcinoma; pregnancy.

Laboratory analyses and instrumental evaluations

Before treatment, patients underwent routine blood tests [including assessment of complete blood count, aspartate aminotransferase (AST), ALT, gamma-glutamyltransferase (GGT), alkaline phosphatase

(ALP), total and fractionated bilirubin, alpha-fetoprotein, INR, creatinine, uric acid, cryoglobulins, thyroid hormones, ferritin, HBV serum profile and HCV-RNA], abdominal ultrasound and fibrosis assessment.

Specific anti-HCV antibodies were assessed by chemiluminescence ("chemiluminescent assay", Architect, Abbott Laboratories, AbbottPark, IL).^[3,4]

Qualitative and quantitative assessment of HCV RNA were performed using the "COBAS Amplicor HCV system" (sensitivity 50 IU/mL, Roche Molecular Systems, INC., Branchburg, NJ) and the bDNA signal amplification test (sensitivity 615 IU/mL, Branched-DNA version 3.0, Bayer Diagnostics Corporation, Tarrington, NY), respectively, in the period 2002-2007, and the qualitative method COBAS AmpliPrep™-COBAS TaqMan™ (CAP/CTM HCV; sensitivity 15 IU/mL) since 2008.^[5-8]

Both HCV RNA genotype and subtype were assessed by reverse hybridization line probe assay (INNO-LiPA, Innogenetics, Ghent, Belgium).^[9]

Hepatic steatosis, assessed by ultrasound, was defined as an increased liver parenchyma echogenicity compared to the spleen or to the right kidney, the attenuation of the ultrasound beam in depth tissues and the loss of echoes in the portal veins walls according to the following grade scoring system: grade 0, normal echogenicity, absence of differences between echogenicity of liver and kidney; grade 1, mild steatosis with increased echogenicity of liver compared to kidney, absence of attenuation of the ultrasound beam, possibility to explore the depth of hepatic parenchyma; grade 2, moderate increase of steatosis with higher echogenicity of the liver, attenuation of ultrasound beam in depth, loss of echoes from the peripheral portal branches; and grade 3, advanced steatosis with marked increase in echogenicity, attenuation of ultrasound beam in depth and loss of echoes from the major portal branches.

Fibrosis was assessed by elastography (FibroScan elastography) and defined as follows: F0 (up to 5 KPa), F1 (5 to 8.9 KPa), F2 (8.9 to 11 KPa), F3 (11 to 14.5 KPa), F4 (> 14.5 KPa).^[10]

In subjects not underwent to FibroScan ($n = 33$, males = 18), fibrosis was estimated by the FIB-4 method, according to the formula: $[\text{age (years)} \times \text{AST (U/L)}] / \text{PLTs (10}^9\text{/L)} \times [\text{ALT (U/L)}]^{1/2}$ and defined

according to the score: < 1.45 was considered as FO-F1; $1.45-3.25$ was considered as F2; > 3.25 was considered as F3-F4.

Treatment

Standard treatment consisted in pegylated IFN alfa-2a $180 \mu\text{g}$ s.c. once a week or pegylated IFN alfa-2b $1-1.5 \mu\text{g/kg}$ s.c. once a week plus ribavirin (800 mg/day for patients weighing $< 70 \text{ kg}$, $1,000 \text{ mg/day}$ for patients weighing $70-80 \text{ kg}$, $1,200 \text{ mg/day}$ for patients weighing $> 80 \text{ kg}$) for 48 weeks.

In the presence of adverse events, both IFN and ribavirin doses were reduced by 25% and down to 200 mg , respectively; both were stopped when hemoglobin $< 8.5 \text{ g/dL}$ and/or leukocytes count $< 2,000 \text{ cells/mm}^3$ and/or PLTs count $< 50,000/\text{mm}^3$.

Treatment efficacy was assessed according to rapid virological response (RVR, undetectable HCV RNA at week 4 of treatment); early virological response (EVR), including complete EVR (cEVR, undetectable HCV RNA at week 12 of treatment in the absence of RVR) and partial EVR (pEVR, $\geq 2 \log$ reduction of serum HCV RNA at week 12 of therapy compared with the baseline level, in the absence of RVR or cEVR); end-of-treatment virologic response (ETVR, undetectable HCV RNA at the end of treatment); sustained virological response (SVR, HCV RNA negativity at the end of treatment and in the after 24 weeks); relapse was defined as undetectable HCV RNA at end of treatment and detectable HCV RNA during follow-up.

Subjects were followed monthly, until the end of therapy. Thereafter, subjects showing ETVR were observed during the following 24 weeks, in order to verify either the persistence SVR or the loss of response.

Statistical analysis

Data were expressed as means \pm SD (continuous variables) or proportions (categorical values). *T*-test and Chi-square test were used to evaluate group differences in means and proportions, respectively. Univariate analysis was performed on baseline parameters to identify factors potentially related to SVR. All *P* values were two sided, considering statistically significant a *P* value < 0.05 . All analyses were performed with Statistical Package for the Social Science version 20.0.

RESULTS

Assessment of baseline characteristics

Baseline characteristics, laboratory data and the degree of steatosis and fibrosis are summarized in Tables 1-6. Baseline characteristics, laboratory data and the degree of both steatosis and fibrosis were comparable in men and women, excepted for haemoglobin, GGT and uric acid values, resulted significantly higher in men. Similar results were obtained after stratification of participants by gender and age $<$ or > 50 years; haemoglobin and GGT values were significantly higher in men compared to women both aged less and more than 50 years. Cryoglobulins positivity occurred more frequently in women aged more than 50 years ($P = 0.05$).

Table 1: Comparison of baseline serum chemistry parameters between males and females in the whole sample

| | Whole sample ($n = 100$) | | Student's <i>T</i> -test | <i>P</i> value |
|---------------------------------------|----------------------------|---------------------|--------------------------|----------------|
| | Male ($n = 55$) | Female ($n = 45$) | | |
| Age (years) | 45.6 ± 11 | 48.8 ± 11.6 | -1.43 | 0.156 |
| PLTs ($\times 10^9/\text{L}$) | 202 ± 46 | 220 ± 69 | -1.534 | 0.128 |
| Hb (g/dL) | 15.2 ± 1.38 | 13.78 ± 1.36 | 4.774 | 0 |
| WBC ($\times 10^9/\text{L}$) | 6 ± 1.6 | 5.5 ± 1.5 | 1.318 | 0.191 |
| AST (U/L) | 56 ± 46 | 56 ± 44 | 0.03 | 0.976 |
| ALT (U/L) | 95 ± 72 | 72 ± 64 | 1.653 | 0.102 |
| GGT (U/L) | 96 ± 87 | 42 ± 31 | 4.568 | 0 |
| ALP (U/L) | 89 ± 36 | 98 ± 48 | -0.327 | 0.746 |
| Total bilirubin (mg/dL) | 0.96 ± 0.43 | 0.79 ± 0.26 | 1.878 | 0.065 |
| INR | 1.1 ± 0.3 | 1 ± 0.1 | 1.954 | 0.059 |
| Uric acid (mg/dL) | 5.6 ± 1.3 | 4.3 ± 1 | 3.854 | 0 |
| AFP (ng/mL) | 9.3 ± 9.9 | 6.7 ± 4.8 | 1.026 | 0.312 |
| HCV RNA ($\log_{10} \text{ UI/mL}$) | 5.7 ± 0.64 | 5.8 ± 0.64 | -0.445 | 0.657 |
| Cryoglobulins (+/-) | 3/52 | 6/39 | Chi = 1.88 | 0.17 |
| Ferritin (ng/mL) | 308 ± 469 | 134 ± 115 | 1.624 | 0.111 |

Data are shown as mean \pm SD. PLT: platelet; Hb: hemoglobin; WBC: white blood cell; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyltransferase; ALP: alkaline phosphatase; INR: international normalized ratio; AFP: alpha-fetoprotein; HCV: hepatitis C virus

Table 2: Comparison of baseline serum chemistry parameters between males and females respectively in the < 50 and > 50 year-aged patient samples

| | < 50 years sample (n = 55) | | | | > 50 years sample (n = 45) | | | |
|-----------------------------------|----------------------------|-----------------|------------------|---------|----------------------------|-----------------|------------------|---------|
| | Male (n = 34) | Female (n = 21) | Student's T-test | P value | Male (n = 21) | Female (n = 24) | Student's T-test | P value |
| Age (years) | 38.2 ± 5.7 | 38.1 ± 7.1 | 0.053 | 0.958 | 57.5 ± 5.6 | 58.2 ± 4.1 | -0.467 | 0.643 |
| PLTs (× 10 ⁹ /L) | 212 ± 38 | 235 ± 55 | -1.235 | 0.222 | 187 ± 54 | 206 ± 78 | -0.901 | 0.373 |
| Hb (g/dL) | 15.5 ± 1.37 | 13.8 ± 1.1 | 4.371 | 0 | 15 ± 1 | 13.74 ± 1.58 | 3.021 | 0.005 |
| WBC (× 10 ⁹ /L) | 6 ± 1.5 | 6 ± 1.48 | 0.077 | 0.939 | 5.8 ± 1.74 | 5.1 ± 1.3 | 1.409 | 0.168 |
| AST (U/L) | 55 ± 54 | 43 ± 35 | 0.877 | 0.384 | 56 ± 27 | 66 ± 49 | -0.879 | 0.384 |
| ALT (U/L) | 98 ± 82 | 62 ± 52 | 1.769 | 0.083 | 89 ± 48 | 80 ± 73 | 0.474 | 0.638 |
| GGT (U/L) | 105 ± 91 | 27 ± 20 | 4.583 | 0 | 75 ± 75 | 54 ± 34 | 1.194 | 0.239 |
| ALP (U/L) | 85 ± 42 | 97 ± 52 | -0.361 | 0.724 | 94 ± 28 | 100 ± 45 | -0.303 | 0.766 |
| Total bilirubin (mg/dL) | 0.94 ± 0.47 | 0.77 ± 0.25 | 1.294 | 0.203 | 1 ± 0.3 | 0.8 ± 0.2 | 1.659 | 0.108 |
| INR | 1.1 ± 0.3 | 1 ± 0.0 | 1.053 | 0.301 | 1.1 ± 0.2 | 1 ± 0.1 | 1.503 | 0.16 |
| Uric acid (mg/dL) | 5.6 ± 1.3 | 4.6 ± 0.7 | 1.032 | 0.314 | 5.6 ± 1.2 | 4.61 ± 1.1 | 3.036 | 0.006 |
| AFP (ng/mL) | 5.2 ± 2.9 | 4.1 ± 3.2 | 0.694 | 0.498 | 14.5 ± 13.2 | 8.3 ± 5 | 1.194 | 0.272 |
| HCV RNA (log ₁₀ UI/mL) | 5.66 ± 0.71 | 5.63 ± 0.75 | 0.094 | 0.926 | 5.86 ± 0.52 | 5.98 ± 0.48 | -0.737 | 0.466 |
| Cryoglobulins (+/-) | 3/31 | 2/19 | Chi = 0.008 | 0.93 | 0/21 | 4/20 | Chi = 3.84 | 0.05 |
| Ferritin (ng/mL) | 307 ± 77 | 26 ± 60 | 1.363 | 0.183 | 310 ± 191 | 357 ± 130 | 0.997 | 0.331 |

Data are shown as mean ± SD. PLT: platelet; Hb: hemoglobin; WBC: white blood cell; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyltransferase; ALP: alkaline phosphatase; INR: international normalized ratio; AFP: alpha-fetoprotein; HCV: hepatitis C virus

Table 3: Degree of fibrosis and steatosis in the whole sample considering males and females, value of the Chi-square test and the related P value

| | Whole sample (n = 100) | | Chi-square test | P value |
|-------------------|------------------------|-----------------|-----------------|---------|
| | Male (n = 55) | Female (n = 45) | | |
| Fibrosis, n (%) | | | 0.069 | 0.966 |
| 0-1 | 34 (61.8%) | 28 (62%) | | |
| 2 | 12 (21.8%) | 9 (20%) | | |
| 3-4 | 9 (16.4%) | 8 (18%) | | |
| Steatosis, n (%)* | | | 0.488 | 0.921 |
| Assessment | 19 (35.85%) | 13 (31%) | | |
| Light | 13 (24.5%) | 12 (28.6%) | | |
| Mild | 19 (35.85%) | 16 (38%) | | |
| Advanced | 2 (3.8%) | 1 (2.4%) | | |

*Steatosis degree has been assessed only in 95 subjects (53 males and 42 females), resulting 5 ultrasonographic investigations poorly reliable

Table 4: Degree of fibrosis and steatosis considering males and females, value of the Chi-square test and the related P value respectively in the < 50 and > 50 year-aged patient samples

| | < 50 years sample (n = 55) | | | | > 50 year sample (n = 45) | | | |
|-------------------|----------------------------|-----------------|-----------------|---------|---------------------------|-----------------|-----------------|---------|
| | Male (n = 34) | Female (n = 21) | Chi-square test | P value | Male (n = 21) | Female (n = 24) | Chi-square test | P value |
| Fibrosis, n (%) | | | 4.954 | 0.0839 | | | 0.145 | 0.929 |
| 0-1 | 27 (79.4%) | 21 (100%) | | | 7 (33.3%) | 7 (29.2%) | | |
| 2 | 4 (11.8%) | 0 (0%) | | | 8 (38.1%) | 9 (37.5%) | | |
| 3-4 | 3 (8.8%) | 0 (0%) | | | 6 (28.6%) | 8 (33.3%) | | |
| Steatosis, n (%)* | | | 1.8127 | 0.612 | | | 3.1 | 0.375 |
| Assessment | 11 (33.3%) | 9 (45%) | | | 8 (40%) | 4 (18.2%) | | |
| Light | 8 (24.2%) | 5 (25%) | | | 5 (25%) | 7 (31.8%) | | |
| Mild | 12 (36.4%) | 6 (30%) | | | 7 (35%) | 10 (45.5%) | | |
| Advanced | 2 (6.1%) | 0 (0%) | | | 0 (0%) | 1 (4.5%) | | |

*Steatosis degree has been assessed only in 95 subjects (33 males < 50 years, 20 females < 50 years, 20 males > 50 years, and 22 females > 50 years), resulting 5 ultrasonographic investigations poorly reliable

Assessment of virological response

Forty patients reached a SVR (SVR rate 40%); and 60 patients were negative at the end of the treatment (ETVR rate 60%), but among these 20 fell in the later 24 weeks, with a 33.3% relapse rate.

In the whole sample < 50 years patients showed a significant rate of SVR ($P = 0.040$) [Table 6], due to < 50 years women who achieved significant higher rates of both ETVR ($P = 0.001$) and SVR ($P = 0.01$) compared to males of similar age [Table 7, Figures 1

Table 5: Comparison of virologic response rates between males and females in the whole sample

| | Whole sample (n = 100) | | Chi-square test | P value |
|--------------------------------|------------------------|-----------------|-----------------|---------|
| | Male (n = 55) | Female (n = 45) | | |
| RVR | 5/55 (9.1%) | 6/45 (13.3%) | 0.455 | 0.5 |
| cEVR | 13/55 (23.6%) | 16/45 (35.6%) | 1.708 | 0.191 |
| pEVR | 16/55 (29.1%) | 13/45 (28.9%) | 0 | 0.982 |
| Absence of RVR or cEVR or pEVR | 21/55 (38.2%) | 10/45 (22.2%) | 2.947 | 0.086 |
| ETVR | 30/55 (54.5%) | 30/45 (66.7%) | 1.515 | 0.218 |
| Relapse rate | 11/30 (36.7%) | 9/30 (30%) | 0.30 | 0.584 |
| SVR | 19/55 (34.5%) | 21/45 (46.7%) | 1.515 | 0.218 |

Data are shown as n/N (%). RVR: rapid virological response; EVR: early virological response; cEVR: complete EVR; pEVR: partial EVR; ETVR: end-of-treatment virologic response; SVR: sustained virological response

Table 6: Comparison of virologic response rates between males and females respectively in the < 50 and > 50 year-aged patient samples

| | < 50 year sample (n = 55) | | | | > 50 year sample (n = 45) | | | |
|--------------------------------|---------------------------|-----------------|------------|---------|---------------------------|-----------------|------------|---------|
| | | | Chi-square | | | | Chi-square | |
| | Male (n = 34) | Female (n = 21) | test | P value | Male (n = 21) | Female (n = 24) | test | P value |
| RVR | 4/34 (11.8%) | 5/21 (23.8%) | 1.376 | 0.241 | 1/21 (4.8%) | 1/24 (4.2%) | 0.009 | 0.923 |
| CEVR | 11/34 (32.4%) | 12/21 (57.1%) | 3.279 | 0.070 | 2/21 (9.5%) | 4/24 (16.7%) | 0.494 | 0.48 |
| PEVR | 4/34 (11.8%) | 2/21 (9.5%) | 0.067 | 0.796 | 12/21 (57.1%) | 11/24 (45.8%) | 0.573 | 0.44 |
| Absence of RVR or cEVR or pEVR | 15/34 (44.1%) | 2/21 (9.5%) | 7.275 | 0.007 | 6/21 (28.6%) | 8/24 (33.3%) | 0.118 | 0.73 |
| ETVR | 19/34 (55.9%) | 19/21 (90.5%) | 7.275 | 0.007 | 11/21 (52.4%) | 11/24 (45.8%) | 0.192 | 0.66 |
| Relapse rate | 6/19 (31.6%) | 5/19 (26.3%) | 0.128 | 0.7206 | 5/11 (45.5%) | 4/11 (36.4%) | 0.188 | 0.66 |
| SVR | 13/34 (38.2%) | 14/21 (66.7%) | 4.199 | 0.04 | 6/21 (28.6%) | 7/24 (29.2%) | 0 | 0.964 |

Data are shown as n/N (%). RVR: rapid virological response; EVR: early virological response; cEVR: complete EVR; pEVR: partial EVR; ETVR: end-of-treatment virologic response; SVR: sustained virological response

Table 7: Comparison of virologic response rates between < 50 year-aged and > 50 year-aged male sample and between < 50 year-aged and > 50 year-aged female sample

| | Male sample (n = 55) | | | | Female sample (n = 45) | | | |
|--------------------------------|----------------------|---------------------|-----------------|---------|------------------------|---------------------|-----------------|---------|
| | < 50 years (n = 34) | > 50 years (n = 21) | Chi-square test | P value | < 50 years (n = 21) | > 50 years (n = 24) | Chi-square test | P value |
| RVR | 4/34 (11.8%) | 1/21 (4.8%) | 0.77 | 0.38 | 5/21 (23.8%) | 1/24 (4.2%) | 3.73 | 0.0531 |
| cEVR | 11/34 (32.4%) | 2/21 (9.5%) | 3.74 | 0.053 | 12/21 (57.1%) | 4/24 (16.7%) | 8 | 0.004 |
| pEVR | 4/34 (11.8%) | 12/21 (57.1%) | 12.95 | 0.0003 | 2/21 (9.5%) | 11/24 (45.8%) | 7.187 | 0.007 |
| Absence of RVR or cEVR or pEVR | 15/34 (44.1%) | 6/21 (28.6%) | 1.329 | 0.24 | 2/21 (9.5%) | 8/24 (33.3%) | 3.67 | 0.0553 |
| ETVR | 19/34 (55.9%) | 11/21 (52.4%) | 0.064 | 0.8 | 19/21 (90.5%) | 11/24 (45.8%) | 10.04 | 0.001 |
| Relapse rate | 6/19 (31.6%) | 5/11 (45.5%) | 0.577 | 0.44 | 5/19 (26.3%) | 4/11 (36.4%) | 0.33 | 0.56 |
| SVR | 13/34 (38.2%) | 6/21 (28.6%) | 0.536 | 0.464 | 14/21 (66.7%) | 7/24 (29.2%) | 6.32 | 0.01 |

Data are shown as n/N (%). RVR: rapid virological response; EVR: early virological response; cEVR: complete EVR; pEVR: partial EVR; ETVR: end-of-treatment virologic response; SVR: sustained virological response

and 2]. On the other hand, frequency of subjects not achieving RVR or EVR was significantly higher in men > 50 years than in females [Table 7]. No significant differences existed in virological responses in subjects > 50 years.

Influence of age among patients of the same gender

Analysis performed in subjects of the same gender stratified by age showed significantly higher rates

of pEVR in males > 50 years compared to < 50 years males. Furthermore, women < 50 years were characterised by significantly higher rates of cEVR, ETVR, SVR and by significantly lower rates of pEVR compared to women > 50 years [Figure 3].

Univariate analysis

In univariate analysis, factors associated with SVR were presence of RVR, a lower level of GGT, a degree

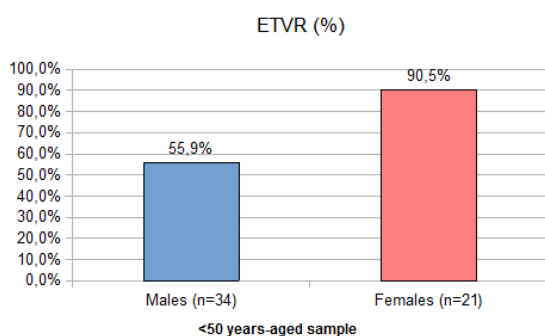


Figure 1: End-of treatment virological response (ETVR) in the < 50 year-aged sample

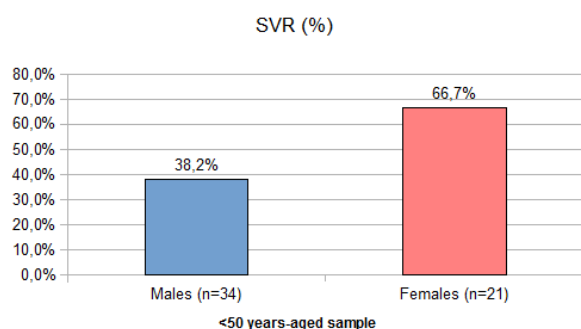


Figure 2: Sustained virological response (SVR) in the < 50 year-aged sample

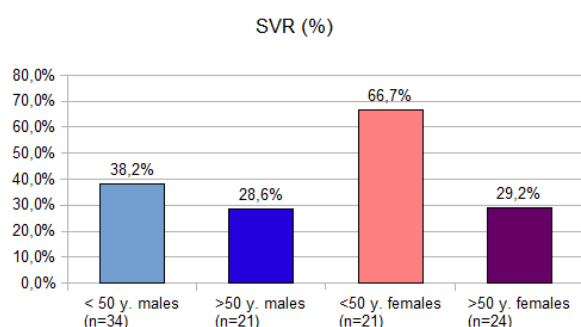


Figure 3: Sustained virological response (SVR) in < 50 year-aged and > 50 year-aged male and female sample

of fibrosis F0-2. Both age and female gender were associated with SVR within the subgroup of subjects < 50 years [Figure 3].

DISCUSSION

Our survey has compared men and women before considering the overall sample and afterwards analyzing separately a group of patients younger than 50 years with a group of patients older than 50 years.

This study considered biochemical and ultrasonographical characteristics, presence of fibrosis at baseline, several types of virological response.

Few meaningful differences in biochemical characteristics between genders have been found. Haemoglobin was significantly lower in women compared with men either in < 50 or in > 50 years patients; the uric acid was significantly higher in men either in the whole sample and in the > 50 years group; GGT-set was significantly higher in men in the overall sample and in the < 50 years group.

In women with menopause, hepatic steatosis was more frequent and severe than in men:^[11] menopause may correlate with necro-inflammation, steatosis and metabolic alterations (high levels of cholesterol and glycemia). Steatosis showed a higher prevalence in chronic-HCV patients in post-menopause (> 55 years); moreover the pro-inflammation state related with menopause may cause a moderate to severe fibrosis progression, leading to an inefficient response to antiviral therapy.^[12,13]

In our sample, the pre-treatment steatosis level did not differ meaningfully in two genders either in the whole sample or in younger and older than 50 years groups. The presence of fibrosis at baseline was not associated to gender either in the overall sample or in two examined groups.

Studies on natural history and predictors of severity disease showed that the evolution of sickness presented a high inter-individual variability and several factors were associated to progression in fibrosis.

Rigamonti *et al.*^[14] stressed as the gender may influence the progression of CHC only in young patients: in < 50 years women emerged lower necro-inflammation and fibrosis than in same aged men, whilst in > 50 years women and men authors did not noticed differences in the disease severity.

In literature effects of gender remain a controversial topic not only as regards the therapy outcome but also relatively to the spontaneous clearance of infection, to the developments of infection linked complications, to the outcomes after liver transplantation.^[13,15]

Several studies demonstrate a higher clearance in women than in men; steroid hormones would play a role for the gender-specific susceptibility of infection even though any sufficiently exhaustive model has not been submitted yet.^[16]

In order to identify factors able to predict SVR, our univariate analysis considered biochemical

and ultrasonographical parameters, and presence of fibrosis at baseline: beyond age and gender, the factors appeared associated to SVR were RVR, GGT levels and 0-2 fibrosis. RVR was a powerful predictive factor of SVR in previous studies, showing as patients with RVR had ratio of SVR meaningfully higher than others; moreover some studies suggest RVR as the most important SVR predictor.^[17-20]

Villela-Nogueira *et al.*^[21] identified that higher levels of GT during a pre-treatment may be a independent negative predictive factor of response to treatment: being a biochemical parameter easily available and at low cost, it may be incorporated in evaluation of response to treatment alongside with other predictive factors.

In conclusion several studies demonstrated that higher degrees of fibrosis have been associated with lower rates of response. To evaluate the effectiveness of treatment, several indicators of response were analysed, in particular SVR which represents the optimal outcome of treatment. There is no concordance of opinion concerning the gender role on the response to the treatment.

In literature there are few studies which identify alike responses in two genders after making comparisons between men and women in patients younger than 40-50 years. Two recent works do not detect a significant influence of genre even though both identified a meaningfully greater response in women younger than 40-50 years compared to the eldest ones. Other studies consider the male gender as one of the strongest factors to predict SVR.^[17,25] Furthermore data concerning rates of SVR in women are conflicting; studies which identified female gender as an independent factor linked to SVR or which noticed as not exist meaningful differences in genders were not stratified by age and considered not differences in female hormonal status;^[20,22,23] other studies suggested a better response in women even after splitting the sample into age groups.^[13,19,23,24]

Few studies lead to identify alike responses in < 40-50 year-old patients of both genders: recent works detected not a significant influence of gender even though in presence of a better response in < 40-50 year-old women compared to the elder ones; other studies considered the male gender as a strong factors to predict SVR.

Our outcomes did not identify meaningful association

between virological responses and gender considering the whole sample: RVR, cEVR, ETVR and SVR frequencies were higher in woman and the relapse rate was higher in men even though no statistically significant difference resulted, so indicating as the gender influences not the therapy outcome.

Nevertheless meaningful gender differences emerged after stratification by age (< and > 50 years). We noticed < 50 years aged women had a higher frequency of response and a lower relapse rate compared to men belonging to the same age group, differences appearing statistically meaningful due to the absence of RVR, cEVR, ETVR, and SVR, suggesting as female gender would be a positive predictive factor of response to the therapy.

Otherwise, in the > 50 years aged group frequency of RVR and ETVR appeared higher in men whilst SVR was slightly higher in women: men had higher relapse ratio compared to women and so reaching less frequently SVR. The evidence of a better response to therapy in < 50 year-old females than in co-aged men and of an alike response in the > 50 years in both groups leads to formulate several hypotheses. It may be supposed only a worsening in women older than 50 years compared to those younger ones linked to an alike response among men before and after 50 years, or we may assume a deterioration with age in both genders even though it is more accentuated in women. Another theory considered the possibility of a rapprochement between sexes with age linked to a worsening in female gender and an improvement in > 50 year-old males. Finally, it could exist an improvement in men with age up to the level of women younger than 50 years without a real worsening of women older than 50 years; this condition may be true whether there is a meaningful improvement in men older than 50 years and an alike response in > 50 year-old women compared with younger and same gender patients.

Comparing the response frequencies in younger and older than 50 year-old males, we could exclude the last two hypothesis, having observed a less response in > 50 year-old patients in both genders without an improvement in these men compared to the < 50 year-old ones.

Since women responded to the treatment differently by age and they achieved the viral clearance more frequently than men, the hormonal activity and especially oestrogen levels may be associated to

SVR;^[11,26] many metabolic processes may be involved, related to the reduction in the oestrogen serum concentration after menopause,^[27] although it has been not separately considered in our study; many data from literature suggested as the reproductive state may be an important factor in predicting the response to antiviral therapy.^[26] These observations suggest as women in reproductive age with CHC should be treated even if liver disease is moderate, being this condition linked to oestrogens exposure.^[28]

Results obtained from our comparison between younger and elder women and younger and elder men showed in the group of women a meaningful worsening in > 50 year-old patients compared to younger ones whilst this difference was not so significant in the group of men although there was a worse response after 50 years. This suggested the lost of the advantage in female gender after 50 years without having a worsening in both genders with age.

In summary, even if affected by limitations related to retrospective and in subgroups analysis, the outcomes we obtained reveal not meaningful differences between men and women when the whole sample is examined without stratification by age whilst an influence of gender on the response to the treatment is identified when patients were divided in two groups younger or elder than 50 years. Despite the grade of influence of gender on standard treatment is still debated, we noticed as the female gender may be considered a positive predictor of response to therapy, taking into account its strong interaction with age and inserting in a broader context made of several modifiable and non-modifiable predictive factors related to the host and virus. Considering both high efficacy and costs of new antiviral drugs therapy protocols, the evidence of a gender- and age- different response to the standard treatment may play a role in changing epidemiologic characteristics of eligible patients and asks the question if certain groups of patients should be primarily treated.

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Conflicts of interest

There are no conflicts of interest.

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Physiological potential of cytokines and liver damages

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ABSTRACT

Cytokines are soluble extracellular small molecular weight protein or peptide. They are produced by virtually every nucleated cell type in response to injurious stimuli to control body metabolism, infection, inflammation and tissue or neuronal damage; therefore acting as messengers between tissues and the immune system; and participating in many physiological processes through their either anti-inflammatory or pro-inflammatory characteristics. Many cytokines have multiple cellular sources and targets, as well as many natural inducers and inhibitors. In pathophysiological conditions and during the early phase of chronic liver diseases, agent like virus, bacteria, parasites, ethanol, or toxins, induce secretion of cytokines at high levels. The presence of cytokine antagonists and soluble cytokine receptors, often released in concert with their respective cytokine agonist, presents additional complexity to interpretation.

Key words: Cytokines; liver diseases; oxidative stress; inflammation

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INTRODUCTION

Cytokines are small molecular weight proteins or peptides messengers between tissues and the immune system^[1] and participate in many physiological processes.^[2] They are either poor anti-inflammatory, suppress the activity and production of pro-inflammatory signals limiting inflammation and host damage; or pro-inflammatory, induce inflammation as a result of infection or injury.^[3] Different cytokine combinations give rise to distinct

consequences, such as inflammation, proliferation, and angiogenesis.^[4]

Many cytokines have multiple cellular sources and targets, as well as many natural inducers and inhibitors.^[5] They are produced to control infection, inflammation and tissue or neuronal damage. The inflammatory ones are fundamental regulators of body metabolism.^[6]

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On several bases, cytokines may be classified depending on their (1) cell of origin; (2) spectrum of activity; (3) the category of activity they influence; (4) the cells that are their targets; or (5) on specific features of their ligand-receptor interaction,^[7] although the nomenclature is somewhat arbitrary, having arisen in different branches of biology [Table 1].^[8] Extensive genetic polymorphisms have also been described, which in many cases appear to play an important part in their level of expression and have been linked to a variety of diseases,^[6] as a variety of experiments has shown that either excessive or insufficient production of cytokines may contribute significantly to the pathophysiology of a range of diseases^[2] including hepatic diseases.^[9]

GENERAL PROPERTIES OF CYTOKINES

Cytokines can be produced by virtually every nucleated cell type in response to injurious stimuli

Table 1: Important classes of cytokines (Ikram *et al.*^[8])

| Cytokines classes |
|--|
| Growth factors |
| Haemopoietic growth factors; granulocyte-colony stimulating factor; granulocyte macrophage-colony stimulating factor; erythropoietin; thrombopoietin; stem cell factor or c-kit ligand |
| Epidermal growth factor |
| Platelet derived growth factor |
| Transforming growth factor β |
| Fibroblast growth factor |
| Insulin like growth factor |
| Nerve growth factor |
| ILs |
| IL-1 to IL-18, <i>etc.</i> |
| IFNs |
| IFN- α |
| IFN- β |
| IFN- γ |
| Miscellaneous |
| Tumour necrosis factor, <i>etc.</i> |

IL: interleukin; IFN: interferon

Table 2: Cytokine properties (Oppenheim^[10])

| Cytokine properties |
|--|
| Low molecular weight protein/glycoproteins |
| Almost all cells produce some cytokines |
| A single cytokine may be produced by many cell types |
| Cytokine expression is usually induced, not constitutive |
| Have a pleiotropy: one cytokine may exhibit many biological activities |
| Have redundancy: several cytokines may share the same/similar activities |

[Table 2].^[10] Mostly, cytokines are produced and act locally. A minority enter the systemic circulation in biologically relevant amounts and a few have an important physiological role there. However, their “endocrine” role is subtly different from that of classical endocrine hormones. Whereas the purpose of endocrine hormones is to ensure the efficient function of normal tissues and the whole organism, cytokines with a physiological role in the circulation are concerned with restoring normal function to the tissue in which they were produced. Indeed, when tissues are severely challenged, and larger amounts of cytokines do enter the circulation, they may be responsible for upsetting systemic homeostasis, inducing fever, sickness behavior, cachexia and a variety of endocrine hormone imbalances.^[11] Individual cytokine either in tissues or in the circulation may exhibit a range of activities and many of these overlap with activities of other cytokines.^[12]

STRUCTURAL ORGANIZATION OF THE LIVER AND CYTOKINES POTENTIAL IN ITS DAMAGE

The liver consists of several cell types that under normal circumstances produce only minimal levels of cytokines. When liver cells, particularly immune cells called Kupffer cells (KC), become activated cytokine production increases dramatically; therefore, if the liver has been damaged, cytokines mediate the regeneration of liver tissue. Also, KC can be activated by diseases caused by microorganisms or substances (i.e. pathogens). In this case, cytokines produced and released by the KC induce an inflammatory response in the liver (hepatitis), which is required to start the healing process. However, if the inflammation does not subside after a short time, persistent production of these same cytokines may lead to formation of fibrosis and cirrhosis. Thus, cytokine production can have both beneficial and harmful effects, depending on the amount and duration of cytokine release. The architecture and cellular composition of the healthy liver in numbers indicating the estimated frequency of each population relative to the total number of parenchymal and nonparenchymal cells in the liver is shown in Figures 1 and 2. This discontinuous structure allows contact between hepatocytes and lymphocytes. The contact can either be produced through hepatocyte microvilli protruding into the lumen or by lymphocyte pseudopod extensions penetrating into the space of Disse. The space of Disse contains hepatic stellate cells (HSCs, fat storing). KC reside within the liver sinusoidal vascular space, predominantly in the

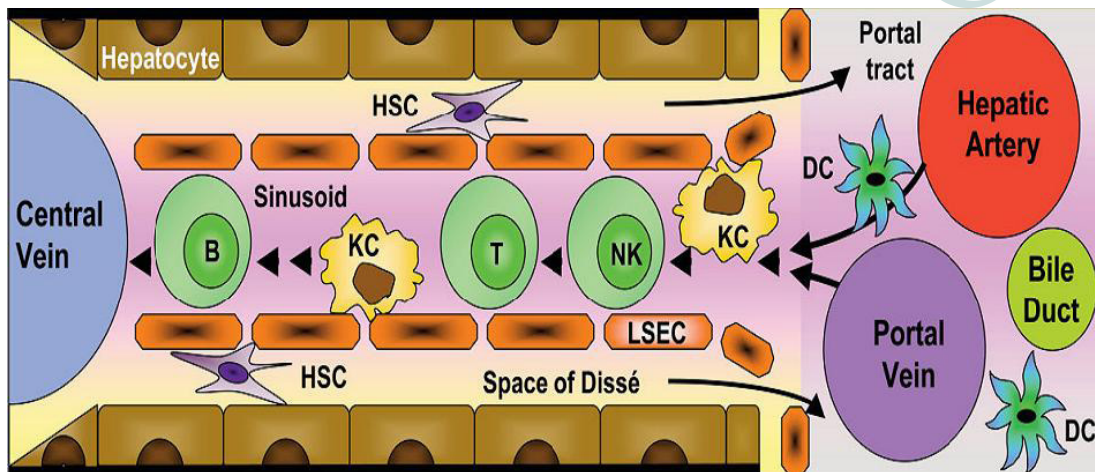


Figure 1: Architecture of the liver: sinusoids, hepatocytes and immune cells. LSEC form a fenestrated monolayer within the sinusoidal endothelium. HSC: hepatic stellate cells; NK: natural killer; KC: Kupffer cells; LSEC: liver sinusoidal endothelial cells; DC: dendritic cells

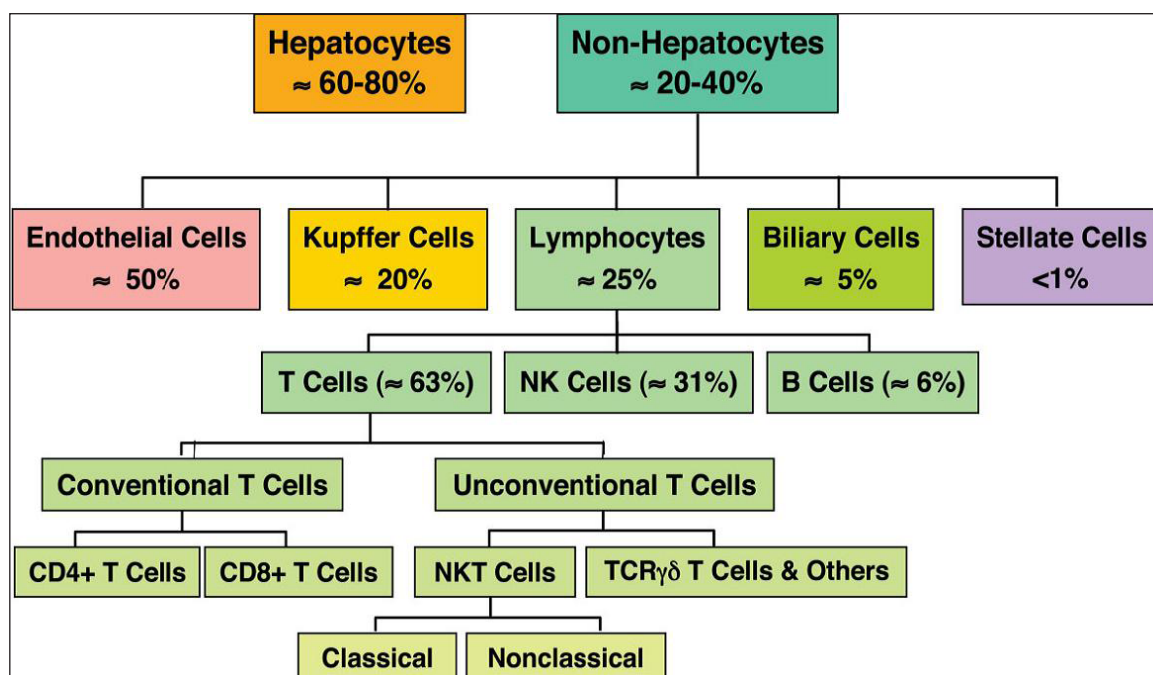


Figure 2: The estimated frequency of each population relative to the total number of parenchymal and nonparenchymal cells in the liver. NK: natural killer; TCR: T-cell receptor

periportal area. Together sinusoidal endothelial cells and resident dendritic cells represent the liver antigen presenting cells. Lymphocytes are scattered throughout the parenchyma and portal tracts, and include conventional and unconventional T cells. A low frequency of B cells and abundance of natural killer (NK) are also characteristic of the liver immune microenvironment.^[13]

Cytokine inhibitors, regardless of the mechanism by which they block the action of cytokines, appear to be the host's own defense against the cytokines. The finding of elevated plasma concentrations of cytokines antagonist in patients with various diseases suggests that antagonism to cytokines is part of the host's

natural response to illness. One might therefore ask what the balance is between the amount of cytokines and these natural cytokine inhibitors in disease and whether disease can result, at least in part, from the failure to produce sufficient amounts of these inhibitors. For instance, the principle agonist forms of the interleukin (IL)-1 family are IL-1 α and IL-1 β . A third member of the IL-1 family, IL-1 receptor antagonist (IL-1ra), is also induced during inflammatory responses [Figure 3]^[14] but has preventive effect against the binding of IL-1 α and β to their receptors IL-1ra is required to be in approximately 100-fold excess to inhibit IL-1 α or IL-1 β effectively; that is why IL-1ra is produced in greater amounts and is present at greater concentration matched with rare occurrence

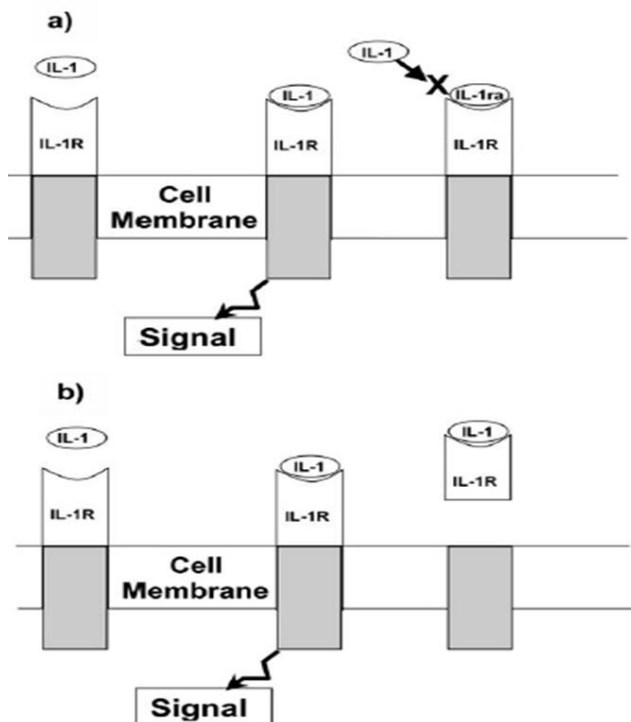


Figure 3: Interleukin 1 (IL-1) antagonism: IL-1 activity at the receptor may be blocked either by (a) competitive inhibition by IL-1 receptor antagonist (IL-1ra) or by (b) soluble forms of the type II receptor that bind to free IL-1

of α and β in the circulation. Additionally, cellular receptors for both IL-1 and tumor necrosis factor (TNF) exist in soluble forms, after being cleaved from the cell surface, and are able to bind and neutralize the cytokine [Figure 4].^[15]

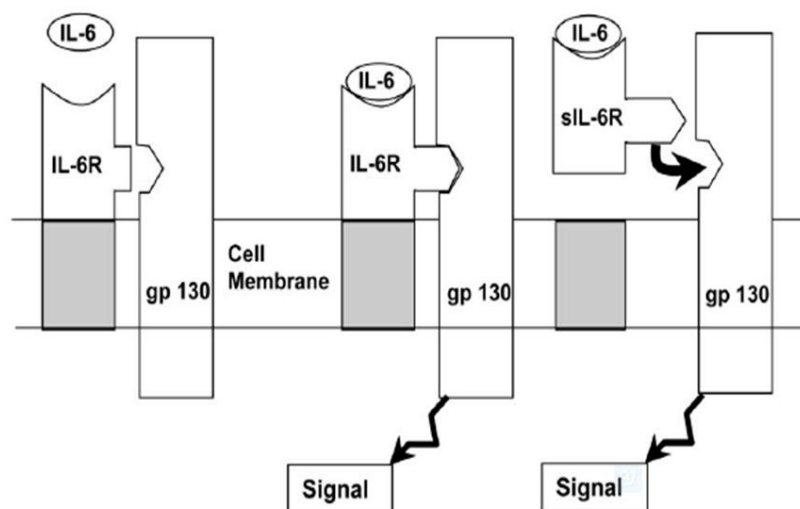


Figure 4: Maintained activity by soluble interleukin 6 receptor (sIL-6R); ligation of IL-6 with its membrane binding protein (IL-6R) results in association of the complex with a 130 kDa signal transduction glycoprotein (gp130). When cleaved from the cell surface, the IL-6/sIL-6R complex remains able to bind and activate gp130

The goal of this review is to highlight, in brief account, the major cytokines involved in different liver damage and discuss their basic biology and clinical applications.

Cytokines and alcoholic liver disease

Alcohol-related liver disease (ALD) is a major cause of morbidity and mortality worldwide. Chronic alcohol consumption leads to hepatocellular injury, fat accumulation, and liver inflammation and sometimes leads to liver cirrhosis or hepatocellular carcinoma (HCC) [Figure 5].^[16] In the liver, TNF- α is mainly produced by KC.^[17] The role of TNF- α as a critical inflammatory cytokine in the progression of ALD is well known.^[18] KC secrete inflammatory cytokines^[19] and reactive oxygen species (ROS)^[20] which activate cells such as hepatocytes, HSCs, and endothelial cells.^[21] After chronic alcohol consumption, KC exhibit enhanced sensitivity to lipopolysaccharide (LPS)-stimulated TNF- α production.^[22] Elevated serum levels of TNF- α inducible cytokines or chemokines, including IL-6, IL-8, and IL-18, have also been reported in patients with alcoholic hepatitis.^[23] Serum TNF- α is increased in patients with ALD and correlates with mortality. Treatment with pentoxifylline (an inhibitor of TNF- α synthesis) improved the survival of patients with severe alcoholic hepatitis (AH).^[24] Anti-TNF- α antibody, infliximab, is also effective in severe AH patients.^[25] These results suggest that TNF- α plays an important role in the progression of ALD.

IL-6 appears to have some beneficial effects on the liver. IL-6 may protect against hepatocyte apoptosis and participates in mitochondrial DNA repair after alcoholic liver injury.^[26] IL-6 may promote human

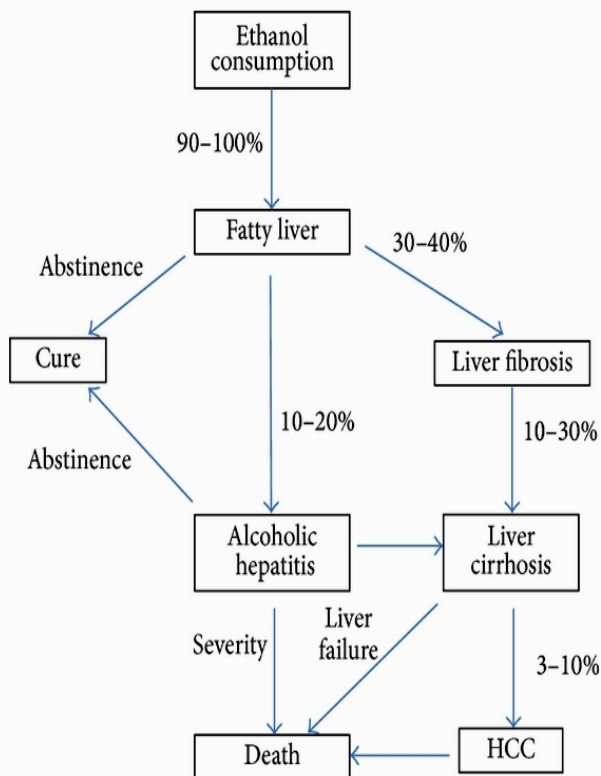


Figure 5: The natural history of alcoholic liver disease. Chronic ethanol consumption leads to fatty liver for more than 90%. But only up to 40% of this population develops more severe forms of alcoholic liver disease, including fibrosis and alcoholic hepatitis. Continuous ethanol consumption finally leads to liver cirrhosis or HCC and leads to death. HCC: hepatocellular carcinoma

thymus derived monocytes helper 17 (Th17) differentiation and IL-17 production, therefore contributing to ethanol induced liver inflammation. IL-6 is also released along with IL-10, TNF- α and other cytokines by KC after alcohol consumption. IL-6 and IL-10 are two cytokines that play roles in reducing alcoholic liver injury and inflammation.^[27] Elevated IL-6 is found in patients with ALD.^[28] On the other hand, IL-6 knockout mice fed chronic alcohol showed increased liver fat accumulation, lipid peroxidation, mitochondrial DNA damage, and sensitization of hepatocytes to TNF- α induced apoptosis, which was prevented by the administration of recombinant IL-6.^[29] These findings suggest that IL-6 has a protective effect at the early phase of ALD. Furthermore, IL-17 can act with other cytokines to activate NF- κ B which plays a central role in regulating genetic transcription and encoding of inflammatory cytokines, and induce IL-8. Recently it was shown that patients with ALD had higher IL-17 plasma levels compared with healthy subjects.^[30] IL-1 α is also a potent proinflammatory cytokine. In both animal model and patient with ALD, the levels of pro-IL-1 β are significantly increased in the liver and serum.^[31]

Cytokines and fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is now the most frequent chronic liver disease that occurs across all age groups and is recognized to occur in 14-30% of the general population, representing a serious and growing clinical problem due to the growing prevalence of obesity and overweight.^[32] The first manifestation of hepatic injury is the accumulation of fat within hepatocytes (steatosis), this is followed by the development of necroinflammatory (steatohepatitis) activity that leads to cirrhosis.^[33]

The importance of cytokines as molecular effectors in liver damage has been particularly well demonstrated in patients and animals ranging from steatosis to cirrhosis. TNF- α is involved in the progression from steatohepatitis to cirrhosis, since it promotes activation of stellate cells, matrix-gene expression, and matrix remodeling.^[34] Recent studies have indicated that deficiency of IL-1 α in KC reduces liver inflammation and expression of inflammatory cytokines, which may implicate KC-derived IL-1 α in steatohepatitis development.^[35]

Obesity, especially visceral adiposity, is a major risk factor for NASH in humans.^[36] Adipose tissue is a source of free fatty acids (FFA) that are delivered to the liver and a depot for triglycerides that are synthesized by hepatocytes and released into the blood. As producers of TNF- α and IL-6, adipocytes are considered a component of the immune system.^[37] Visceral fat, which appears to be less “mature” than subcutaneous fat, produces more TNF- α and free fatty acids but less adiponectin than subcutaneous fat. Adiponectin antagonizes both the production and activity of TNF- α ; thus the effect of this cytokine is potentiated when adiponectin is scarce. In addition, TNF- α inhibits adiponectin. Adiponectin also inhibits synthesis and uptake of FFA by hepatocytes, while stimulating FA oxidation enhancing their sensitivity to insulin. The combination of low adiponectin and high TNF- α levels in the context of increased hepatic exposure to FFA results in hepatic steatosis and severe hepatic insulin resistance.^[38]

Leptin, as one of adipocyte secretions, together with its receptor share structural and functional similarities with the IL-6 family of cytokines, and leptin appears to play a critical role in the inflammatory response by stimulating leukocyte proliferation and the resulting increased plasma levels of the proinflammatory cytokines such as IL-6 and TNF- α .^[39] These cytokines influence nitric oxide^[40] that induces free radicals

production and lipid peroxidation. Elevation of the inflammatory markers above normal levels is an independent predictor of several chronic diseases, including coronary heart disease, stroke, diabetes, atherosclerosis and insulin resistance.^[41]

Cytokines and hepatic cholestasis

Cholestasis is defined as a decrease in canalicular bile flow that results in accumulation of bile in hepatocytes and canaliculi.^[42] Hepatocellular cholestasis may be due to functional or structural alterations in the biliary tree. The clinical consequences of prolonged cholestasis are due to the failure of bile acids to reach the duodenum with subsequent malabsorption of fat and fat-soluble vitamins A, D, E and K as well as the accumulation of biliary constituents such as bile acids, bilirubin and cholesterol in the liver. Bile acids retention causes liver cell damage and pruritus.^[43]

TNF- α plays a critical role in epithelial cell injury as well as in immune-mediated cholangiocyte injury.^[44] Systemic levels of TNF- α are increased following biliary obstruction in experimental cholestasis produced by ethinylestradiol in rats.^[45] Furthermore, TNF- α (in combination with other inflammatory cytokines) inhibits cholangiocyte secretory function *in vitro*.^[44]

In cholestatic diseases, the intrahepatic bile acids induce hepatocellular apoptosis by stimulating Fas (a surface receptor that mediates apoptosis upon oligomerization by its ligands) translocation from the cytoplasm to the plasma membrane where self-aggregation occurs to trigger apoptosis.^[46] Apoptosis is known to be the mechanism leading to progressive inflammation and destruction of bile ducts.^[47] Also, bile acids can induce hepatic inflammatory response via the activation of hepatic macrophages^[46] that follows the activation of the transcription factor NF- κ B, since NF- κ B activation has been shown to have a key role in the inflammatory process.^[48] It is well known that NF- κ B is activated by a wide range of agents and cytokines including TNF- α and IL-1 α secreted from the injured hepatic macrophages.^[48]

Proinflammatory cytokines were reported to stimulate the biliary epithelium to generate nitric oxide (NO), via nitric oxide synthase induction. NO causes ductular cholestasis by a reactive nitrogen oxide species mediated inhibition of adenylate cyclase and cAMP-dependent HCO₃⁻ and Cl⁻ secretory mechanisms. This pathogenetic sequence may contribute to ductal cholestasis in inflammatory cholangiopathy.^[44]

Cytokines and hepatic HCV infection

In HCV infection, the production of abnormal cytokine levels appears to contribute in the progression of the disease, viral persistence, and affects response to therapy. Cytokine genes polymorphisms located within the coding/regulatory regions have been shown to affect the overall expression and secretion of cytokines.^[6]

The pathogenesis of liver cell damage in HCV infection may be related to several immunologic mechanisms and the subsequent T-cell responses.^[49] Patients with chronic HCV infection, viral persistence which is a characteristic feature of chronic hepatitis C may be due to selective immune responses deficiencies and the production of inappropriate cytokine patterns.^[50]

The involvement of macrophage derived cytokines such as TNF- α and IL1 β in the production of inflammation has been described.^[51] TNF- α acts as important mediator in liver injury and generally associated with several known cirrhosis-related complications. Moreover, TNF- α is positively related with the extent of liver necrosis.^[52]

Adhesion molecules are necessary for leucocytes to adhere tightly to endothelial cells and have been reported to be cytokine-induced. Intercellular adhesion molecule-1 (ICAM-1) is one of the principal adhesion molecules expressed on sinusoidal and venular endothelial cells and involved in firm adhesion and trans endothelial cell migration.^[53] Consequently, The predominant features of HCV-C are more related with those that allow viral evasion of the immune defenses, especially although not exclusively, inhibition of interferons secretion, natural killer cells activation and T cell-mediated cytotoxicity.^[54]

Several researchers have suggested that an adequate T-helper 1 (Th1) response [i.e. high interferon (IFN)- γ secretion by peripheral blood mononuclear cells] may be associated with a protective antiviral immune response,^[55] while insufficient systemic Th1 cytokine secretion may be associated with increased viral load and disease progression.^[56] Indeed, serum samples from HCV patients contain significantly lower level of soluble IFN- γ compared with controls.^[57] In this sense, it has been reported that the IL-18 and IFN- γ mRNA expression in the liver were significantly correlated with each other and both upregulated in chronic HCV patients.^[58] It was suggested that inheritance of IL-28B CT and TT, transforming growth factor (TGF)- β 1 CT and TT and TNF- α AG and AA genotypes which

appear to affect the cytokine production may be associated with susceptibility to HCV infection and resistance to combined antiviral therapy.^[6]

Mitochondria are a major source of ROS under physiologic conditions, because 2% to 3% of the $O_2^{\cdot-}$ consumed is converted to $O_2^{\cdot-}$ mainly by auto oxidation of ubiquinone which transfer electrons from complexes I and II to complex III. Hepatocyte ischemia described in chronic liver pathology, enhances $O_2^{\cdot-}$ production by impairing function of complex III.^[59] TNF- α as one of the cytokines released from endotoxin-stimulated KC, through intracellular signaling, leads to decreased function of complex III.^[60] Endotoxemia has been described in chronic hepatitis. Furthermore, activation of sinusoidal inflammatory cells such as lymphocytes and KC has been described in chronic hepatitis C virus infection.^[61] Therefore, ROS serve as signaling molecules for the initiation and perpetuation of the inflammatory process that occurs with conditions of oxidative stress. This involves genetic regulation. Transcription factors that are directly influenced by reactive species and proinflammatory signaling include NF- κ B. NF- κ B plays a central role in regulating genetic transcription and encoding of inflammatory cytokines, growth factors, acute phase proteins, adhesion molecules, other transcription factors, and cell death regulators. These NF- κ B regulated genes are important in regulating genetic activity during critical illness, inflammatory diseases, and cancer.^[62]

Cytokines and hepatic hepatitis B virus infection

Hepatitis B, which is caused by hepatitis B virus (HBV) infection, remains a major health threat worldwide. Hepatic injury and regeneration from chronic inflammation are the main driving factors of liver fibrosis and cirrhosis in chronic hepatitis B.^[63]

During HBV infection, intrahepatic production of Th1 inflammatory cytokines and type-I IFNs activates two functionally independent pathways: an early elimination of HBV nucleocapsid particles from the hepatocytes; and a later post-transcriptional downregulation of viral RNA. Most of these effects are mediated direct or indirectly by IFN- α , β and γ .^[64] Additionally, chronic HBV patients who clear the virus have higher levels of IL-12 than patients who remain HBV positive.^[65] IL-12 can inhibit the replication of HBV through the induction of IFN- γ .^[66]

Cytokines and hepatitis E virus

Hepatitis E virus (HEV) is a small non enveloped single-

stranded positive-sense RNA virus and is one of the major causes for acute hepatitis worldwide. C-X-C motif ligand 8 (CXCL-8) is a small multifunctional proinflammatory chemokine. It was reported recently that HEV infection significantly upregulates CXCL-8 gene expression.^[67]

The severity of HEV infection and associated adverse outcome might be mediated by cytokine1. In a pregnant and non-pregnant HEV infected women study, HEV viral load in acute viral hepatitis and fulminant hepatic failure were comparatively higher levels of TNF- α , IL-6, IFN- γ and TGF- β 1 than those in controls; moreover TNF- α , IL-6 and IFN- γ had significant positive correlation with viral load, serum bilirubin and prothrombin time within infected women.^[68]

Cytokines and hepatic schistosoma infection (Schistosomiasis)

Schistosomiasis is a chronic and debilitating disease that affects over 200 million people worldwide.^[69] The pathology, resulting from infection with the helminth parasite *Schistosoma mansoni* or *Schistosoma japonicum*, is predominantly caused by the host immune response to parasite eggs that are laid in the portal venous system and then become trapped in hepatic sinusoids and sequestered within granulomatous lesions.^[70] Cytokines, which communicate between the fibrotic areas and the immune system, form a network of host-parasite responses. Nevertheless, the mechanisms involved in the pathogenesis and progression of hepatic fibrosis in patients with schistosomiasis have not yet been fully elucidated.^[71]

Studies on certain-cytokines knockout mice which had been infected with *Schistosoma mansoni* showed that egg granulomas and the hepatic fibrosis are dependent on the regulation of cytokines.^[72] Higher levels of eosinophil-derived cytokines were observed in periportal fibrosis. A mixed cytokine pattern, characterized by positive correlation between TNF- α , IL-4 and IL-5 was observed in periportal fibrosis. Also, the positive association between lymphocyte-derived IL-10 and the eosinophils cytokine profile was observed exclusively in intestine further emphasize the hypothesis that immunoregulatory events take place controlling disease morbidity in human schistosomiasis^[73] or in experimental models.^[74] However, in human and animal schistosomiasis, studies have shown that high levels of TNF- α produced by peripheral blood mononuclear

cells stimulated with schistosome antigen (Ag) are significantly associated with the presence of hepatosplenomegaly.^[75,76] As hepatosplenic disease is a long-term complication of schistosomiasis and is considered to be indicative of severe hepatic and periportal fibrosis, it is conceivable that the immune mechanisms responsible for this lesion occur much earlier during infection and precede the downstream development of hepatosplenomegaly.^[75] In addition *Schistosoma japonicum* significantly activates collagen deposition and hepatic stellate cell in the liver, however, fibrosis was accompanied by increased IFN- α , IFN- β , IFN- γ , IL-12, TNF- α , and IL-10 mRNA expression as well as decreased the expression of IL-4, IL-5 mRNA, natural killer group 2 member D (NKG2D) mRNA and tumor necrosis factor related apoptosis-inducing ligand (TRAIL).^[77]

Cytokines and autoimmune hepatitis

Autoimmune hepatitis (AIH) is an inflammatory liver disorder, characterized by female preponderance, hypergammaglobulinaemia and interface hepatitis on histology. AIH is associated with impairment of regulatory T-cells,^[78] a lymphocyte subset key in maintaining immune-tolerance to autoantigens.^[79]

Limited data are available for the participation of cytokines in the development of AIH. In a previous study, Chernavsky *et al.*^[80] analyzed the expression of cytokines in liver biopsies from pediatric autoimmune hepatitis (PAIH) patients in comparison with liver control samples obtained from cadaveric liver donors. While the expression of IFN- γ and IL-12p40 was not detectable in control livers, it was clearly unregulated,^[80] and showed an increased expression of IL-18, IL-4 and the IL-12 β 2 chain receptor in PAIH patients. The unexpected increase of mRNA for IL-4, a typical Th2 cytokine, was found in conjunction with a severe histological inflammation in AIH. The up regulation of IL-4 in PAIH but not in another disease clearly suggests a more complex immunopathologic mechanism.

Th2 cytokines activate B cells and induce their differentiation into antibody-producing cells. Liver-infiltrating autoreactive B cells, in addition to their role in producing autoantibodies, also play a critical role in the development of fibrosis. The mechanism of suppressing fibrosis by B-cell depletion is independent of antibodies or T cells, raising the possibility that cytokines, produced or induced by autoimmune B cell, are responsible for fibrosis in autoimmune diseases targeting the liver.^[81]

Human liver contains an uncommonly high number of NKT cells that participate in the early regulation of Th1/Th2 cell differentiation through the release of IFN- γ and IL-4. Moritoki *et al.*^[81] and Solari *et al.*^[82] found an increased number of V α 24 positive cells and transcripts coding for this invariant V α 24 chain in the liver of PAIH patients, pointing to a probable involvement of these regulatory cells as mediators of the hepatocellular injury in PAIH.

Cytokines and hepatic fibrosis, cirrhosis and cellular carcinoma

Chronic hepatic injury is associated with both liver cirrhosis and liver cancer.^[71] Several cytokines and ROS, produced in the injured liver by resident macrophages and infiltrating leukocytes during inflammatory conditions, cause transformation of the quiescent HSCs into the activated phenotype, which is responsible for fibrosis, cirrhosis and cancer.^[71,83]

The perisinusoidal retinoid- storing quiescent HSCs physiologically regulate liver architecture and blood flow by producing components of extracellular matrix and contractility respectively. During hepatic injury, HSCs transform into retinoid-free proliferating myofibroblast-like cells (activated HSCs, aHSCs), which express α -smooth muscle actin (α -SMA). aHSCs are highly fibrogenic and contractile, and play major role in causing architectural damage and portal hypertension.^[83]

A phenomenon of aHSCs rapid apoptosis was observed among the proliferating cells during CCl₄-induced active fibrosis.^[84] During inflammatory liver injury, ROS are produced by resident macrophages and infiltrating blood cells, particularly neutrophils.^[85] ROS-induced increased expressions of α -SMA, collagen I and collagen III in rat and human HSCs^[86] indicate their role in HSC activation and fibrosis. While investigating the actions of ROS on aHSCs it is noted that superoxide (SO) reduced their viability revealing that SO causes apoptosis of aHSCs that involves mitochondrial release of cytochrome-C, activation of caspase-3 and increased expression of Bax.^[87] During inflammation, the activated inflammatory cells produce fibrogenic cytokines and growth factors that activate HSCs.^[88] The role of cytokine gene polymorphism in the progression of liver fibrosis or development of cirrhosis in patients with hepatic diseases has been investigated extensively. Yee *et al.*^[89] indicated that TNF2 (-238A) and TNF3 (-308A) alleles are frequently found in patients with cirrhosis in chronic HCV infection.

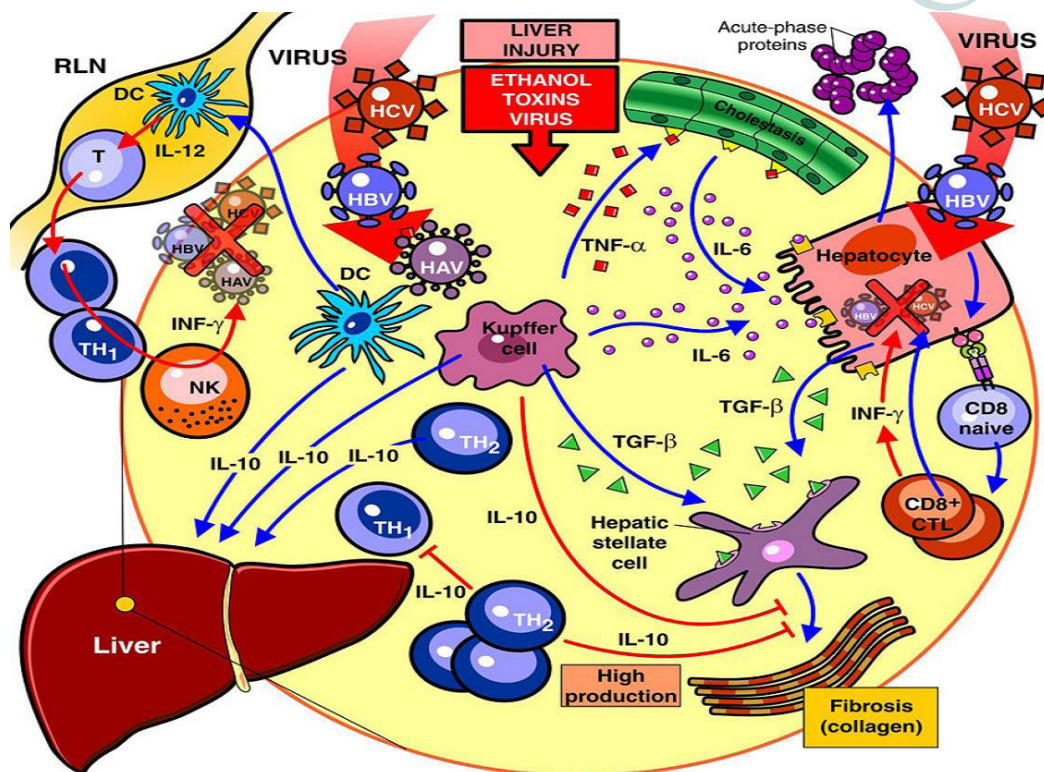


Figure 6: Overview of immune and parenchymal cells during liver injury. A steady-state migration of immature DC to the RLN and the production of IL-10 by KC and resident DC are involved in the phenomenon of tolerance to self-antigens within a healthy liver. After a virus infection, viral particles are incorporated into DC either because they become infected or through cross-priming and then migrate to the RLN, where they differentiate and activate naive T cells. Effector CD4+ T cells return to the liver and through secretion of Th1 cytokines and collaboration with activated NK cells, might contribute to the virus clearance. In an alternative view, exogenous antigen (Ag) expressed in hepatocytes can be presented to naive CD8+ T cells which after clonal expansion become efficient CTLs and secrete Th1 cytokines. Under conditions of liver injury, KC play a critical role through secretion of TNF- α , TGF- β and IL-6. The latter acting on hepatocytes induces the production of the acute phase proteins. TGF- β activates the induction of fibrosis through the action of stellate cells and TNF- α plays a critical role in the induction of cholestasis. A high production of IL-10 is able to modulate the development of fibrosis. IL: interleukin; DC: dendritic cell; RLN: regional lymph node; NK: natural killer; CTL: cytotoxic T lymphocytes; KC: Kupfer cell; TNF- α : tumor necrosis factor- α ; TGF- β : transforming growth factor- β ; HAV: hepatitis A virus; HBV: hepatitis B virus; HCV: hepatitis C virus (Fainboim *et al.*^[104])

Polymorphisms of TGF- β gene are thought to be one of the determinants of fibrosis progression in viral hepatitis.^[90] HSCs play an important role in hepatic fibrogenesis and that IL-1 is a potent cytokine that induces the myofibroblastic activation of HSCs. IL-1 is also implicated in the proliferation of HSCs and the regulation of the expression of various matrix metalloproteinases, which play a key role in the turnover and the deposition of extracellular matrix (ECM). Therefore, it is possible that genetic polymorphism of IL-1B gene may influence the progression of hepatic fibrosis by affecting the hepatic expression of IL-1 during the process of liver injury.^[91] TGF- β has been implicated in hepatic fibrogenesis; as it stimulates the production of extracellular matrix proteins and their receptors, and inhibits the synthesis of matrix-degrading proteolytic enzymes in chronic HCV; moreover, its serum or liver level has a positively correlation with the fibrosis score in both untreated patients or those respond to IFN- α treatment.^[92]

IL-10 has a protective role in hepatic fibrogenesis,

as it showed a decreased hepatic inflammation and increased serum levels of HCV-RNA matched with reduced liver fibrosis score either in chronic HCV-infected patients who received a short or after 12 months therapy with recombinant IL-10.^[93]

A characteristic feature of HCV infection is a high frequency of persistence and progression to chronic liver disease (CLD). Persistent infection upsets the balance between immunostimulatory and inhibitory cytokines, which can prolong inflammation, and lead to necrosis, fibrosis, and CLD.^[94] Elevated concentrations of cytokines also represent a characteristic feature of CLD, regardless of underlying etiology, which may represent a consequence of liver dysfunction instead of inflammatory disorder.^[95]

T lymphocytes and immunoregulatory cytokines are of critical importance in the host defense against HCV infection. T-helper type 1 (Th1) cytokines (IL-2, IFN- γ) are required for host anti-viral responses, while T-helper type 2 (Th2) cytokines (IL-4, IL-10) can inhibit the development of these effectors.^[96] Significant

elevations in circulating Th22 cells, Th17 cells, Th1 cells, IL-22, IL-17A, and IFN- γ were observed in the hepatic fibrosis groups compared with the control group.^[97]

It has been demonstrated that the proinflammatory IL-6 and IL-10 have been implicated to associate with certain human cancers and HCC. Previous study indicated that both IL-6 and IL-10 levels were elevated in HCC patients compared to normal controls, and the high levels would invariably decrease after surgical resection.^[98] In addition, a high IL-10 level predicted a poor disease-free survival in patients undergoing curative surgery.^[99] Hsia *et al.*^[98] found that both IL-6 and IL-10 expression were more often higher in HCC patients compared to patients in other disease categories.

It has been postulated that an imbalance between Th1 and Th2 cytokine production is implicated in disease progression or inability to clear infections. It was reported that HCV-infected patients who develop chronicity have a predominant Th2 response, but a weak Th1 response, suggesting that this immune response imbalance can result from HCV interaction with dendritic cell functions.^[100] These results support the notion that Th-lymphocyte polarization may play an important pathophysiologic role in influencing the outcome of HCV infection. All these immunological findings are mostly due to HCV infection rather than schistosomal infection, because patients with no schistosomal antibody had the same elevation of the same cytokines, late *Schistosoma mansoni* cases showed a suppressed cell-mediated immunity and a significant depletion of T-helper/inducer subset.^[101]

In the majority of cases, HCC is found in conjunction with cirrhosis of the liver. Chronic inflammation and cirrhosis, accompanied by regenerative process, function as a tumor promoter, providing a common pathway from chronic HBV or HCV-infection to HCC. The direct etiologic role of HBV and HCV for HCC is obscure. Tumor progression may be brought about in HCC by mutation of p53 tumor suppressor gene. The prevalence of p53 mutations is similar in HBV-associated and HCV-associated HCCs. Other mechanisms of host defense are the production of TGF- β 1, and the induction of cytotoxic T lymphocytes; the failure of these mechanisms permits the process of hepatocarcinogenesis. Treatment with alpha interferon of chronic hepatitis is necessary to delay or prevent the progression to liver cirrhosis and development of HCC.^[102]

To date, two cytokines have achieved Food and Drug Administration approval as single agents for cancer treatment: high-dose, bolus IL-2 for metastatic melanoma and renal cell carcinoma and IFN- α for the adjuvant therapy of Stage III melanoma.^[103]

The classical and current view of the cytokines role and mechanisms in both healthy and diseased liver is presented in Figure 6.^[104]

SUMMARY AND CONCLUSION

Cytokines are a large family of small proteins secreted by leukocytes and having an essential role in mediating the immune function. Many cytokines have multiple cellular sources and targets, as well as many natural inducers and inhibitors. Cytokines are produced to control body metabolism, infection, inflammation and tissue or neuronal damage. The pharmacological agents that can either suppress the production of the cytokines or block its biological actions may have potential therapeutic value against a wide variety of liver diseases. However, a stress is needed for a better knowledge about the adverse side effects for the anti-cytokine agents on the autoimmune responses; therefore future studies, leading to a combination of drugs that modulate the cellular immunity system but selectively block cytokines action, may be more useful for use to overcome the side effects of anti-cytokine therapy in the long-term. Again, proinflammation and prooxidation is the main cause of the complications of various inflammatory diseases. Since the high levels of cytokines directly induces the oxidative stress of the cells by depleting the vital antioxidant substances (such as glutathione) of the body and therefore elevate the ROS levels of the cells, it would be interesting to check the effectiveness of combination of drugs including antioxidant enhancer to effectively combat the side effects of anti-cytokine therapy.

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Conflicts of interest

There are no conflicts of interest.

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Hepatoprotective and antioxidant activity of *Bombax ceiba* flowers against carbon tetrachloride-induced hepatotoxicity in rats

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ABSTRACT

Aim: The flowers of *Bombax ceiba* are traditionally used as home remedy in the treatment of jaundice and spleen enlargement. The present work investigated the effect of aqueous extract of flowers of *Bombax ceiba* (BCAE) on experimentally induced hepatotoxicity in rats to substantiate its traditional use as hepatoprotective agent. **Methods:** Hepatotoxicity was induced in rats by carbon tetrachloride (CCl₄) treatment; at the same time vehicle or BCAE (250 or 500 mg/kg) or silymarin (25 mg/kg) were administered daily orally for seven days. Hepatotoxicity was assessed by estimating the activities of marker enzymes and by histological studies. The antioxidant effect of BCAE was assessed by measuring amount of antioxidant phytochemicals (total phenolics and flavonoids), and DPPH free radical scavenging assay of the extract. **Results:** BCAE treatment significantly prevented the CCl₄-induced elevations in levels of glutamate oxaloacetate transaminase, glutamic pyruvic transaminase, alkaline phosphatase, bilirubin, and triglycerides, and decreased the total protein levels. Treatment with BCAE attenuated the CCl₄-induced cytotoxic damage to liver. BCAE exhibited presence of antioxidant phytochemicals and showed scavenging action on DPPH radicals. The hepatoprotective effect of BCAE was comparable to that of the standard antioxidant hepatoprotective agent, silymarin. These findings indicated that BCAE showed hepatoprotective effect against CCl₄-induced hepatotoxicity and exhibited *in vitro* antioxidant effects. **Conclusion:** *Bombax ceiba* flowers exhibited hepatoprotective effect which may be attributed to antioxidant potential. This study also validated their traditional medicinal use in liver disorders.

Key words: Semal; liver disorders; liver function test; free radical scavenging; silymarin

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INTRODUCTION

The liver is exposed to many kinds of xenobiotics and therapeutic agents and has large capacity for metabolic conversions. As the liver is largely responsible for the biotransformation of many complex molecules, it is always at the risk of detrimental physiological and pathological alterations characterized as liver diseases. Various types of liver disorders include cirrhosis, jaundice, cancer, metabolic and degenerative lesion, liver cell necrosis, and hepatitis.^[1] Steroids, vaccines and anti-viral drugs, which have been employed as a therapy for liver diseases, have potential adverse side effects especially when administered for long term.^[2] Hepatoprotective agents of plant origin have attracted special interest, and numerous medicinal plants and their formulations have been used for liver disorders in the Ayurvedic system of medicine. These medicinal plants have been studied for their influence on liver dysfunction.^[3]

Bombax ceiba Linn. (Family: Bombacaceae), is a large, deciduous tree commonly known as Silk Cotton Tree, Indian Red Kapok tree, Semal, Shimul and Shalmali. It is found throughout India and other parts of tropical and sub-tropical Asia, Australia, and Africa. The plant has both economic and medicinal value. It yields gum and cotton. It is a large and long-living tree species which gives strength to the body, mind, and heart.^[4] The plant is popular among the tribal communities for the treatment of various diseases. Almost every part of the plant, the seeds, flowers, roots, and barks of *Bombax ceiba* have a long history of medicinal uses. The paste of flowers and leaves are applied externally to relieve swellings, boils, and various skin conditions. The traditional healers of Chhattisgarh Plains boiled the flowers throughout the night, and gave them with mustard seeds orally as treatment of enlarged spleen.^[5] The decoction of the semal flowers is used as home remedy for the treatment of jaundice. The flowers, leaves, and stem of *Bombax ceiba* have been evaluated for various pharmacological actions. The various extract of *Bombax ceiba* have shown analgesic, oxytocic^[6] hypotensive, hypoglycemic,^[7] antimicrobial,^[8,9] antioxidant,^[10-12] antiangiogenic^[13] activities.

Despite the traditional use of this plant in the treatment of jaundice and splenic enlargement, very few scientific studies have been carried out to delineate its influence on experimentally induced hepatotoxicity. A recent study has reported hepatoprotective effect of the *Bombax ceiba* flowers in anti-tubercular drugs-induced toxicity.^[14] However, the effects were limited to reversal of drug-induced necrosis. Water is an extraction solvent to extract the hydrophilic antioxidants present in the plants. For use in foods, plant extracts made with water are nutritionally more relevant and would have obvious advantages in certification and safety.^[15] The present study was undertaken to validate the traditional use of *Bombax ceiba* in jaundice and to confirm earlier studies. Furthermore, we demonstrated the role of free radicals in hepatotoxicity, and the *in vitro* antioxidant activity of the

flowers of *Bombax ceiba*.

METHODS

Plant material

The flowers of *Bombax ceiba* were collected from the Medicinal Garden of the National Research Institute for Ayurveda-Siddha Human Resource Development, Gwalior in April 2011. The flowers were identified by Dr. N.K. Pandey, Research Officer (Botany), National Research Institute for Ayurveda-Siddha Human Resource Development, Gwalior, Aamkho, Gwalior, India. A voucher specimen (Accession no. 410) of the authenticated *Bombax ceiba* flowers has been deposited in the herbarium of the Institute.

Drugs and chemicals

Carbon tetrachloride (CCl₄) was purchased from Qualigens Fine Chemicals, Mumbai, India. Olive oil (Figaro, Spain), ascorbic acid, and tannic acid were purchased from local market of Gwalior. Quercetin and DPPH (2, 2-Diphenyl-1-picrylhydryl) were obtained from Sigma Chemicals, USA. Glutamic-oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), and alkaline phosphatase (ALP) estimation kits (Erba-Mannheim) were procured from Transasia Biomedicals Pvt. Limited, Mumbai while total bilirubin (T) estimation kit was procured from Siemens Medical Solution Diagnostic Ltd. Baroda India. Triglycerides (TG), total protein, and albumin estimation kits were procured from Span Diagnostic Pvt. Ltd., Surat, India. All remaining chemicals used in the experiment were of the highest grade commercially available.

Preparation of aqueous extract of flowers of *Bombax ceiba*

The dried flowers were subjected to size reduction to a coarse powder by using dry grinder. This powder (100 g) was soaked in 1 L purified water, mixed, and kept in dark and dry place for 48 h. Chloroform was added in quantity of 1% total mixture to prevent microbial growth. After 48 h, the mixture was filtered initially by Muslin cloth and after that with Whatman Filter paper No.1. The filtered extract was dried using a rotary evaporator. After drying, a light brown extract was obtained (20% w/w).

Preliminary phytochemical screening

Preliminary phytochemical screening of aqueous extract of flowers of *Bombax ceiba* (BCAE) was carried out to detect the presence of various phytochemicals by standard procedures^[16] [Table 1].

Animals

Healthy adult Wistar rats of either sex weighing about 200-250 g, between 2-3 months of age were used in the study. They were housed in groups in polypropylene cages, under standard conditions (12:12 h light:dark cycle; 22 ± 3 °C; 40-60% humidity) and had free access to standard rat pellet diet (Ashirwad brand, Chandigarh, India) and filter water, *ad libitum*. The experiments were carried out in accordance with

Table 1: Phytochemical screening of the BCAE

| Phytoconstituents | Phytochemical test | Inference | BCAE |
|------------------------------|-----------------------------|---|------|
| Carbohydrates | Molisch's test | Formation of violet ring at junction | + |
| Proteins | Biuret test | Appearance of violet color | - |
| | Xanthoproteic test | Formation of white precipitate | |
| Amino acids | Ninhydrin test | Appearance of Purple color | - |
| Triterpenoid sterols | Salkowski reaction | Appearance of red color in chloroform layer while greenish yellow in acid layer | + |
| Fats, oils and volatile oils | Solubility test | Solubility in water, ether, benzene and chloroform | - |
| Fats and oils | Saponification test | Formation of soap | - |
| Glycosides | Keller Killiani test | Formation of reddish brown colour at junction | + |
| Flavonoids | Shinoda test | Formation of reddish to pink color | + |
| Alkaloids | Dragendroff's test | Formation of orange colour precipitate | + |
| | Wagner's test | Formation of reddish brown precipitate | |
| Phenolic compounds | Lead acetate test | Formation of white precipitate | + |
| and tannins | Test with FeCl ₃ | Appearance of bluish black color | |

+: present; -: absent; BCAE: aqueous extract of *Bombax ceiba*

guidelines prescribed by The Committee for the Purpose of Control and Supervision of Experiments on Animals and the use of animals was approved by the Institutional Animal Ethics Committee of the Institute (Proposal No. CRI-GWL/IAEC/2010/08).

Acute toxicity study

Healthy Wistar rats, starved overnight, were subjected to acute toxicity studies to determine non-observable adverse effect dose level (NOAEL) by acute toxic class method of oral toxicity as per Organization for Economic Co-operation and Development 423 guidelines.^[17] The rats ($n = 3$) were administered BCAE in the limit test dose of 2000 mg/kg and observed continuously for behavioral, neurological, and autonomic profiles for 2 h, and after a period of 24, 72 h and thereafter up to 14 days for any lethality, moribund state, or death. The limit test was repeated in another group of rats ($n = 3$) for confirmation and approximate LD₅₀ determination.

Experimental induction of hepatotoxicity

Hepatotoxicity was induced in Wistar rats by intraperitoneal (i.p.) administration of CCl₄ in olive oil in the ratio of 1:1 at the dose of 1 mL/kg for two continuous days as described previously with modifications.^[18,19] After 48 h of the last dose of CCl₄, blood was withdrawn from retro-orbital plexus by capillary puncture method.^[20] Plasma was separated and analyzed for the various biochemical markers of hepatotoxicity and hepatic damage.

Grouping and treatments

The rats were divided into five groups ($n = 5$ each). Group I received only olive oil (1 mL/kg, i.p.), and remaining groups (group II, III, IV and V) received 1 mL/kg, i.p. CCl₄ in olive oil for two continuous days. While group II (control) received the vehicle of the extract (5 mL/kg, distilled water, orally), group III and IV received BCAE (250 and 500 mg/kg orally, respectively). Group V received silymarin suspension (25 mg/kg, orally), a known antioxidant and hepatoprotective agent.^[21,22] The vehicle/drugs were administered daily orally for seven days and CCl₄ administration was done on the 5th and 6th day of vehicle/drug treatments.

Assessment of liver function test and hepatic damage

On the eighth day of the experiment, blood was withdrawn by micro-capillary technique from the retro-orbital plexus under light ether anesthesia. This technique is used with recovery in experimental circumstances and this method is also called periorbital, posterior-orbital and orbital venous plexus bleeding. Briefly, a capillary is inserted into the medial canthus of the eye (30 degree angle to the nose) with a slight thumb pressure to puncture the tissue and enter the plexus/sinus. Once the plexus is punctured, blood will come through the capillary tube which was collected in 1.5 mL Eppendorf tubes from the plexus. The capillary tube is then gently removed and wiped with sterile cotton. Bleeding can be stopped by applying gentle finger pressure.^[20] Blood was centrifuged at 3,000 g to obtain plasma, which was used to assess liver function parameters (GOT, GPT,^[23] ALP,^[24] T,^[25] total protein,^[26] albumin and TG) using semi-autoanalyser (Microlab 300, Merck Specialities Pvt. Ltd. New Delhi).

Histological studies

After the withdrawal of blood, the animal was sacrificed by cervical dislocation. Abdomen was cut opened and aorta was cut to washout the blood from tissues. The liver was dissected out. A piece of liver was fixed in 10% v/v neutral buffered formalin. Serial sections (4-5 μ m thick) of the paraffin-embedded tissue blocks were cut with a Microm HM 360 microtome and processed for hematoxylin and eosin (HE), Masson's trichrome (Accustain Trichrome Stains, Sigma-Aldrich Inc, USA). Staining was done as per manufacturer's protocol. The sections were studied under microscope.

Assessment of antioxidant activity

Quantitative estimation of antioxidant phytochemicals

The total phenolic content of the extracts was determined spectrometrically^[27] and expressed as milligrams of tannic acid equivalents (TAE) per gram of extract. Total flavonoid content was measured by aluminum chloride colorimetric assay^[28] and expressed as milligrams of quercetin equivalent per gram of extract.

DPPH (1, 1-Diphenyl-2-picryl-hydrazil) free radical scavenging activity

The free radical scavenging activity of extract was measured by 1, 1-diphenyl-2-picryl-hydrazil (DPPH•) using the method previously described.^[29] Briefly, 0.1 mmol/L solution of DPPH in ethanol was prepared, and 3.5 mL was added to 0.5 mL of extract solution of different concentrations in water. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm by using a spectrophotometer (UV 1800, Shimadzu Corporation, Japan). Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. Ascorbic acid was taken as standard antioxidant. The percent DPPH scavenging effect was calculated using the following equation: $\text{DPPH}^\bullet \text{ scavenging effect (\%)} = 100 \times A_i/A_0$ (where A_0 was the absorbance of the control reaction and A_i was the absorbance in the presence of the test).

Statistical analysis

The data were analyzed by one-way ANOVA followed by Tukey's multiple comparisons *post hoc* test. A statistical difference of $P < 0.05$ was considered significant in all cases.

RESULTS

Phytochemical screening of BCAE

The qualitative tests for identifying the nature of phytochemicals in BCAE revealed the presence of flavonoids, carbohydrates, sterols, glycosides, alkaloids, volatile oils, and phenolic compound. However, proteins were found to be absent in the extract [Table 1].

Acute toxicity study of BCAE

Acute oral toxicity studies revealed that the BCAE was safe up to a dose level of 2,000 mg/kg of body weight (limit test) and NOAEL dose is more than 2,000 mg/kg. No lethality or any toxic reactions or moribund state were observed up to the end of the observation period of 14 days.

Effect of CCl₄ treatment on liver function test

One-way ANOVA showed that the CCl₄ treatment (1 mL/kg, i.p. on continuous two days) significantly influenced the liver functions parameters ($P < 0.0001$ in all cases). Post hoc

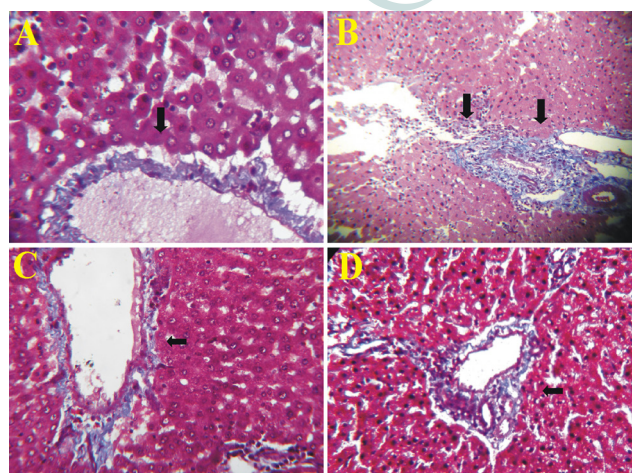


Figure 1: Effect of aqueous extract of *Bombax ceiba* on histopathology of liver. Histological sections of liver stained with Masson's trichrome stain from olive oil treated control rats (A) shows normal hepatic architecture with central canal having radiating hepatocytes. Minimal amount of collagen tissue (arrow) stained blue with Masson's stain in the portal triad. Liver section from CCl₄ treated rats (B) that received vehicle showed hepatocellular degeneration with moderate amount of collagen tissue (arrow) stained blue with Masson's stain in the portal triad. Section of liver of CCl₄ treated rat which concurrently received silymarin (C) and the aqueous extract of flowers of *Bombax ceiba* (500 mg/kg) (D) respectively, shows lesser amount of collagen and was comparable to control (A), showing minimal amount of collagen tissue (arrow) stained blue with Masson's stain in the portal triad.

test indicated CCl₄ treatment significantly ($P < 0.001$ in all cases) elevated plasma levels of GOT, GPT, ALP, and T while decreased the albumin and total protein and TG as compared to olive oil control [Table 2].

Effect of BCAE treatment on liver function test

One-way ANOVA showed that BCAE (250 or 500 mg/kg per day, orally) or silymarin (25 mg/kg per day, orally) treatment for seven days significantly influenced the liver functions parameters ($P < 0.0001$) in CCl₄ treated rats. The BCAE or silymarin significantly ($P < 0.05$ - 0.001) attenuated the elevation in levels of GOT, GPT, ALP, T, and TG while increased total protein without affecting the levels of albumin [Table 2]. The effect of BCAE was lesser than that of standard drug silymarin.

Effect of BCAE treatment on histology of liver of CCl₄ treated rats

Treatment with CCl₄ caused marked liver damage and fibrosis characterized by hepatocellular degeneration with moderate

Table 2: Effect of BCAE on liver function parameters

| Treatments | Liver function parameters | | | | | | |
|------------------------------|---------------------------|-----------------|-----------------|--------------------------|-------------------------|-------------------|-----------------|
| | GOT (U/L) | GPT (U/L) | ALP (U/L) | Bilirubin (T) (mg/dL) | Total protein (g/dL) | Albumin (g/dL) | TG (mg/dL) |
| Olive oil | 134.0 ± 12.69 | 48.60 ± 2.29 | 137.80 ± 10.18 | 0.21 ± 0.04 | 5.10 ± 0.30 | 4.64 ± 0.19 | 156.30 ± 17.01 |
| CCl ₄ + vehicle | 306.8 ± 24.50* | 202.2 ± 10.34* | 255.20 ± 32.87* | 1.18 ± 0.01* | 2.30 ± 0.21* | 4.04 ± 0.30 | 76.77 ± 6.40* |
| CCl ₄ + BCAE 250 | 271.0 ± 19.25 | 189.0 ± 14.39 | 155.60 ± 15.60# | 0.73 ± 0.06# | 2.74 ± 0.15 | 3.58 ± 0.57 | 139.0 ± 9.02# |
| CCl ₄ + BCAE 500 | 205.8 ± 10.01# | 153.8 ± 16.78\$ | 147.6 ± 15.42# | 0.60 ± 0.09@ | 2.26 ± 0.42 | 3.24 ± 0.06 | 143.20 ± 11.94# |
| CCl ₄ + silymarin | 134.6 ± 8.06@ | 58.00 ± 5.04@ | 69.20 ± 5.85@ | 0.35 ± 0.04@ | 5.50 ± 0.20@ | 3.27 ± 0.30 | 126.10 ± 7.88\$ |

Rats were treated for 7 days with vehicle or BCAE (250 and 500 mg/kg, i.g.) or silymarin (25 mg/kg i.g.) along with olive oil or CCl₄ in olive oil (1 mL/kg, i.p.) treatment on day 5 and liver functions markers (GOT, GPT, ALP, T, total protein, albumin and TG) were assessed on day 8. Results are expressed as mean ± SEM ($n = 5$) * $P < 0.001$ vs. olive oil or \$ $P < 0.05$, # $P < 0.01$, @ $P < 0.001$ vs. CCl₄ treated vehicle control (one-way ANOVA followed by Tukey's multi-comparison post hoc test). GOT: glutamic oxaloacetic transaminase; GPT: glutamic pyruvic transaminase; ALP: alkaline phosphatase; TG: triglycerides; BCAE: aqueous extract of *Bombax ceiba*

Table 3: Effect of BCAE on DPPH radical scavenging

| | Concentration ($\mu\text{g/mL}$) | % DPPH inhibition | IC ₅₀ value |
|---------------|------------------------------------|-------------------|------------------------|
| BCAE | 10 | 15.53 \pm 1.85 | 50.21 $\mu\text{g/mL}$ |
| | 20 | 31.76 \pm 2.25 | |
| | 40 | 36.07 \pm 71.35 | |
| | 60 | 71.96 \pm 1.76 | |
| | 80 | 73.16 \pm 2.15 | |
| | 100 | 80.16 \pm 1.07 | |
| Ascorbic acid | 5 | 24.92 \pm 1.33 | 3.35 $\mu\text{g/mL}$ |
| | 10 | 54.67 \pm 2.89 | |
| | 20 | 68.83 \pm 1.68 | |
| | 40 | 86.73 \pm 2.46 | |
| | 50 | 91.86 \pm 1.75 | |
| | 100 | 93.63 \pm 0.86 | |

Results are expressed as mean \pm SEM ($n = 3$); IC₅₀ = 50% inhibitory concentration. BCAE: aqueous extract of *Bombax ceiba*

amount of collagen tissue (arrow) stained blue with Masson's trichrome stain in the portal triad [Figure 1B]. Liver section of olive oil treated animals [Figure 1A] showed normal hepatic architecture with central canal having radiating hepatocytes. Minimal amount of collagen tissue (arrow) stained blue with Masson's stain was evident in the portal triad.

The BCAE treatment (500 mg/kg) or silymarin showed significant protection against CCl₄-induced hepatic damage as indicated by lesser amount of collagen tissue vascular as compared to vehicle [Figure 1B]. Treatment with 500 mg/kg dose of BCAE exhibited comparable protection [Figure 1D] to that offered by silymarin (25 mg/kg) [Figure 1C].

Antioxidant effect of BCAE

Quantitative estimation of antioxidant phytochemicals

The total flavonoid content of BCAE was found to be 5.79 mg quercetin equivalents/g of extract, while the total phenolic content was found to be 0.225 mg tannic acid equivalent/g of extract, respectively.

DPPH free radical scavenging activity

The BCAE in concentration range of 10-100 $\mu\text{g/mL}$ inhibited DPPH radical formation as indicated by concentration-dependent decrease in the purple color of the solution. Similar effect was obtained with ascorbic acid, the standard antioxidant, in the concentration range of 5-100 $\mu\text{g/mL}$. The linear regression analysis of concentration vs. percent DPPH inhibition was carried out. The IC₅₀ value of BCAE and ascorbic acid, obtained from regression analysis, were 50.21 and 3.35 $\mu\text{g/mL}$, respectively [Table 3].

DISCUSSION

Acute toxicity study of the BCAE (2,000 mg/kg, orally) revealed that there was no toxicity of any nature or moribund stage during the observation period. This illustrates that the NOAEL of BCAE is more than 2,000 mg/kg. Based on this, the BCAE was administered in the dose range of 200 mg/kg (one tenth of the limit test dose level). The previous studies have also used extract of *Bombax ceiba* in the similar dose range.^[14]

In accordance with earlier reports,^[30-32] the present investigations revealed that administration of CCl₄ caused a marked impairment in liver function, as indicated by significant increase in plasma levels of marker enzymes; and produced extensive histological damages to liver. CCl₄ undergoes metabolism in liver to form trichloromethyl peroxy (CCl₃O₂) radical^[33] and several lines of evidences suggest that the free radicals oxidize the essential macromolecular structures, that is, DNA, proteins, and lipids, and eventually produce cytotoxicity.^[34,35] In addition, higher levels of lipid peroxidation are clinically evident in liver disorders^[36] and the antioxidant therapy was found to ameliorate these effects.^[37]

It was observed that treatment with BCAE ameliorated the CCl₄-induced impairments in the liver functions except total protein and albumin. BCAE in the dose 500 mg/kg offered moderate degree of attenuation in the elevated GOT, GPT, and TG, but with very remarkable prevention of ALP and T. The lower dose of 250 mg/kg was almost ineffective in normalizing the liver markers except for a few. BCAE also showed lesser degree of collagen fiber as compared to vehicle control [Figure 1] which suggests the preventive nature of the extract on liver tissue fibrosis. These findings confirmed that BCAE exerts moderate hepatoprotective effect. Previously the hepatoprotective effect of *Bombax ceiba* flowers was demonstrated in isoniazid plus rifampicin induced hepatotoxicity^[14] and supports the findings of the present study.

Phytochemical analysis of BCAE revealed the presence of the antioxidant phytochemicals flavonoids, terpenes and phenolic compounds. It has been earlier reported that the flowers and other parts of this plant contains flavonoids and sesquiterpenes, etc.^[38,39] The present study also revealed that BCAE has fair amount of flavonoids and phenolics.

BCAE was further tested for its antioxidant activity. The results revealed that BCAE has significant free radical scavenging property [Table 3] with IC₅₀ of 50.21 $\mu\text{g/mL}$. The antioxidant activities of the flavonoids are well

demonstrated and they are often found effective in hepatic disorders.^[40-42] Previous studies have reported that *Bombax ceiba* extract possesses *in vitro* antioxidant activity.^[10-12] Based on this, it can be hypothesized that the observed hepatoprotection offered by BCAE may be ascribed to its antioxidant activity. Furthermore, this was supported by the observation that daily treatment with silymarin, a well proven antioxidant, showed similar effects on CCl₄-induced changes in the levels of hepatic function markers and similarly prevented the CCl₄-induced damage to the liver. The *in vivo* antioxidant activity and hepatoprotective effect of silymarin are well demonstrated in earlier studies^[21,22] and corroborate with the present findings. The observed hepatoprotective effect of BCAE was comparable to that of silymarin.

In conclusion, BCAE exhibited protective effect on CCl₄-induced free radical mediated hepatotoxicity. The observed hepatoprotection by BCAE may be a consequence of its antioxidant effect due to the presence of flavonoids or other phenolic compounds in BCAE. The present investigations scientifically validate the traditional use of flowers of *Bombax ceiba* in hepatic disorders.

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Conflicts of interest

There are no conflicts of interest.

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Identifying microRNA panels specifically associated with hepatocellular carcinoma and its different etiologies

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ABSTRACT

Aim: Deregulation of microRNAs (miRNAs) expression has been identified in hepatocellular carcinoma (HCC), but few results are consistent. The objective of this study is to investigate “HCC tumor type specific” and “tumor common” miRNA panels. **Methods:** The authors integrate and analyze clinical, etiologic and miRNA profiles data from 9 types of solid tumors in The Cancer Genome Atlas (TCGA) and HCC data from Columbia University Medical Center (CUMC). **Results:** Levels of 33 miRNAs were significant different between HCC tumor and paired non-tumor tissues (over 2-fold changes) after Bonferroni correction for multiple comparisons, and most (28 miRNAs) were down-regulated in HCC tumors. Using this panel, the authors well classified HCC tumor tissues with 4 misclassifications among 48 paired tissues. Validating this panel in an additional 302 HCC tumor tissues, the authors almost perfectly distinguished tumor from non-tumor tissues with only two misclassifications (99% of HCC tissues correctly classified). Evaluating miRNA profiles in 32 independent HCC paired tissues from CUMC, the authors observed 40 miRNAs significantly deregulated in HCC with over 2-fold changes; 14 overlapped with those identified in TCGA. Subgroup analyses by HCC etiology found that 4 upregulated and 8 downregulated miRNAs were significantly associated with alcohol-related HCC. There were 7 and 4 miRNAs significantly associated with hepatitis B virus- and hepatitis C virus-related HCC, respectively. Data for the first time revealed that miR-24-1, miR-130a and miR-505 were significantly down-regulated only in HCC tumors; miR-142 and miR-455 were significantly down-regulated in HCC, but up-regulated in 5 other solid tumors; suggesting their HCC “tumor type specific” characteristics. A panel of 8 miRNAs was significant in at least 5 tumor types, including HCC, and was identified as “tumor common” marker. **Conclusion:** The authors concluded that aberrant miRNA panels have HCC “tumor type specificity” and may be affected by etiologic factors.

Key words: MicroRNA; hepatocellular carcinoma; etiologies; The Cancer Genome Atlas

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INTRODUCTION

MicroRNAs (miRNA) are important biological regulators and play a critical role in controlling protein-coding genes' expression at the post-transcriptional level. It is estimated that one third of human genes are directly or indirectly governed by miRNAs and they impact multiple cellular pathways involved in tumorigenesis.^[1,2] Anomalous expression of miRNAs have been implicated in a wide variety of cancers, including hepatocellular carcinoma (HCC), one of the most common cancers and the third leading cause of cancer death worldwide. The incidence of HCC has tripled over the past 30 years in the United States,^[3,4] which may be attributed to increased hepatitis C virus (HCV) infection and obesity-related nonalcoholic fatty liver disease (NAFLD).^[4,5] Other established etiologies of HCC are hepatitis B virus (HBV) infection, alcohol abuse and aflatoxin B₁ (AFB₁) exposure.^[6,7] Most previous studies examining miRNA profiles in HCC tumor tissue or blood focused on investigation of the main effects of aberrant miRNAs associated with cancer status without consideration of the potential influence of etiologic risk factors on miRNA levels that may bias the miRNA patterns observed in HCC tumors with heterogeneous etiologies. That may be one reason for the discrepant results of previous miRNA marker studies of HCC. Although some studies do examine miRNA profiles in HCC patients carrying specific etiologies,^[8-10] it is still unclear whether the identified miRNAs are etiology-specific due to lack of transverse comparisons with HCC patients carrying other etiologic factors. Another challenge is the lack of a comparison of miRNA panels between HCC and other solid tumor types, and no evidence to indicate HCC tumor type specific miRNA alterations that may limit future clinical application. In the current study, we integrate HCC etiologies and miRNA sequencing data from HCC and 8 other types of solid tumors in The Cancer Genome Atlas (TCGA) resource, and investigate whether miRNA panels identified in HCC tumor are organ specific and affected by important etiologic factors. These results can be used for more precise clinical early diagnosis of HCC subtypes and screening of high risk populations with specific HCC etiologies.

METHODS

Demographic, etiologies, clinical and miRNA data in HCC patients from TCGA dataset

TCGA is a comprehensive and coordinated project supported by the National Cancer Institute (NCI) and the National Human Genome Research Institute

(NHGRI) to characterize the genomic data of more than 30 different types of cancers, and accelerate understanding of the molecular basis of cancer. Currently, there are 366 cancer patients in the cancer type of liver hepatocellular carcinoma, who provided demographic, etiologic and clinical data, as well as tissue samples for TCGA study. The miRNA expression and corresponding etiologies and clinical data were downloaded (up to June 16, 2015) from TCGA data portal (<https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp>), and a total of 414 samples from 366 histologically confirmed liver cancer patients have completed miRNAs, etiologic and clinical data. After checking histologic diagnosis and tissue types, we excluded 10 non-HCC cases (either mixed hepatocholangiocarcinoma or fibrolamellar carcinoma); 5 recurrent HCC tumors and 1 HCC non-tumor tissue without relevant paired tumor tissue. Finally, data from 48 HCC patients with paired tumor and non-tumor tissues and 302 HCC patients with tumor tissues alone were analyzed in the current study.

Demographic, etiologic and clinical data include co-variables of age, gender, race/ethnicity, height (m) and weight (kg) at cancer diagnosis, body mass index (BMI, kg/m²), HCC risk factors (alcohol consumption, HBV, HCV, NAFLD, mixed and none), tumor status (free vs. not free), family history of cancer (no vs. yes), alpha fetoprotein (AFP, ng/mL), histologic tumor grade (G1-G2 vs. G3-G4), the American Joint Committee on Cancer (AJCC)^[11] tumor-node-metastasis (TNM) (T0-T2 vs. T3-Tx), lymph node involvement (N0-N1 vs. Nx), pathological stage (stage I-II vs. stage III-IV), metastasis status (M0-M1 vs. Mx), vital status (alive vs. dead), survival days (either days to last follow-up or days to death). Other clinical variables (Child-Pugh classification, vascular tumor invasion, adjuvant treatment, surgical types and new tumor event after initial treatment) were not analyzed in the current study due to either a large amount of missing data or small sample sizes in subgroups.

The level 3 (archive type) miRNA expression data were generated from the Illumina HiSeq 2000 platform (Illumina Inc., San Diego, CA) and annotated to reference miRBase v16 of UCSC hg19 alignments.^[12] A total of 1,046 unique mature miRNAs were obtained. The sequencing data are presented as raw read counts and reads per million (RPM) mapped miRNAs reads. The RPM indicates the expression level of miRNA and is calculated according to the formula: $RPM = (N_{miR} / N_{all}) \times 10^6$, N_{miR} : number of reads mapped to the specific miRNA reference; N_{all} : total number of reads mapped in the sample. Because all demographic, clinical

and miRNA data are derived from the de-identified publically available TCGA dataset, it is not possible to link to any individual. Therefore, no Institutional Review Board (IRB) approval was required.

MiRNA sequencing data from 8 other solid tumors in TCGA dataset

MiRNA sequencing and clinical data from other solid cancers were also downloaded from TCGA data portal. Eight solid tumors with available miRNA and clinical data in over 40 paired tumor and non-tumor tissues were considered in the final statistical analyses, including female breast invasive carcinoma (BRCA), head and neck squamous cell carcinoma (HNSC), kidney renal cell carcinoma (KIRC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), prostate adenocarcinoma (PRAD), stomach adenocarcinoma (STAD), and thyroid carcinoma (THCA). The samples sizes (pairs) were 102 for BRCA, 71 for KIRC, 59 for THCA, 52 for PRAD, 46 for LUAD, 43 for HNSC, and 41 for both STAD and LUSC.

HCC patients and miRNA data used as the validation set

For the first set of validation, we used 32 HCC frozen tumor and adjacent non-tumor tissues (16 pairs) that were collected by the Center for Liver Disease and Transplantation, and stored in the Molecular Pathology Shared Resource of the Herbert Irving Comprehensive Cancer Center, Columbia University Medical Center (CUMC). This study has been approved by the IRB of CUMC. Total RNA, including miRNAs was isolated from HCC tissues by RNeasy Microarray Tissue Mini Kits (Qiagen, Frederick, MA) according to the manufacturer's protocol. TaqMan Low Density Arrays (TLDA, Applied Biosystems, Foster City, CA), covering 733 miRNAs (670 unique human mature miRNAs), were used to generate miRNA profiles that were deposited in NCBI's Gene Expression Omnibus database (accession number GSE54751).^[13] TaqMan MicroRNA assays were used to further evaluate the consistence of candidate miRNA expression patterns in 66 paired HCC tumor and non-tumor tissues from CUMC. U6 snRNA stable in liver tumor/adjacent tissues (Ct: 21.19 vs. 21.08, $P = 0.398$) was used as an endogenous control to normalize the expression of miRNAs using the $2^{-(\Delta\Delta Ct)}$ approach.^[14]

Statistical analysis

We applied stringent criteria to filter available miRNA sequencing data before performing any statistical analysis to ensure the reliability and abundance of candidate miRNAs in the target tissues. MiRNAs were excluded from further data analyses if the RPM was

less than 10 counts and missing data exceeded 10% of all subjects. MiRNAs with less than 10 counts per million may be due to sequencing errors.^[15] A low missing value ($< 10\%$) provides the most reliable and consistent result without the need for further normalization.^[16] A total of 153 miRNAs passed the filtering criteria and data were log2-transformed for final statistical analysis in HCC.

Paired *t*-test with Bonferroni correction for multiple comparisons was used to identify miRNAs that were significant different ($P < 0.0001$) with at least a 2-fold expression change between the 48 paired HCC tumor and adjacent non-tumor tissues. The volcano plot and hierarchical clustering were performed using the panel of significant miRNAs to describe the distribution of miRNAs and tumor classification, respectively. The same miRNA panel was used to construct a heat-map and classify the 302 unpaired tumor tissues. The general linear model was used to compare miRNAs expression levels between unpaired HCC tumor and non-tumor tissues adjusted for covariates significantly different between groups. Prediction analysis of microarrays using the nearest shrunken centroid methodology was used to separately evaluate the classification of tissues (tumor vs. non-tumor) for paired and unpaired tumors by those significantly altered miRNAs, and estimate prediction error, sensitivity, specificity, positive predictive value and negative predictive value via cross-validation.^[17] Two-sample *t*-tests were applied to identify significant miRNAs ($P < 0.0001$) with over 2-fold changes by age group (< 60 vs. ≥ 60 years), gender (male vs. female), BMI (≥ 25 vs. < 25), etiologies [alcohol vs. hepatitis B surface antigen (HBsAg) positive vs. anti-HCV positive], AFP (≥ 400 vs. < 400 ng/mL), and other clinicopathological covariates described above. Subgroups analyses were further conducted among HCC tumor and non-tumor tissues carrying one specific risk factor (alcohol, HBsAg or anti-HCV) to identify etiologic-specific miRNA panels.

Similar stringent filtering criteria and statistical analysis strategies were used to identify aberrantly expressed miRNA profiles from the other 8 different solid tumors. The identified miRNA panels from different tumors were compared to each other to discover "tumor type specific" or "tumor common" miRNA panels. We define "tumor common" miRNAs as those significant for at least 5 tumor types, including HCC, and with fold-changes in the same direction. "Tumor type specific" miRNAs are defined as only significant for one type of tumor among the 9 investigated tumors. If miRNAs are significant for several different tumor types, but the direction in

one type of tumor is opposite to all others, we also define them as “tumor type specific” miRNAs. The most commonly or uniquely expressed miRNAs were selected as “tumor common” or “tumor type specific” markers, respectively, for further bioinformatics validation.

All statistical data analyses were performed using BRB-ArrayTools (version 4.4) developed by Dr. Richard Simon and the BRB-ArrayTools Development Team (<http://linus.nci.nih.gov/BRB-ArrayTools.html>)^[18] and Statistical Analysis System 9.0 (SAS Institute). TCGA data used in this study meet the publication guidelines provided by TCGA (<http://cancergenome.nih.gov/publications/publicationguidelines>).

Bioinformatics analyses of miRNA targets and pathways enrichment

The targets of the miRNAs were predicted by mirsystem, which integrates seven well known miRNA target gene prediction programs (<http://mirsystem.cgm.ntu.edu.tw/index.php>)^[19] as well as the experimentally validated miRNA-target data from miRecords (<http://c1.accurascience.com/miRecords/>) and TarBase (<http://www.hsls.pitt.edu/obrc/index.php?page=URL1237572545>). The seven predictive tools include DIANA (<http://diana.imis.athena-innovation.gr/DianaTools/index.php>),

miRanda (<http://www.microrna.org/microrna/home.do>), mirBridge (<http://mirbridge.org/>), PicTar (<http://pictar.mdc-berlin.de/>), PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html), RNA22 (<https://cm.jefferson.edu/rna22v2/>), and TargetScan v6.2 (<http://www.targetscan.org/>). The concordant targets in the current study were defined as genes predicted by at least 5 out of 7 algorithms or validated by functional experiment. These genes were the most likely miRNA targets that were further evaluated by ToppGene (<https://toppgene.cchmc.org/prioritization.jsp>)^[20] to identify significant biological processes, pathways, molecular functions and cellular components after Bonferroni correction $P < 0.05$.

RESULTS

Demographic and clinical characteristics of HCC patients

We compared the demographic and clinical characteristics between 48 HCC patients with paired tumor and non-tumor tissues, and 302 patients with tumor tissue alone [Supplementary Table 1]. There were no significant differences for the co-variables of age (means of 61.1 vs. 59.5 years), gender, etiology, BMI, AFP level, tumor grade, lymph node involvement,

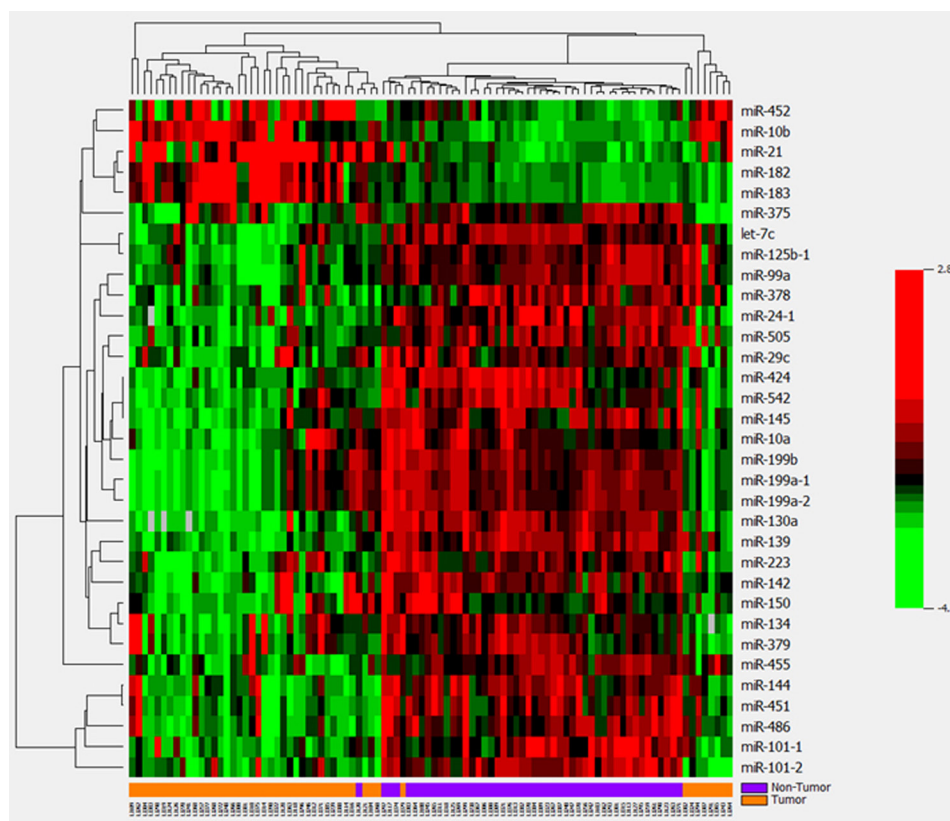


Figure 1: Hierarchical cluster analysis of 33 significantly differentially expressed miRNAs between 48 paired tumor and adjacent non-tumor tissues. Most miRNAs are down-regulated (green), while a few are up-regulated (red) in tumor tissues. The panel of miRNAs can well classify tissue types with 4 misclassified tumor and non-tumor tissues

Table 1: Differentially expressed miRNAs between 48 paired hepatocellular carcinoma tumor and non-tumor tissues

| miRNAs | Geometric mean of RPM in tumor tissue | Geometric mean of RPM in non-tumor tissue | Fold-change | P-value | FDR |
|----------------------|---------------------------------------|---|-------------|----------|----------|
| Upregulated | | | | | |
| miR-10b | 15,976.87 | 1,435.06 | 11.13 | < 1e-07 | < 1e-07 |
| miR-183 | 1,451.95 | 261.13 | 5.56 | < 1e-07 | < 1e-07 |
| miR-182 | 4,034.39 | 971.8 | 4.15 | 1.00E-07 | 4.50E-07 |
| miR-452 | 161.94 | 52.46 | 3.09 | 6.00E-07 | 2.30E-06 |
| miR-21 | 165,875.22 | 73,641.4 | 2.25 | < 1e-07 | < 1e-07 |
| Downregulated | | | | | |
| miR-199a-1 | 242.98 | 1,308.64 | -5.26 | < 1e-07 | < 1e-07 |
| miR-199a-2 | 404.21 | 2,176.44 | -5.26 | < 1e-07 | < 1e-07 |
| miR-199b | 490.46 | 2,611.64 | -5.26 | < 1e-07 | < 1e-07 |
| miR-139 | 101.67 | 426.13 | -4.17 | < 1e-07 | < 1e-07 |
| miR-375 | 1,338.06 | 5,364.67 | -4.00 | 1.87E-05 | 5.50E-05 |
| miR-424 | 133.58 | 537.65 | -4.00 | < 1e-07 | < 1e-07 |
| miR-130a | 37.45 | 140.38 | -3.70 | < 1e-07 | < 1e-07 |
| miR-451 | 382.94 | 1,433.49 | -3.70 | < 1e-07 | < 1e-07 |
| miR-144 | 94.72 | 315.05 | -3.33 | < 1e-07 | < 1e-07 |
| miR-142 | 996.3 | 2,968.42 | -2.94 | < 1e-07 | < 1e-07 |
| miR-486 | 94.12 | 279.26 | -2.94 | < 1e-07 | < 1e-07 |
| miR-99a | 535.01 | 1,568.16 | -2.94 | < 1e-07 | < 1e-07 |
| let-7c | 1,921.73 | 5,196.5 | -2.70 | < 1e-07 | < 1e-07 |
| miR-101-2 | 67.78 | 176.16 | -2.63 | < 1e-07 | < 1e-07 |
| miR-145 | 830.74 | 2,177.06 | -2.63 | < 1e-07 | < 1e-07 |
| miR-379 | 470.22 | 1,209.95 | -2.56 | 2.00E-07 | 8.27E-07 |
| miR-150 | 218.83 | 546.98 | -2.50 | 5.20E-06 | 1.69E-05 |
| miR-223 | 90.56 | 228.55 | -2.50 | < 1e-07 | < 1e-07 |
| miR-24-1 | 34.72 | 85.92 | -2.50 | < 1e-07 | < 1e-07 |
| miR-125b-1 | 445.23 | 1,078.99 | -2.44 | < 1e-07 | < 1e-07 |
| miR-542 | 195.84 | 462.64 | -2.38 | < 1e-07 | < 1e-07 |
| miR-101-1 | 18,399.45 | 42,453.11 | -2.33 | < 1e-07 | < 1e-07 |
| miR-10a | 10,426.51 | 23,842.5 | -2.27 | 6.00E-07 | 2.30E-06 |
| miR-134 | 138.39 | 318.13 | -2.27 | 9.10E-06 | 2.90E-05 |
| miR-378 | 767.99 | 1,756.29 | -2.27 | < 1e-07 | < 1e-07 |
| miR-455 | 681.31 | 1,547.61 | -2.27 | 3.40E-06 | 1.13E-05 |
| miR-505 | 66.11 | 149.37 | -2.27 | < 1e-07 | < 1e-07 |
| miR-29c | 2,061 | 4,281.77 | -2.08 | < 1e-07 | < 1e-07 |

RPM: reads per million mapped miRNAs reads; FDR: false discovery rate

metastasis status, AJCC pathological stage and TNM. The 48 HCC patients were more often white, had less surgical remove of their tumors, more frequently had a cancer family history and had a longer survival time compared to the 302 patients.

MiRNA abundance and classification of tumor tissues

A pie graph shows the distribution of the most abundant

miRNAs (top 20) in HCC tumor and non-tumor tissues [Supplementary Figure 1]. The most abundant 5 miRNAs are miR-21, miR-22, miR-143, miR-148a and miR-192; they account for more than 58% of all detectable miRNAs. Paired *t*-test analysis revealed that 33 miRNAs were significantly differentially expressed between the 48 paired HCC tumor and non-tumor tissues with over 2-fold changes at the significance level of $P < 0.0001$ [Table 1, Supplementary

Table 2: Accuracy of hepatocellular carcinoma tumor tissues classification by 33 significant miRNAs panel

| Classification | Sensitivity | Specificity | PPV | NPV | Correct classification (%) | Misclassification (%) |
|---------------------|-------------|-------------|-------|-------|----------------------------|-----------------------|
| 48 paired tumors | 0.917 | 1.000 | 1.000 | 0.923 | 95.9 | 4.1 |
| 302 unpaired tumors | 0.990 | 0.979 | 0.997 | 0.940 | 98.5 | 1.5 |

PPV: positive predictive value; NPV: negative predictive value

Table 3: Aberrant miRNAs associated with etiologic specific HCC tumors

| Etiologies | miRNAs | Geometric mean of RPM in tumor tissue | Geometric mean of RPM in non-tumor tissue | Fold-change | P-value | FDR |
|------------------------|-----------|---------------------------------------|---|-------------|----------|----------|
| Alcoholic HCC | | | | | | |
| (n = 79) | miR-10b | 12,283.98 | 1,395.27 | 8.80 | 2.60E-06 | 7.96E-05 |
| | miR-21 | 189,758.19 | 68,433.86 | 2.77 | 3.00E-07 | 1.15E-05 |
| | miR-500a | 423.56 | 165.36 | 2.56 | 5.73E-05 | 7.57E-04 |
| | miR-532 | 1491.55 | 607.79 | 2.45 | 2.47E-05 | 4.20E-04 |
| | miR-424 | 98.38 | 483.13 | -5.00 | 1.00E-07 | 7.65E-06 |
| | miR-3607 | 43.77 | 221.60 | -5.00 | 5.60E-06 | 1.22E-04 |
| | miR-139 | 101.16 | 487.34 | -4.76 | 2.00E-07 | 1.02E-05 |
| | miR-130a | 48.83 | 152.77 | -3.13 | 8.60E-06 | 1.64E-04 |
| | miR-24-1 | 32.81 | 96.18 | -2.94 | < 1e-07 | < 1e-07 |
| | miR-29c | 1,510.76 | 4,355.13 | -2.86 | 4.31E-05 | 6.59E-04 |
| | miR-101-1 | 16,403.75 | 45,299.71 | -2.78 | 3.60E-06 | 9.18E-05 |
| | miR-101-2 | 81.66 | 189.96 | -2.33 | 7.73E-05 | 9.10E-04 |
| HBV-related HCC | | | | | | |
| (n = 79) | miR-532 | 1,665.49 | 605.92 | 2.75 | 6.40E-05 | 1.40E-03 |
| | miR-93 | 5448.5 | 2,193.92 | 2.48 | 5.11E-05 | 1.30E-03 |
| | miR-21 | 205,313.3 | 84,355.47 | 2.43 | 1.60E-06 | 8.16E-05 |
| | miR-424 | 85.19 | 535.38 | -6.25 | < 1e-07 | < 1e-07 |
| | miR-139 | 104.28 | 383.68 | -3.70 | 2.22E-05 | 6.79E-04 |
| | miR-24-1 | 28.91 | 73.80 | -2.56 | 1.50E-06 | 8.16E-05 |
| | miR-26b | 865.69 | 2,027.85 | -2.33 | 1.04E-05 | 3.98E-04 |
| HCV-related HCC | | | | | | |
| (n = 31) | miR-93 | 5,978.72 | 1,423.17 | 4.20 | 1.99E-05 | 1.60E-03 |
| | miR-500a | 457.17 | 125.83 | 3.63 | 9.53E-05 | 3.65E-03 |
| | miR-424 | 91.62 | 611.42 | -6.67 | 3.13E-05 | 1.60E-03 |
| | miR-3607 | 46.28 | 249.57 | -5.26 | 2.90E-05 | 1.60E-03 |

RPM: reads per million mapped miRNAs reads; HCC: hepatocellular carcinoma; HBV: hepatitis B virus; HCV: hepatitis C virus; FDR: false discovery rate

Figure 2]. However, only 5 overlap with the top 20 most abundant miRNAs, suggesting the most significant miRNAs are infrequently expressed in liver tissues a more sensitive approach for their detection is needed. Five miRNAs (miR-10b, miR-182, miR-183, miR-21 and miR-452) were significantly up-regulated in HCC tumor tissue with fold changes ranging from 11.13 to 2.25, while 28 miRNAs showed significant down-regulation in tumor tissue (fold-change from -5.26 to -2.08). The same expression patterns for the 33 significant miRNAs were also observed in additional 302 HCC

tissues comparing with unpaired 48 non-tumor tissues [Supplementary Table 2]. After adjusting for covariates of race, survival time, tumor status and family history of cancer, these miRNAs still kept significance with over 2-fold changes, indicating the aberrant miRNAs mainly caused by tumor itself. Using the 33 significant miRNAs as a panel to generate a hierarchical heat map, only 4 tumor/non-tumor tissues were misclassified among the 48 paired tissues [Figure 1]. The same panel of miRNAs was used to classify the additional 302 HCC patients with tumor tissues

alone, and excellent clustering was observed with only two misclassifications [Supplementary Figure 3]. Percentages of correctly classified HCC tissues were 96% and 99% for the 48 paired and 302 unpaired tumor tissues respectively [Table 2], suggesting the promise of aberrantly expressed miRNAs as HCC biomarkers.

We validated the findings from TCGA data by measuring miRNA profiles in 32 paired HCC tissues from CUMC. We observed 40 miRNAs significantly deregulated in HCC tumors ($P < 0.05$) with over 2-fold changes [Supplementary Figure 4], and 14 (let-7c, miR-21, miR-99a, miR-125b, miR-130a, miR-139, miR-144, miR-145, miR-150, miR-199a, miR-223, miR-378, miR-455 and miR-486) overlap with those identified in TCGA data. Eight miRNAs (miR-122, miR-1180, miR-199a, miR-182, miR-152, miR-125b, miR-18a and miR-10a) with various expression levels in TCGA data were randomly selected and evaluated by TaqMan quantitative reverse transcription polymerase chain reaction (RT-PCR) in 66 paired HCC tissues from CUMC. Seven out of 8 miRNAs had consistent fold-changes as in TCGA data [Supplementary Table 3]. Only miR-152 showed an inconsistent fold-change (1.01 in

RT-PCR and -1.03 in TCGA). The raw expression data of 8 miRNAs were showed in Supplementary Table 4.

Aberrant miRNAs panels associated with etiology-specific HCC

Subgroup analyses for three HCC-specific major etiologic factors (alcohol abuse, HBV and HCV infection) by two-sample *t*-tests, we identified 4 upregulated (miR-10b, miR-21, miR-500a and miR-532) and 8 downregulated miRNAs panel significantly associated with alcohol-related HCC [Table 3, Supplementary Figure 5A]. The 12-miRNAs panel can distinguish alcohol-related HCC tumor from non-tumor with 3 misclassifications [Supplementary Figure 5B]. There were panels of 7 and 4 significant aberrantly expressed miRNAs observed in HBV- or HCV-related HCC, respectively, with over 2-fold expression changes [Table 3, Supplementary Figure 6]. These miRNA panels can also correctly classify HBV- or HCV-infected tumors with 1-2 misclassifications [Supplementary Figure 7]. Comparison of significant miRNAs for HCCs with different etiologies and overall HCCs, only miR-424 was consistently down-regulated among all HCC groups; miR-6b was only significantly

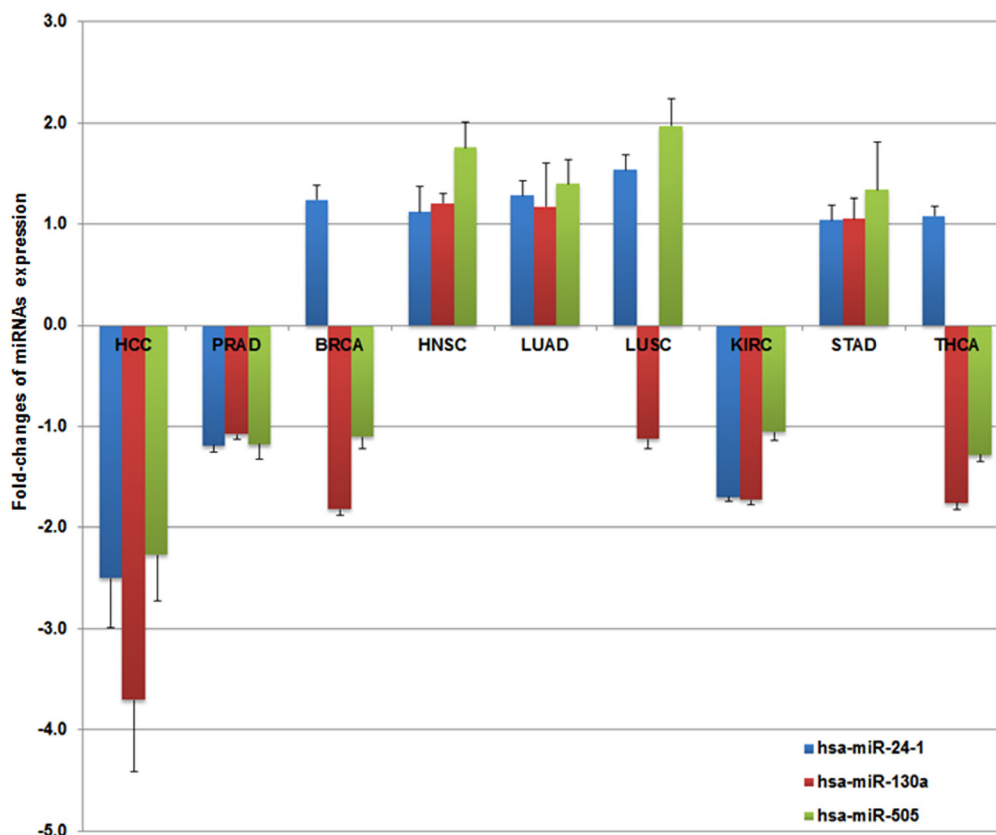


Figure 2: Hepatocellular carcinoma (HCC) “tumor specific” miRNA expression patterns (fold-changes and standard errors) compared to 8 other types of solid tumors. Three miRNAs (miR-24-1, miR-130a and miR-505) were significantly down-regulated in HCC with over 2-fold changes. Although the expression pattern of 3 miRNAs was consistently repressed in kidney renal cell carcinoma (KIRC) and prostate adenocarcinoma (PRAD), none were statistically significant. An up-regulated expression pattern was observed for the 3 miRNAs in head and neck squamous cell carcinoma (HNSC), lung adenocarcinoma (LUAD) and stomach adenocarcinoma (STAD), but also no significant difference. Both up- and down-regulation patterns were obtained for the 3 miRNAs in female breast invasive carcinoma (BRCA), lung squamous cell carcinoma (LUSC) and thyroid carcinoma (THCA), suggesting their tumor specificity

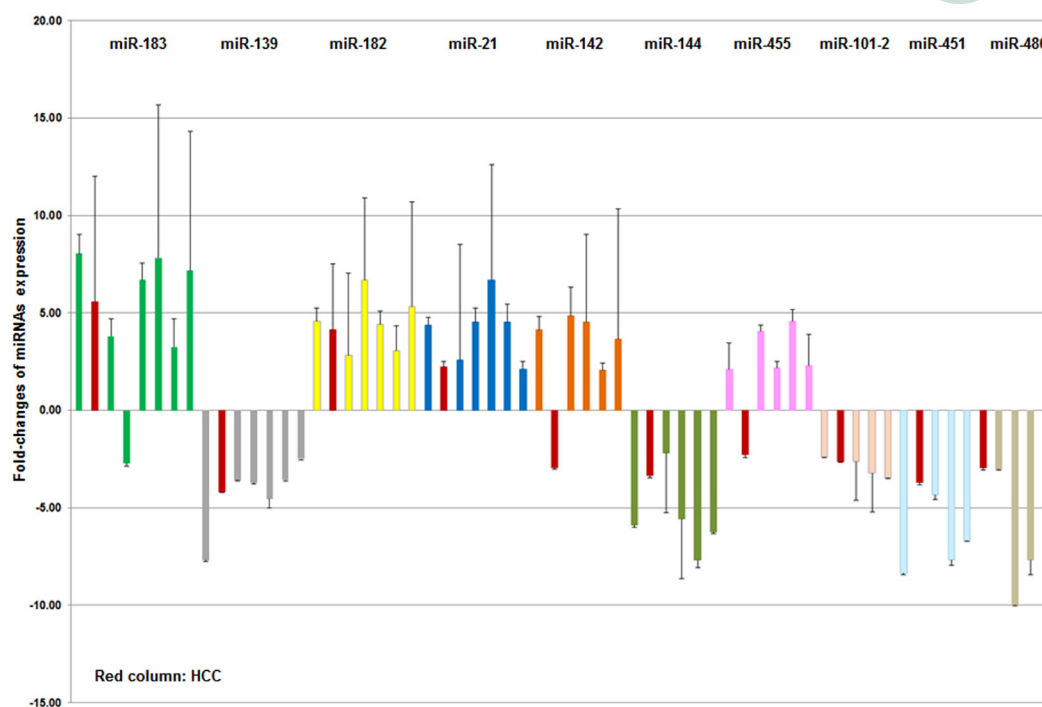


Figure 3: Expression patterns (fold-changes and standard errors) of 10 “tumor common” miRNAs in different types of solid tumors. MiR-182, miR-183, miR-21, miR-142 and miR-455 were significantly up-regulated, and miR-139, miR-144, miR-101-2, miR-451 and miR-486 were significantly down-regulated for most of tumor types. Interestingly, miR-142 and miR-455 were significantly down-regulated in HCC and is in the opposite direction from the five other solid tumors, suggesting their potential as “HCC tumor specific” markers. HCC: hepatocellular carcinoma

repressed in HBV-related HCC; and up-regulated miR-93 was identified in both HBV and HCV related HCC [Supplementary Figure 8A]. Several etiology-specific miRNAs were identified that do not overlap with those found in overall HCC [Supplementary Figure 8B], but most significant miRNAs identified in etiology-specific HCC were also consistently observed in overall HCC, which indicates that the fundamental mechanisms may be similar for hepatocarcinogenesis regardless of etiology. However, the results should be explained with caution because of the small sample sizes in subgroup analyses.

Exploring HCC “tumor type specific” and “common tumor” miRNAs panels

Using the same filtering criteria and statistical analysis strategies as for HCC, we examined miRNA profiles in an additional 8 solid tumor types with TCGA data available on at least 40 paired tumor and adjacent non-tumor tissues. Different panels consisting of 15 to 52 significant miRNAs ($P < 0.0001$) with over 2-fold changes were obtained for these tumors [Supplementary Figure 9]. Many more up-regulated miRNAs were found for LUAD, LUSC, STAD and PRAD compared to HCC which had more down-regulated miRNAs [Supplementary Figures 2 and 4]. Other tumors have similar numbers of up- or down-regulated miRNAs. Our data for the first time revealed that certain miRNAs have “tumor type specificity”

and are only significant in one type of tumor, but not others, or aberrant expression in one tumor type is in the opposite direction compared with all others. Significantly down-regulated miR-24-1, miR-130a and miR-505 were only observed in HCC tumors with fold-changes ranging from -2.27 to -3.7 [Figure 2]. Similar down-regulation was confirmed by TaqMan array in the validation set of HCC patients from CUMC although only miR-24 and miR-130a achieved statistical significance [Supplementary Table 5]. The expression pattern of the 3 miRNAs was consistently repressed in KIRC and PRAD, but was not statistically significant. An up-regulated expression pattern was observed for 3 miRNAs in HNSC, LUAD and STAD, but none was significant. Inconsistent regulation patterns (up- or down-) for the 3 miRNAs were obtained in BRCA, LUSC and THCA, suggesting HCC tumor specificity for these miRNAs. We also identified 2 “tumor specific” miRNAs for LUAD, 3 for PRAD, 4 for HNSC, 5 for BRCA, 6 for KIRC, and 8 for LUSC, STAD and THCA (data not shown). These data provide promising evidence to justify further investigation of “tumor type specific” miRNAs in other types of tumors.

We also identified 8 “tumor common” miRNAs fitting into the above definition including up-regulated miR-21, miR-182, miR-183, and down-regulated miR-139, miR-144, miR-101-2, miR-451, miR-486 [Figure 3]. Interestingly, the expression of miR-142 and miR-

455 were significantly down-regulated in HCC, but significantly upregulated in 5 other solid tumors. The down-regulated expression pattern of miR-142 and miR-455 were confirmed in the CUMC HCC validation set [Supplementary Table 5]. These results suggest that miR-142 and miR-455 may be potential HCC “tumor specific” markers, but they should be considered as “tumor common” markers for 5 other tumor types.

Searching for target genes and biologically enriched pathways

Mirsystem was used to search for target genes of the 5 HCC “tumor specific” miRNAs (miR-24-1, miR-130a, miR-505, miR-142 and miR-455). A total of 2,270 genes (1,937 unique genes) were obtained as the targets of at least one miRNA [Supplementary Table 6]. Among them, 619 genes were identified by 5 out of 7 predictive tools or are experimentally validated miRNA-targets including 577 unique genes because some might be targeted by 2 or 3 miRNAs [Supplementary Figure 10]. Among target genes, 130 genes have been associated with HCC in at least one previous report by comparing with Liverome database (<http://liverome.kobic.re.kr/>).^[21] Upon further evaluation for enriched biological function, we identified several important biologic pathways, including transforming growth factor beta (TGF β) receptor signaling pathway, endocytosis, signaling by epidermal growth factor receptor, signaling by nerve growth factor (NGF), NGF signaling via tropomyosin receptor kinase A from the plasma membrane, BMAL1: CLOCK/NPAS2 activates circadian expression and adherens junction [Supplementary Table 7], which confirmed the potential biological role of HCC specific miRNAs involved in tumorigenesis.

DISCUSSION

The most interesting finding in the current study was significant down-regulation of miRNAs (miR-24-1, miR-130a, miR-505, miR-142 and miR-455) in HCC tumor tissue that showed “tumor type specificity” [Figure 2, Supplementary Table 5]. Additionally, a panel of miRNAs (miR-21, miR-182, miR-183, miR-139, miR-144, miR-101-2, miR-451 and miR-486) was first identified as “tumor common” markers that are significantly altered in most solid tumors by comparing RNA-seq data from 9 different cancer types [Figure 3, Supplementary Table 5]. A few miRNAs were also significantly dysregulated in etiology-specific (alcohol drinking, HBV- or HCV- infection) HCC [Table 3, Supplementary Figures 5 and 6], suggesting the potential impact of different etiologies in addition

to tumorigenesis itself. However, most etiologic relevant miRNAs were also consistently observed in overall HCC, indicating similar fundamental mechanisms involved in hepatocarcinogenesis regardless of different etiologies. These candidate miRNAs may be applied to improve clinical early diagnosis of HCC and more precise prevention and therapy. A similar research strategy can be adopted to discover and verify other “tumor type specific” miRNAs and promote early detection and precise treatment of different types of cancer.

Accumulating evidence based on genome-wide and candidate miRNA approaches have uncovered miRNAs dysregulation in HCC acting as either oncogenes or tumor suppressors.^[22,23] A few but not all studies of the 5 “HCC tumor specific” miRNAs are consistent. The expression of miR-24 was significantly reduced in HCC with cirrhosis compared to adjacent cirrhotic tissue, suggesting an influence on hepatocyte carcinogenic transformation of cirrhotic tissues.^[24] Significant down-regulation of miR-130a was observed in over 75% (78/102) of HCC tumor tissues.^[25] The same repression pattern of miR-130a was also found in HCV-infected human HCC cells^[26] and rat liver tissue after treatment with AFB₁, a strong hepatocarcinogen.^[27] A recent study found that miR-142-3p and miR-142-5p were significantly downregulated in HCC.^[28] The ectopic expression of miR-142 significantly reduced HCC cell migration and invasion, and overexpression both miR-142-3p and miR-142-5p synergistically inhibited HCC cell migration, indicating their cooperative regulatory role.^[28] This result is supported by a mechanistic study demonstrating that miR-142-3p can directly repress the expression of *RAC1* (Ras-Related C3 Botulinum Toxin Substrate 1), which regulates a diverse array of cellular events including increased colony formation, migration and invasion in HCC cell lines.^[29] Only one study observed miR-455 significantly down-regulated in HCC tissue and serum in HCC related to type I glycogen storage disease.^[30] The different up-regulation patterns of miR-130a and miR-505 in other solid tumors (bladder,^[31] breast,^[32] gastric,^[33] ovarian,^[34] colorectal^[35] and non-small cell lung cancers^[36]) provide further evidence for their potential as HCC specific biomarkers.

However, inconsistent results were also observed in previous studies that suggest significant up-regulation for some of “HCC specific” miRNAs. For example, miR-24 was found significantly up-regulated in HCC tumor tissue, cell lines,^[37,38] and serum compared

with healthy controls and/or chronic liver disease patients.^[39,40] Serum miR-505 level was also increased in HCC cases compared to controls.^[41] The expression of miR-130a was significantly higher in HCV-infected hepatocytes and liver biopsy specimens.^[42] While in prostate cancer^[43] and glioblastoma,^[44] miR-130a was significantly down-regulated showing the same pattern as in HCC, indicating possible non-specificity as HCC biomarker. It is known that certain miRNAs may act as both tumor suppressor and oncogene in a cell/tissue specific manner or vary by etiology and cancer stage because they simultaneously regulate multiple target genes involved in different biological pathways. The function of miR-24 as a tumor suppressor can inhibit cell proliferation, migration and invasion by regulating cMyc and E2F2 in HCC-derived HepG2 cell line,^[45] and Fascin homologue 1 (*FSCN1*) in nasopharyngeal carcinoma cell lines.^[46] On the other hand, miR-24 acted as an oncogene directly repressing SOX7 (Sex Determining Region Y-Box 7), a putative tumor suppressor,^[47] and overexpressed miR-24 led to inhibition of hepatocyte nuclear factor 4 α and initiated hepatocellular transformation through an epigenetic positive feedback circuit in the absence of genetic alterations.^[48] Tumor suppressor gene (*p16*)^[49] and pro-apoptotic protein FAF1^[50] can be negatively regulated by miR-24 in cervical carcinoma, prostate, gastric and HeLa cells. These data strongly suggested the complicated network of miRNA alterations in tumorigenesis that needs further clarification.

Several “tumor common” miRNAs have been extensively studied in HCC, as well as in various other cancers, but have not been recognized for their generalizability as “tumor type non-specific” biomarkers. The over-expression of miR-21 has been commonly observed in HCC tumor compared to adjacent non-tumor tissues, as well as in the circulation of patients with HCC. The overall pooled results from a diagnostic meta-analysis of miR-21 revealed a sensitivity of 74% and a specificity of 78%^[51] for HCC classification that is far from ideal for clinical application. Meanwhile, miR-21 was also significantly up-regulated in other cancer types (breast, colorectal, esophageal, gastric, lung, pancreatic and prostate), and the overall predictive sensitivity and specificity were, respectively 76% and 79%,^[52] which were similar to HCC. The cluster of miR-182/miR-96/miR-183 located within 2-4 kb at chromosome 7q32 functions as micro-oncogenes in carcinogenesis and the metastatic cascade. Two members (miR-182, miR-183) of this cluster showed frequent up-regulation in HCC.^[53] The expression of miR-182 was also consistently increased in 14 other cancer types

and miR-183 was up-regulated in 9 others.^[53] Down-regulation of miR-139, miR-144, miR-101-2, miR-451 and miR-486 was also reported in various cancer types besides HCC. These observations are biologically plausible because the “tumor common” miRNAs and their target genes participate in general carcinogenic processes and tumorigenic pathways, such as p53, phosphatase and tensin homolog, fibroblast growth factor receptor 3, DNA damage/repair, apoptosis, angiogenesis, cell cycle, phosphoinositide 3-kinase, mitogen-activated protein kinase, TGF β , NOTCH, and Wnt signaling pathways, *etc.*^[54-56] Therefore, “tumor common” miRNAs aberrantly expressed in various tumors may provide clues to further investigate their common similar underlying mechanisms in tumorigenesis. If verified, the miRNA signatures may be promising targets for precision cancer prevention and therapy. However, these miRNAs may have limited power as diagnostic tools to detect specific cancer type because of their “non-specificity”.

The advantages of the current research include a two-phase study design using a discovery and independent validation sample sets, paired tumor/non-tumor tissues, and unpaired tissues to verify promising miRNAs; simultaneously analyzing miRNA sequencing data in multiple cancer types that allows us to identify “HCC specific” and “tumor common” miRNAs panels. We used the most stringent criteria to select miRNAs for the final data analyses, i.e., RPM ≥ 10 in at least 90% of samples and *P*-value < 0.0001 as the significant level to adjust for multiple comparisons. Other studies, such as Wojcicka *et al.*^[57] analyzed miRNAs (GSE63046) passing the criteria of RPM ≥ 5 in samples with over 50% detectable rate; Zhang *et al.*^[58] excluded miRNAs with missing data exceeding 10% of all subjects but without precluding unreliable sequencing reads less than 10, which may lead to biased results or identify miRNAs with too much missing data, and are unable to be applied in clinical samples.

In interpreting the results, some drawbacks need to be recognized. First, for some miRNAs, the results are not in agreement with previous studies. For example, miR-122 was identified as the most abundant miRNAs in liver tissue previously,^[59,60] but is only the 7th in the TCGA data; miR-3591 has been reported as abundant in liver tissue,^[60] but is not even detectable in TCGA data. So we may miss a few important candidates due to different detection techniques (RNA-seq, microarrays and RT-qPCR); different approaches and criteria for data processing and analysis, and the heterogeneity of tumor tissue

itself. Second, 5 identified “HCC specific” miRNAs were all down-regulated in tumor tissue, which requires more sensitive methods of detection for future clinical application. It is known that RNA-seq has a better sensitivity than RT-qPCR,^[61] but the latter is more accurate and usually used for the validation of candidate miRNAs.^[62] We also observed that the changes of miRNAs in tumor tissue detected by RT-qPCR were minor compared to those by RNA-seq [Supplementary Table 3]. Even more challenging is to measure these miRNAs in circulation in pre-diagnostic samples, which strongly suggests a dire need for development of more sensitive PCR-based assays that can be used in large population studies.

In conclusion, our study identified 33 miRNAs significantly aberrantly expressed in HCC tumors with over 2-fold changes, and for the first time distinguished 5 of them as having “HCC tumor type specificity”, while another 8 are “tumor common” alterations. We also found several etiology-related miRNA panels, but most overlap with those observed in overall HCC. These findings have promising applications to better understand the common mechanisms underline tumorigenesis and improve precision prevention and therapy for specific cancers by targeting tumor specific miRNAs. Large retrospective and prospective studies to evaluate miRNA changes in circulation and trends during cancer development are warranted.

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We used public available miRNAs and clinical data from TCGA in this manuscript. Information about TCGA and the investigators and institutions that constitute the TCGA research network can be found at <http://cancergenome.nih.gov>.

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Conflicts of interest

There are no conflicts of interest.

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Hepatitis B virus molecular biology and pathogenesis

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ABSTRACT

As obligate intracellular parasites, viruses need a host cell to provide a milieu favorable to viral replication. Consequently, viruses often adopt mechanisms to subvert host cellular signaling processes. While beneficial for the viral replication cycle, virus-induced deregulation of host cellular signaling processes can be detrimental to host cell physiology and can lead to virus-associated pathogenesis, including, for oncogenic viruses, cell transformation and cancer progression. Included among these oncogenic viruses is the hepatitis B virus (HBV). Despite the availability of an HBV vaccine, 350-500 million people worldwide are chronically infected with HBV, and a significant number of these chronically infected individuals will develop hepatocellular carcinoma (HCC). Epidemiological studies indicate that chronic infection with HBV is the leading risk factor for the development of HCC. Globally, HCC is the second highest cause of cancer-associated deaths, underscoring the need for understanding mechanisms that regulate HBV replication and the development of HBV-associated HCC. HBV is the prototype member of the *Hepadnaviridae* family; members of this family of viruses have a narrow host range and predominately infect hepatocytes in their respective hosts. The extremely small and compact hepadnaviral genome, the unique arrangement of open reading frames, and a replication strategy utilizing reverse transcription of an RNA intermediate to generate the DNA genome are distinguishing features of the *Hepadnaviridae*. In this review, the authors provide a comprehensive description of HBV biology, summarize the model systems used for studying HBV infections, and highlight potential mechanisms that link a chronic HBV-infection to the development of HCC. For example, the HBV X protein (HBx), a key regulatory HBV protein that is important for HBV replication, is thought to play a cofactor role in the development of HBV-induced HCC, and the authors highlight the functions of HBx that may contribute to the development of HBV-associated HCC.

Key words: Hepatitis B virus; hepatocellular carcinoma; hepatitis B virus life cycle; hepatitis B virus-associated disease

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INTRODUCTION

The discovery by Baruch Blumberg and colleagues of the Australia antigen, which would later be identified as the hepatitis B virus (HBV) surface antigen, was a major breakthrough towards improving global health.^[1,2] For decades prior to Blumberg's discovery, an unknown virus in blood and plasma samples had been the suspected

cause of post-transfusion hepatitis.^[3] Recognition that the Australia antigen was a marker of viral hepatitis facilitated the generation of a blood-screening protocol that led to a two- to three-fold reduction in the incidence of post-transfusion hepatitis,^[4] with the remaining cases likely caused by hepatitis C virus (HCV), which would not be identified for another 23 years.^[5] Retrospective studies

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also associated the presence of the Australia antigen with chronic liver diseases such as cirrhosis and hepatocellular carcinoma (HCC).^[6,7] Finally, the discovery of the Australia antigen also facilitated the eventual development of a vaccine that has greatly reduced the global burden of HBV infection,^[8-10] and Baruch Blumberg was awarded the 1976 Nobel Prize in Medicine for his discovery of HBV.

Despite the decades of work between the discovery of HBV and our current understanding of the virus, many aspects of the HBV life cycle and pathogenesis remain unclear. The fact that it is estimated that as much as a third of the world's population has been infected with HBV at some point, that roughly 5% of the population (350-500 million people) are chronically infected with the virus, and that about 800,000 people die annually from acute or chronic HBV-related consequences underscores the importance of a more complete understanding of HBV biology and pathogenesis.^[11,12]

HEPADNAVIRIDAE

While the discovery of human HBV, hereafter denoted as HBV, occurred in the 1960s, recent research has shown that hepatitis B viruses have actually been present since the time of the dinosaurs. In fact, the earliest known hepatitis B virus is approximately 82 million years old and was identified from the DNA of infected birds from the Mesozoic period.^[13] Although multiple theories of the origins of HBV exist, it appears that the infection of mammals is a much more recent event. The jump into humans, in particular, may have been only about 40,000 years ago.^[14] Despite the evolutionary timeline, modern HBV is remarkably similar to these ancient hepatitis B viruses.^[13]

The present day *Hepadnaviridae* family is a group of small, hepatotropic, DNA viruses that are divided into two distinct genera based on their divergent genomic sequences and narrow host range of infection. The avihepadnaviruses, such as duck HBV (DHBV) and heron HBV, infect birds. In contrast, the orthohepadnaviruses infect mammals and include HBV and woodchuck hepatitis virus (WHV), among others. Each member of the *Hepadnaviridae* family is primarily species specific. For example, the only non-human hosts of HBV are chimpanzee and treeshrew, each of which can be experimentally infected.^[15,16] Additionally, a primate virus similar to HBV, called woolly monkey HBV, has been identified in woolly monkeys and designated as the prototype of a new species of hepatitis B-like viruses. A maximum of 40% sequence divergence exists between orthohepadnaviruses, while only 20% sequence divergence exists among avihepadnaviruses; however, little to no homology exists between the two genera. All mammalian HBV encode an X protein, which has been shown to be required for viral replication and has oncogenic properties (discussed below). This X protein is either lacking or highly divergent in avian viruses,

and the acquisition of this X protein could have been an essential factor for the evolution of hepadnaviruses from avian into mammalian hosts.^[13] Genomic diversity between species of hepadnaviruses is reviewed in detail in the literature.^[11,17]

While significant genomic diversity exists between viral species and particularly between the *hepadnaviridae* genera, all hepadnaviruses share a large number of common features. Among these, all members have an extremely small (3.0-3.3 kb) and compact DNA genome that encodes overlapping open reading frames (ORFs). Additionally, all hepadnaviruses use a genome replication strategy in which the virus replicates its DNA genome by reverse transcription of an RNA intermediate using the reverse-transcriptase activity of the viral polymerase. Hepadnaviruses are also distinguished from nearly all other viruses utilizing reverse transcription for viral replication by a number of unique features, including envelopment of a DNA genome, rather than RNA, and the fact that integration of the hepadnavirus DNA genome into the host-cell genome is not required for viral replication. These features, common to all members of the hepadnavirus family, contributed to the designation of *Hepadnaviridae* as a distinct family of viruses.^[11]

HBV

Studies have identified a minimum of eight HBV genotypes, designated A-H, with genetic differences greater than 8%, but less than 17% between each genotype.^[11,17,18] Two additional potential genotypes have been described. Genotype I has genetic divergence around 8% with a strong homology to genotype C,^[19] making its classification as a distinct genotype more controversial than that of the more well-accepted genotypes.^[20] A potential 10th genotype, genotype J, has also been described recently and is likely the result of recombination of genotype C and gibbon HBV.^[18]

There is a distinct distribution of HBV genotypes within specific populations and geographic locations. Similarly, there is an association between genotype and disease severity and outcome. In the United States, where chronic HBV infection is relatively uncommon, each genotype is present, though not at equal levels. Within the United States population, genotypes A and D are most prevalent overall, and the distribution of genotypes can be further divided based on ethnicity.^[21,22] For example, genotype C is most common in Asian Americans, which correlates with the prevalence of this genotype in much of Asia. This is significant because genotype C has been associated with a more severe disease and a lower response rate to interferon therapy.^[23,24]

HBV GENOME ORGANIZATION

HBV has a small (3.2 kb), partially double-stranded, relaxed-circular DNA genome that encodes four

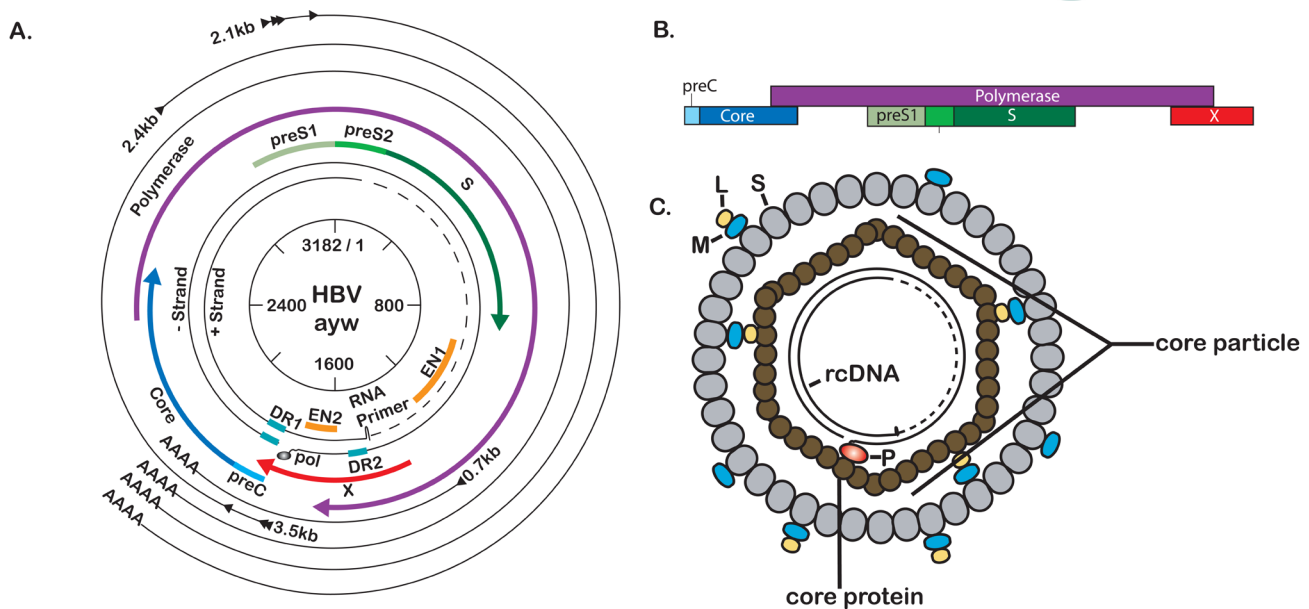


Figure 1: Molecular biology of hepatitis B virus (HBV). (A) Scaled depiction of the HBV (genotype ayw) genome. Internal circle shows genomic position relative to EcoRI site at position 1. Partially double-stranded genome is depicted with attached RNA primer and polymerase protein. Open reading frames (ORFs) are indicated by the thicker, colored lines. The outermost black circles represent the viral transcripts with the shared polyadenylation site; (B) schematic representation of the overlapping nature of the HBV ORFs; (C) the mature HBV virion (Dane particle) consists of two main parts: a nucleocapsid (or core particle) consisting of a partially double-stranded DNA genome bound to polymerase (P) and encapsidated by dimers of core protein, and a viral envelope consisting primarily of S-HBsAg (S), with an intermediate amount of M-HBsAg (M) and lower levels of L-HBsAg (L)

overlapping ORFs [Figure 1a]. The largest ORF encodes the viral polymerase, which also has reverse transcriptase (RT) activity that generates the first strand of the DNA genome from an RNA intermediate. The second largest ORF encodes the three viral envelope proteins: large (L-), middle (M-), and small (S-) surface antigen (HBsAg). Another ORF encodes precore, also referred to as HBV E antigen (HBeAg), and the core protein, which makes up the viral capsid. Finally, the smallest ORF encodes the HBV X protein (HBx), a small regulatory protein that has been shown to be required for HBV replication both *in vitro* and *in vivo*.^[25-29] The viral ORFs are encoded in distinct capped and polyadenylated RNAs that can be divided into genomic and subgenomic transcripts. The subgenomic transcripts act only as templates for HBV proteins and consist of the 0.7 kb transcript, which encodes HBx, and the 2.1 kb and 2.4 kb HBsAg transcripts encoding M- and S-HBsAg, and L-HBsAg, respectively. The genomic transcripts act as mRNAs for precore, core, and polymerase. The genomic transcript that encodes both core and polymerase is multifunctional and referred to as pregenomic RNA (pgRNA). The pgRNA is the template for HBV replication and is reverse transcribed to generate the HBV DNA genome. As the viral genome is only 3.2 kb and the pgRNA is 3.5 kb, the pgRNA is a greater than unit length, terminally redundant copy of the viral genome. All HBV RNA transcripts share the same polyadenylation site, and each of the smaller transcripts makes up the 3' end of each of the larger transcripts. This means that the sequence of the HBx transcript is contained at the 3' end of all HBV mRNA transcripts, while the largest transcript is the only viral transcript to contain sequence that is not shared with the other transcripts.^[11,30,31]

Transcription of HBV RNAs is driven from specific promoter sequences within the viral genome. At least some of the hepatotropic restriction of HBV can be attributed to transcriptional activation by hepatocyte-specific transcription factors. For example, activation of the Enhancer I/HBx promoter is a required first step in viral transcription, as this is believed to enhance transcription from downstream promoters. A number of the transcription factors that have been mapped to the EN1/HBx promoter are liver specific, including hepatocyte nuclear factor (HNF) 1, HNF3, and HNF4. Many of the transcription factor binding sites that have been identified within the 4 promoter regions of HBV are for transcription factors that are activated by HBV proteins, oftentimes HBx, implying a specific cascade of transcription.^[32] Transcription factor-mediated regulation of HBV transcription has been reviewed in more detail elsewhere.^[11,33]

HBV PROTEINS

The HBV genome encodes seven proteins: HBx, core, polymerase, L-, M-, and S-HBsAg, and precore/HBeAg [Figure 1a]. Of these proteins, HBx is a non-structural regulatory protein, HBeAg is not incorporated into virions and is independently secreted from the cells, the polymerase is responsible for genome replication, and the core and HBsAg proteins form the structural aspects of the virion.^[11] Each of these will be discussed in further detail below.

E antigen

HBeAg is the final product of post-translational processing

of the translated precore ORF [Figure 1a and b]. As one of the proteins encoded by a genomic transcript, the genomic promoter drives its expression. The HBeAg ORF encodes an endoplasmic reticulum (ER) targeting sequence that co-translationally traffics the peptide to the ER, where the protein is processed to the final 15 kD HBeAg that is secreted from HBV-infected cells.^[34]

The function of HBeAg remains incompletely defined. Multiple groups have hypothesized that HBeAg can facilitate HBV immune evasion, and studies with HBeAg-transgenic (tg) mice crossed with T cell receptor (TCR)-Tg mice expressing receptors for the HBeAg specifically suggest that a function of HBeAg is to suppress the immune response to the HBV core protein.^[35,36] The secretion of a viral marker that is not present in the HBV infectious virion may help to dampen the neutralizing immune response by diverting this response away from infectious viral particles.^[11] From a diagnostic perspective, HBeAg is an important marker of HBV replication, and the levels of serum HBeAg are generally considered to correlate with viral titer. In fact, HBeAg seroconversion is considered an important aspect of the transition to the inactive carrier state of infection (described below).^[37]

Surface antigens

HBV encodes three envelope proteins, or surface antigens, that make up the viral envelope: large (L), middle (M), and small (S) surface antigen [Figure 1a and c]. The smallest envelope protein, or S (24 kD), is 226 amino acids (aa) in length and makes up a shared C-terminal region of the two longer envelope proteins. The M protein (31 kD) contains the S sequence with a 55aa N-terminal extension known as preS2. Expression of the M- and S-encoding mRNA is driven by the S promoter, with translation initiating from an upstream (M) or downstream (S) AUG. The L protein (39 kD), the largest of the envelope proteins, contains S, preS2, and an additional 108aa or 119aa (depending on the genotype) N-terminal extension known as preS1. L-HBsAg is encoded by its own mRNA transcript that is controlled by the preS1 promoter.

The envelope proteins are synthesized at the ER, where they attain their transmembrane configuration. Because all three proteins contain an identical C-terminal sequence, the transmembrane topology of this region is the same across all three proteins. Specifically, an N-terminal signal sequence initiates insertion of S into the ER membrane, followed by another signal that pushes the downstream peptide sequence into the ER lumen. The sequence upstream of this signal remains in the cytosol, with the signal domain itself acting as a transmembrane anchor domain. This orientation forms two loops; one loop, between aa 23-79, remains on the cytosolic side, while the other loop, between aa 99-169, remains in the ER lumen.^[38] Importantly, the luminal loop contains the major conformational epitope of HBsAg and

is glycosylated in nearly half of all S-protein moieties.^[39] Once budding of the membrane occurs, these epitopes are on the outer surface of the viral particles. The topology of M is identical to that of S, except for the presence of preS2 within the ER lumen.^[40]

A major characteristic of L is that it exists in two conformations that vary in the localization of the N-terminal domain. In the first conformation of L, the preS1 and preS2 domains are present in the cytosol. This conformation of L is essential for binding of capsids and for the assembly of HBV virions. In the second conformation of L, the N-terminus is located in the ER lumen and, as a result, exposed on the surface of viral particles. Thus, this conformation of L plays a role in the infection of hepatocytes. The conformational change is facilitated by interactions of molecular chaperones Hsc70/Hsp40 and BiP with L; however, the exact details of the mechanism underlying this step are not yet understood.^[11] The preS1 domain contains the receptor-binding region for HBV,^[41,42] thus it needs to be exposed out of the cell. A myristylated peptide containing a portion of the N-terminal preS1 region is sufficient to inhibit infection^[41] and is currently being developed as a therapeutic.^[43]

The main function of the surface antigen proteins is to form the HBV envelope. Three different forms of viral particles are secreted from an HBV-infected cell as a result of the unequal expression of each of the three surface antigens. S-HBsAg is the highest expressed of the three envelope proteins and makes up the majority of the viral envelope. Intact, infectious HBV virions, called Dane particles, also include M-HBsAg and L-HBsAg. In addition, an HBV-infected cell produces non-infectious subviral particles (SVP) made primarily of S-HBsAg containing varying (but much lower) amounts of M-HBsAg and little to no L-HBsAg. These SVPs can reach a concentration 10,000-fold higher than infectious HBV particles in the serum of an infected individual.^[44,45] SVPs are produced in two forms: 25 nm spheres, which are almost exclusively made up of S-HBsAg, and 22 nm filaments, which are made up primarily of S-HBsAg, with some M-HBsAg and potentially small amounts of L-HBsAg. It is currently unknown why HBV produces SVPs in such excess compared to the level of infectious virions, but multiple hypotheses have been proposed. For example, it has been suggested that the excess SVPs act to divert neutralizing antibodies away from infectious particles and that SVPs play a role in inducing the immune tolerance required to sustain a long-term chronic infection. A study of DHBV SVPs showed that the SVP-to-infectious-particle ratio plays a role in determining the efficiency of hepatocyte infection, with SVPs acting to either enhance or inhibit infection based on the ratio of SVP-to-infectious-particles.^[46]

Core protein

The 21 kD HBV core protein, or HBeAg, is the organizing framework for the virion [Figure 1c]. When expressed in

cells, core mainly exists as soluble dimers, or in T = 3 or T = 4 icosahedral capsids. About 95% of mature nucleocapsids isolated from Dane particles contain T = 4 capsids made up of 120 core dimers, with the remaining 5% being the smaller T = 3 with 90 dimers.^[47] Core is translated from the pgRNA and the first 149aa of core form the assembly domain, which is sufficient for *in vitro* formation of capsids that are indistinguishable from capsids isolated from Dane particles.^[48] The remaining 34-36aa makes up the arginine-rich C-terminal domain (CTD); phosphorylation of various aa in the CTD regulates multiple stages of the HBV life cycle.^[49-52]

While the best-described role for core protein is assembling the nucleocapsid, the results of recent studies also suggest that the core protein does more than simply act as an inert container for the HBV genome. In fact, core protein binds to HBV covalently closed circular DNA (cccDNA), potentially to regulate spacing of nucleosomes on cccDNA; cccDNA is a nuclear-localized replication intermediate of hepadnaviruses that forms a minichromosome (described in more detail below).^[53] In addition, the CTD is required for pgRNA packaging,^[54] and core protein also plays an active role in initiating reverse transcription^[55-57] and in mature nucleocapsid envelopment.^[58] The many potential roles of core protein in the HBV life cycle were recently reviewed, along with a detailed description of the mechanism of capsid assembly.^[59]

Polymerase/reverse transcriptase

Not long after the identification of an HBV-like virus in ducks,^[60] the DHBV model was used to demonstrate that DHBV genome replication utilizes an RNA intermediate, implying that hepadnaviruses replicate via reverse transcription.^[61] While reverse transcription is a mechanism employed by many viruses, hepadnaviruses approach genome replication with a number of unique features. The 90 kD, 838aa polymerase protein of HBV (reverse transcriptase/RT/Pol/P) is made up of 3 functional domains and a variable spacer region. At the N-terminus is the terminal protein (TP) domain, which is important for multiple facets of the initiation of genome replication. This region, despite its important role in P binding to the pgRNA, RNA packaging, and protein-priming,^[62-64] is a unique domain that is not shared by any non-hepadnavirus RTs. A variable spacer separates the TP domain from the RT domain, and studies have shown that nearly all aa within the variable spacer region can be mutated without altering P function.^[65] In fact, only 3 cysteine residues within the C-terminal end of the spacer region, along with a fourth in the N-terminal side of the RT domain, are thought to be important for RT/pol function.^[66]

The RT domain is responsible for genome replication by reverse transcribing the pgRNA to form the (-)-strand of the DNA genome and subsequent use of the (-)-strand

as a template for synthesis of the (+)-strand of the DNA. This domain shares significant homology to the RT of other retroviruses.^[67] The RT domain is the only current anti-HBV therapeutic target,^[12] which is based on the efficacy of nucleoside analogs to inhibit the human immunodeficiency virus (HIV) RT.^[68] In fact, portions of HBV RT can be replaced by homologous portions of HIV RT; this can generate an active RT that can function to produce mature HBV virions.^[69]

The final domain, P, is the RNase H domain. This domain is responsible for degrading the pgRNA template during synthesis of the (-)-strand of the DNA genome. Coordination of metal ion binding, which is important for RNase H activity, is achieved through 4 conserved carboxylates.^[70] Studies of the RNase H domain have also shown that purified recombinant RNase H domain is functional *in vitro* and that the RNase H domain of P is important for pgRNA packaging.^[71] Further information on the HBV RT/pol, including a detailed description of the RT domain active sites and binding motifs, can be found in the literature.^[68,72]

X protein

HBx is the only regulatory protein encoded by HBV. It is a 154aa, 17 kD protein that is encoded by the smallest HBV ORF. Various studies have provided considerable evidence that HBx plays an essential role during HBV replication. Specifically, studies have shown that HBx is bound to cccDNA,^[73] that HBx is required for transcription from cccDNA,^[28,74] and that downstream HBx-mediated effects are required for HBV replication. Importantly, studies of other mammalian hepadnaviruses have also supported the role of their respective X proteins in viral replication. For example, two different studies demonstrated that the WHV X protein is required for WHV replication *in vivo*,^[25,27] although another study did show a low level of viral replication from a WHx-deficient WHV mutant in infected woodchucks.^[75] Similarly, viral replication was detected from tg mice expressing either wild-type or an HBx-null HBV mutant; however, when the HBx-null mice were crossed with HBx-tg mice, levels of HBV replication surpassed those seen in wild-type HBV-tg mice.^[76] A similar experiment using hydrodynamic tail vein injection of a plasmid encoding either the wild type HBV genome or an HBx-deficient mutant HBV showed a significant decrease in the levels of HBV replication in the absence of HBx, which could be restored through co-injection of the HBx-deficient mutant HBV and a plasmid encoding HBx.^[26] This indicates that while HBx may not be absolutely required for HBV replication in these systems, it undoubtedly enhances the levels of replication. Moreover, studies of direct HBV infection of mice with humanized livers demonstrated that only infection with wild type HBV, and not HBx-deficient virus, could result in HBV replication.^[29,77] A similar requirement for HBx in HBV replication has been shown in human HepG2 hepatoblastoma cells^[78-82] and in primary rat

hepatocytes.^[83-86] Importantly, the requirement for HBx was also confirmed in primary human hepatocytes directly infected with either wild-type or HBx-deficient HBV.^[28]

The lack of a single accepted model for studies of HBV and HBx has created some confusion about the overall consequences of HBx expression for HBV replication and hepatocyte physiology. HBx-related studies have often been performed in transformed or immortalized cell lines and with different levels of HBx expression, leaving the impact of HBx on a normal hepatocyte incompletely understood.^[87] While HBx is generally considered to have oncogenic potential, it is yet to be determined if it is directly oncogenic or simply acts as a co-factor in HCC development, as both effects have been demonstrated in different HBx-tg mouse models.^[88-91] It is important to recognize that a strongly oncogenic HBx would not be consistent with the biology of HBV-associated HCC, which involves decades of a chronic HBV infection, and it is more likely that HBx plays a cofactor role in the development of HBV-induced liver cancer. The hypothesis that HBx-induced subtle changes in hepatocyte physiology sensitize cells to other oncogenic signals, while facilitating HBV replication, is more consistent with the biology of HBV-associated HCC.^[92] Peripheral evidence for the oncogenic potential of HBx comes from the fact that hepadnavirus-associated HCC seems to be restricted to mammalian hepadnaviruses, which each express a form of the X protein. Avian hepadnaviruses, which do develop a chronic infection but do not cause HCC, either do not express an X protein or express a highly divergent form.^[78,93]

HBx is a multifunctional protein that can modulate many hepatocyte signaling cascades and factors that have also been linked to mechanisms that underlie cellular transformation. For example, HBx can modulate calcium,^[84,85] apoptosis,^[83,86] and proliferation signals, among other pathways, and can activate numerous transcription factors, including activator proteins 1^[94] and 2^[95] (AP-1 and AP-2), nuclear factor of activated T cells (NFAT),^[96] and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B).^[97-99] HBx can also regulate cellular signaling factors, such as Wnt/ β -catenin,^[100] p53,^[101] and Akt,^[86,102] that have been implicated in HCC. Recently, modulation of miRNA expression has also been included in the functions of HBx. It is possible that the many functions attributed to HBx could actually be the result of a few fundamental upstream HBx functions that can affect multiple downstream cellular signal-transduction pathways in a context-dependent manner. Interestingly, while HBV replication in established HBV-associated HCCs is typically absent, a number of groups have shown that these tumors can still express HBx from fragments of the HBV genome that have integrated into the host genome. The presence of HBx in these cells could mean that HBx might be active in these HCC cells,

even in the absence of replicating HBV, and potentially contribute to HCC development or maintenance.

HBV LIFE CYCLE

Studies have shown that the species specificity and hepatotropic nature of HBV are due to at least two different layers of cellular factors. The first is the hepatocyte-specific expression of the recently described HBV receptor, human sodium taurocholate cotransporting peptide (hNTCP/SLC10A1) [Figure 2]. hNTCP is only expressed on human hepatocytes, and mouse NTCP cannot be bound by HBV, which correlates with the inability of HBV to directly infect mouse hepatocytes.^[42] The second level of cell-specificity of an HBV infection is controlled by hepatocyte-specific transcription factors such as HNF1 α and HNF4 α ; these control post-entry, downstream stages of the HBV life cycle. Evidence for the additional role of intracellular factors for controlling the cell-specificity of an HBV infection comes from the observation that humanized-mouse NTCP, in which the binding residues from mouse NTCP are replaced by hNTCP, allows binding of HBV to the receptor but does not result in a productive HBV infection when expressed in mouse cells.^[103] Studies using hepatitis D virus (HDV), which is a satellite virus requiring HBV envelope proteins for entry into a cell, demonstrated that the 75 aa at the N-terminal portion of the PreS1 domain of L-HBsAg are required residues responsible for binding to the viral receptor.^[104] In addition, it was shown that N-myristylation of the PreS1 domain is required for infectivity, but not HBV virion assembly.^[105] In fact, a myristylated peptide consisting of only the first 47 aa of the preS1 domain is able to bind to hNTCP and inhibit the binding of HBV.^[41] Additional studies have suggested a role for heparin sulfate proteoglycans in the initial stages of HBV binding to hepatocytes,^[106] including the recent identification, using an RNAi-based screen in Huh7 cells stably expressing hNTCP, of glypican 5 as an HBV and HDV entry factor.^[107]

Although amino acid sequences of both preS1 and hNTCP that affect binding of HBV to hNTCP are known, the lack of an effective model system that mimics a robust natural infection has hampered a complete understanding of aspects of the HBV life cycle immediately following receptor-binding. The observation that preS1 binds to clathrin heavy chain and the adapter protein AP-2 in immortalized primary human hepatocytes, and that knockdown of these proteins inhibits infection, suggests that the HBV-hNTCP complex may enter the cell through clathrin-mediated endocytosis.^[108] Once in the cell, the HBV DNA is delivered into the nucleus by mechanisms that remain unclear. One potential mechanism is the active transport of the nucleocapsid through nuclear pores.^[109] Another potential mechanism involves CTD phosphorylation of the core protein, which is thought to expose nuclear localization signals,^[49] leading to

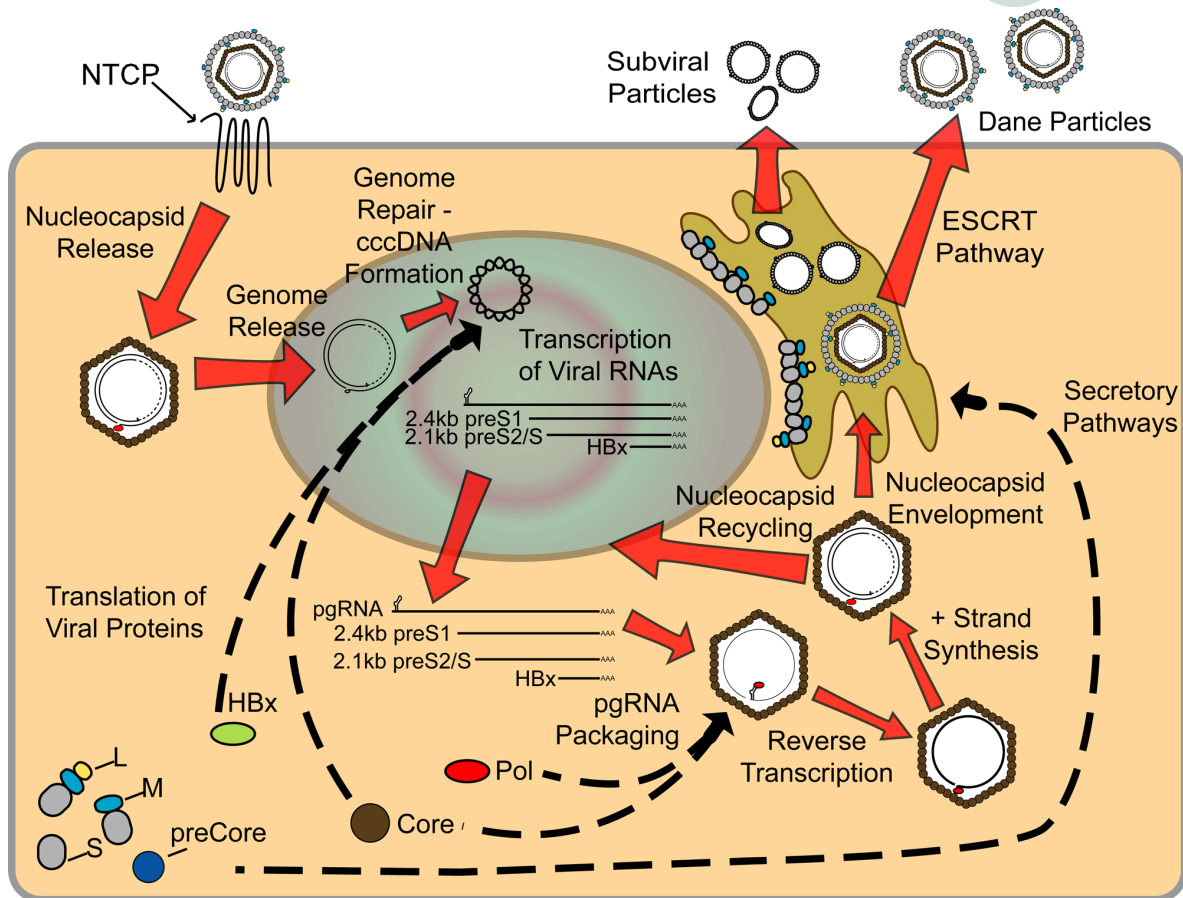


Figure 2: Life cycle of hepatitis B virus (HBV). Mature HBV virions enter hepatocytes through the sodium taurocholate cotransporting polypeptide receptor on the cell membrane. After release from the viral envelope, the nucleocapsid is then transported to the nucleus where the genome is repaired to form covalently-closed circular DNA (cccDNA). Using cccDNA as the template, viral RNAs are transcribed and exported into the cytoplasm where they are translated to form the viral proteins. Additionally, pregenomic RNA (pgRNA) is packaged by core protein, along with the polymerase protein, and the viral genome is replicated through reverse transcription of the pgRNA to form the - strand, followed by partial synthesis of the + strand. Mature nucleocapsids can then either be recycled back to the nucleus to maintain a pool of cccDNA, or enveloped and secreted through the ESCRT pathway. See text for a more detailed description of viral life cycle

nucleocapsid disintegration at the nuclear pore and transfer of the polymerase-bound, relaxed circular (rc) form of the HBV DNA into the nucleus.^[110,111] The single-stranded gaps in the rcDNA are repaired either through (+)-strand extension by the HBV polymerase or through repair activity of host proteins, and cccDNA is formed as a nucleosome-bound minichromosome in the nucleus. The observations that some HBV-tg mice do not produce cccDNA,^[112] and that nucleoside analogues that inhibit the RT function of polymerase do not prevent cccDNA formation,^[113] suggest that the production of cccDNA likely involves specific host factors. In addition to studies suggesting a role for cellular histones in cccDNA formation, evidence also exists showing that cccDNA is bound to both core protein^[53] and HBx^[73] and that this influences the structural arrangement of the cccDNA episome and the epigenetic regulation of cccDNA. Although multiple studies have suggested that HBx is not required for cccDNA formation, transcription of viral RNA from cccDNA is lost in the absence of HBx,^[28,114] and HBx has been suggested to regulate levels of cccDNA histone acetylation and methylation.^[115] Host RNA polymerase II uses cell-specific transcription factors and cccDNA, which

serves as the template for all viral transcripts, to produce 5'-capped and 3'-polyadenylated RNA transcripts. Translation of the viral transcripts occurs in the cytoplasm following nuclear export.

While a portion of the pgRNA is translated, forming the pool of core and polymerase proteins, pgRNA also serves as the template for reverse transcription [Figure 2]. This requires encapsidation of pgRNA by 120 dimers of core protein to form the nucleocapsid. This occurs through a complex cascade of events involving multiple viral and host proteins. Specifically, the 5' end of the pgRNA contains an encapsidation signal, termed ϵ , which is recognized and bound by polymerase. Studies have also shown that the 5' cap structure is required for packaging of the pgRNA;^[116] however, polyadenylation is not required.^[117] In addition, interaction of pgRNA-bound polymerase with the 5' cap and host eIF4E leads to encapsidation of this entire RNP complex,^[118] resulting in cellular eIF4E within the viral nucleocapsid. Cellular heat shock proteins have also been suggested to play a similar role in stabilizing the binding of polymerase to ϵ .^[119]

Once packaged, reverse transcription is initiated through priming by the polymerase from a specific tyrosine residue within the N-terminal, TP domain of the polymerase^[63,64] [Figure 1a]. A bulge region within ϵ supplies the template for the first 3-4nt of the (-)-DNA strand before translocation to a matching acceptor motif in the 3' direct repeat 1* (DR1*).^[120] This strand is then extended until completion, resulting in a unit length (-)-DNA strand copy of the pgRNA that contains an additional 10nt terminal redundancy (r). The majority of pgRNA is degraded during DNA synthesis by the RNase H activity of polymerase, with the remaining bases serving as the 5' primer for synthesis of the (+)-strand.^[121] Direct extension of this primer from its 5' position results in a double-stranded linear form of the genome that is replication incompetent.^[122] This double-stranded linear form does, however, seem to play a role as the main form of HBV DNA that can be integrated into the host genome.^[123] Instead of direct extension of the RNA primer from its 5' location, successful rcDNA formation can occur only after the RNA primer is translocated to the 3' DR2 sequence. Once on DR2, the RNA primer is extended towards the 5' end of the (-)-DNA strand. Because r on the other end has the same sequence, exchange of the two ends allows (+)-strand synthesis to continue. As with the previous translocations, additional cis-acting elements are likely playing a role in long-range base-pairing, which allows the close juxtaposition of these donor and acceptor sites that can otherwise be separated by kilobases of sequence.^[124,125] In addition, recent evidence has suggested a role for core protein in regulating DNA synthesis, as mutations in core protein inhibit the synthesis of the (+)-strand of DNA.^[126] The complex process of reverse transcription has been reviewed in more detail elsewhere.^[30,31,68]

Replication occurs in core particles in the cytosol of an HBV-infected hepatocyte, and the final product of DNA synthesis is the encapsidated, partially double-stranded rcDNA with the polymerase still bound to the 5' end of the (-)-DNA strand [Figure 2]. This nucleocapsid can then proceed in one of two directions. The first is shuttling of the nucleocapsid back to the nucleus to amplify and maintain a stable pool of cccDNA.^[127,128] The levels of envelope proteins influence this recycling, with decreased amounts of HBsAg promoting shuttling of the nucleocapsid to the nucleus.^[129] In particular, levels of L-HBsAg directly influence shuttling back to the nucleus,^[130] and these findings correlate well with the early establishment of a cccDNA pool, followed by identification of secreted infectious HBV.^[127] The result is a pool of cccDNA that contains a fluctuating number of copies (typically less than 10) of cccDNA per cell,^[131-133] which can be maintained in the cell for years. Additionally, it has been suggested the half-life of a single cccDNA molecule is between 33 and 57 days,^[132,134] underscoring the role of cccDNA in maintaining HBV persistence.

The second potential HBV nucleocapsid-associated process is envelopment by HBV envelope glycoproteins residing in the ER membrane [Figure 2]. Interestingly, mechanisms exist that may limit envelopment of capsids containing immature HBV genome; however, these mechanisms remain incompletely understood. For example, it has been suggested that only mature rcDNA-containing nucleocapsids are enveloped, while ssDNA or RNA containing nucleocapsids are not secreted from the cell.^[135] Studies utilizing an RNase H-deficient polymerase, which renders the virus unable to initiate (+)-DNA strand synthesis, have suggested that only completion of the (-)-DNA strand is required for envelopment,^[136] and specific mutations in core protein can allow envelopment of immature nucleocapsids.^[137] The mechanisms associated with this selectivity are unknown, although the phosphorylation state of core protein, likely influenced by the nucleic acid species inside the capsid, could be playing a role. Specifically, studies have shown that core protein isolated from DNA-containing capsids is dephosphorylated (after the prior phosphorylation required for pgRNA packaging and reverse transcription) in a specific C-terminal region, while immature nucleocapsids remain phosphorylated at at least 6 different sites.^[50] The overall secretion of infectious HBV Dane particles has been hypothesized to be as little as 1-10 virions per cell per day,^[138] which, because of the large number of cells in the liver, can account for high *in vivo* HBV titers, but can hinder *in vitro* research requiring isolation of large amounts of infectious virus. Secretion of Dane particles was originally thought to follow the same secretory pathway as the much more abundant SVP, with the envelope proteins residing within the ER-golgi intermediate compartment from where they could bind the DNA-containing capsid, enter the lumen, and be secreted from the cell. Recent evidence has suggested, however, that mature HBV virions are secreted from the cell using a pathway that is dependent on proteins involved in the endosomal sorting complex required for transport (ESCRT) pathway, which forms multivesicular bodies.^[139] One characteristic that needs to be considered regardless of the pathway of HBV secretion is the seemingly contradictory conformations of L-HBsAg, with the domains required for both interaction with the nucleocapsid and hNTCP being needed on opposite sides of the membrane. This is addressed by the fact that nearly half of L-HBsAg changes transmembrane conformation after translation, to expose the preS domains within the ER lumen.^[140]

MODEL SYSTEMS USED IN THE STUDY OF HBV

Each member of the hepadnavirus family has a narrow host range that is thought to be defined primarily by the interaction between the virus and a specific cell-surface receptor that is present on host hepatocytes.^[11,90] Available cell culture systems for studying the life cycle of the *Hepadnaviridae* are limited. Typically, members of a

hepadnavirus family can only directly infect hepatocytes within the liver of their respective avian or mammalian hosts or cultured, well-differentiated primary hepatocytes that are derived from these hosts. This has hampered the capabilities of researchers to study a natural HBV infection. An overview of the *in vivo* model systems that exist for studying HBV biology is provided below.

Due to the limited host range of HBV, few suitable animal models exist for studying an *in vivo* HBV infection. Closely related viruses, such as DHBV^[60] and WHV^[141] have been used in their respective host animals as surrogate models for understanding overall hepadnavirus biology. These studies have been instrumental in establishing our understanding of the viral life cycle, including the identification of DNA replication through an RNA intermediate,^[61] the establishment of a pool of cccDNA as a mechanism for maintaining chronic infection,^[142,143] and the course of both acute^[144-146] and chronic^[147-149] infection.

The treeshrew, *Tupaia belangeri*, is a small animal model and is one of the very few animals that can be experimentally infected with HBV.^[150] Genomic analysis has placed the treeshrew phylogenetically between humans and rodents,^[151,152] and this similarity to primates has spurred its use as a model for a broad range of studies, including as a model for viral hepatitis.^[16,153] Specifically, *Tupaia belangeri* has been used as a model to study the immediate effects of HBV infection on gene expression in the liver^[16] and to identify genes potentially contributing to the development of HBV-associated HCC.^[154] In fact, freshly isolated primary treeshrew hepatocytes were recently used in multiple studies in which hNTCP was identified as the HBV receptor.^[42,155] Recent studies also suggest that neonatal exposure of treeshrews to HBV can lead to a disease progression similar to what is seen in humans, with development of a chronic infection leading to the eventual development of HCC.^[156] Unfortunately, a relatively low HBV infection efficiency and lack of genetically uniform tree shrew strains has limited their use.^[157]

The chimpanzee is the only non-human primate model for HBV infection and, along with the tree shrew, represents one of the only animal models that can be directly infected with HBV. HBV can establish both acute^[158] and chronic infections^[159] in chimpanzees, and this model has been used most often for modeling the immune response to HBV and the interaction between the virus and host.^[160-164] Studies in chimpanzees have helped to establish the relationship between the innate and adaptive immune response to HBV infection, demonstrating minimal early activation of innate immune mediators^[160] and a reliance on CD8⁺ T cells for viral clearance through interferon γ - and tumor necrosis factor α -dependent mechanisms,^[161] in agreement with previous work in HBV-tg mice.^[165,166] Another important use of the chimpanzee model has been as a surrogate model for preclinical drug and

vaccine testing.^[167-170] The ethical issues and high costs associated with non-human primate use, however, have limited the use of this model and the recent reevaluation in the United States (one of only two countries to allow chimpanzee research) of the need for chimpanzees in preclinical research^[171] will likely diminish their future use even further.

A number of mouse models exist for the study of HBV, and have been reviewed more extensively in the literature.^[157,172] Typically these models can be separated into two categories: HBV/HBx-tg mice, which constitutively express HBV or HBx, respectively, and mice that are delivered the HBV genome or an HBx-expressing plasmid by hydrodynamic tail-vein injection. Although mouse hepatocytes cannot be directly infected with HBV, the use of tail-vein-delivered DNA or HBV-tg mice allows studies of the impact of HBV replication on hepatocyte physiology; HBx-tg mice similarly aid in the study of HBx-mediated effects. While these mouse models are valuable tools, they do have their drawbacks. For example, there is no inflammatory response against HBV in an HBV-tg mouse, which could alter the cellular pathways activated in these models compared to a natural HBV infection. Additionally, because some HBV-tg mice do not produce HBV cccDNA, there is some concern over whether this system accurately mimics all aspect of HBV replication in humans.^[112] Despite this, mouse models have been instrumental in determining a number of important aspects of the HBV life cycle, including the requirement for HBx in HBV replication^[26] and the oncogenic potential of HBV^[173] and HBx.^[89,91]

More recently, two additional mouse models have been described that may greatly enhance our understanding of the HBV life cycle and HBV-associated disease. The first of these systems is the humanized mouse model, in which the majority of the mouse liver is repopulated with either primary human hepatocytes or human induced pluripotent stem cells. The use of these animals represents a significant advancement, as they support direct infection with HBV and can develop a chronic HBV infection,^[174,175] thereby allowing studies of the impact of an HBV infection on the liver in a cellular context more similar to that seen in the human liver. Some of these chimeric mouse models include both a humanized liver and humanized immune cells, offering the unique opportunity to study the human immune response in a small animal model. Although different techniques for the development of the humanized mouse model exist, a number of groups have adapted their use for the study of HBV. These studies cover a broad range of aspects of HBV biology, including studies of the HBV-mediated immune response,^[175,176] investigation of potential HBV therapeutics,^[177,178] and aspects of the HBV life cycle such as particle formation,^[179] receptor binding,^[180] and cccDNA regulation.^[181]

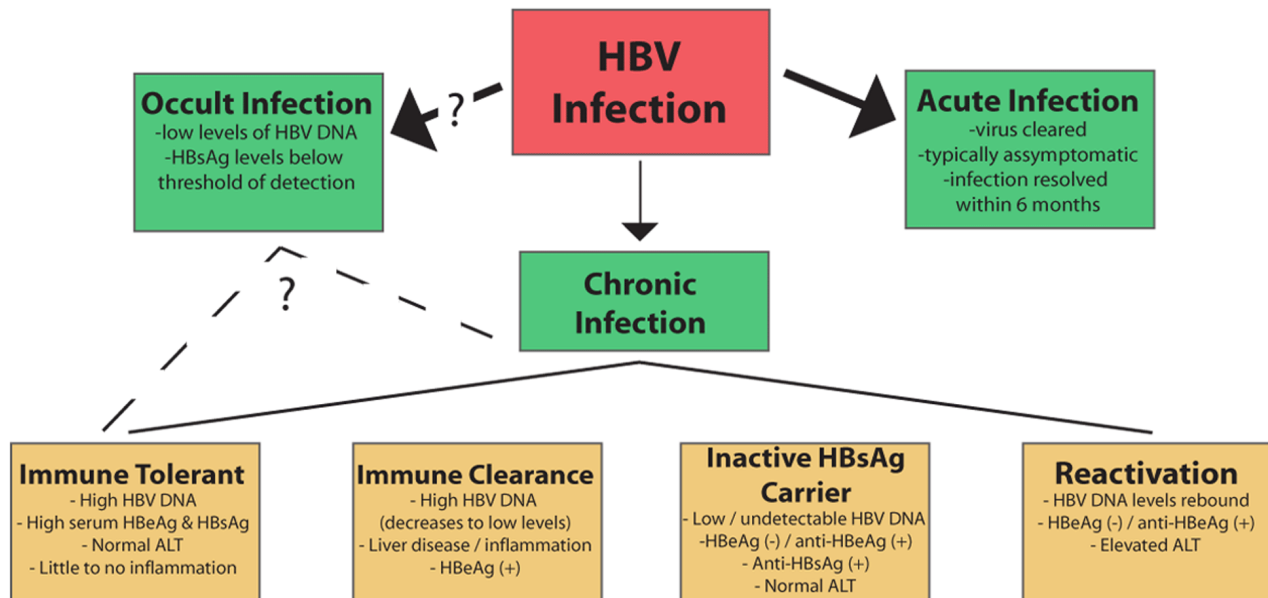


Figure 3: The natural history of an hepatitis B virus (HBV) infection. Infection with HBV can result in an acute, self-clearing, or chronic HBV infection; the development of a chronic HBV infection positively correlates with younger age. A chronic infection usually follows a long-term course in which the virus replicates at high levels, followed by immune-mediated control of viral replication associated with liver inflammation. Seroconversion and maintenance of undetectable or low levels of viral replication are markers of a favorable prognosis, but long-term disease can lead to the development of cirrhosis and hepatocellular carcinoma. (See text for additional details)

Another recently described mouse model expresses hNTCP to allow receptor binding by HBV. Currently these mice are limited in their utility for studying HBV, as multiple groups have shown that while HBV can bind to hNTCP expressed in mouse cells, the HBV life cycle does not appear to proceed beyond receptor binding.^[103,182,183] Conversely, HDV utilizes HBV envelope proteins for its envelopment, and hNTCP-expressing mice have been used for the study of HDV infection.^[184] Further work with hNTCP-expressing systems may help to determine species-specificity factors that could ultimately lead to the development of an hNTCP-expressing mouse model useful for the study of HBV infection. Together with the humanized-liver model, these mouse models could greatly contribute to our understanding of the early stages of an HBV infection, including entry, HBV genome transport to the nucleus, and genome repair.

The paucity of *in vivo* models for studying direct HBV infections, and the limited availability of cultured primary human hepatocytes, has lead many researchers to study HBV replication and the activities of HBV-encoded proteins in immortalized or transformed liver cell lines and in cultured primary hepatocytes derived from small-animal models such as rats or mice.^[11,92] Use of these systems necessitates bypassing the initial receptor-mediated infection of the cell by direct transfection of the HBV DNA genome. Although primary hepatocytes derived from small-animal models, namely rat and mouse, cannot be directly infected with HBV, they can support HBV replication and serve as a surrogate model system for studying the effects of HBV replication and HBV proteins on cellular physiology.^[86,185,186] Here we provide a

summary of the available cell culture model systems that are used to study HBV biology, each one possessing its own benefits and limitations.

Most hepatocyte cell lines that are used in HBV-related studies are tumor-derived and thus are transformed. Since cellular signaling pathways are significantly altered in cancer, cell lines derived from tumors do not recapitulate the physiology of normal hepatocytes. While the results obtained by using transformed cell lines may be valid in a specific cellular context, caution should be exercised in the interpretation of such results because they may not necessarily represent the effects of HBV on cellular physiology in normal, untransformed hepatocytes, the authentic site of an HBV infection. In addition to tumor-derived cell lines, some cell lines have been specifically derived from HBV-positive tumors. Examples of cell lines isolated from HBV-positive tumors include the PLC/PRF/5 cell line and the Hep3B cell line, which are human HCC-derived cell lines containing multiple copies of HBV DNA integrated into the host DNA. While the PLC/PRF/5^[187] and the Hep3B^[188] cells are active in HBsAg production, they do not produce HBV virions and display no indicators of HBV replication,^[187,189-192] so results of studies using these cell lines require careful interpretation.

In an attempt to establish a system to study the biology of HBV, and specifically HBV replication, the results of numerous studies demonstrated that HBV DNA could be transfected into many different cell lines, including the hepatoblastoma-derived cell line HepG2^[15,193] and the hepatoma-derived cell line Huh7^[188,194,195] and that HBV could replicate efficiently in these cells. Consequently,

HepG2 and Huh7 cells expressing HBV DNA are frequently used in the study of HBV biology. While HepG2 cells and Huh7 cells support HBV replication, similar to nearly all existing human liver cell lines, they cannot be directly infected with HBV, partly due to the low expression levels of hNTCP, the functional cellular receptor for HBV.^[42] In order to analyze differences between cells with and without replicating HBV, HepG2.2.15 cells have sometimes been compared to HepG2 cells. HepG2.215 cells were originally derived from HepG2 cells and stably express HBV from two integrated head-to-tail dimers of the HBV genome.^[196] Results obtained by comparing HepG2.215 cells to the parental HepG2 cells, however, need to be interpreted with caution; because of the continuous passaging of HepG2.215 cells since their development in 1987, dissimilarities beyond the expression of HBV may exist between HepG2.2.15 cells and the parental HepG2 cells. Due to these dissimilarities, phenotypic differences that are observed between HepG2.215 and HepG2 cells might not be an exclusive consequence of replicating HBV.

Together, the use of exogenously delivered HBV DNA into established cell lines, such as HepG2 and Huh7, and the use of cell lines stably expressing HBV, such as the HepG2-derived cell lines HepG2.2.15 and HepAD38,^[196,197] make up the majority of the studies that have been conducted to understand HBV biology. While these cell culture models have proven extremely valuable to study HBV DNA replication, viral assembly, and virion secretion, they have limitations that prevent them from recapitulating all the aspects of an authentic human HBV infection.^[198]

Some recent developments have lead to increased optimism for the development of an effective *in vitro* system to study the complete HBV life cycle. For example, fusion of primary human hepatocytes with hypoxanthine-guanine phosphoribosyltransferase null [HGPRT(-)] HepG2 cells led to the establishment of the immortal cell line, HepCHLine-4/-7, that may provide a model system for HBV infection. This cell line supports HBV replication and is susceptible to HBV infection when incubated with serum from HBV-positive patients.^[199,200] However, an uncertain genetic stability during maintenance hampers the use of this system.^[198] In addition, the HepaRG cell line, a human hepatoma cell line, can also be directly infected with HBV and supports HBV replication. While this cell line is often used in studies of HBV infection, its use is limited by a low HBV infection efficiency of only about 10-20% and the need to induce differentiation prior to infection, which involves the maintenance of cells in 2% DMSO for 2 weeks before the induction of differentiation.^[198,201]

The recent discovery of hNTCP as the functional HBV receptor has important implications for basic HBV research and antiviral development. In particular, identification of hNTCP has opened new avenues for the establishment of novel cell culture model systems that

can be utilized to understand the effects of natural HBV infection. HepG2 cells expressing hNTCP (HepG2-hNTCP), theoretically, provide a convenient *in vitro* system for HBV infection. The exogenous expression of hNTCP in HepG2 cells does render them susceptible to HBV infection; however, low levels of infection, typically around 10%, and a requirement for large viral inoculums limit their use. Infection-based studies are hampered even further by the low levels of virus released by HBV-infected cells, believed to be around 1 virion per day, making it difficult to produce the large quantities of virus needed for these types of studies. Despite these issues, HepG2-hNTCP cells provide a valuable *in vitro* model system for elucidating the effects of natural HBV infection, investigating the complete HBV life cycle including the early steps of an HBV infection, and identifying novel therapeutic options.^[42,103,172,198,202-204]

As the natural target of an HBV infection, primary human hepatocytes would be the ideal *in vitro* system for studies of HBV. Unfortunately, cultured primary human hepatocytes lose susceptibility to HBV infection within days of isolation and culture, potentially because hNTCP expression rapidly decreases over time in culture.^[42,198,205] Interestingly, Rice and colleagues recently reported that induced pluripotent stem cell-derived hepatocytes (iPSC-derived iHeps) can support HBV infection, opening potential new avenues to study HBV biology and virus-host interaction and to test antiviral candidates. However, a long induction process involving differentiation of the iPSCs is required prior to HBV infection, and viral markers of infection can only be detected more than a week after inoculation.^[198,205,206]

Although studies in immortalized or transformed cells have served as powerful models for studying various aspects of HBV replication and the functions of HBV-encoded proteins, these studies have also demonstrated that HBV-mediated activities, particularly those associated with HBV proteins such as HBx, may vary in different cellular contexts.^[92,207] Alternatively, studies in cultured primary hepatocytes have begun to clarify HBV replication strategies and the function of HBV proteins in a more relevant context.^[92] Recently, cultured primary rat hepatocytes have been used to study HBV replication and functions of the HBx protein.^[83-86,208] HBx activities in cultured primary rat hepatocytes were similar to HBx activities in cultured primary human hepatocytes, supporting the use of cultured primary rat hepatocytes as a model system for studying the impact of HBV on hepatocyte physiology.^[86,185,186]

HBV NATURAL HISTORY

HBV infection can lead to high viral titers in the blood of HBV-infected individuals, with levels of HBV virions reaching as high as 10^{10} particles/mL.^[209] Because of these high titers of HBV in blood, the main mechanism for the transmission of the virus is through the blood.

In particular, exposure during childbirth from an HBV-infected mother is the leading global cause of HBV infections, with the potential of vertical transmission being as high as 90% in some parts of Asia. Additional routes of exposure to bodily fluids from infected individuals, such as sexual contact or sharing of needles, are also common routes of transmission.^[11]

The natural history of HBV has been divided into two types of infection [Figure 3]. For about 90-95% of HBV infections in adults, the result is “acute hepatitis” where the infected individual resolves infection to the point of undetectable viral DNA and the presence of antibodies against HBsAg. Symptomatic HBV-infected individuals present with inflammation of the liver, which is known as hepatitis, and associated nausea, jaundice, abdominal pain, and vomiting. For many cases of HBV infection, the infected person is asymptomatic, and acute infections are generally cleared within 6 months. In models of acute infection in WHV-infected woodchucks and HBV-infected chimpanzees, the first several weeks of infection are typically characterized by minimal innate^[160,210] or adaptive^[211] immune activation, with viral spread throughout the entire hepatocyte population.^[145,211] Eventually, the activation of an effective antiviral response, including activation of cytotoxic T lymphocytes (CTLs), results in inflammation in the liver and killing of the majority of HBV-infected hepatocytes over the length of a few weeks. Interestingly, studies of integrated WHV DNA in woodchucks treated with clevudine, a viral polymerase inhibitor, demonstrated that repopulation of the liver seems to occur from the population of infected hepatocytes and not from a smaller population of uninfected hepatocytes.^[212] Clearance appears to be mostly mediated by antiviral cytokines, with CTLs directly killing HBV-infected hepatocytes once the viral load has dropped below specific levels.^[213,214]

Approximately 5-10% of cases of HBV-infected adults, and a significantly higher percentage of HBV-infected infants and children, develop a chronic HBV infection^[215] as indicated by continued, detectable expression of HBsAg for at least 6 months after the initial infection. More recently, the application of more sensitive detection techniques, such as polymerase chain reaction (PCR)-based methods that can detect < 250 HBV virions/mL, has also shown that many individuals who were believed to be HBV-free following purported HBV clearance (indicated by the absence of detectable levels of HBsAg expression) actually have low levels of detectable serum and liver HBV DNA. In fact, low levels of HBV DNA can be detected in up to 30% of patients with liver disease of previously unknown etiology.^[215-217] This result has led to the recognition of occult HBV infections, in which the level of virus is persistently low and below the level of detection by traditional HBsAg detection techniques. Because of the relatively recent identification of this group of HBV-infected individuals, the risk factors associated

with an occult HBV infection remain incompletely understood, although some evidence does suggest that occult infections retain much of the same risk factors as chronic HBV infection.^[218]

Clinically, a chronic HBV infection can be divided into multiple phases,^[11,209,215,219] though not all patients progress through each stage. The “immune tolerant” phase is characterized by high titers of HBV DNA (> 100,000 copies/mL), the presence of HBeAg, and little liver disease. This phase can last decades, especially in perinatally infected patients, but is typically short or absent in childhood- and adult-acquired HBV.

The “immune clearance” phase also has high levels of HBV, though usually less than is present in the immune tolerant phase, as well as HBeAg expression, but is also characterized by more advanced liver disease with increased inflammation and progression of fibrosis. In addition, this phase is associated with spikes in levels of aminotransferases, which are believed to be a result of an HBV-specific cytotoxic T-cell-mediated immune response and destruction of HBV-infected hepatocytes.^[220] This is important, as a longer duration of this phase and higher frequency/severity of the HBV flares are associated with the development of cirrhosis and HCC.^[221] Typically this phase can last from several weeks to years and likely represents immunological attempts to control HBV levels. Seroconversion from HBeAg to anti-HBe is considered an important clinical outcome of the immune clearance phase, with immune control of the virus leading to very low or undetectable levels of serum HBV along with normal aminotransferase levels. Importantly, HBeAg seroconversion is associated with a favorable long-term outcome and with decreased risk of developing cirrhosis or HCC.^[37]

The “inactive HBsAg carrier” phase is characterized by multiple changes to the disease state. Specifically, there is a loss of HBeAg expression corresponding to an increased presence of anti-HBe. Spontaneous seroconversion from HBsAg to anti-HBs and low to undetectable levels of serum HBV DNA are also hallmarks of this phase. Additionally, aminotransferase levels remain consistently normal; low to mild hepatitis and fibrosis may be observed based on the length of the immune clearance phase. The inactive carrier phase could potentially be maintained indefinitely and is associated with a favorable clinical outcome;^[215,219] however, some individuals in the inactive HBV carrier phase enter a “reactivation/HBeAg-negative chronic hepatitis B” phase during which HBV replication rebounds either spontaneously or as a result of immune suppression. These patients are HBeAg negative/anti-HBe positive and have elevated liver enzymes with increased necroinflammation. Serum HBV DNA levels can reach as high as 10⁸-10⁹ copies/mL, though levels are typically lower than in HBeAg-positive patients.^[44,209,215,219]

Ultimately, for many patients the end result of a chronic HBV infection is the development of HBV-associated HCC. While seven therapeutics are currently approved for the treatment of chronic HBV, none has proven successful at achieving an “absolute cure” or a complete loss of HBV DNA and a lifetime risk of development of HCC equal to natural clearing of the infection. Five of these therapeutics are nucleoside analogs, designed to directly inhibit the RT. The other two, standard and pegylated interferon- α , function as antiviral cytokines, signaling through the interferon receptor to activate the JAK-STAT pathway.^[11,219] While generally effective at lowering viral load, the fact that none of these anti-HBV therapies is curative means these therapies must be life-long treatments, which eventually leads to the development of HBV mutants that are resistant to these therapies. Because of this, specific guidelines have been developed for when to use antiviral therapy and which therapeutic to use.^[219]

HBV AND HCC

HCC, which accounts for 80-90% of all liver cancers, is one of the most common and most deadly cancers worldwide. Globally, liver cancer is the sixth most common and second deadliest cancer, with an incidence to mortality rate near 1.^[222] Epidemiological studies have identified chronic HBV infection of the liver as the leading risk factor for HCC development.^[223,224] Despite the availability of a vaccine, 350-500 million people worldwide are chronically infected with HBV and, depending on age and route of infection, as many as 25% of these individuals could go on to develop HBV-associated HCC.^[224,225] The number of cases of HCC that are attributed to HBV will likely increase as occult infections become both better reported and better understood.

The molecular mechanisms that link a chronic HBV infection to HCC development are incompletely understood but are likely subtle considering that HBV-associated HCC usually occurs in the context of a decades-long chronic HBV infection. Studies have focused on three main factors that may be involved in the development of HBV-associated HCC: chronic inflammation accompanied by destruction and regeneration of hepatocytes, consequences of HBV DNA integration into host genome, and the potential effects of HBV proteins such as HBx.^[88,92,93,225,226] Some potential mechanisms that might link an HBV infection to HCC development have already been described above. Here we summarize additional mechanisms that have been suggested to link a chronic HBV infection to the development of HCC.

One particularly important intermediate aspect of a decades-long chronic HBV infection includes the development of HBV-associated cirrhosis prior to HCC development.^[219] It is generally accepted that the majority, potentially as much as 70-90%, of all HCC occurs in the

context of decompensated cirrhosis,^[224] and a strong relationship exists between chronic HBV infection and cirrhosis. In fact, a recent cohort study demonstrated that the cumulative lifetime risk of developing HBV-associated cirrhosis is 41.5% for chronically infected patients, with a cumulative risk of developing HCC of 21.7%.^[209] Therefore, establishing a clearer understanding of the cellular mechanisms associated with the intermediate stages of chronic disease, particularly development of cirrhosis, could enhance the overall understanding of causes of HBV-associated HCC.

Numerous aspects of an HBV infection could be playing a role in the development of HCC. It is logical to assume that hepatotropic viruses such as HBV, which alter hepatocyte physiology as part of, or a consequence of their replication, may disrupt normal hepatocyte and overall liver functions. Many of these disruptions and alterations, either through viral replication or activities of viral proteins such as HBx, could be playing a role in the development of downstream HBV-associated HCC and have been extensively reviewed elsewhere in the literature. For example, HBV has been shown to disrupt cell cycle regulation,^[227,228] alter apoptotic pathways,^[229] alter hepatocyte metabolism,^[33] and alter miRNA expression and miRNA-mediated regulation.^[230] Many of these studies have focused on multiple cellular signal transduction pathways, including those involving Ras/mitogen-associated protein kinases (MAPK),^[231] mechanistic target of rapamycin (mTOR),^[232] PI3K/Akt,^[86,233] and NF κ B,^[229] among many others. Each of these pathways and factors, while also important for HBV replication, are main mediators of hepatocyte functions. As such, disruption can have a major impact on hepatocyte physiology, which has generated a considerable amount of interest in their potential role as mechanisms for the development of HBV-associated HCC. The results of some of these studies are summarized here.

HBV and the cell cycle

As with many viruses, HBV must optimize the cellular environment for viral replication. In the case of HBV, this involves inducing hepatocytes to exit quiescence and enter into an active cell cycle, and the status of cell proliferation pathways can have a significant impact on HBV replication.^[91] For example, in primary rat hepatocytes, HBV moved cells from G₀ into and through G₁, but stalled progression before the hepatocytes were able to reach S phase, and this regulation of the cell cycle is required for HBV replication in primary rat hepatocytes.^[85] Studies in cell lines suggest a similar HBV-mediated regulation of cell cycle progression, with HBV stalling progression of the cell cycle before entry into S phase in both Huh7 cells expressing HBV and the HBV-expressing HepG2.2.15 cells.^[234] Interestingly, studies have also shown decreased proliferation of HepG2.2.15 cells, in comparison to HepG2 cells, along with HBV-mediated alteration of cell cycle regulators leading to a G1

phase arrest.^[235] Another study, however, in Huh7 cells and primary marmoset hepatocytes, demonstrated an HBV-mediated stall in the G2 phase of the cell cycle.^[236] While somewhat contradictory, these results together correlate well with the results of other studies showing that HBV replication is increased when the cell cycle is arrested in either G₁ or G₂, but HBV replication is decreased during S phase, when cellular DNA synthesis would be higher, potentially depleting the pool of nucleotides that would be available for HBV replication.^[237,238]

Much of the HBV-mediated regulation of the cell cycle appears to be through the activity of the HBx protein. Multiple studies in primary hepatocytes have demonstrated that HBx alters cell cycle regulators, including decreasing p15 and p16 expression, decreasing DNA synthesis, and increasing p21, p27, cyclin D1, and cyclin E expression.^[85,185,239] Together these results suggest that HBx drives hepatocytes into the cell cycle but increases expression of inhibitors that prevent progression beyond G₁. This HBx-mediated regulation of the cell cycle could have a long-term impact on hepatocyte physiology, altering hepatocyte proliferation pathways and contributing to the development of HBV-associated disease and HCC.

HBV and metabolism

Because of the primary role of the liver as a metabolic organ, a growing body of research has begun to investigate the impact of HBV infection on metabolic pathways in HBV-infected cells. In fact, HBV has been referred to as a “metabolovirus” due to the perceived intersection between HBV gene expression and control of cellular metabolism.^[206,240] Specifically, a number of groups have examined the role of HBV in lipid metabolism, especially considering the well-established link between hepatocyte lipids and various stages of the HCV life cycle^[241] and the recent identification of a bile salt transporter as a functional receptor for HBV.

The influence of HBV infection on hepatocyte metabolism was recently brought to the forefront with the identification of hNTCP, the primary bile salt transporter in hepatocytes, as a functional HBV receptor. Interestingly, the binding of HBV, specifically the preS1 domain of L-HBsAg, to hNTCP directly interferes with the normal function of hNTCP suggesting competition for binding motifs within the receptor. Furthermore, point mutations in hNTCP that abolished binding of preS1 also blocked the ability of the receptor to bind taurocholate,^[242] suggesting that by binding to hNTCP, HBV could dramatically alter hepatic bile acid uptake.

Recently, HBV-mediated inhibition of normal hNTCP function was extended further using a biochemical profiling approach in which human liver chimeric mice were infected with HBV, and the impact on cholesterol metabolism was determined. Indeed, this study was able

to demonstrate overall modest HBV-mediated changes in lipid metabolism, but specific factors involved in both cholesterol and bile acid metabolism were significantly altered. Interestingly, similar results were seen in a comparison of HBV-infected humanized mice, mice treated with the HBV entry inhibitor Myrcludex-B, which mimics the preS1 domain and binds to hNTCP to block HBV infection, and liver biopsy samples from chronically HBV-infected individuals.^[180] Together these results indicate that the binding of HBV to hNTCP inhibits bile acid uptake, which stimulates bile acid synthesis pathways. One interesting caveat to these studies is the relatively novel use of a direct infection system, which drastically alters the question being asked in the experiment. For example, by also using the preS1 mimic Myrcludex-B, the studies are specifically addressing the impact of receptor binding by HBV, and not the cellular impact of HBV replication. This is in contrast to some previous work, which has typically been done using systems that bypass the infection step. An example of the importance of this distinction is that while the study using HBV-infected human-liver chimeric mice demonstrated that by binding to hNTCP, HBV alters the levels of nuclear farnesoid X receptor (FXR) and small heterodimer partner (SHP), previous work in an HBV-tg mouse model (which bypasses the infection step, among other differences) showed that depletion of FXR and SHP signaling did not diminish viral replication or transcription.^[243] This suggests that although HBV binding to its receptor alters the expression of these transcription factors, this alteration might not affect HBV replication. Further research would be needed to determine the relevance of similar contrasting results within different model systems.

In addition to the functional inhibition of a major bile salt transporter, evidence from other studies has suggested that HBV replication may be intimately associated with central metabolic pathways. For example, multiple transcription factors associated with hepatic metabolic processes, including HNFs,^[244,245] peroxisome proliferator-activated receptors (PPARs),^[245-247] and FXR^[248-250] can all be recruited to the HBV genome.^[33] Moreover, studies *in vitro* have shown that exogenous addition of bile acids to HBV-expressing cells can increase HBV replication.^[250,251]

Induction of gluconeogenesis enhances HBV replication,^[252] and HBx has been shown to increase expression of multiple gluconeogenic genes,^[253] potentially contributing to the central role of HBx in HBV replication. Recent RNA-seq analyses of HBV-expressing Huh7 cells^[254] and primary rat hepatocytes^[186] also detected decreased expression of GLUT2, the main hepatic glucose transporter. Investigation of the effect of fasting glucose levels on HBV replication revealed a link, albeit minor, between the metabolic state of the cell and the level of HBV replication,^[245] and both gluconeogenesis and lipogenesis are under the same transcription factor control as HBV replication.^[255] Studies

have linked metabolic changes to effects of HBV proteins. For example, some of the earliest functions attributed to HBx involved its regulation of metabolic pathways, such as HBx-mediated activation of the Ras-Raf-MAPK pathway,^[256,257] a central pathway involved in the response to nearly all changes that affect metabolism.^[258] Protein kinase C (PI3K) is also activated by HBx,^[259,260] which correlates with recent results suggesting HBV and HBx activate the PI3K/Akt pathway, reducing HBV replication.^[86,233] In addition, studies have shown that mutant L-HBsAg can activate mTOR signaling,^[261-263] ultimately causing increased lipogenesis.^[262]

When considered in combination, these studies support the characterization of HBV as a “metabolovirus”, and HBV responds to and causes significant metabolic changes in the cell. While the clinical impact of this altered metabolic regulation remains unknown, some studies have suggested that HBV can “help” reduce fatty liver disease.^[264] The overall link between HBV and metabolic syndrome remains less clear; however, studies that consider the impact of direct infection through a major metabolic receptor may help to enhance our understanding of the link between HBV and metabolic pathways, and how this relationship may impact the metabolic state of the liver and the development of HBV-associated disease and HCC.

HBV and apoptosis

Despite the many studies that have investigated the effect of an HBV infection and expression of HBV proteins on hepatocyte pro- and anti-apoptotic signaling pathways, the interplay of an HBV infection and hepatocyte apoptotic signaling pathways remains incompletely understood. Because an HBV infection is non-cytopathic, it would be expected that HBV either inhibits or has little effect on apoptotic pathways. Evidence has suggested, however, an HBV-mediated effect on cellular apoptosis that is cell-type- or cell-context-dependent. Some of these differing effects can be attributed to HBx activities, which often have divergent functions depending on context. In the case of apoptosis, some studies have shown that HBx can inhibit apoptosis^[86,265-269] or have no effect on apoptosis,^[99,270,271] while other studies have shown that HBx can activate apoptotic pathways^[272-276] or sensitize cells to pro-apoptotic stimuli.^[277-279] The context-dependent apoptotic effects of HBx were clearly shown by studies demonstrating that HBx sensitized dedifferentiated hepatocytes to apoptosis, while HBx-expressing hepatocytes that remained differentiated were resistant to apoptotic stimuli.^[279] This underscores the importance of using relevant cell systems for studying the cellular impact of HBV replication and protein expression on cell physiology. HBx was also shown to have divergent apoptotic functions in the context of HBV replication. Studies in primary hepatocytes demonstrated that HBx can have both a pro- and anti-apoptotic effect, depending on the cellular context of

HBx expression. Specifically, inhibition of apoptosis was linked to HBx-mediated activation of NFκB; however, when activation of NFκB was blocked, HBx induced apoptosis through pathways involving the mitochondria permeability transition pore (MPTP), a critical pore that spans the inner mitochondrial and outer mitochondrial membranes and affects numerous mitochondrial functions, including mitochondrial control of apoptosis.^[83] Whether HBV, through functions of HBx, regulates apoptosis as a mechanism for regulating viral replication or enhancing viral spread is currently unknown. Although activation of apoptosis may impact both viral spread,^[280,281] and immune evasion,^[93] recent evidence suggests that alteration of apoptosis during HBV infection is unlikely to impact viral spread.^[282] Due to the regenerative nature of hepatocytes, it is also possible that the impact of HBV on apoptosis may fluctuate during the course of infection, as regenerating hepatocytes have different active signaling pathways than quiescent cells, and these could have differing influences on apoptotic stimuli.^[280,283,284] Interestingly, both the activation and the inactivation of apoptosis could be playing a role in the long-term development of HBV-associated HCC: enhanced regeneration associated with HBV-mediated activation of apoptosis could lead to selection of apoptosis-resistant cells,^[285] while inhibition of apoptosis could lead to unchecked proliferation and the accumulation of transforming mutations.^[93] Although the exact mechanisms that underlie HBV and HBx regulation of apoptosis remain incompletely understood, the cellular impact of altered apoptotic signaling could significantly contribute to the downstream development of HBV-associated disease and HCC.

HBV and microRNAs

Potentially spurred by the discovery of the required role of miR-122 in successful HCV replication,^[286,287] multiple groups have begun to investigate how cellular miRNAs may impact various aspects of HBV biology, and alternatively, how HBV may impact the expression of cellular miRNAs. These effects have been reviewed in more detail elsewhere.^[230,288]

While a wide range of cellular miRNAs has been investigated for their role in regulating or being regulated by HBV, none has been studied as extensively as miR-122. This miRNA, which makes up 50-70% of the total miRNA pool in hepatocytes^[289,290] has been shown to have many different roles in the context of HBV replication. Although conflicting reports do exist, it seems that miR-122 functions as an antiviral miRNA, potentially through multiple mechanisms. These mechanisms include direct targeting of viral RNAs through miR-122 recognition sites in the HBV genome^[291,292] and altered miR-122-mediated regulation of cellular factors involved in regulation of HBV replication, such as heme oxygenase-1,^[293] cyclin G1,^[294,295] and pituitary tumor-transforming gene 1

binding factor (PBF).^[292] Importantly, multiple studies have also shown an HBV-mediated decrease in the levels of functional miR-122,^[291,292,296-299] although the mechanism for this reduction remains unclear. One potential mechanism is a sponge effect, where the HBV transcripts act as a sponge to divert miR-122 away from endogenous targets,^[291,292] although it is unclear whether the levels of HBV transcripts in the cell reach the high levels of target required for this sponge effect to have a biological impact.^[300] Interestingly, primary tree shrew hepatocytes, which can be directly infected with human HBV, showed an increase in the levels of miR-122 in response to HBV infection.^[301] Further research will be needed to determine if this effect is the result of using a more biologically relevant system, with direct infection, or is an inherent difference between tree shrew and human hepatocytes.

Other miRNA families have also been assessed for their role in HBV replication, including miRNAs with well-established roles as either oncomirs or tumor suppressors. For example, the let-7 family, which function as tumor suppressor miRNAs and are downregulated in multiple cancers including HCC,^[302] are decreased in the context of HBV replication, HBx expression, and HBV-associated HCC.^[303-307] The miR-15 family,^[305,307-312] mir-125 family,^[303,305,310,313,314] miR-17/92 cluster,^[289,303,307,310,315] and miR-199a-3p^[289] are all HCC-related miRNAs that multiple groups have studied in the context of HBV. Although the field is still developing and contradictory reports exist, when taken together these reports support the overall impact of HBV on cellular miRNAs. How these miRNAs impact HBV replication, and ultimately HBV-associated disease and development of HBV-associated HCC, remains incompletely understood.

CONCLUSION

Hepatocytes are the main target of an HBV infection, and a chronic HBV infection is the major global cause for the development of HCC.^[92,207] While the association between chronic HBV infections and HCC is well established, there are still gaps in our understanding of how a chronic HBV infection can lead to HCC development. The high worldwide prevalence of chronic HBV infections, the limited therapeutic options currently available for the treatment of chronic HBV infections, the increased global incidence of HCC, the high mortality rate of individuals with HCC, and the close correlation between chronic HBV infections and HCC development have generated considerable interest in understanding HBV biology and elucidating the molecular mechanisms that underlie the development of HBV-associated HCC. In this article, we provided a review of HBV biology and highlighted the potential mechanisms that could underlie the development of HBV-associated HCC. These mechanisms are thought to involve a combination of continuous immune-mediated destruction of HBV-infected

hepatocytes and concomitant hepatocyte regeneration, the activities of certain HBV proteins such as the HBx, and potential consequences of HBV genome integration into the host genome.^[92,207]

Although there are treatments for a chronic HBV infection, resistance to currently available anti-HBV drugs, which develops due to the emergence of HBV mutants, is one major drawback of continuing nucleoside analog therapy. Moreover, existing antiviral treatments can control but not entirely eliminate HBV because of the persistence of HBV nuclear-localized cccDNA, and the persistence of cccDNA remains a major obstacle for the treatment and cure of chronic HBV infections.^[316-320] While there has been substantial progress in identifying mechanisms that underlie HBV infection, replication and clearance, there are still gaps in our understanding of the HBV lifecycle. The paucity of cell culture model systems that can recapitulate all the aspects of a human HBV infection and the scarcity of *in vivo* models for studying direct HBV infections has impeded our understanding of HBV biology. The recent discovery of hNTCP as the functional HBV receptor has provided new opportunities for the creation of novel cell culture model systems that can be used to understand the outcomes of a natural HBV infection. It would also be interesting to utilize tg mice expressing a hNTCP to study HBV biology and examine the activities of HBV-encoded proteins. However, currently, hNTCP tg mice do not permit the establishment of a productive HBV infection and it is likely that identification of additional species-specific determinants of HBV infection will be required before small rodent models of HBV infection and pathogenesis can be fully utilized.^[103,182,183] Although mice with humanized-livers and immune systems provide another promising model for studying HBV infection and pathogenesis, the complexity of generating these models have limited their use for studying HBV biology.^[175] Overall, studies aimed at enhancing our current understanding of the HBV life cycle and identifying central factors involved in the development of HBV-associated HCC are still needed and remain critical for the generation of novel therapeutics to inhibit HBV replication and the development of HBV-associated HCC.

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Conflicts of interest

There are no conflicts of interest.

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Preoperative liver functional volumetry performed by 3D-99mTc-GSA scintigraphy/vascular fusion imaging using SYNAPSE VINCENT: a preliminary study

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ABSTRACT

Aim: The present study was designed to evaluate the feasibility of preoperative liver functional volumetry performed by 3D-technetium-99m-diethylenetriaminepentaacetic acid-galactosyl-human serum albumin (99mTc-GSA) scintigraphy/vascular fusion imaging using SYNAPSE VINCENT and to examine the discrepancy between conventional and functional volumetry. **Methods:** The study group comprised 15 patients who underwent preoperative 3-dimensional (3D)-99mTc-GSA scintigraphy/vascular fusion imaging using SYNAPSE VINCENT software before hepatectomy between July 2014 and August 2015. The diagnosis was hepatocellular carcinoma ($n = 4$), metastatic liver tumor ($n = 10$), or intrahepatic cholangiocarcinoma ($n = 1$). Right hepatectomy was performed in 2 patients, left hepatectomy in 3 patients, right posterior sectionectomy in 3 patients, segmentectomy in 2 patients, and partial hepatectomy in 4 patients. 99mTc-GSA scintigraphy and computed tomography (CT) were performed to construct 3D-99mTc-GSA scintigraphy/vascular fused images. The conventional volume ratio of the planned resection region without tumor (% CT), and the functional volume ratio of the planned resection region without tumor (% GSA) were calculated. The discrepancy ratio was calculated as follows: discrepancy ratio = $100 - \% \text{GSA} / \% \text{CT} \times 100$ (%). **Results:** The % GSA ($17.9 \pm 16.7\%$) was significantly lower than the % CT ($21.5 \pm 17.6\%$) ($P < 0.036$). In all except 2 patients, the % GSA was lower than the % CT. The discrepancy ratio ranged from -4% to 75% (median, 20.7%). **Conclusion:** 3D-99mTc-GSA scintigraphy/vascular fused images constructed using SYNAPSE VINCENT were useful for noninvasively performing functional liver volumetry in patients scheduled to undergo various patterns of hepatectomy. In planned resection regions without tumor, the functional volume ratio was about 20% lower than the conventional volume ratio.

Key words: Functional volumetry; 99m-diethylenetriaminepentaacetic acid-galactosyl-human serum albumin; SYNAPSE VINCENT; fusion image; 3-dimensional; computed tomography

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INTRODUCTION

In liver surgery, preoperative treatment planning is defined in the context of the anatomical structure and the functional reserve of the liver. In patients who have damaged liver function or who are scheduled to undergo extended hepatectomy, the postoperative residual liver volume with adequate preservation of blood supply and drainage vessels is very important for the prevention of liver failure.^[1]

Virtual reality simulation on computed tomography (CT), magnetic resonance imaging (MRI), or ultrasonography (US) plays an important role in examining the anatomical structure of the liver. Recently 3-dimensional (3D) imaging techniques, such as 3D CT, 3D MRI, and 3D US, have been developed. To date, a number of methods and software systems have been developed for 3D surgical planning before liver surgery.^[2-6] Remnant liver volume can thus be determined (volumetry) before hepatectomy.

Technetium-99m-diethylenetriaminepentaacetic acid-galactosyl-human serum albumin (^{99m}Tc-GSA) is an analog ligand of asialoglycoprotein. ^{99m}Tc-GSA binds to the asialoglycoprotein receptor (ASGP-R) located specifically on hepatocytes. The ASGP-R concentration is helpful in evaluating the extent and progression of liver disease, so the hepatic uptake of ^{99m}Tc-GSA reflects the number of functioning hepatocytes.^[7-11] Before hepatic resection, however, it is difficult to correctly estimate the functional hepatocyte mass of the remnant liver.

Thus, ^{99m}Tc-GSA scintigraphy combined with single-photo emission computerized tomography (SPECT) and CT fused imaging has been used to estimate the future remnant liver function before hepatic resection.^[12-16] However, the planned resection region had to be set manually using a 2-dimensional CT display. It was difficult to estimate the local remnant liver function in detail.

The volume analyzer software SYNAPSE VINCENT (Fujifilm Medical Co., Tokyo, Japan) is a high-speed 3D image analysis system. Using previously captured CT or MRI, high-definition 3D images of organs and vessels can be reconstructed quickly.^[4] It has become easy to grasp the complex anatomical relations between the portal triad, hepatic veins, and local tumor by volume rendering. With this software, the surgeon can simulate various patterns of planned hepatectomy.^[4-6]

In the present study, we performed preoperative liver functional volumetry by 3D-^{99m}Tc-GSA scintigraphy/vascular fused imaging using SYNAPSE VINCENT and examined the discrepancy between conventional and functional volumetry.

METHODS

This retrospective study was approved by our institutional

review board; informed consent was obtained from all patients before ^{99m}Tc-GSA scintigraphy and CT. We retrospectively reviewed patients who had undergone liver surgery between July 2014 and August 2015 in the Department of Surgery of our hospital. Twenty-five patients preoperatively underwent 3D-^{99m}Tc-GSA scintigraphy/vascular fused imaging using SYNAPSE VINCENT.

The following exclusion criteria were applied: (1) a history of hepatectomy or portal embolization; (2) hilar cholangiocarcinoma with unilateral biliary drainage; and (3) hepatectomy for benign disease. A total of 15 patients (10 men and 5 women; age, 60 to 81 years; mean, 72.7 years) who agreed to undergo preoperative 3D-^{99m}Tc-GSA scintigraphy/vascular imaging using SYNAPSE VINCENT were studied. The diagnosis was hepatocellular carcinoma in 4 patients, metastatic liver tumor in 10 patients, and intrahepatic cholangiocarcinoma in 1 patient. Based on the Brisbane 2000 classification criteria,^[17] right hepatectomy was performed in 2 patients, left hepatectomy in 3 patients, right posterior sectionectomy in 3 patients, segmentectomy in 2 patients, and partial hepatectomy in 5 patients. Background of the liver was liver cirrhosis in 2 patients, chronic hepatitis in 1 patient, and normal liver in 12 patients. The planned resection region of the liver could be similarly resected in all patients.

3D-vascular imaging using SYNAPSE VINCENT

Preoperative enhanced CT was performed with a 64-multidetector-row CT scanner (Discovery CT 750 HD, GE Healthcare Japan, Co. Ltd., Tokyo, Japan) at 0.625-mm intervals. Four-phase contrast-enhanced CT was performed 30, 60, 90, and 180 s after initiating the injection of contrast media to obtain hepatic arterial, portal venous, hepatic venous, and equilibrium phase images, respectively. A total of 100 mL of nonionic contrast material containing 370 mg of iodine per milliliter (Iopamidol, Bayer Yakuhin, Osaka, Japan) was injected intravenously at a rate of 3.3 mL/s using an automatic power injector. With the use of a workstation, a routine preoperative CT workup was performed in the axial and coronal imaging planes. The data were obtained in Digital Imaging and Communications in Medicine format and transmitted to a workstation running SYNAPSE VINCENT.

Liver Analysis Application uses Dynamic-CT imaging of the liver. After data for the hepatic arterial, portal venous, and hepatic venous phases are obtained, operative simulation by 3D-vascular images is performed. After selection of the portal venous branch of the planned resection area, surgical simulations can be displayed. This system also can calculate the total liver volume, tumor volume, and volume of planned resection region.^[4]

^{99m}Tc-GSA scintigraphy

A bolus of 1 mL of ^{99m}Tc-GSA (185 MBq, Nihon Medi-physics Co. Ltd., Nishinomiya, Japan) was intravenously injected

into an antecubital vein. Images were obtained with the patient in the supine position, using a gamma camera over a large field of view in which a high-resolution, all-purpose parallel-hole collimator (Infinia; GE Healthcare Japan Co. Ltd., Tokyo, Japan) was centered over the liver and precordium. Computer acquisition of gamma camera data was initiated simultaneously with injection of ^{99m}Tc -GSA and stopped at 30 min. Digital images (128×128 pixels) were acquired in the byte mode at a rate of 2 frames/min for 20 min after the injection. Hepatic SPECT data were obtained for 15 min after the end of the dynamic scintigraphic study.

3D- ^{99m}Tc -GSA scintigraphy/vascular fusion imaging using SYNAPSE VINCENT

Data obtained by ^{99m}Tc -GSA scintigraphy and CT imaging are composited by adjusting the axial and coronal images, and 3D- ^{99m}Tc -GSA scintigraphy/vascular fused images are constructed. The 3D-vascular images are used to select the portal venous branch to be resected and to calculate the extraction volume and ratio of the dominant region of the branch (conventional volumetry). In 3D- ^{99m}Tc -GSA scintigraphy, the functional volume and ratio of the same region are calculated (functional volumetry) concomitantly. Count-rates are displayed on the images, which can be saved.^[4]

Image analysis

In ^{99m}Tc -GSA scintigraphy, regions of interest (ROI) over the entire liver and heart are delineated. Time-activity curves are generated for the ROI.

An index of clearance of ^{99m}Tc -GSA from the blood is calculated as the quotient of the radioactivity of the heart ROI 15 min after injection (H15) divided by the radioactivity of the heart ROI 3 min after injection (H3), ($\text{HH15} = \text{H15}/\text{H3}$). Hepatic uptake ratio of ^{99m}Tc -GSA is calculated by dividing the radioactivity of the liver ROI at 15 min (L15) by the sum of H15 and L15 ($\text{LHL15} = \text{L15}/[\text{H15} + \text{L15}]$).^[10,11,18]

The 3D- ^{99m}Tc -GSA scintigraphy/vascular fusion images obtained using SYNAPSE VINCENT are used to calculate the total liver volume without tumor, the conventional volume ratio of the planned resection region without tumor (% CT), and the functional volume ratio of the planned resection region without tumor (% GSA). The discrepancy ratio is calculated as follows: discrepancy ratio = $100 - \% \text{GSA} / \% \text{CT} \times 100$ (%).

Case 9

A 71-year-old woman was admitted with a diagnosis of hepatocellular carcinoma concomitant with liver cirrhosis due to autoimmune hepatitis. CT revealed hepatocellular carcinoma (hypervascular tumor) in Segment 6 [Figure 1]. The % CT and % GSA were 3.0% and 2.2% in partial hepatectomy, 12.2% and 11.7% in segmentectomy, 27.5% and 28.4% in

right posterior sectionectomy, and 63.4% and 68.5% in right hepatectomy, respectively. Segmentectomy was performed to treat the hepatocellular carcinoma.

Statistical analysis

Values are expressed as means \pm standard deviation (range). The Student's *t* test was used to compare differences between two groups. A *P* value of < 0.05 was significant. All analyses were performed with SPSS version 17 (SPSS, Chicago, IL).

RESULTS

The clinical characteristics of the 15 patients are summarized in Table 1. The mean values of HH15 and LHL15 were 0.64 ± 0.10 and 0.90 ± 0.06 , respectively. The % GSA ($17.9\% \pm 16.7\%$) was significantly lower than the % CT ($21.5\% \pm 17.6\%$) ($P < 0.036$). In all except 2 patients, the % GSA was lower than the % CT. The discrepancy ratio ranged from -4% to 75% (median, 20.7%).

DISCUSSION

Our study demonstrated that 3D- ^{99m}Tc -GSA scintigraphy/vascular fusion imaging using SYNAPSE VINCENT is useful for noninvasive functional liver volumetry in patients scheduled to undergo various patterns of hepatectomy.

Postoperative liver failure remains a life-threatening complication after hepatectomy. Conventionally, traditional liver function tests and CT volumetry have been used to evaluate patients before hepatic surgery.

The use of ^{99m}Tc -GSA scintigraphy to evaluate liver function was initially introduced by investigators in Japan. ^{99m}Tc -GSA binds to the ASGP-R located specifically on hepatocytes. The function of ASGP-R remains normal even in regenerating hepatocytes, and the ASGP-R density per hepatocyte is constant. Therefore the total amounts of ASGP-R are lower in cirrhotic liver patients than in normal liver patients according to the hepatocyte theory.^[19-23] We previously reported that, with progression of hepatic functional degeneration, ASGP-R density per hepatic volume decreases, especially in the right lobe.^[9] The hepatic accumulation of ^{99m}Tc -GSA thus reflects the functional liver volume.^[24] The hepatic uptake image of ^{99m}Tc -GSA at 15 min or later reflects the ASGP-R population.^[25] An index of clearance of ^{99m}Tc -GSA (HH15) is calculated as the quotient of the radioactivity of the heart ROI 15 min after injection (H15) divided by the radioactivity of the heart ROI 3 min after injection (H3), ($\text{HH15} = \text{H15}/\text{H3}$). Hepatic uptake ratio of ^{99m}Tc -GSA (LHL15) is calculated by dividing the radioactivity of the liver ROI at 15 min (L15) by the sum of H15 and L15 ($\text{LHL15} = \text{L15}/[\text{H15} + \text{L15}]$).^[10,11,18] HH15 and LHL15 reflect the hepatic function. Various studies of ^{99m}Tc -GSA have examined hepatic function.^[9,10,26-28]

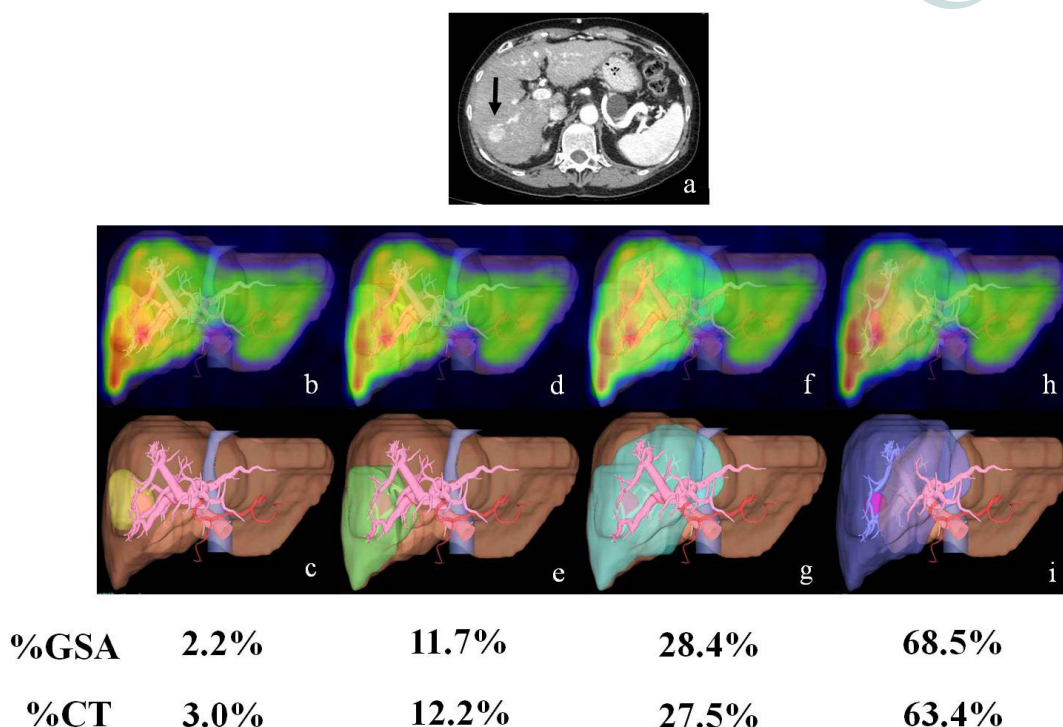


Figure 1: Various patterns of % GSA and % CT on 3D-99mTc-GSA scintigraphy/vascular fusion images in case 11. (a) CT image (planned resection region); (b) partial hepatectomy; (c) partial hepatectomy; (d) segmentectomy (S6); (e) segmentectomy (S6); (f) right lateral sectionectomy; (g) right lateral sectionectomy; (h) right hepatectomy on 3D-99mTc-GSA scintigraphic image; (i) right hepatectomy on 3D-vascular image. % CT: conventional volume ratio of planned resection region without tumor; % GSA: functional volume ratio of planned resection region without tumor

Table 1: Discrepancy ratio between % CT and % GSA

| Case | Age | Gender | Disease | Liver | Operation | HH15 | LHL15 | % CT | % GSA | Discrepancy ratio% |
|------|------|--------|---------|--------|-------------------------------|------|-------|------|-------|--------------------|
| 1 | 60 | M | HCC | CH | Right hepatectomy | 0.71 | 0.84 | 33.2 | 33.7 | -2 |
| 2 | 76 | M | Meta | Normal | Right hepatectomy | 0.74 | 0.94 | 66.3 | 66.4 | 0 |
| 3 | 72 | M | HCC | Normal | Left hepatectomy | 0.44 | 0.94 | 42.7 | 19.0 | 56 |
| 4 | 65 | F | ICC | Normal | Left hepatectomy | 0.56 | 0.92 | 21.6 | 12.5 | 42 |
| 5 | 78 | F | Meta | Normal | Left hepatectomy | 0.62 | 0.90 | 32.9 | 29.1 | 12 |
| 6 | 68 | M | Meta | Normal | Right posterior sectionectomy | 0.57 | 0.96 | 28.9 | 25.5 | 12 |
| 7 | 74 | M | Meta | Normal | Right posterior sectionectomy | 0.70 | 0.86 | 27.5 | 23.5 | 15 |
| 8 | 77 | M | Meta | Normal | Right posterior sectionectomy | 0.70 | 0.92 | 25.5 | 23.2 | 9 |
| 9 | 71 | F | HCC | LC | Segmentectomy (S6) | 0.74 | 0.88 | 12.2 | 11.7 | 4 |
| 10 | 77 | M | Meta | Normal | Segmentectomy (S8) | 0.61 | 0.92 | 17.0 | 12.0 | 29 |
| 11 | 81 | F | HCC | LC | Partial hepatectom (S8) | 0.88 | 0.72 | 6.6 | 5.1 | 23 |
| 12 | 77 | F | Meta | Normal | Partial hepatectomy (S6) | 0.56 | 0.96 | 1.3 | 1.1 | 15 |
| 13 | 78 | M | Meta | Normal | Partial hepatectomy (S6) | 0.54 | 0.94 | 1.6 | 0.4 | 75 |
| 14 | 69 | M | Meta | Normal | Partial hepatectomy (S8) | 0.60 | 0.92 | 2.6 | 2.7 | -4 |
| 15 | 68 | M | Meta | Normal | Partial hepatectomy (S8) | 0.68 | 0.88 | 2.9 | 2.2 | 24 |
| AV | 72.7 | | | | | 0.64 | 0.90 | 21.5 | 17.9 | 20.7 |
| SD | 5.6 | | | | | 0.10 | 0.06 | 17.6 | 16.7 | 21.4 |

HCC: hepatocellular carcinoma; Meta: metastatic liver tumor; ICC: intrahepatic cholangiocarcinoma; LC: liver cirrhosis; CH: chronic hepatitis; % CT: conventional volume ratio of planned resection region without tumor; % GSA: functional volume ratio of planned resection region without tumor; Discrepancy ratio: $(1 - \% \text{GSA} \% \text{CT}) \times 100$; AV: average value; SD: standard deviation

The SYNAPSE VINCENT system has already been tested in various surgical fields,^[29-32] and techniques for its use have been refined. The system has been greatly helpful in previsualizing intraoperative scenarios. In fact, the time required for intraoperative confirmatory US was considerably shortened after the introduction of this technology. This system is relatively easy to operate, allowing even a novice user to create 3D images.

3D-^{99m}Tc-GSA scintigraphy/vascular fusion images could also be easily created with the use of SYNAPSE VINCENT. It was possible to select the portal venous branch to be resected on the 3D-vascular images, and the conventional

volume could be compared with the functional volume of the planned resection region of the liver. The % GSA and % CT could also be calculated and compared.

In conclusion, 3D-^{99m}Tc-GSA scintigraphy/vascular fusion imaging performed with the use of SYNAPSE VINCENT is useful for non-invasive functional liver volumetry in patients scheduled to undergo various patterns of planned hepatectomy. There was a discrepancy between the results of conventional and functional volumetry. In the planned resection region without tumor, the functional volume ratio estimated with SYNAPSE VINCENT was about 20% lower than the conventional

volume ratio.

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Conflicts of interest

There are no conflicts of interest.

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Hemorrhagic cardiac tamponade after percutaneous laser ablation of a liver metastasis in segment II

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ABSTRACT

Despite percutaneous laser thermal ablation (LTA) of liver tumors being regarded as a safe technique, major complications can occur. We report the first case of hemorrhagic cardiac tamponade after LTA of a colorectal metastasis in segment II of the liver. Unpredictable heat diffusion causing indirect thermal injury to the pericardium with resultant hemorrhagic reaction was hypothesized as the most likely cause of tamponade. A pericardial drain was emergently placed, 200 mL of bright red blood were drained, and the patient showed rapid hemodynamic improvement. For lesions located in segment II of the liver and strictly close to the pericardium, a careful risk/benefit analysis should be made by the multidisciplinary team to identify the best treatment option, taking into account both effectiveness and complications of each available technique.

Key words: Laser thermal ablation; liver tumors; complications; cardiac tamponade

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INTRODUCTION

Percutaneous laser thermal ablation (LTA) of liver malignancies is a well established treatment for both primary and secondary liver tumors, with its effectiveness and safety being proven over the last several years.^[1] Among all thermal treatment modalities, LTA enables the use of finer needles than radiofrequency ablation (RFA) and microwave ablation (MWA), and allows one to tailor


the ablation volume by using one to four laser fibers, and thus sparing the normal parenchyma as much as possible. These attributes make LTA an attractive option for the treatment of nodules in high-risk locations, and/or multiple nodules differing in size.^[2]

Although RFA is the most commonly used ablation technique worldwide, the safety profile reported for LTA is comparable to RFA. Mortality rates for both RFA and LTA

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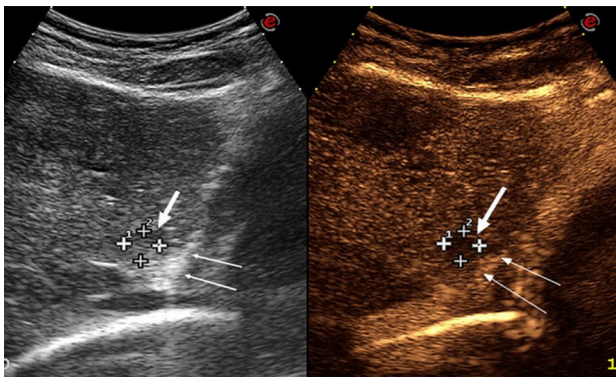


Figure 1: Oblique sub-costal contrast-enhanced ultrasound scan of the left lobe of the liver, showing an 11 mm metastasis in segment II (large arrows), at close proximity to the diaphragm and pericardium (thin arrows)

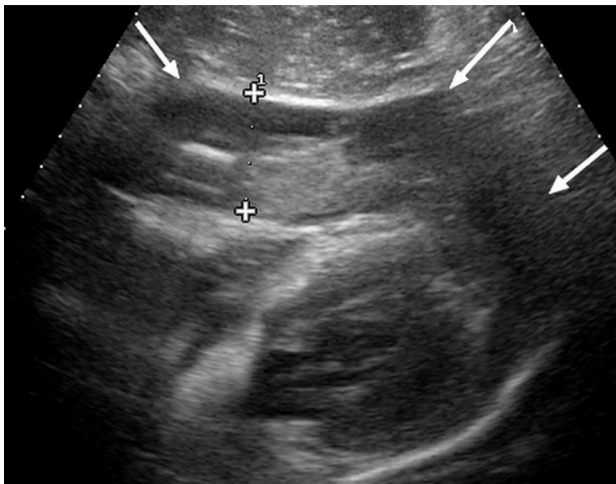


Figure 2: Subxiphoid ultrasound scan showing a large, partially hyperechoic pericardial effusion (arrows) surrounding the cardiac cavities

are less than 1%, and major complication rates range from 3.3% to 5.1%, and from 1.9% to 3.5%, respectively.^[3-5]

Hemorrhagic cardiac tamponade is a very uncommon but potentially fatal complication that has been sporadically reported during RFA of nodules located in the left lobe of the liver, close to the diaphragm and pericardium.^[6-8]

We report the first case of acute hemorrhagic cardiac tamponade occurring after LTA of a small liver metastasis from colorectal cancer in segment II.

CASE REPORT

This is a retrospective report of a clinical case, and was exempted from Institutional Review Board approval. The patient gave his written informed consent prior to the interventional procedure.

A 41-year-old man underwent LTA of a small, 11 mm colorectal metastasis in segment II of the liver, in close proximity to the diaphragm and pericardium [Figure 1]. Four liver metastases in the right lobe and one metastasis in segment III had been successfully ablated by LTA three

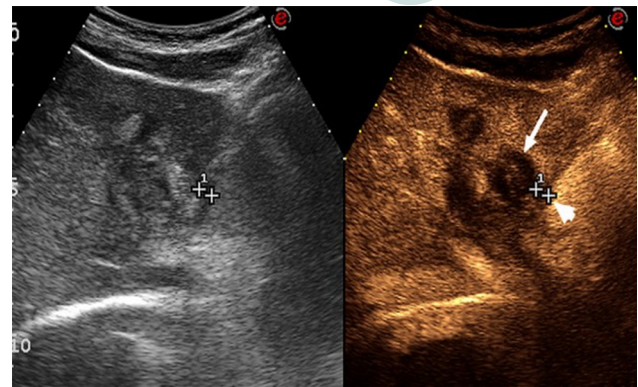


Figure 3: Oblique subcostal contrast-enhanced ultrasound scan of the left lobe of the liver performed a few days after laser thermal ablation, showing complete ablation of the metastasis with a 24 mm x 22 mm avascular area in segment II (arrows), at a distance of 4 mm from the diaphragm and pericardium (arrowheads)

months prior, without any complication. The procedure was performed under conscious sedation according to the technique proposed by Pacella *et al.*^[9] and modified by Di Costanzo *et al.*^[10] by using a diode laser unit (Echolasar, Elesta srl, Florence, Italy). Under sonographic guidance, two 21-gauge Chiba needles were placed 12 mm apart from each other along the anterior border of the tumor. Subsequently, two bare-tip 300 μ m in diameter laser fibers were introduced through the needles and advanced until the tip of the fibers was placed 1 cm beyond the tip of the needle into the deepest part of the tumor. Eighteen hundred Joule per fiber were delivered in 6 min. Immediately at the conclusion of the procedure, the patient had a sudden episode of tachycardia to 140 beats/min, followed by cardiogenic shock. Ultrasound (US) showed a large amount of partially hyperechoic pericardial fluid [Figure 2]. Cardiopulmonary resuscitation of the patient was initiated, and a 6-French pericardial drain was emergently placed via the paraxiphoid approach by an experienced cardiologist. Two hundred milliliter of bright red blood were drained, and the patient showed rapid hemodynamic improvement. After hemodynamic stabilization, abdominal artery angiography was performed in order to exclude vascular damage to the diaphragmatic arteries and left hepatic artery. No vascular injury was observed, and the patient was admitted to the cardiology unit. He remained asymptomatic, the drainage catheter was removed, and he was discharged after 5 days. Contrast-enhanced US (CEUS) performed before the discharge from the hospital showed complete ablation of the metastasis with a 24 mm x 22 mm avascular area in segment II [Figure 3]. No lesion or injury of the diaphragm was observed. Echocardiography showed resolution of the pericardial effusion.

Clinical follow up was performed weekly for the first month after discharge, and no further complication was observed.

DISCUSSION

Acute cardiac tamponade is an extremely infrequent,

life-threatening complication of thermal ablation treatments. To date, 4 cases of cardiac tamponade have been reported in literature as a complication of percutaneous thermal ablation.^[6-8] In all cases, the complication occurred after RFA of liver nodules performed by using expandable radiofrequency needles. The authors hypothesized two possible explanations for the occurrence of cardiac tamponade.^[6-8] First, the exact position of expandable RFA needles is more complicated to track at any time than that of the non-expandable RFA probes, MWA antennas, or LTA fibers. Therefore, a RFA hook could have inadvertently been placed in the diaphragm or in the pericardium, causing direct injury to these structures. Indeed, in 2 cases the presence of a RFA hook in the pericardial fat was documented by computed tomography.^[8] Secondly, in some unclear circumstances, the distribution of heat *in vivo* may be unpredictable, and the pericardium can become injured by heat conduction. Indeed, tissues exposed to elevated temperature may react with an inflammatory or hemorrhagic response, and such an injury has been observed in other viscera such as the gall bladder or colon.^[11]

Although LTA has been less investigated than the other ablation techniques, it seems to have the same efficacy and safety profile as RFA. By using one to four fibers according to the tumor size, the reported complete response rates range from 82% to 97% (hazard ratio). Mortality rate is < 1%, and major complication rate ranges from 1% to 3.5%.^[5]

To the best of our knowledge, this is the first case of cardiac tamponade following LTA reported in literature. LTA was preferred to other ablation techniques for lesions with small diameters, and those with difficult location. The procedure was performed under US-guidance, which enables one to check the position of the needle in real-time, minimizing the risk of incorrect placement and direct injury to the diaphragm. Moreover, unlike RFA and MWA where the ablation device is advanced through the entire lesion, using LTA technique the advancement of the needle tip was stopped 1 cm from the deepest part of the tumor, and just the very flexible, flat-tip fibers were placed close to the diaphragm, making direct injury to the diaphragm or pericardium by the needle tip very unlikely. Furthermore, no damage to diaphragmatic arteries or left hepatic arterial vessels was documented by abdominal artery angiography. For all these reasons, even though we cannot exclude with absolute certainty direct damage of pericardium, we believe that the most likely explanation for cardiac tamponade in our patient was unpredictable heat diffusion that caused indirect thermal injury to the pericardium with hemorrhagic reaction. Indeed, CEUS performed a few days after LTA documented successful ablation with a coagulation area 24 mm × 22 mm in size as expected, indirectly confirming that both needles and laser fibers had been correctly placed into the tumor.

Regardless of the exact mechanism responsible for hemorrhagic cardiac tamponade in our patient, this case report highlights some issues that should be considered in future similar cases. First, in all four cases previously reported in literature as well as in our patient, such a life-threatening complication occurred with tumors located in segment II of the liver.^[6-8] Although cardiac tamponade is an extremely infrequent complication of thermal ablation and is more likely to occur when expandable RFA needles are used,^[6-8] our experience shows that it may also occur with other theoretically safer techniques, such as LTA. Therefore, tumor location in segment II must be considered a major risk factor for cardiac tamponade during ablation procedures regardless of technique used, and according to Moumouh *et al.*,^[6] we wonder: “was percutaneous thermal ablation the best therapeutic option in this case?” A careful risk/benefit analysis must be made ideally by the multidisciplinary team before treating tumors located in segment II. Surgical resection or thermal ablation with open or laparoscopic approach could be considered, as they may be easier for isolating the lesion from adjacent critical structures and potentially provide better control of bleeding.^[11] However, these approaches are more invasive and not always simple, and the risk of complications due to an open or laparoscopic approach should be weighed against the risk of cardiac tamponade. In addition, alternative locoregional treatments such as transarterial chemoembolization or stereotactic radiotherapy, or non thermal ablation techniques such as ethanol injection in presence of primary liver tumors should be considered.

Second, early detection of cardiac tamponade is pivotal to minimize its clinical magnitude, and US scans of the pericardial space should be promptly performed when blood pressure suddenly drops during thermal ablation of nodules located in segment II. Likewise, careful consideration should be given to the location where the procedure is performed, in order to ensure rapid availability of emergency personnel and emergency resuscitation equipment to properly manage major complications when they occur.

Finally, the treatment planning of a nodule in segment II should include the presence, or at least the immediate availability, of an interventional radiologist or cardiologist very experienced in the placement of pericardial drains.

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Conflicts of interest
There are no conflicts of interest.

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Percutaneous hepatic perfusion with melphalan for unresectable liver metastasis

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ABSTRACT

Percutaneous hepatic perfusion (PHP) is an investigative technique for treating patients with diffuse unresectable metastatic liver disease. The technique has been clinically evaluated and shows great treatment potential for regional therapy to the liver. The advantage of PHP lies in its minimally invasive approach and ability to be repeated when compared to isolated hepatic perfusion. In a literature search, 135 publications were screened and 16 of these publications, including clinical trials and reviews, contributed to this review of PHP with melphalan. Melphalan is an alkylating agent that, when used as the chemotherapeutic agent in PHP, has shown potential for significant control of tumor burden in the liver, especially in metastatic ocular melanoma. In the current landscape of liver directed therapy, PHP is a viable option for those with unresectable metastatic disease to the liver. This article will focus on the technical aspects of PHP and describe the current data available from clinical trials, including outcomes of patients treated with this minimally invasive approach.

Key words: Percutaneous hepatic perfusion; melphalan; unresectable liver metastasis; metastatic melanoma to the liver; ocular melanoma

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INTRODUCTION

What is percutaneous hepatic perfusion

The treatment of metastatic disease to the liver is an evolving paradigm that has been evaluated with increasing potential over the past few decades. Though there are treatment options for solitary or localized liver lesions, there is no treatment consensus when multiple metastatic lesions are found throughout the liver.^[1] It is estimated that approximately 80% of people with liver metastasis are

considered unresectable due to excessive tumor burden, tumor location, effect on inflow or outflow, an insufficient liver remnant, or a significant comorbidity.^[2] Most patients with liver-only unresectable metastatic disease have options of directed treatment. Percutaneous hepatic perfusion (PHP) is one of these novel techniques for patients with diffuse liver-only metastatic disease.

PHP is a minimally invasive procedure which allows for regional therapy to the liver. Arterial cannulation of the

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hepatic artery via a femoral artery puncture is used to selectively administer an anti-neoplastic agent directly to liver tumors. By endovascular venous cannulation, a unique double balloon catheter (Delcath catheter) is inserted into the inferior vena cava (IVC) to capture the hepatic venous outflow from the liver. Using veno-venous bypass, the chemotherapy laden blood can be captured at the hepatic vein confluence and filtered before returning to the systemic circulation by a central venous line. This novel treatment technique has evolved from original operative liver isolation techniques, which capitalized on the hepatic anatomy for inflow and surgical outflow control in liver directed perfusion.^[3]

History and development

The first use of hepatic perfusion was reported by Dr. Robert Ausman in 1961 as a surgery resident at the Roswell Park Cancer Institute where he developed the technique. His initial studies were performed on animal models, and once the technique was standardized it was tested on 5 patients with different types of hepatic malignancies. Though there was no long term follow-up and significant toxicity noted with the procedure, there was a therapeutic effect described in 2 patients.^[3] This initial study helped lay the foundation for isolated hepatic perfusion (IHP) which has been refined over 60 years. Multiple centers have evaluated IHP with various chemotherapy agents, various tumor histologies, hyperthermic perfusion, and improved techniques.^[4]

With data from isolated limb perfusion by Lienard *et al.*^[5] in 1992, melphalan was initially tested in combination with tumor necrosis factor alpha (TNF α). This regimen was used for IHP to treat liver disease. Early results at the National Cancer Institute showed a 75% radiographic response rate

with this combination and no diminishment of antitumor activity with advanced disease burden in the liver.^[3] However, due to the unavailability of TNF α for continued clinical testing in the United States, melphalan has been the most widely used chemotherapeutic agent in current trials. Through these early studies of the operative technique for IHP, key elements and principles were noted and carried over to the minimally invasive PHP technique in use today.

PHP was initially reported approximately 20 years ago by 2 centers. The largest study described by Ravikumar *et al.*^[6] involved 28 patients who were treated with escalating doses of doxorubicin or 5-fluorouracil. Through the catheter based approach, the chemotherapy was administered via a hepatic artery catheter and collected and filtered using veno-venous bypass from the venous outflow of the liver. Concurrently, a phase I study by Curley *et al.*^[7] was being performed in patients with hepatocellular carcinoma. Similar to the early use of IHP, no long term follow-up data was published and these studies were not continued at these centers. However, these studies described the potential use of this procedure and contributed to the refinement of its technical feasibility.

In 2005, the comprehensive evaluation of PHP was conducted as a phase I trial at the National Cancer Institute where 28 patients were treated with melphalan PHP, for 74 treatments in a dose escalation format. The overall radiographic response rate was observed to be 30% (RECIST criteria), with rates as high as 50% in 10 patients with metastatic ocular melanoma. Though transient hepatic toxicity and some hematologic toxicity were observed, this study helped determine the maximum tolerated dose of melphalan (3.0 mg/kg) and established the groundwork for a multicenter trial.^[8] After

Percutaneous Hepatic Perfusion

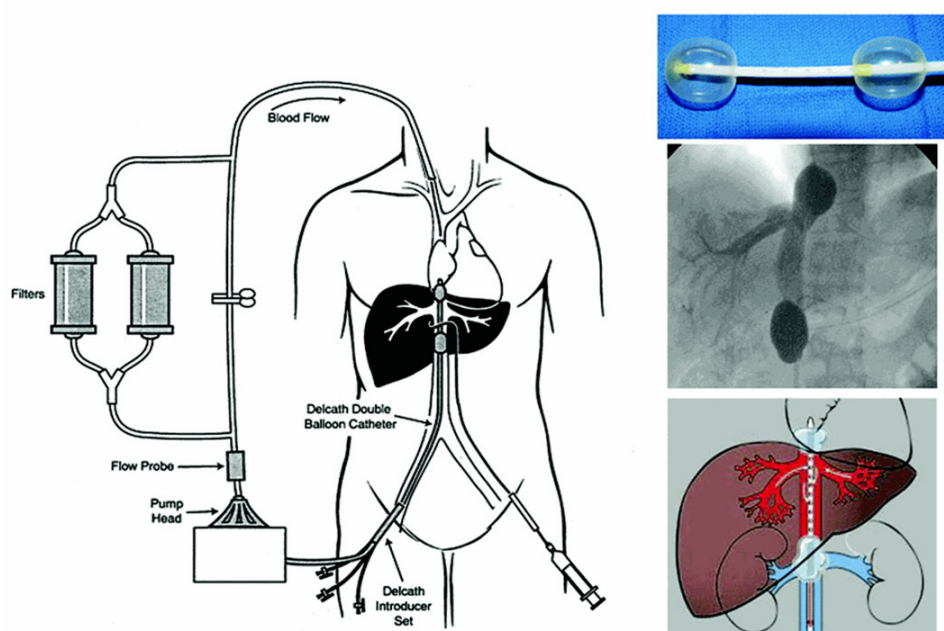


Figure 1: Diagram of the percutaneous hepatic perfusion system. This Delcath[®] Catheter System is used to infuse melphalan into the hepatic artery percutaneously (syringe) via the femoral artery. A double balloon catheter (shown in the upper right) is placed in the retro-hepatic inferior vena cava under fluoroscopic guidance (middle right image) to isolate the hepatic venous outflow. The multiple fenestrations along the balloon catheter then draw out the isolated blood which then is directed into the extracorporeal system. The blood is then pumped thorough a pair of activated charcoal filters, which extract the melphalan, before being returned to the systemic circulation. (This image has been reproduced with permission and purchase from *The Cancer Journal*)

publication of this phase I data, a multi-institutional phase III random assignment control trial was started in 2005, where PHP with melphalan was compared with the current best available care (systemic chemotherapy, embolization, supportive care) in patients with metastatic melanoma with the majority of tumor contained in the liver.^[3] This trial was completed in 2010 and the results have recently been published, with analysis showing an increase in hepatic progression-free survival in the melphalan PHP arm compared to the best available care.^[9] Currently, there are numerous centers throughout the world evaluating PHP and improving the technical aspects and treatment outcomes.

Evaluation

We evaluated data using previous publications on methods of liver perfusion, ranging from reviews to clinical trials. An initial PubMed search with the keyword “percutaneous hepatic perfusion” was performed yielding 135 publications. Publications were excluded if they were not in English, had no mention of liver metastasis or liver tumors, or were not available online or through an easily accessible source. We then screened 25 publications relating to PHP using the addition of the keyword “melphalan”. This search yielded 17 publications, only those that linked or contained primary data relating to PHP or IHP were selected, and ultimately 16 publications contributed to this review.

TECHNICAL ASPECTS

Procedure

As mentioned previously, PHP is a technique where a chemotherapeutic or biologic agent is delivered via catheterization of the hepatic artery. The hepatic venous

circulation is isolated via a special patented double balloon catheter directed via venous cannulation and fluoroscopically guided placement in the IVC (Delcath Catheter Systems, Delcath Inc., New York, NY). This allows for capture of the chemotherapy-laden effluent from the liver, which is filtered via veno-venous bypass prior to returning to the systemic circulation.^[3,8] PHP takes advantage of the tumor blood supply in which 90% of the tumor is supplied by hepatic artery inflow. In contrast, normal hepatocytes receive over 50% of their blood flow from the portal venous inflow. By isolating the hepatic arteries, infusion of chemotherapeutic agents are able to take the most direct circulatory pathway to liver tumors while somewhat sparing normal hepatocytes. It is critical to ensure that flow is isolated to the liver to avoid inadvertent chemoperfusion of non-target organs. Once the agent has completed its hepatic circulation, it is collected via fenestrations situated between patented double balloons of the catheter, from the hepatic veins as it enters the IVC. This catheter is initially placed and tested under fluoroscopy in the retrohepatic IVC so that the balloons are carefully seated cephalad and caudad to the hepatic veins. The blood is then directed through an extracorporeal filtration system (containing activated charcoal filter cartridges) which removes the agent prior to return to the systemic circulation via an internal jugular venous catheter [Figure 1].

The procedure is usually performed using general anesthesia with arterial line access placed for blood pressure monitoring, as well as internal jugular venous access for infusion from the veno-venous bypass circuit. The extracorporeal pump is primed with normal saline, and during the procedure, heparin is administered to maintain an activated clotting time at therapeutic levels. Percutaneous access of the right common



Figure 2: A 51-year-old female with a history of pancreatic neuroendocrine tumor and metastatic disease to the liver. (a) Common hepatic artery cannulated and filled with contrast defining the vascular anatomy of the liver. Visible are the numerous metastatic lesions which are contrast enhancing; (b) gastroduodenal artery coiled after contrast evaluation; (c and d) intra-procedural images of hepatic venous system isolation

femoral artery and vein are obtained, and an angiogram is performed via the celiac and superior mesenteric arteries to define the arterial anatomy. The gastroduodenal artery is usually embolized to minimize any extrahepatic perfusion and a catheter is positioned in the hepatic artery proper under fluoroscopic visualization. A double balloon catheter is then introduced percutaneously via the right common femoral vein. The cephalad balloon is inflated with contrast until it is maximally inflated while in the right atrium and then withdrawn until indentation of the diaphragmatic hiatus is visualized under fluoroscopy. The caudal balloon is then inflated until the balloon wall is deformed indicating a seal. Hepatic venous isolation is obtained both superiorly and inferiorly to the hepatic veins. Given the IVC will be blocked by the balloon, a bypass circuit is needed. The bypass circuit is composed of a venous Delcath 16F polyethylene catheter with one large fenestrated lumen and 3 accessory lumens, flushed bypass tubing, 2 filters, and the internal jugular central venous return line. Contrast is injected via the fenestrated lumen to confirm that the hepatic outflow is sealed and there is no leakage of hepatic outflow into the systemic circulation. All of this is critical to be accomplished prior to administration of any chemotherapeutic agents. Since venous return from the lower extremities is blocked at this time, veno-venous bypass is initiated. Just prior to initiation of this bypass circuit and filter activation, some patients can experience a transient drop in blood pressure requiring additional fluid and infrequent vasopressor support.

After confirmation of vascular outflow isolation, chemotherapy is given for a 30 min continuous infusion via the proper hepatic artery catheter. Occasionally due to anatomy, the chemotherapy infusion must be split between the right and the left hepatic artery to avoid any chemotherapy infusion to organs other than the liver. The filtration circuit is continued for an additional 30 min after the chemotherapy is infused to ensure adequate removal of the agent. Reversal of anticoagulation after the procedure is achieved via protamine administration, along with fresh frozen plasma, as necessary for safe catheter removal. After the start of reversal, the balloons are deflated and the IVC and hepatic artery catheters are removed. However, the venous and arterial sheaths and internal jugular catheter are not removed until coagulation normalizes. The patient is placed in a monitored setting for a minimum of 12 h and is maintained on bedrest for 4 h post-procedure. Postoperative laboratory studies are usually assessed daily while the patient is in the hospital, and once a patient's liver function tests and complete blood count stabilize, they are discharged. Labs are repeated within 5-7 days after discharge and weekly due to delayed hematologic changes secondary to melphalan exposure, which generally has a nadir of 7-10 days post-procedure [Figure 2].

What is melphalan

L-phenylalanine mustard (melphalan) is an alkylating agent. It has been attractive for use in PHP because as an agent used for regional therapy, its peak perfusate concentrations are 10- to 100-fold higher than maximally tolerated peak levels with systemic intravenous administration.^[10] Melphalan is

active against both resting and rapidly dividing tumor cells. The maximum level of melphalan-induced DNA crosslinks is reached within 4 h of regional perfusion and declines thereafter.^[11] Side effects and toxicities observed from a Phase I trial are described in detail below.

PHP vs. IHP

There are some advantages to PHP when compared with IHP. Multiple infusions can be administered via PHP, which may improve the duration of responses compared to a single infusion using IHP. A percutaneous approach also avoids the morbidity of an open surgical procedure. However, the complications resulting from this type of procedure are those commonly associated with vascular procedures, including, but not limited to, hepatic artery dissection, hematoma, pseudoaneurysm, pneumothorax from line placement, and possible device failure. Specifically, deep venous thrombosis, heparin induced thrombocytopenia, anaphylaxis to protamine have been observed.^[8] In comparison to PHP, IHP has the advantage of the ability to administer hyperthermic chemotherapy up to a temperature of 40 °C, which would otherwise be fatal if systemically administered; this can be accomplished in IHP due to the complete surgical isolation of hepatic blood flow in a closed circuit.^[2]

One must have experience with PHP as it can result in transient hemodynamic changes, such as decreased mean arterial blood pressure and venous return secondary to initiation of extracorporeal filtration and mechanical occlusion of the inferior vena cava. Acidosis has also been observed requiring the administration of intravenous sodium bicarbonate.^[12] Therefore, PHP must be done with a well-trained, experienced, and coordinated multidisciplinary team consisting of a vascular surgeon or interventional radiologist, anesthesiologist, and physicians that can safely manage the effects from the procedure and chemotherapy in a closely monitored setting.

DATA AND OUTCOMES OF TRIALS

Phase I dose escalation trial

The initial study evaluating the feasibility of hepatic arterial melphalan infusion using PHP for unresectable hepatic malignancies was completed by Pingpank *et al.*^[8] The phase I study treated an initial cohort of 12 patients at 2.0 mg/kg, followed by an additional 16 patients treated with escalating doses to the maximum tolerated dose (MTD) of 3.0 mg/kg. A total of 78 treatments were administered to 28 patients.^[8] The histologies of patients with metastatic liver disease included: ocular melanoma, neuroendocrine neoplasms, colorectal cancer, cutaneous melanoma, adrenocortical carcinoma, pancreatic adenocarcinoma, retroperitoneal sarcoma, breast adenocarcinoma, and renal cell carcinoma. Three patients with unresectable primary hepatobiliary tumors also received treatment. At 3.5 mg/kg, a dose limiting toxicity of neutropenia and/or thrombocytopenia was observed in 2 of 6 patients. Many patients who were treated experienced transient hepatic and systemic toxicities.

Pharmacokinetic analysis revealed that there was no degradation of melphalan during the 30 min infusion and rapid intrahepatic clearance occurred within 10 min of completing the infusion. No renal, cardiac, or pulmonary complications were observed in patients after treatment with melphalan in PHP. The treatment course for this study was planned approximately every 4 to 6 weeks for a total of 4 treatments, and patients were required to recover from the previous treatment toxicity to grade II or less prior to embarking on the next perfusion. The investigators evaluated responses in the 27 evaluable patients using standard RECIST criteria. Reported antitumor activity included minor responses ($n = 10$), partial responses (PR) ($n = 6$) and complete responses (CR) ($n = 2$). At the time of the trial's publication the duration of responses included 2 PRs ongoing for 9 and 11 months and 2 CRs at 10 and 12 months. The overall radiographic objective response rate was found to be 30%, and impressively in a subgroup of patients with ocular melanoma the overall objective response rate was found to be 50%. The authors concluded that PHP, as a regional treatment of hepatic metastasis, can be safely performed with predictable and manageable toxicity.

Moffitt cancer center experience

Another trial described by Forster *et al.*^[13] retrospectively reviewed patients treated with PHP at their single institution over a 7 years period. The patients included those with unresectable melanoma or sarcoma hepatic metastases. Between 2008 and 2013, 10 patients were treated - a total of 27 PHP treatments were administered with the median number of treatments reported at 3 per patient. Nine of 10 (90%) patients treated had stable disease (SD) or a PR, with a median partial response of a 33% decrease in tumor burden from baseline.^[13] The median follow up for the evaluation was 11.5 months in which the hepatic progression free survival (hPFS) was 240 days. At last 60% of the patients treated at the institution died from their disease. The median overall survival from time of diagnosis of hepatic metastases was 12.6 months and from time of first PHP was 8.7 months. They also reported a median postoperative hospital stay of 3 days following PHP. The most common adverse event was myelosuppression which was treated on an outpatient basis. Seven of the patients in the cohort experienced a mild elevation in their serum troponin levels with the 1 patient having a value greater than 1.0 ng/mL. There was no electrocardiography or echocardiographic evidence of myocardial ischemia, dyskinesia or dysfunction. The authors concluded from these results that for select patients with unresectable melanoma or sarcoma hepatic metastases, PHP is a safe and promising management option.^[13]

European experience

Vogl *et al.*^[14] reported a European experience of patients with hepatic metastases treated with PHP using melphalan. Fourteen patients were treated between January 2012 and February 2013 at 2 centers with the following histologies: ocular or cutaneous melanoma, breast cancer, gastric cancer, and cholangiocarcinoma. These patients received 3.0 mg/kg of melphalan similar

to the phase I trial reported by Pingpank *et al.*^[8] The tumor response included 1 CR seen in the cholangiocarcinoma patient, and 6 PRs (ocular melanoma: $n = 3$, cutaneous melanoma: $n = 3$). Stable disease was observed in 5 patients (ocular melanoma: $n = 3$, breast cancer and gastric cancer). Toxicity was similar to that seen in previous series including melphalan-related thrombocytopenia, anemia and pancytopenia. In this series, second generation filters were used in a select number of patients. These filters are reported to have increased melphalan extraction efficiency.^[15] In the portion of patients treated with the second generation filters, the toxicity was found to be milder and patients experienced a faster recovery.^[14] Similar to other groups with experience in PHP, Vogl *et al.*^[14] concluded that PHP for non-resectable liver metastasis is a feasible treatment.

Phase III multicenter trial

These single-center phase I and II studies established the framework for a multicenter phase III trial with melphalan in 2005. Hughes *et al.*^[16] published results of the phase III, multicenter randomized trial comparing PHP with melphalan (PHP-Mel) to best alternative care (BAC) for patients with cutaneous or ocular melanoma metastatic to the liver. The trial accrued 93 patients between February 2006 and July 2009. Those enrolled were randomized to PHP-Mel ($n = 44$) or BAC ($n = 49$). Primary BAC treatment included systemic chemotherapy, chemoembolization, radioembolization, immunoembolization and supportive care. The trial design allowed for crossover to PHP-Mel for patients who experienced hepatic progression in the BAC arm, provided they still met enrollment criteria. The percutaneous procedure involved delivery of high dose melphalan directly to the liver via the hepatic artery over 30 min. The initial dose of melphalan administered was 3.0 mg/kg based on ideal body weight. If a dose-limiting toxicity was encountered, the melphalan dose was decreased to 2.5 mg/kg in subsequent PHPs. Those randomized to PHP-Mel received treatment every 4-8 weeks when hematologic toxicity resolved to a grade 2 or less. Patients were eligible to receive up to 6 PHP procedures in the absence of progressive disease.^[9]

The primary endpoint reported by Hughes *et al.*^[16] includes hPFS, with secondary endpoints including xPFS (date of randomization to the first observation of extrahepatic disease progression or death due to any cause), hepatic objective response (hOR), objective response rate (ORR), overall progression-free survival (oPFS), overall survival (OS), and safety. The results of the trial include a median hPFS in PHP-Mel of 7 months compared to 1.6 months in BAC. The median oPFS was 5.4 months and 1.6 months in PHP-Mel and BAC, respectively. The hOR for PHP-Mel was noted to be 36.4% with a SD rate of 52.3%; hepatic disease control was observed in 75% of patients. The authors report a significant improvement in response favoring PHP-Mel patients including an ORR of 27.3% (median duration 6.3 months) in the PHP-Mel group compared to 4.1% (median duration 3.7 months) in those who received BAC. There was no significant difference

in OS observed between the 2 groups - the median OS of 10.6 months observed in PHP-Mel vs. 10.0 months in BAC was due to the built in crossover design.^[16]

Hughes *et al.*^[16] described immediate peri-procedural events (within 72 h) observed in 90% of PHP-Mel treated patients to include mostly self-limited thrombocytopenia and anemia. These events were attributed to platelet sequestration in the filters and/or hemodilution. The delayed post-procedural events, defined as occurring between 3 to 20 days after the melphalan exposure or until the next treatment cycle, were thought to be hematologic due to imperfect filtration. Neutropenia, thrombocytopenia and anemia were observed in most PHP-Mel patients and thought to be related to the effects of bone marrow suppression. Hyperbilirubinemia was observed in 10 patients. Some fatalities were observed on this trial and each death lead to further safety maneuvers in the development of improved filters.^[15,16]

The authors concluded that the results of their phase III study demonstrate the efficacy of PHP-Mel. They report that the toxicity is significant but manageable in order to provide effective therapy for this select cohort of patients. Overall, given the improved hepatic PFS, oPFS, and hOR, Hughes *et al.*^[16] conclude that PHP with melphalan should provide a new treatment strategy for patients with unresectable metastatic melanoma to the liver.

CONCLUSION

PHP has been shown to be an innovative and promising technique for delivering regional chemotherapy to the liver. The evaluation of its use for different tumor histologies, has been, and continues to be studied in numerous trials. PHP has significant potential for the control of tumor burden in metastatic melanoma, particularly for ocular melanoma, which seems to be less responsive to checkpoint inhibition and other immunotherapies in comparison to cutaneous melanoma.^[17] The advantage of PHP lies in the ability to administer multiple therapies using a less invasive approach, in contrast to the laparotomy required for a single therapy with IHP. Currently, PHP in the United States is only available on study or compassionate use, however it does have the European mark and is being aggressively evaluated in seven different European countries.^[14] In the current landscape of liver directed therapy, PHP is a viable option for those with unresectable metastatic disease to the liver.

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Conflicts of interest

There are no conflicts of interest.

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Hepatic disorder in Zika virus infection

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ABSTRACT

Zika virus infection is the present global problem. This arbovirus infection can cause acute illness and affect fetus in utero. However, there can be other additional clinical manifestation including to the hepatic disorder. In this short commentary article, the author briefly discusses on the liver problem due to Zika virus infection.

Key words: Zika virus; liver disorder; infection

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INTRODUCTION

Zika virus is an arbovirus that can cause acute febrile illness. At present, it is the big public health threat.^[1] The infection can be serious and can cause neurological complication. In addition, the serious effect on development of fetus in utero can be seen. Hence, World Health Organization document Zika virus infection as an important problem that needs urgent attention and management.^[2]

Briefly, Zika virus infection can cause a dengue like illness and can be easily misdiagnosed.^[3] The acute hemorrhagic fever can be the first presentation of Zika virus infection. Nevertheless, there can be other atypical manifestations. The atypical clinical manifestations can add difficulty in diagnosis of the Zika virus infection. Of several atypical clinical problems, liver disorder can be seen and this is an issue that is less mentioned. In this short commentary, the authors discusses on the liver disorder seen in Zika virus infection.

EVIDENCE OF LIVER DISORDER IN ZIKA VIRUS INFECTION

There are limited reports on liver pathology in Zika virus infection. Most reports showed no abnormality in liver. In

the clinical report of new epidemics, Deng *et al.*^[4] and Zheng *et al.*^[5] mentioned for no abnormal liver function in infected cases. In infected death fetus, the molecular pathology also revealed no observed virus in liver tissue.^[6] However, there was an interesting report at the time when the Zika virus had just been discovered by Macnamara that Zika virus could be isolated from the cases presenting with jaundice during the outbreak of jaundice in Africa.^[7] In addition, the recent animal mice model study revealed that the Zika virus RNA can be seen in Zika virus infected mice.^[8,9] In fact, Zika virus is usually included in differential diagnosis of acute febrile illness due to arbovirus infections including to yellow fever.^[10] Hence, the question whether there is any interrelationship between Zika virus infection and liver pathology is still a topic for further research.

CO-INFECTION WITH HEPATITIS VIRUS: A TOPIC THAT IS STILL A MYTH

Finally, it should be noted that Zika virus can be concomitantly seen with other infections (such as dengue^[11] and human immunodeficiency virus^[12]). In hepatology, the topic that is still a myth is the concomitance between Zika virus infection and viral hepatitis. Although there has never been report on this issue, it is no doubt that the co-infection already occurred in many tropical countries that presently have the problem of Zika virus epidemic. How the Zika virus infection superimpose

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to viral hepatitis and how viral hepatitis superimpose to Zika virus infection is another topic for further research.

ZIKA VIRUS, CHRONIC LIVER PROBLEM AND HEPATOMA

An important concern is on the Zika virus infection in the cases with underlying chronic liver problem. As already noted, the evidence on liver pathology in Zika virus infection is extremely limited. For the affect fetus, the recent investigation showed no liver problem.^[13] In fact, the relationship between Zika virus infection and cancer is very interesting. Recently, Benelli *et al.*^[14] noted that “basic epidemiological knowledge on the relationships occurring between mosquito vector activity and the spread of cancer is urgently needed, as well as detailed information about the ability of Culicidae to transfer viruses or tumor cells among hosts over time.” Nevertheless, the long term follow-up of Zika virus affected patients, especially for those with underlying chronic hepatitis is suggested. The observation on the possible emerging hepatoma among these cases is recommended.

CONCLUSION

It is still inconclusive on the exact effect of Zika virus infection on human liver. The further research on this area is recommended for hepatologists.

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Conflicts of interest

There are no conflicts of interest.

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Comment on “A series of microRNA in the chromosome 14q32.2 maternally imprinted region related to progression of non-alcoholic fatty liver disease in a mouse model”

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Okamoto K, Koda M, Okamoto T, Onoyama T, Miyoshi K, Kishina M, Kato J, Tokunaga S, Sugihara TA, Hara Y, Hino K, Murawaki Y. A series of microRNA in the chromosome 14q32.2 maternally imprinted region related to progression of non-alcoholic fatty liver disease in a mouse model. *PLoS One* 2016;11:e0154676.

Non-alcoholic fatty liver disease (NAFLD) is a liver disease related to metabolic syndrome with rising socio-economic impact worldwide. NAFLD is defined by significant lipid deposition in hepatocytes that is unrelated to alcohol consumption. This high prevalence of liver disease occurs after a protracted inflammatory status caused by insulin resistance derived from high consumption of fructose-rich goods^[1] as shown by the multi-parallel hit theory.^[2,3]

NAFLD is currently classified in simple steatosis (SS) and non alcoholic steatohepatitis (NASH). NAFLD is a benign condition without histological signs of inflammation and could be reversed by change of life style, recovering from hyperinsulinism and the metabolic syndrome. However, a protracted inflammation and elevated serum transaminases determine a severe stage of disease, so called NASH, that affects the liver irreversibly leading to liver fibrosis, cirrhosis and cancer.^[4]

MicroRNAs (miRNAs) represent one of the key regulators of epigenetic modifications. They are normally expressed in clusters and their mature forms are able to combine together with proteins and form the RNA-inducing silencing complex (RISC).^[5] Once the RISC is formed, miRNAs bind the high

affinity mature mRNAs forming a double RNA sequence. The duplex impedes the translational machinery and stabilizes the mRNA or promote its degradation.

So the exact role exerted by miRNAs is based on the inhibition of gene products expression.^[6] This fine regulatory mechanism is responsible of several cellular processes and can be altered in several diseases including NAFLD.^[7-10]

Okamoto *et al.*^[11] present an outstanding study concerning a broad range analysis of miRNAs characterizing NAFLD mouse model and serum from patients affected by this disease.

They performed a microarray in order to identify the expression variation of miRNAs and their possible identification with NAFLD. The data obtained in the closest mouse model for NAFLD fatty liver shionogi ob/ob characterized by mice bearing a spontaneous obesity mutation of the leptin gene (Lepob, commonly known as ob) were processed for similarity with human expressed miRNAs.


Interestingly, analysis of similarity conservation of miRNAs between rodent and human confirmed the expression of the same miRNAs in patient affected by SS and NASH. These miRNAs were identified at the maternally imprinted region (mat) of the chromosome 14q32.2.

Seven miRNAs were identified as markers for NAFLD, especially for NASH, all belonging to the Dlk1-Dio3 mat cluster.

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Here, we highlight the importance that have these miRNAs as repressor of factors coordinating the cell fate by triggering pro-death mechanisms e.g. apoptosis and autophagy.

In particular, the authors reported that AMP-activated protein kinase (AMPK) is a target of the majority of the identified miRNAs. AMPK is responsible of metabolic processes as mentioned by the authors, thus conferring it also a key role during autophagy.^[12] In particular, AMPK is responsible of ULK1 (serine/threonine-protein kinase) phosphorylation with consequent mammalian target of rapamycin complex (mTORC) inhibition and autophagy activation during nutrient starvation.^[12] Autophagy represents a fine regulated mechanism to overcome cellular stress and promote cell death in case of protracted cellular stress. It has been shown that its modulation can be a promising target for cancer therapy in liver cancer.^[13] The expression of miRNAs repressing autophagy regulators like AMPK could highlight the variations occurring at epigenetic level conferring to cells an altered metabolism that irreversibly modifies the liver cells and tissue. These alterations could be responsible to trigger further pathological cellular features leading to cirrhosis and furthermore liver carcinogenesis.^[14,15] For this reason it will be interesting to further focus on the expression of the miRNAs localized at mat 14q32.2 in patients affected by cirrhosis and liver cancer, as it has been already shown for other miRNAs in liver cancer cells and thyroid cancer.^[16-18] The miRNAs discovered in this study can represent valid targets for the diagnosis of NAFLD and could be furthermore adopted as biomarkers for patients affected by cirrhosis and liver cancer.

Finally, inhibition of mTORC by the use of biguanides (metformin),^[19] a well known mTOR inhibitors currently used for the treatment of type 2 diabetes,^[20] could represent a therapeutic target for NASH^[21] in a translational setting defining mTORC as a major target of NASH related miRNA.

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Diet and nutrition therapy in pre-liver transplant patients

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ABSTRACT

Malnutrition is universally prevalent among pre-liver transplantation patients. Malnutrition among cirrhotic patients had been associated to increased morbidity and mortality rates. Also, severely malnourished patients before the transplant surgery have a higher rate of complications and a decreased overall survival rate after liver transplantation. In light of the high incidence of malnutrition and associated complications, it is essential to initiate treatment as early as it is assessed. This review addresses the aetiologies of malnutrition and appropriate treatment strategies to correct it in pre-liver transplant phase. Treatment should focus on maintaining nutrient intake and correcting various nutritional deficiencies. The dietician plays an integral role as part of the transplant team by providing appropriate nutrition therapy for solving various nutrition problems.

Key words: End stage liver disease; liver transplantation; pre-liver transplant

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INTRODUCTION

Liver transplantation (LT) revolutionized the management of liver disease. LT is the only option for those with end stage liver disease (ESLD).^[1] According to Institute of Health Metrics and Evaluation of Global Burden of Disease, deaths from cirrhosis in all age groups is ranked 12th globally and 19th in South Asia in 1990 and was ranked 12th globally and 11th in South Asia in the year 2010. Hence, an increasing death from cirrhosis is seen in South Asia over a period of time.^[2]

METABOLIC CHANGES IN ESLD

Various metabolic changes that occur in ESLD patients are presented in Table 1^[3-7] which affect the nutrition state of pre-LT recipients. These factors are inadequate dietary intake, increased intestinal protein losses, malabsorption, low protein synthesis, hypermetabolism and disturbed substrate utilization.^[8,9]

CONSEQUENCES OF PREOPERATIVE MALNUTRITION ON LIVER TRANSPLANTATION OUTCOME

Survival in cirrhosis decreases according to the severity of malnutrition.^[10,11] Preoperative hypermetabolism and body cell mass depletion was proven to be better predictors of the outcome of LT than the traditional Child-Pugh score.^[12] Undernutrition may induce an exaggerated cytokine response favouring postoperative systemic inflammatory response syndrome and multi-organ failure in these patients.^[13] Zinc deficiency is a precipitating factor for hepatic encephalopathy.^[14] Deficiencies of water-soluble vitamins (B and C) and fat soluble vitamins (A, E, D, and K) may occur in patients with cirrhosis which increases the susceptibility of cell membranes to lipid peroxidation.^[8] Low retinol levels leads to an increased risk of developing hepatocellular carcinoma.^[15]

Hence, careful nutritional assessment of candidates for LT is very important because the nutritional status of these patients may ultimately influence morbidity and mortality. Unfortunately, no gold standard exists to determine the extent of malnutrition in this population.^[16] An suitable nutritional

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Table 1: Metabolic changes in ESLD for liver transplant candidates^[5-7]

| Metabolic changes | Abnormalities |
|--------------------|---|
| Glucose metabolism | Insulin resistance; depleted hepatic glycogen stores; fat is utilized as the main substrate for energy, increased gluconeogenesis, lipid oxidation and protein catabolism |
| Protein metabolism | Increased protein catabolism; amino acid metabolism alterations; imbalance of BCAA and aromatic amino acids |
| Lipid metabolism | Polyunsaturated fatty acids deficiency; deficiency of essential fatty acid and long-chain polyunsaturated fatty acids |

ESLD: end stage liver disease; BCAA: branched-chain amino acids

Table 2: Formula for REE^[18,19]

| Gender | Formula |
|-------------|--|
| For males | REE (kcal) = $66 + 13.7 \times W$ (kg) + $5 \times H$ (cm) - $6.8 \times A$ (years) |
| For females | REE (kcal) = $655 + 9.6 \times W$ (kg) + $(1.7 \times H$ (cm) - $4.7 \times A$ (years) |

REE: resting energy expenditure

assessment can include combination of nutrition tools like anthropometry, body composition analysis, subjective global assessment, and hand grip strength to formulate a composite score for assessment of malnutrition.^[17]

NUTRITION TREATMENT FOR PRE-LIVER TRANSPLANT PATIENTS

The goals of nutritional therapy are to improve protein energy malnutrition and correct nutrient deficiencies. This can be accomplished by meeting nutrient requirements.

Energy requirement

When energy expenditure is related to lean body mass, patients with advanced liver disease have increased resting energy expenditure (REE).^[18,19] Despite the usually offsetting errors of excess total body water in estimation of REE from the Harris-Benedict equation [Table 2],^[18,19] it is still considered useful to measure the REE by way of indirect calorimetry in some patients with severe liver disease. Increased REE (hypermetabolic) was found over controls in patients with cirrhosis. But this is not a uniform finding since hypometabolism as well as normometabolism have been observed in patients with cirrhosis.^[19-21] When related to predicted energy expenditure among stable cirrhotics, a subgroup of 15-20% may be considered as hypermetabolic, 25-30% as hypometabolic and the large majority as normometabolic.^[21] Increased REE has also been observed during complications of liver disease, such as acute hepatic failure,^[18] high volume ascites,^[22] or presence of hepatocellular carcinoma.^[23] Measurements of total energy expenditure in patients with cirrhosis indicate that the 24 h energy requirement of cirrhosis patients amounts to about 130% of the basal metabolic rate (BMR).^[24] Diet-induced

Table 3: Nutrition in chronic liver disease-recommendations 1997^[45]

| Clinical condition | Non-protein energy (kcal/kg per day) | Protein or amino acid (g/kg per day) |
|-----------------------|--------------------------------------|---|
| Compensated cirrhosis | 25-35 | 1.0-1.2 |
| Complications | | |
| Inadequate intake | 35-40 | 1.5 |
| Malnutrition | | |
| Encephalopathy I-II | 25-35 | Transiently 0.5, then 1.0-1.5 if protein intolerant: vegetable protein or BCAA supplement |
| Encephalopathy III-IV | 25-35 | 0.5-1.2 BCAA-enriched solution |

BCAA: branched-chain amino acids

thermogenesis^[25,26] and the energy cost of defined physical activity in stable cirrhosis patients^[27,28] and it also shows no deviation from values obtained in healthy patients. The spontaneous physical activity level is also low in cirrhotics.^[5,28]

In cirrhotics without ascites, the actual body weight should be used for the calculation of the BMR using Harris and Benedict formulae. In patients with ascites the ideal weight according to body height should be used. In general, non-protein energy provision of $1.3 \times$ REE is sufficient.^[29,30] For most patients, the daily caloric need equals $(1.2-1.4) \times$ REE (25-30 kcal/kg body weight).

Administration of adequate calories is critical for the efficient use of protein sources, particularly when patients are protein restricted. Excess calories particularly from carbohydrate, should be avoided because it promotes hepatic lipogenesis, liver dysfunction and increased carbon dioxide production leading to increased work of breathing.^[31]

For patients with steatorrhea, it is important to limit long-chain fatty acids and increase short-chain and medium-chain fatty acids in the formula. Pancreatic enzymes should be supplemented, especially in patients with alcohol-related cirrhosis.^[32] The serum lipid variables appeared to be more useful indicators of functional liver improvement than the classic liver function tests.^[33]

Protein requirements

In clinical intervention trials proteins were given in amounts of 0.6-1.2 g/kg per day for patients with cirrhosis and severe encephalopathy^[34] and 0.5-1.6 g/kg per day in patients with alcoholic hepatitis with or without low grade encephalopathy.^[35] Patients with stable cirrhosis appear to have increased protein requirements of 1.2 g/kg per day to maintain nitrogen homeostasis as opposed to 0.8 g/kg per day in normal individuals.^[36] The reasons for this phenomenon are not yet clear, but the increased protein requirement seems to be due to increased whole body protein degradation which may be due to low plasma levels of insulin-like

Table 4: Nutrition recommendations for a liver transplant candidate^[58-63]

| Nutrient | General recommendations |
|---------------|---|
| Calories | Energy needs vary with each individual; 30-35 kcal/kg dry weight for maintenance; 35-40 kcal/kg dry weight for malnourished patients; 25-35 kcal/kg dry weight for hepatic encephalopathy; 150-175% of predicted basal energy expenditure (calculated on dry weight) |
| Proteins | 0.8-1.0 g/kg dry weight in compensated liver disease; 1.5-2.0 g/kg dry weight in decompensated liver disease; 0.6-1.0 g/kg dry weight for hepatic encephalopathy, BCAA-enriched formulas |
| Fats | 25-40% of calories, moderate amounts of medium chain triglycerides oil when steatorrhea present |
| Carbohydrates | Restrict simple carbohydrate if glucose intolerance is present |
| Sodium | 2-4 g/day depending upon level of fluid retention |
| Fluid | 1,000-1,500 mL/day if fluid retention or hyponatremia is present |
| Vitamins | Fat malabsorption leads to malabsorption of fat-soluble vitamins; vitamin A: liver unable to synthesize retinol-binding protein; vitamin D: decreased biliary excretion of 1,23-dihydroxycholecalciferol; vitamin E: cholestatic liver disease affect vitamin E because it is carried by lipoproteins; B vitamins: excess losses due to alcohol abuse |
| Minerals | Mineral bioavailability, tissue distribution, and toxicity can be affected by decreased liver production of their protein carriers; manganese and copper excretion in bile affected by an interruption in enterohepatic circulation; Serum potassium, magnesium, and phosphorus levels may decrease as a result of diuretic administration, refeeding syndrome, malabsorption, or alcoholism; 800-1,200 mg calcium/day |

growth factor (IGF)-1.^[19]

According to Morgan *et al.*^[37] (2006) whole protein formula providing 35-40 kcal/kg per day energy and 1.2-1.5 g/kg per day protein is recommended for enteral feeding. Standard preparation contains approximately 100 kcal, 4 g protein, and 3.5 mmol of sodium and potassium per 100 mL. Concentrated high energy (1.5 kcal/mL) and protein formulas may be preferable in patients with hyponatremia and ascites to regulate fluid balance. This may also improve treatment adherence because less volume needs to be consumed.

A study by Nielsen *et al.*^[38] (1995) showed protein balance in a subgroup of patients did not change protein balance values.

Protein intake increased from 1.0 g/kg per day to 1.8 g/kg per day. With increasing protein intake, 84% of the increase in intake was retained. The rate of protein retention was not saturated at the intakes obtained in this study.

Protein requirement and protein utilization were investigated further by measuring protein synthesis and degradation. In 2 separate studies, patients with cirrhosis of the liver were refed on a balanced diet for an average of 2-4 weeks. Protein and energy intakes were doubled in both studies. Refeeding caused a statistically significant increase of about 30% in protein synthesis in both studies while protein degradation was only slightly affected. The increase in protein synthesis was associated with significant increases in plasma concentrations of total amino acids while insulin, growth hormone, IGF-1 and IGF-3 were not changed significantly. The results indicate that the efficient protein utilization is due to increased protein synthesis, rather than decreased protein degradation.^[5]

Value of branched-chain amino acids

Branched-chain amino acids (BCAAs) (leucine, isoleucine, valine) are essential amino acids. In cirrhosis, there is a likely reduced total body pool of BCAAs due to reduced lean muscle mass and defective use secondary to hyperinsulinemia.^[39]

BCAAs compete with the serotonin precursor tryptophan for the same amino acid transporter in the blood-brain barrier, and the imbalance between the 2 in cirrhosis influences brain ammonia levels directly or indirectly.^[40] So supplementation with BCAAs may reduce brain uptake of tryptophan and improve encephalopathy.^[41,42] Furthermore, BCAA supplementation by both enteral and parenteral routes of feeding has shown improved in cerebral perfusion by which encephalopathy may get improved but still basic mechanism is unclear. A large multicenter study showed that oral BCAAs given for 1 year improved the Child score, reduced hospital admissions, and prolonged/improved event-free survival.^[43] However, there have been no controlled studies and no mention of the timing of BCAA supplementation in cirrhotic patients.^[44] At 3 months, a significant increase in serum albumin level was observed in patients who were administered with nocturnal BCAAs but not daytime BCAAs. It is hypothesized that BCAAs when consumed in daytime are utilized as calories, whereas nocturnal BCAAs are utilized for protein synthesis.^[39] European Society for Parenteral and Enteral Nutrition (ESPEN) guidelines [Table 3]^[45] recommends use of enteral feed enriched with BCAAs for patients who develop encephalopathy. The use of solutions rich in BCAA and low in aromatic acids and tryptophan in encephalopathy has been proposed.^[46] However, a Cochrane analysis based on 11 trials found no convincing evidence regarding benefit from BCAA. The use of BCAAs remains controversial, and they are not widely available in many centres due to their expense and unpalatability.^[47]

According to ESPEN Guidelines, for a positive effect on liver function and clinical outcome, non-protein energy

Table 5: Major studies recommending use of nutrition supplementation

| Study | Recommendations | Outcomes |
|---|--|--|
| Bories and Campillo ^[32] (1994) | 40 kcal/kg per day | Protein and energy intakes were significantly higher; improved nutritional status; improved biochemical parameters |
| Hirsch <i>et al.</i> ^[81] (1993) | 1,000 kcal and 35 g of nitrogen/day for 1 year | Need for hospitalization was significantly lower in the supplemented; reduction of infectious complications; a lower mortality in the therapeutic group |
| Mendenhall <i>et al.</i> ^[82] (1993) | > 2,500 kcal/day | 51% mortality in severe malnourished patients with inadequate caloric intake; 19% mortality in patients who received adequate oral nutrition |
| Le Cornu <i>et al.</i> ^[83] (2000) | Nutritional supplementation to pre-transplant candidates | Did not increase overall dietary energy or protein intake and did not significantly improve post-transplant outcome; regular dietary counselling is as effective in increasing energy intake |
| Kawaguchi <i>et al.</i> ^[84] (2008) | 200-kcal nutritional supplement | Stress scores for physical and mental symptoms were significantly lower compared to those in the fasting group |

was given in amounts of 35-40 kcal/kg per day plus protein up to 1.6 g/kg per day. In patients with encephalopathy, transient protein restriction can be instituted, but after a few days adequate nutrition should be reinstituted. Patients in coma (encephalopathy grade III-IV) can safely be given total parenteral nutrition (TPN) regimens providing 25-30 kcal/kg per day from non protein energy plus 1.0 g/kg per day using BCAA-enriched solutions. Fasting periods should not exceed 6 h due to the limited glycogen stores in malnourished cirrhotic patients. Generally, the oral or enteral routes are preferred. Parenteral nutrition should only be used when enteral feeding is not possible or impracticable [Table 3].^[45]

Micronutrients requirements

Micronutrient deficiency has been observed in 10-50% of patients with cirrhosis. Multivitamin supplements may be considered in these patients.^[48]

Vitamins

Various vitamins deficiency occurs in LT recipients like folate deficiency is due to a combination of decreased intake, decreased absorption, as well as losses from renal excretion and poor hepatic storage. Supplementation of folate and B12 is crucial in alcoholic hepatitis to protect uninjured hepatocytes and stimulate the repair/replacement of damaged cells [Table 4]. The common recommendation for folate supplementation is 1 mg/day orally.^[48] Vitamin B1 deficiency is linked to primary tissue damage such as alcoholic polyneuropathy and also Wernicke's encephalopathy. Usual supplementation is 100 mg/day orally or subcutaneously initially for 2 weeks or until repleted, the amount in a standard multivitamin should be sufficient.^[48] Deficiency of vitamin B6 (pyridoxine) is due to decreased intake or altered metabolism and storage. Standard supplementation is 50-100 mg/day orally, or more in severely depleted individuals.^[49] Liver stores are often depleted even in the setting of normal

serum levels.^[50] Hypovitaminosis A has been linked to night blindness, impairment in immune function, and also to an increased risk of hepatic fibrosis [Table 4]. If malabsorption is suspected as a prime contributor to depletion, doses of 25,000-50,000 IU 3 times per week may be needed for repletion. Vitamin A supplementation improves the sense of taste and thereby may also improve dietary intake of the patients.^[14] Inadequate intake of calcium and vitamin D and losses from malabsorption and renal excretion are related to lower serum levels of albumin and magnesium.^[49] If the individual is unable to increase dietary intake to a consistent, adequate level of 1,000-1,500 mg/day, supplementation should be initiated, especially in those with suspected low bone mineral density. Osteoporosis has been confirmed in 17-23% of patients with liver disease. The role of vitamin D and calcium on bone mass in the setting of liver disease is unclear.^[51] Serum levels should be monitored in 3 months to assess tolerance and success of repletion. Low serum levels of vitamin D are thought to be the result of poor dietary intake, malabsorption from cholestasis, pancreatic insufficiency, and decreased sunlight exposure.^[52] Supplementation usually begins at 400 IU per day, with some patients requiring up to 800 IU per day of vitamin D or 12,000-50,000 IU per day of ergocalciferol, with serum levels reassessed in 2-3 months.^[53] Serum vitamin E levels are typically decreased in alcoholic patients, pancreatitis or fat malabsorption [Table 4]. A dose of 400 IU per day either as standard vitamin E or as α -tocopherol, if malabsorption is suspected, should provide for adequate supplementation in most individuals.^[49]

Minerals

During the pre-LT phase patients suffer from various mineral deficiencies because of metabolic changes due to liver impairment. Zinc deficiency is very common in cirrhotics.^[54] Zinc supplementation may also be used for those patients with hepatic encephalopathy, with refractory response to vitamin A supplementation for night blindness, and

Table 6: Guidelines for pre-transplant nutrition support^[31,37,63,89,90]

| ESPEN Guidelines | Recommendations for nutrition |
|--|--|
| For organ transplantation 2006 | Under nutrition majorly influence outcome after LT; use additional oral nutrition supplementation or even tube feeding; EN improves nutritional status and liver function, reduces the rate of complications, cost and prolongs survival; assess nutritional status regularly |
| For enteral nutrition for liver disease 2006 | Use high-energy formulae in patients with ascites; increased protein requirements; use BCAA-enriched formulae (hepatic encephalopathy); EN and probiotic formula reduces the incidence of infections; hepatic encephalopathy must be treated with lactulose or rifaximin; normal protein diets can be given safely to patients with hepatic encephalopathy; recommended protein supplementation is based on “dry” body weight; recommended to insert fine bore nasogastric tubes in patients with esophageal varices |
| For parenteral nutrition in hepatology 2006 | PN is indicated in unprotected airways, encephalopathy and moderately or severely malnourished cirrhotics; cirrhotics who have to abstain from food temporarily for > 12 h should be given i.v. glucose at 2-3 g/kg per day. When this fasting period lasts longer than 72 h TPN is required; the i.v. provision of all macro- and micronutrients must be ensured from the beginning of PN; carbohydrate should be given as glucose to cover 50-60% of non-protein energy requirements; in case of hyperglycaemia glucose infusion should be reduced to 2-3 g/kg per day and i.v. insulin infusion should be used; lipid should be provided using emulsions, should cover 40-50% of non-protein energy requirements |

ESPEN: European Society for Parenteral and Enteral Nutrition; BCAA: branched-chain amino acids; LT: liver transplantation; EN: enteral nutrition; TPN: total parenteral nutrition

for potential improvement in immune function and taste perception.^[49] Supplementation in the form of 220 mg zinc sulphate is given in 1-3 divided doses per day. Zinc and selenium deficiency has been observed in both alcoholic and non-alcoholic liver disease and may be associated with neurological symptoms.^[55] Depleted serum iron levels, blood losses can cause deficiency in LT patients.^[49] Hepatic iron overload is common and often secondary to increased intestinal iron absorption and transfusions, and may imitate hemochromatosis as well as increase the risk of developing progression of liver disease.^[56] Patients undergoing LT are prone to hypomagnesemia, with potential deleterious effects. A study evaluated the efficacy and safety of routine intraoperative magnesium supplementation to prevent hypomagnesemia. The results^[57] showed lower prevalence of postoperative hypomagnesemia in patients administered magnesium supplementation of 3 g [Table 4]^[58-63] but may not affect the occurrence of arrhythmias.

CHALLENGE IN PRE-TRANSPLANT NUTRITION SUPPORT

Ascites, defined as the accumulation of fluid within the peritoneal cavity as a direct consequence of portal

hypertension, is a common complication of ESLD and associated with a poor prognosis.^[64] The squeal of impaired renal perfusion and fluid volume expansion can precipitate hyponatremia as well.^[65] Spontaneous bacterial peritonitis may develop which is associated with increased mortality.^[66] Nutrition issues may occur in cirrhotics with ascites due to decreased intake from early satiety, increase in REE before paracentesis. Also, imposing dietary restrictions of sodium and fluid reduces the palatability of food.^[22]

The American Association for the Study of Liver Disease practice guidelines (2004), recommend sodium restricted diet and diuretic therapy as the mainstay of treatment for ascites, with their effectiveness demonstrated in about 90% of patients. A dietary sodium restriction of 2 g/day appropriately balances the need for adequate nutrition and fluid status. The reduction in ascitic fluid through careful diuresis can relieve early satiety. A 24-h urinary sodium excretion with a goal of ≥ 78 mEq urinary sodium per day can be measured to follow compliance to a sodium-restricted diet. A fluid restriction is appropriate in cirrhotic patients with dilutional hyponatremia or serum sodium levels < 125 mg/dL [Table 4]. Small, frequent feedings and an adequate intake of protein, in addition to the sodium restriction, are important dietary

measures for the patient. Contraindication of these measures lead to large-volume paracentesis (intravenous albumin) or transjugular intrahepatic portosystemic shunt placement may be necessary.^[66,67]

OTHER NUTRITIONAL FACTORS

Probiotics

Current evidences have shown the advantages of probiotic use in preventing post LT infection, as well as improving the hyperdynamic circulatory state of cirrhosis, hepatic encephalopathy, and Child-Pugh class.^[68,69] Its evaluated that neutrophil phagocytic capacity improved in cirrhotic and hepatic encephalopathy patients after probiotics supplementation which prevents infections by altering gut microbiota, preventing bacterial translocation and decreasing endotoxin levels which leads to the restoration of the immune system.^[70-72] The effect of probiotic mix (8 strains of *Lactobacillus*, *Bifidobacterium* and *Streptococcus*) for 2 months was assessed on portal hypertension, which showed no reduction on hepatic venous pressure gradient or bacterial translocation in patients with compensated or early decompensated cirrhosis.^[73] But, Lata *et al.*^[74] (2007) observed a trend towards decreased endotoxemia and an improvement in Child-Pugh scores (results not statistically significant) with use of the *Escherichia coli* Nissle [(2.5-25) × 10⁹ bacteria in 1 capsula, for 42 days] in 39 cirrhotic patients.

Immunonutrition

The impact of nutritional interventions with immune modulating enteral diets in patients' pre- and post-LT showed possibility of improved preoperative nutritional status of ESLD patients, thus reducing infectious complications after transplantation.^[75] Qiu *et al.*^[76] (2009) investigated the effect of TPN supplemented with alanyl-glutamine dipeptide in cirrhotic patients undergoing LT. Within 9 days, the group supplemented had a significant increase in the prognostic nutrition index and prealbumin levels compared with day 2 levels. It was observed better improvement in aspartate amino transferase and reduced hepatic cell injury compared with the traditional TPN group and a significant decrease in postoperative hospital stay.

Nocturnal meals

A study by Plank *et al.*^[77] (2008) showed the effects of night-time and day time nutritional supplementation over a 12-month period on body protein stores in cirrhotic patients. Significant accretion of total body protein equivalent to about 2 kg of lean tissue was seen in patients having night-time supplementation. In the daytime group, no significant accretion was seen. Confirming this, a classical study showed nocturnal supplementation in cirrhotic patients would improve and prevent catabolic states and under nutrition.^[78]

ROUTES OF FEEDING

Nutrition supplementation

Oral intake, including supplements, is the first line therapy

to prevent and treat malnutrition in liver diseases. The data suggested that by providing medical nutrition therapy, nutrition status may be improved and complications of cirrhosis may be decreased (less hospital admissions, decreased hepatic encephalopathic symptoms, infections, gastrointestinal bleeding, ascites), although the true impact on survival is still unclear.^[79,80] Various studies recommending use of nutrition supplementation for LT patients are depicted in Table 5.^[32,81-84]

Enteral and parenteral nutrition

Studies show an increased dietary intake by oral nutrition, improves in liver function and lower hospital mortality than enteral and parenteral.^[31,85] Most of the well-nourished patients admitted with variceal bleeding and other complications failed to show benefit in nutritional status or disease-related morbidity and mortality. In hospitalized patients with poor dietary intake, enteral nutrition (EN) should be initiated in about 24-48 h of admission.^[86] Hasse *et al.*^[87] demonstrated early enteral feeding benefits like improved nitrogen balance and fewer viral infections after LT.

PN should be used as a second line approach in those who cannot be fed adequately by the oral or enteral route, patients with unprotected airways and advanced hepatic encephalopathy, after visceral surgery in cirrhotics, a lower complication rate was observed when postoperative PN was given instead of just fluid and electrolytes; usually standard amino acid formula is recommended.^[16,88] In a direct comparison between PN and early EN, both strategies proved to be equally effective with regard to the maintenance of nutritional state.^[89]

DISCUSSION

Different mechanisms are known for the nutritional derangement in ESLD patients. These include malabsorption, poor dietary intake, low protein synthesis, higher intestinal protein losses, disturbances in substrate utilization, and hypermetabolism.^[8] Poor dietary intake is one of the major contributors to ESLD malnutrition. Also, various metabolic disturbances like increased REE, insulin resistance, and low respiratory quotient which indicates decreased glucose and increased lipid oxidation which can contribute to nutritional depletion in liver disease.^[9] Early nutrition therapy intervention can improve response to treatment; alleviate symptoms, and quantity of life of ESLD patients.^[90] In this review, medical nutrition therapy goals for pre-LT patients are discussed. Various guidelines have been established for pre-LT nutrition care. ESPEN guidelines for chronic liver disease showed increased calorie and protein requirement in malnourished liver disease patients (30-35 kcal/kg per day and 1.5 g/kg per day).^[45] Also malabsorption of other nutrients increases requirements of other vitamins and minerals like Ca, Mg, vitamin A, B, D, E and complications like ascites recommends use of low sodium diet which can lead to hyponatremia.^[64] ESPEN guidelines for organ transplantation recommends enteral nutrition or oral nutritional supplementation which

can improve nutritional status and survival in severely malnourished ESLD patients.^[31,37,63,90,91] In patients with cirrhosis, enteral feeding improves nutritional status and liver function and reduces the rate of complications and prolongs survival.^[91] Another guideline that is ESPEN Guidelines for enteral nutrition for liver disease recommends use of more concentrated high-energy formulae in patients with ascites, BCAA-enriched formulae in hepatic encephalopathy patients.^[87] Administration of enteral nutrition has been shown to reduce the incidence of viral and bacterial infections.^[69,87] Protein restriction is rarely required for encephalopathy patients, if necessary, usually for not more than 48 h. The recommended protein supplementation should be based on “dry” body weight and may need alteration in edematous patients [Table 6].^[87]

ESPEN guidelines for parenteral nutrition in hepatology recommend indication and timing of PN in cirrhosis. Immediate commencement of PN is recommended in moderately or severely malnourished cirrhotics who cannot be nourished sufficiently by either oral or enteral route or patients who have to abstain from food temporarily (including nocturnal fasting), when this fasting period lasts longer than 72 h total PN is required. Carbohydrate should be given as glucose to cover 50-60% of non-protein energy requirements. PN related hyperglycaemia should be avoided by all means. In case of hyperglycaemia glucose infusion should be reduced to 2-3 g/kg per day and i.v. insulin infusion should be used. Lipids should be provided using emulsions with a content of n-6 unsaturated fatty acids lower than in traditional pure soybean oil emulsions and should cover 40-50% of non-protein energy requirements. PN should be considered in patients with unprotected airways and encephalopathy when cough and swallow reflexes is compromised [Table 6].^[92]

These recommendations clearly portray the need for nutrition intervention among ESLD patients at the earliest to treat the nutrition mediated complications before LT for improved outcomes after the surgery and overall wellbeing of the ESLD patient.

CONCLUSION

Malnutrition is a well-known complication of ESLD and is associated with detrimental consequences if left untreated. It is, therefore, of critical importance to assess the nutritional status of all patients with ESLD and to optimize nutritional support in these patients. Treatment should focus on maintaining adequate protein and caloric intake and correcting nutrient deficiencies. The dietician plays an integral role as part of the transplant team by providing appropriate nutrition therapy for solving various nutrition problems. Strategies include the consumption of frequent small meals and a late evening snack to reduce protein breakdown. When oral intake is insufficient, early implementation of enteral feeding should be considered. The use of BCAAs remains controversial, but the most recent data promote their therapeutic potential.

Malnutrition is a potentially reversible condition that, when identified and treated appropriately, can lead to improved outcomes hence, more nutrition interventions should be planned with motive of attaining positive nutrition balance in patients undergoing LT.

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Role of natural antioxidants in the therapeutic management of hepatocellular carcinoma

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ABSTRACT

Hepatocellular carcinoma (HCC) is a growing health problem in humans. HCC is considered the most common of internal malignancy which cause the death of human, but in the developed Western world, HCC is less common accompanied by increasing essentially in incidence, due to it occurs specially in chronic liver disease. HCC associated with various risk factors including hepatitis B virus infection; hepatitis C virus infection; prolonged aflatoxin exposure; and alcoholic cirrhosis. Overall, one-third of cirrhosis patients will develop HCC during their life time. Also, chemical carcinogens cause tumor promotions through free radical metabolites result in many biochemical and molecular changes that induces oxidative stress. The identify of HCC stage and underlying liver status then choosing the most appropriate line of therapy (surgical, loco regional, radiological and medical) can be improve the survival and/or the quality of life of the patient. Taken into the account of the nutritional value of some natural antioxidant agents that support the function of the body resulting an improvement of the health and protection from different diseases, our review will provide an up-dated status of the different aspects of HCC management through covering the efficacy and the beneficial effects of different natural agents and their mechanism of action against HCC for the future therapy modalities.

Key words: Hepatocellular carcinoma; risk factors; natural antioxidants

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INTRODUCTION

Hepatocellular carcinoma (HCC) incidence is the most common tumor in worldwide.^[1] HCC involves major changes in multiple molecular pathways, genetic and epigenetic factors, which consequently leads to the malignant transformation and HCC progression.^[2] Chronic liver disease and cirrhosis of patients cause HCC. HCC has major risk factors for developing cirrhosis such as, alcoholic consumption, hepatitis B virus (HBV) and hepatitis C virus (HCV) and nonalcoholic steatohepatitis.^[2] Additionally, the contamination of water by chemicals, diabetes, obesity and genetic factors including hemochromatosis, and some physiological disorders act as risk factors for developing HCC.^[3] Cirrhosis is the most dangerous factors

for HCC, especially cirrhosis which caused by hepatitis virus infections.^[4] Therefore, increasing HCC risks occur in the acquired HBV during the childbirth and early childhood.^[5] The patients with HCC present with one or more of several clinical features as weight loss, right upper. HCC causes acute disaster of abdominal by bleeding intra-abdominal or extra hepatic appearance.^[6] Also, patients have HCC with cirrhosis cause palmarerythema, obstructive jaundice, gynecomastia and portal hypertension.^[7,8] HCC is associated with hypoglycemia, erythrocytosis, hypercalcemia, hypercholesterolemia and diarrhea.^[9]

ETIOLOGY OF HCC

The distributions of HCC are largely result from various

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risk factors particularly the majority of hepatitis B and C viral infection and alcoholic liver disease.^[10] Chronic HBV infection cause of HCC in different area, where the virus is largely endemic and vertical transmission common.^[11,12] High alcohol consumption; smoking of cigarette; obesity; and diabetes have also been associated with an increased risk of developing HCC.^[13-15] Previous studies have reported a close correlation with obesity and diabetes and an increased risk of HCC progression.^[16] Also, there are common environmental factor associated with HCC development such as aflatoxin, a product of the *Aspergillus* fungus.^[17] Several physiological disorders of the liver have been implicated in the HCC development, including α -1 antitrypsin deficiency; certain porphyrias; Olchi's disease; and hereditary hemochromatosis, each typically in the setting of cirrhosis.^[18] Additionally, an autoimmune disorders have been implicated in HCC pathogenesis, including primary biliary cirrhosis and autoimmune hepatitis.^[19]

PATHOPHYSIOLOGY OF HCC

HCC majority occurs in the setting of liver cirrhosis. The accumulations of genetic and epigenetic changes related to hepatocarcinogenesis disease are well known. However, the regulating cell cycle and suppressing apoptosis used for maintenance the survival of cancerous cells. Retinoblastoma and *p53* genes responsible for the oncogenes activation and tumor suppressor genes are the good markers that understand the molecular, physiological mechanisms and disorders in the cellular signaling pathways of HCC incidence growing.^[20] When the liver gets injured, necrosis will appear in the liver accompanied by the subsequent hepatocyte proliferation, after continuous cycles of destructive-regenerative process. The hyperplastic nodules will turn into dysplastic nodules inducing a high risk of developing HCC.^[21]

Furthermore HCC well associated with various metabolic changes including biochemical alterations. Alfa-fetoprotein (AFP) is a glycoprotein in serum that was first recognized as a major marker for HCC. AFP elevation indicating to malignant after pathological diagnosis and endodermal lining tumor of the stomach, pancreas, and biliary tree.^[22] Moreover, HCC development has also been associated with plasma lipid and lipoprotein alterations.^[23] This alterations result in cellular dysfunction, reduction in the membrane integrity, fluidity and regulation of cellular processes related to growth and cell survival causing cancer development.^[24,25] The cirrhosis and HCC characterized by a decrease of total protein and impair hepatic function indicating by increasing hepatic enzymes (aspartate aminotransferase, alanine transaminase, alkaline phosphatase, and gamma glutamyl transferase) activity through the loss of functional integrity of the cell membrane in liver resulting liver damage.^[26-28] Furthermore, the development and progression of HCC are well associated with the oxidative stress status that produced by increasing level of reactive oxygen species (ROS) resulting distortion and decrease the antioxidant activity in

the tissues.^[29,30] Lipid peroxidation (LPO) is responsible for formation of many toxic products, such as 4-hydroxynonenal and malondialdehyde MDA which attack cellular targets, thereby inducing carcinogenicity.^[31-33] Many biochemical and molecular changes leads to free radical metabolites causing the chemical carcinogens induce oxidative stress leading to tumor promotion.^[34,35] The failure of antioxidant defense mechanism and tissue damage were enhanced by increasing LPO. Glutathione (GSH) is present in high concentration of liver and widely distributed in cells.^[36] It has many properties as, protects the cell against free radical, peroxides and other toxins, so after decreased of GSH level in tissue causing DNA damage, protein oxidation and LPO of the cell membrane biomolecules lead to hepatocyte damage.^[37] However, the decrease of the antioxidant enzymes activity (superoxide dismutase and catalase) caused the increase of hepatocytes in the cirrhotic livers. The production of cytokines, ROS, and inflammation-mediated events leads to tumor formation.^[38] The inflammatory diseases of cell, is produced by many pro-inflammatory cytokine as $\text{TNF-}\alpha$ and structural cells especially the pathogenesis of asthma.^[39] Liver cirrhosis causes elevated in the pro-inflammatory cytokine $\text{TNF-}\alpha$ as a major marker for inflammatory state in the cirrhotic liver.^[40] HCC has an anti-apoptotic genes expression and rapid cell proliferation,^[41] due to apoptosis resistance under conventional therapies and incomplete cell cycle arrest.^[42] HCC increased apoptosis by the down-regulation of the Bcl2 level, the activation of caspase cascade, and the up-regulation of Bax and the *p53* level.^[43-45] Additionally, HCC contains various histological changes such as: (1) pseudoglandular pattern including gland-like dilatation of the canaliculi in tumor cells; and (2) trabecular pattern of growth.^[46] Cytologically; polygonal and displaying of tumoral hepatocytes; smaller tumor cell; granular eosinophilic cytoplasm; vesicular nuclei; giant tumor cells; and conspicuous nucleoli are associated with HCC.^[46-48]

MANAGEMENT AND PROGNOSIS OF HCC

There is a wide heterogeneity in HCC pattern, patient variations as candidates for recommended treatments, and increasingly complex available therapeutic options with diverse responses to these therapies in clinical practice.^[49] Also HCC is highly associated with variable biologic behavior and the frequent coexistence of chronic liver disease and cirrhosis.^[50] So, it is important to manage HCC patients by multidisciplinary HCC teams including hepatologists; medical and surgical oncologists; transplantation surgeons; diagnostic and interventional radiologists; pathologists; nurses and nurse practitioners.^[51] The most commonly used treatment by the enhancement of latent antitumor immune response through chemotherapy.^[52] Chemotherapy has varying effects, and work is underway in the search for active chemotherapy and appropriate for chemo-embolization, an intensive localized chemotherapy method by using improvement prognosis.^[53] However, chemotherapy still has severe side effects and low survival rates.^[54] As a recent reports, a large number of natural antioxidant extracts

have been suggested to induce beneficial effects on human health and disease control.^[55] The beneficial effects of many medicinal plants may be due to the presence of antioxidative, antibacterial and antimicrobial components. Antioxidants such as flavonoids, phenolic acids and diterpenes can be used to treat the undesirable and harmful action of the free radicals related to various diseases.^[30]

THERAPEUTIC MANAGEMENT OF HCC BY NATURAL ANTIOXIDANTS

Natural agents are alternative therapeutic agents to control different diseases including cancer progression through their antioxidant activity. They stimulate the normal metabolic function in cancer cells and regulate the tumor suppressor genes and immunity. These natural products control the over expression of metabolic enzymes and tumor growth factors in cancer cell.^[56] Also they have the ability to control DNA damaging factors in cancer cells and regulate DNA transcription in tumors. Moreover, they possess numerous therapeutic benefits such as anti-obesity effects; anti-diabetic effects; immune enhancement; and anti-inflammatory effects.^[57] Previous studies recorded that natural extracts, herbs and spices have been used for controlling diseases, including cancer through different mechanisms such as prevention of tumor initiation; delay or arrest of the development of tumors; extension of cancer latency periods; reduction in cancer metastasis and mortality and prevention of recurrence of secondary tumors.^[58,59] Vegetables and fruits rich with polyphenol plays a crucial role in the protection of liver against hepatitis due to its potential activity in the reduction of early pro-inflammatory cytokines, activation of anti-inflammatory IL-10, and inhibition of lipo-polysaccharide induced activation of nuclear factor kappa B (NF- κ B) in hepatocytes.^[60-62] Furthermore, flavonoids are a group of polyphenolic compounds, different in chemical structure and characteristics, naturally founded in plants. They showed versatile health benefits such as anti-inflammatory; antioxidant; anti-proliferative and anticancer activity; free radical scavenging activity; and antihypertensive effects.^[63,64]

Chicory

Chicory (*Cichorium intybus* L.) has been reported in medicine from North Africa to South Asia for several 100 years.^[65] It contains many useful compound such as anthocyanins, vitamins A and C, potassium, calcium, and phosphorus and rich chioric acid.^[66] It act as anti-inflammatory, anti-bacterial agent as well as it has immune-modulatory effects.^[67] Many types of edible plants and vegetables contain high level of chicoric acid.^[68] Chicoric acid have essential properties as antioxidant, antiviral and immunoregulation.^[69] Chicory has a many properties as antioxidant, hepatoprotective, hypoglycemic, diuretic, and anti-testicular toxicity.^[70-73] Also, chicory is a good source for inulin.^[74,75] Inulin is a hepatoprotective compound that prevent of the tissue from demolition by inhibited oxidative degradation of DNA in liver mice.^[74] In addition, inulin has hypolipidemic effect where it

is not affected by digestive enzymes due to it is expected to behave like a soluble fiber.^[76] Moreover it has prebiotic effect by decreasing the activity of growth pathogens and harmful microorganisms as well as increase the activity of growth colonic of beneficial bacteria to the host.^[77,78]

Milk thistle

Milk (*Silybum marianum*) is one of the most famous herbal agents that act as hepato-reno protective agent from 16th century due to it contains approximately 4-6% silymarin and 20-35% fatty acids, particularly linoleic acid.^[79,80] Silymarin composed of both polyphenolic molecules, including flavanolignans (silybin A, silybin B, isosilybin A, isosilybin B, silychristin, isosilychristin, and silydianin) and one flavonoid (taxifolin), silibinin, a semipurified. These components have the beneficial effects, including liver protection and antioxidant, anti-viral, and anti-inflammatory properties.^[81] Silybum is effective in the treatment of liver diseases (cirrhosis, jaundice and hepatitis).^[82] Various studies including *in vitro* and animal research suggest that silybum may have hepatoprotective and antihepatotoxic properties that protect liver cells against toxins through its ability in the reduction of ROS and LPO production, as well as the rebalancing of cellular REDOX status.^[81,83] Moreover its role in inhibition of pro-inflammatory signals, cellular proliferation and expression of survival proteins, resulting a significant protecting the liver.^[81]

Glycyrrhizin

Glycyrrhizin is the active constituent obtained from aqueous extraction of root liquorice (*Glycyrrhiza glabra*). It has been used in traditional medicine to reduce bronchitis, jaundice as well as gastritis. Its major constituents are glycyrrhetic acid; flavonoids; hydroxycoumarins; and beta-sitosterol.^[84] Licorice and their products have been reported to be useful in the treatment of human hepatitis; animal inducible hepatocarcinogenesis; and attenuating titanium dioxide nanoparticles-induced hepatotoxicity.^[85] Glycyrrhizin has pharmacologic roles such as anti-inflammatory; antiviral; antioxidant; immunomodulatory; hepatoprotective and cardioprotective activities through the inhibition of beta-hydroxysteroid dehydrogenase enzyme.^[86] Also it blinded to high mobility group box 1 (HMGB1) directly to suppress HMGB1-induced injury, inhibit toll-like receptor-4 pathway, lower ucLEAR factor- κ B (NF- κ B) concentration and inhibit the production of inflammatory cytokines.^[87,88]

Ginseng

Ginseng (*Panax ginseng*), a valued Chinese and Korean traditional medicinal herb, has been clinically used in China, Europe, United States and North America for thousands of years.^[89-91] Ginseng is one of the well-known medicines in alleviating the development of HCC in chronic hepatitis patients.^[92,93] Ginseng extract has an antioxidant activity due to its ability to scavenge free radicals and suppression of lipid peroxidation.^[94] It has been shown to improve general conditions and non-specific complaints due to the exhaustive and feverish illness through enhancement of

natural healing power. Also, it has been shown to prevent cancer development and inhibit carcinogenesis in several organs.^[95,96] Recent studies suggested the efficacy of ginseng extract to induce apoptosis so it has antitumor activity.^[97-99]

Dandelion

Dandelion (*Taraxacum officinale*) has been used in traditional medicine in the treatment of inflammation and several diseases including cancer.^[100] It has anti-rheumatic and anti-inflammatory.^[101-104] Moreover it has antioxidant properties as well as hepatoprotective activity and success in promotion of liver detoxification and support kidney function. All of these beneficial effects may be attributed to their several flavonoids including caffeic acid; chlorogenic acid; luteolin; and luteolin-7-glucoside.^[105] Additionally it is a rich source of vitamins A, B complex, C, and D, as well as minerals such as iron, potassium, and zinc.^[106-109] Dandelion extract showed a protective effect against membrane fragility consequently, and minimizing the leakage of liver enzymes into the blood circulation and suppressed the production of tumor necrosis factor (TNF)- α by inhibiting interleukin-1 production.^[107,110] Also it has been shown to have stronger free radicals scavenging activity due to its high polyphenol content.^[101]

Garlic

Garlic (*Allium sativum*) has been widely used as a food stuff and a traditional medicine throughout the world. Garlic is available in different forms such as powder or garlic oil. Garlic has a beneficial value such as anti-atherosclerotic, antihypertensive, antimicrobial, anticancer, immunomodulatory, antioxidant, and radioprotector effects.^[111] These beneficial effects may be attributed to its components including organo-sulfur compounds such as diallyl sulfide; diallyl disulfide; diallyl trisulfide; S-allylcysteine (SAC) and S-allylmercaptocysteine.^[112] Several studies evidences have proved that SAC is an anti-tumor agent against different human cancers such as prostate,^[113] breast,^[114] oral,^[115] neuroblastoma^[116] and non-small-cell lung carcinoma.^[117] On the other hand, allicin (diallyl thiosulfonate), which is the main biologically active component of freshly crushed garlic cloves, has anti-hepatocarcinogenic effect through the *p53* gene modulating apoptosis.^[118] Garlic oils have capable for promotion apoptotic signaling as evidenced by the upregulation of Bax and Caspase-3.^[119] Also, garlic oil exhibited potent antioxidant capacity were it reduced the generation of ROS, suggesting that the different mechanisms of hepatocarcinoma prevention.^[120] Additionally, the major role of garlic extract rich in SAC against hepatocarcinogenesis as well as organs tumors may be due to its effect on inhibition of proliferation, induction of apoptosis and suppression of invasion and adhesion.^[115,121]

Curcumin

Curcumin (*Curcuma longa*) is turmeric spice derived from the rhizome of the East Indian plant *Curcuma longa*.^[122] It is a polyphenol contains a class of compounds such as curcuminoids, demethoxycurcumin and

bisdemethoxycurcumin.^[123] Turmeric has also been widely used in medicine for cardioprotective, hepatoprotective, carcinoprotective, and neuroprotective in addition it acts as anti-oxidant, antiseptic, analgesic, antimalarial and anti-inflammatory.^[124] Curcumin rich in curcuminoids are known to inhibit oxidation owing to their methoxy group, 1, 3 B-diketone moiety and phenolic hydroxyl group so it can decrease the free radicals generation, which it was an important step in tumor formation.^[125] Curcumin was found to inhibit NF- κ B, which activates inflammatory cytokines and chemokines, leading to several inflammatory conditions.^[126] NF- κ B activation promotes cellular proliferation, angiogenesis, and invasion and inhibits apoptosis.^[127] In addition, curcumin also inhibits IL1, IL1B, IL6, IL8, tumor necrosis alpha, and cyclooxygenase pathways.^[128] Several studies have supported curcumin's antioxidant and anti-inflammatory, particularly in HCC in addition to its ability to control the cellular signal transduction pathways pertinent to growth, differentiation, and malignant transformation.^[129,130]

Thyme

Thyme (*Thymus vulgaris*) is widely used in folk medicine for the treatment of a variety of diseases including gastroenteric and bronchopulmonary disorders specially in almost everywhere in the world. It is effective as anthelmintic, antispasmodic, carminative, sedative, diaphoretic, antimicrobial, antioxidant, and antifungal agents because its contents of essential oils and anti-oxidative phenolic compounds [geraniol (G), α -terpineol (A), thuyanol-4 (U), linalool (L), carvacrol (C), and thymol (T)].^[131,132] The volatile oils of thyme has been shown to exhibit anti-microbial, anti-mutagenic, anti-platelet, analgesic, anti-inflammatory, anti-angiogenic, anti-oxidant, anti-elastase, insecticidal, anti-parasitic, cell-protective, and anti-tumor activity.^[133,134] Recently, the various studies showed that the beneficial effects of thyme was based on the activation of the apoptosis response, including reduction in mitochondrial membrane capacity and Bcl-2/Bax ratio as well as elevation in cytochrome release from mitochondria and caspase activity. Furthermore, it increases the cleavage of PARP and fragmentation of DNA, which belong to the mitochondrial pathway of the apoptosis pathway.^[135]

CONCLUSION

In conclusion, HCC is the common malignancy of the liver that considered one of death reasons in the worldwide. Chronic infection of HBV and HCV and subsequent liver injury regeneration cycle are considered a major etiology of HCC. HCC is well accepting for multi-drug resistance and not response to current chemotherapeutic agents. Nowadays, no single or combined chemotherapy regimen has been found yet to be effective in HCC. Traditional medicine, especially the herbal medicine plays a vital role in the management of various liver disorders. In this era of science and technology the demand for therapeutic drugs from natural products is increasing day by day, due to their effective therapeutic action and lack of side effects. Recent

studies have shown that medicinal herbs and natural agents rich in antioxidants and other safety micronutrients protect against hepatic dysfunction, carcinogenesis, mutagenesis, DNA-damage and LPO. The greatly positive effect of natural antioxidants on membrane stabilizing by mechanisms that include up-regulation of the key apoptotic regulators, modulate cell cycle arrest and improvement of DNA content by the free radical scavenging, the antimutagenic and antioxidant properties. Thus, it was recommended that the supplementation with edible natural agents may help in safe application of cancer technology in medicine as well as in many other aspects of nowadays life.

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Efficacy of sorafenib therapy in patients with advanced hepatocellular carcinoma in Indian population

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ABSTRACT

Aim: Hepatocellular carcinoma (HCC) is the fourth most common type of cancer and the third leading cause of cancer-related mortality. Sorafenib is an oral multikinase inhibitor that is used for unresectable advanced HCC. It is only approved systemic therapy for advanced HCC. **Methods:** A retrospective prospective study conducted in a multispeciality hospital with 50 patients who received sorafenib. The primary outcome of the study was to find out the survival rate of patients treated with sorafenib. The secondary outcome of the study was to explore the efficacy and safety of sorafenib in a progression of HCC. **Results:** The median overall survival in the Indian population was found as 114 days (3.8 months) after sorafenib therapy. The efficacy of the drug sorafenib was assessed by the survival days which were based on the changes in laboratory values such as haematological and clinical biochemistry. The adverse drug reaction documented in this study was vomiting, abdominal pain; fatigue; anorexia; hyperbilirubinemia; diarrhoea; hand-foot syndrome; rash; rectal bleeding; insomnia; constipation; thrombocytopenia and abdominal discomfort. **Conclusion:** Sorafenib improves the overall survival of the patients with advanced HCC in Indian population up to 3.8 months. It is a safe and effective treatment for patients with advanced HCC in Indian population. The survival of patients was found to be depended on the liver function.

Key words: Hepatocellular carcinoma; sorafenib; Indian population

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fourth most common type of cancer and the third leading cause of cancer-related mortality.^[1] Approximately 4% of new cases diagnosed worldwide. About 782,000 new cases are diagnosed in 2012. In India, the age-adjusted incidence rate of HCC for men ranges from 0.7-7.5 and for women 0.2-2.2 per 100,000 of population per year. The male: female ratio for HCC in India is 4:1. The age-standardized mortality rate for HCC in India for men is 6.8/100,000 and for women is 5.1/100,000. The incidence of HCC is increasing in India. India is one of the developing countries among the worldwide and the incidence of HCC is being increased in

the current decades.^[2] The main cause of HCC is cirrhosis, hepatitis B virus infection, chronic hepatitis C virus infection, and alcohol abuse and aflatoxin exposure. Other risk factors include non-alcoholic steatohepatitis, alcohol abuse, obesity, fatty liver, hemochromatosis, Wilson's disease; type 2 diabetes mellitus, haemophilia alpha 1 antitrypsin deficiency, autoimmune hepatitis, smoking and tobacco, and diabetes.^[3] Advanced HCC is the multinodular/unresectable HCC, HCC with extrahepatic spread or HCC with vascular invasion.^[4]

The treatment options for HCC include surgical resection, liver transplantation, transarterial chemoembolization or

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Table 1: Baseline characteristics along with log rank significance

| Baseline characters | Patients <i>n</i> (%) | Median overall surveillance | 95% CI | <i>P</i> value |
|---------------------|-----------------------|-----------------------------|---------|----------------|
| Age | | | | |
| > 63 years | 19 (46.3%) | 114 | 82-67 | 0.708 |
| < 63 years | 22 (53.7%) | 108 | 100-290 | |
| Gender | | | | |
| Male | 33 (80.5%) | 117 | 41-116 | 0.673 |
| Female | 8 (19.5%) | 108 | 31-77 | |
| Alcohol | | | | |
| Alcoholic | 9 (22%) | 125 | 92-59 | 0.086 |
| Non alcoholic | 32 (78%) | 108 | 95-205 | |
| Smoking | | | | |
| Smoker | 6 (14.6%) | 61 | 10-467 | 0.947 |
| Non smoker | 35 (85.4%) | 117 | 128-248 | |
| Child-Pugh class | | | | |
| B | 23 (56.1%) | 125 | 147-362 | 0.013 |
| C | 18 (43.9%) | 66 | 122-252 | |
| PVT | | | | |
| Present | 19 (46.3%) | 117 | 93-267 | 0.982 |
| Absent | 22 (53.7%) | 108 | 91-289 | |
| Reason | | | | |
| Hepatitis | 13 (31.7%) | 71 | 31-111 | 0.146 |
| Others | 28 (68.3%) | 121 | 83-157 | |

CI: confidence interval; PVT: portal vein thrombosis

Table 2: Adverse drug reaction

| Adverse drug reaction | Number of patients |
|-----------------------|--------------------|
| Anorexia | 10 |
| Abdominal pain | 16 |
| Vomiting | 19 |
| Insomnia | 3 |
| Hyperbilirubinemia | 10 |
| Diarrhoea | 9 |
| Thrombocytopenia | 2 |
| Rectal bleeding | 4 |
| Fatigue | 16 |
| Hand foot syndrome | 6 |
| Constipation | 2 |
| Rash | 6 |
| Abdominal discomfort | 1 |
| Aniemia | 1 |
| Weight loss | 1 |
| Alopecia | 1 |
| Body pain | 2 |

radioembolization, radiofrequency ablation, and sorafenib.^[5]

Sorafenib is an oral multikinase inhibitor that is used for unresectable advanced HCC. It is only approved systemic therapy for advanced HCC.^[6] It affords a modest gain in survival by delaying the progression of HCC and improves the survival of the patient up to 3 to 6 months.

Sorafenib inhibits tumour cell proliferation and tumour angiogenesis and increases the rate of tumour apoptosis. It is made by inhibiting the tyrosine protein kinases (vascular endothelial growth factor receptor 1, 2, 3 and platelet-derived growth factor receptor β). And it also inhibits some intracellular serine/ threonine kinases Raf kinases (Raf 1 and more actively C-Raf than B-Raf).^[7]

But how long a person will survive after the sorafenib

therapy in the Indian population has not been established. Since sorafenib is a costlier therapy, whether the treatment with sorafenib therapy is safe and effective among the Indian population with advanced HCC has not been demonstrated.

METHODS

Patients

This retrospective-prospective observational study was conducted in the Department of Gastroenterology at the multispecialty teaching hospital of P.S.G Medical Science and Research Institute, Coimbatore. Fifty patients who had received sorafenib from January 2009 to December 2014 were collected from the hospital database.

Patient selection was based on patients treated with sorafenib; patients with advanced HCC; and the patient with cirrhosis, hepatitis B and C, steatohepatitis and alcoholic liver disease. Demographic details, disease condition, treatments and adverse drug reaction (ADR) were collected from the hospital database and the Medical Record Department. Survival rate was collected by the phone call. The laboratory investigations such as hematology, serology, microbiology, radiology, computed tomography scan, ultrasound-guided, cytology, urology, biochemistry details of the patients were gathered. Out of 50 patients, 41 patients were recruited for the study.

Treatment

Usually, sorafenib was given as 400 mg bid.^[8] In our patient population mainly three types of dosing regimen were used. They are 400 mg bd, 600 mg daily, and 200 mg bid with good compliance. Dose reduction and treatment regimen were based on the recommendations.

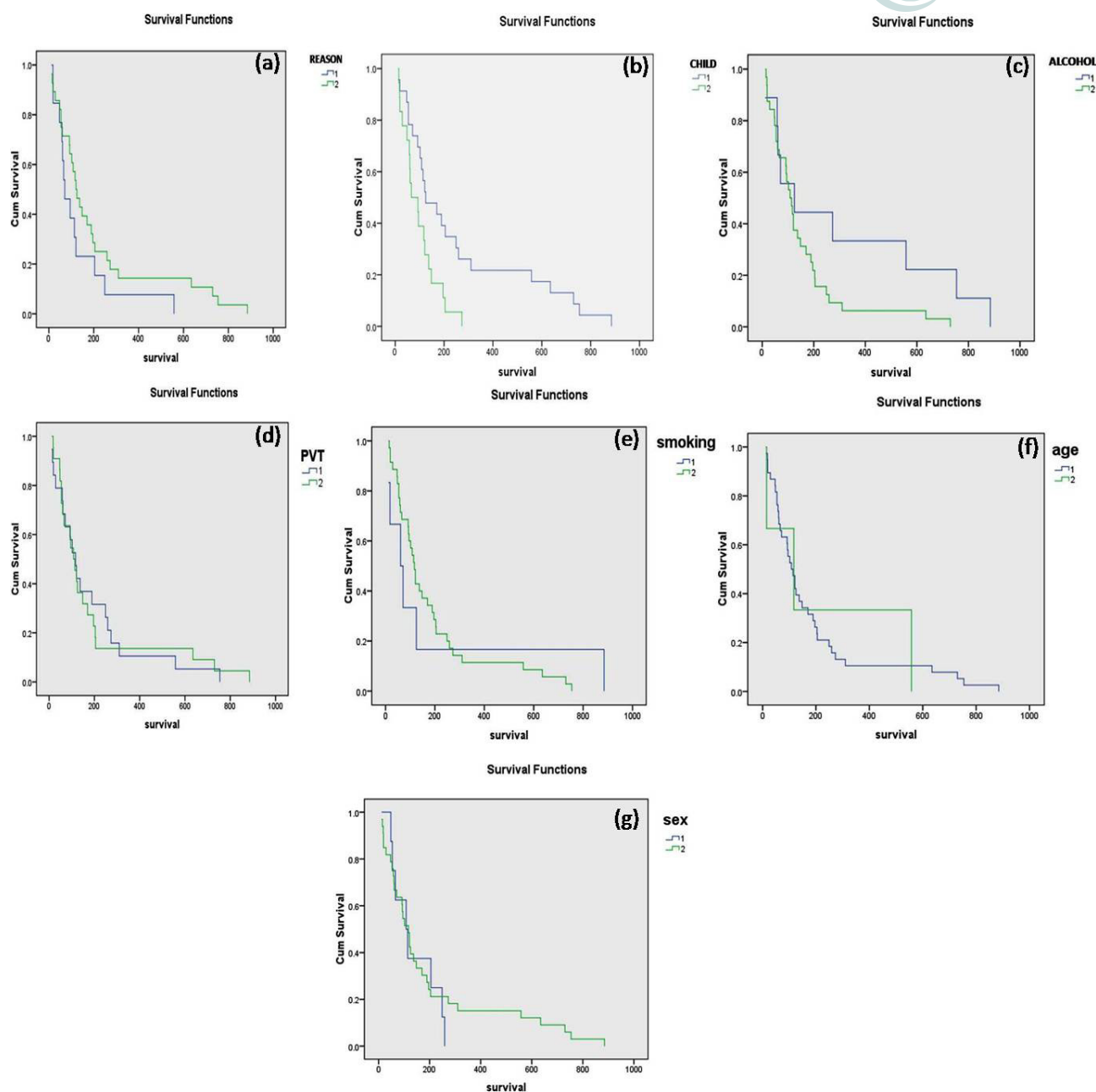


Figure 1: Kaplan-Meier curve. (a) Curve for reasons (1: hepatitis; 2: others); (b) curve for Child-Pugh class (1: class B; 2: class C); (c) curve for alcohol (1: alcoholic; 2: non alcoholic); (d) curve for PVT (1: present; 2: absent); (e) curve for smoking (1: smokers; 2: non smokers); (f) curve for age (1: > 63 years; 2: < 63 years); (g) curve for gender (1: female; 2: male). PVT: portal vein thrombosis

At the end of the study, 7 patients were alive and 34 patients died. The survival days were calculated till April 2015.

Efficacy

The efficacy of the drug was based on the changes in laboratory investigations such as haematological parameters like haemoglobin, platelets, white blood cell (WBC) level as after the therapy and liver function parameters like serum bilirubin, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and serum albumin levels which was monitored after the therapy. Adverse drug reactions were collected from the case files.

Statistical analysis

The primary outcome was the overall mean survival which was calculated for all the patients using Kaplan-Meier method.^[9] All the statistical analysis was done in SPSS software version 16.00. The log-rank analysis was used to determine the significance at the confidence interval was 95%.

RESULTS

Patient's characteristics

A total of 50 patients were found to be treated with sorafenib in our hospital between January 2009 and December 2014. In that, 1 was found to be taken up renal cell carcinoma,

so he/she was excluded from the study, we were unable to contact either 8 since no contact details were available, the rest of 41 patients were included in the study. They were followed up for about 4 months that is January 2015 to April 2015. Median overall survival was 3.8 months, ranging from 12 to 885 days. The baseline characteristics and median overall surveillance are listed in Table 1.

For some patients physicians done ablative therapies like transarterial chemoembolization (TACE) and radiofrequency ablation (RFA) were done, the details regarding that were like as follows, for around 3 patients RFA was done, around 4 were undergone for TACE, for around 2 patients were done with both TACE and RFA. For the rest of their details was not available. Usually, sorafenib was given as 400 mg bid. In our patient population mainly 3 types of dosing regimen were used. They are 400 mg bd, 600 mg daily, and 200 mg bid. They were given to the patients in the following manner 30 (73.17%), 1 (2.44%), and 10 (24.39%) respectively. Among this population due to the ADR, dose reduction was done in 3 patients from 400 mg bid to 200 mg bd, and 2 was ceased and continued. Apart from this, all the patients showed good compliance with the therapy.

Treatment outcome

The overall survival in our population was estimated using the Kaplan-Meier method using statistical software SPSS 16.0 for Windows. The overall survival was found as 114 days. The Kaplan-Meier curve for each criterion was illustrated in Figure 1.

The efficacy of the drug sorafenib was assessed by the changes in laboratory values such as haematological and clinical biochemistry. The haemoglobin level was found to be increased in 34 (82.9%) patients and no changes were observed in 7 (17.1%) patients. The platelet count was found to be raised in 30 (73.2%) patients and no observed changes were found in 11 (26.8%) patients. The WBC count was found to be decreased in 30 (73.2%) and no observed change was found in 11 (26.8%) patients. While looking to clinical liver parameters the bilirubin level was reduced for 19 (46.3%) patients and increased in 22 (53.7%) patients. That may be due to adverse reaction to the drug (hyperbilirubinemia). The albumin level was found to be increased in 26 (63.4%) patients and no observed changes were found in 15 (36.6%). The AST level was found to be decreased in 32 (78%) patients and no observed changes were found in 9 (22%) patients. The ALP level was found to be decreased in 32 (78%) patients and no observed changes were found in 9 (22%) patients. The ALT level was found to be reduced in 33 (80.5%) patients and no observed changes were found in 8 (19.5%) patients. Therefore the efficacy of the drug sorafenib has been proved from the favourable changes in the laboratory values of the patients. ADRs found was illustrated in Table 2.

DISCUSSION

In Indian population, therapy with sorafenib was indicated

in patients with HCC of Barcelona Clinic Liver Cancer (BCLC) stage C. The combination of sorafenib with transplantation or resection, either sequential or concomitant, cannot be recommended outside clinical trials; however, sorafenib can be given for residual/recurrent disease after surgery/transplant/TACE/RFA.^[2]

Køstner *et al.*^[10] conducted a retrospective study with 76 patients in 2011 in Denmark found that patients in performance status 0-1 had a median overall survival of 6.2 months compared to 1.8 months in patients with poorer performance status. Child-Pugh A patients had median overall survival of 6.6 months versus 3.6 months among patients with Child-Pugh B or C.

In this study, we found that the efficacy was depending upon the survival rate which has depended on upon the liver functions which can be determined through liver function test and haematological parameters.

Ji *et al.*^[11] conducted an open-label randomized study with 189 patients with advanced HCC Child-Pugh B or C HCC patients into 2 groups, one with sorafenib and other with best supportive care. Median overall survival was 4 months and 3.5 months in the sorafenib group and best supportive care group respectively. In the sorafenib group, the median performance status and overall survival were significantly longer in patients with BCLC stage B and Child-Pugh class B liver function.

In this study, Child-Pugh B has more median overall survival when compared to Child-Pugh C, 125 days and 66 days respectively. The overall median survival was shown to be similar to the above study that was 3.8 months.

Llovet *et al.*^[12] conducted randomized double-blind placebo-controlled phase 3 trial at 121 centres in 21 countries in Europe, North America, South America, and Australia in 602 patients. The study shows that the median overall survival was 10.7 months in sorafenib group and 7.9 months in the placebo group. The median time to radiological progression was 5.5 months in sorafenib group and 2.8 months in the placebo group.

In this study about 8 patients had good survival (558 days to 885 days, in which sorafenib was highly effective.

Køstner *et al.*^[10] conducted a retrospective study in 2011 in Denmark with 76 patients in that they found that fatigue (68%) was the main ADR followed by anorexia (47%); diarrhoea (42%); rash (33%); nausea (32%); and hand-foot syndrome (28%). Sorafenib is generally tolerable also in more compromised patients as the number and grade of adverse events did not differ significantly between the patients with good versus poor performance status and liver function.

In this study, main ADRs were fatigue, anorexia, diarrhoea, rash, nausea, hand-foot syndrome. Dose reduction was made

to 2 patients in order to overcome the ADR. One patient ceased and continued therapy. Further recommendations were made to overcome the ADR but we have not included that recommendation in our study.

Llovet *et al.*^[12] conducted the sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol study shows that the adverse event is more common in sorafenib group compared to the placebo group, were diarrhoea ($n = 39$), fatigue ($n = 22$), hand-foot syndrome (HFS) ($n = 21$). Sorafenib associated adverse event led to dose reductions and interruptions in a subgroup of patients. The study enrolled 602 patients; this was phase 3 double blind placebo controlled trial.

Expect from the usual adverse drug reactions sorafenib was found as a good choice for treating patients with advanced HCC.

Limitations, all the data were collected retrospectively except survival rate, no computed tomography scan details available so that it was unable to assess the tumour size, less population was involved there were only 41 samples were available, no proper contact details of the patients were available because of that some patients survival days were excluded, lack of follow-up of some patients after treatment.

In conclusion, sorafenib improves the overall survival of the patients with advanced HCC in Indian population. The median overall survival was found to be 114 days (3.8 months). It is a safe and effective treatment for patients with advanced HCC in Indian population. The survival of patients was found to depend on the liver function. The adverse effect was found to be almost same as other countries apart from that we found insomnia in our population. The adverse effects include fatigue, diarrhoea, HFS, hyperbilirubinemia, nausea and vomiting. Finally, we conclude that sorafenib is best oral systemic therapy for advanced HCC in Indian population.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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Hepatocellular carcinoma and type 2 diabetes mellitus: cytokeratin 8/18 expression in hepatocellular carcinoma and glycogen-storing hepatocytes

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Sir,

We have reported two patients with hepatocellular carcinoma (HCC) and type 2 diabetes mellitus (T2DM), who showed abundant glycogen in their liver parenchyma but a marked reduction of glycogen content in HCC.^[1] It was suggested that the latter was associated with appearance of a Warburg type glycolysis^[1] and discussed in some detail.^[2]

Cytokeratins (CKs), the intermediate filament (IF) proteins of epithelia, are sub-divided into type I (CK9-20) and type II (CK1-8) and expressed as type I/II pairs in a cell differentiation manner. In adult liver, hepatocyte IF comprise only CK8/18.^[3] CK8/18 expression in normal and diseased liver has been reported, including positive expression in alcoholic steatohepatitis (ASH) and/or non-alcoholic steatohepatitis (NASH) and HCC.^[3]

We examined the expression of CK8/18 in the liver to investigate cytoskeletal alterations in hepatocytes, possibly related to changes in hepatocellular glycogen content during hepatocarcinogenesis. Our studies revealed that immunoreactivity for CK8/18 was reduced or frequently even negative in glycogen-rich hepatocytes of background liver [Figure 1b and d], but moderately positive in normal hepatocytes and glycogen-poor cells in HCC [Figure 1a, c, e and f]. Overexpression of CK8/18, as Malory Denk bodies, which are hallmark lesions in ASH and NASH,^[3] was not detected [Figure 1b and d]. The

results provide evidence for reduced to negative CK8/18 expression in glycogen-rich hepatocytes.

The mechanism of alteration of CK8/18 expression in glycogen-rich hepatocytes has not been elucidated. Su *et al.*^[4] demonstrated that CK8/18 expression was reduced in excessively glycogen-storing (glycogenotic) clear hepatocytes, which also showed a relative reduction of cytoplasmic organelles as demonstrated by electron-microscopic studies. Given simple CK8/18 expression patterns, hepatocytes are sensitive to alterations of cytokeatin architecture.^[3] Using hepatic cell culture systems, Mathew *et al.*^[5] reported recently that CK8/18 is involved in the interplay between glucose utilization and insulin signaling. The authors demonstrated that insulin stimulates glucose uptake, glucose-6-phosphatase formation, lactate release, and glycogen formation in hepatocytes via the PI-3 kinase dependent signaling pathway, and that CK8/18 IF loss makes them more efficient glycogen producers.^[5] This is in line with the notion that an insulinomimetic effect of oncogenic agents is responsible for the preneoplastic hepatocellular glycogenosis,^[2] which is associated with a reduced or negative expression of CK 8/18 in glycogenotic clear cells appearing in chronic human and woodchuck hepadnaviral infection.^[4] CK8/18 immunohistochemistry may allow distinct recognition of the glycogen-rich hepatocytes as shown in glycogenotic clear cells under various conditions.^[4]

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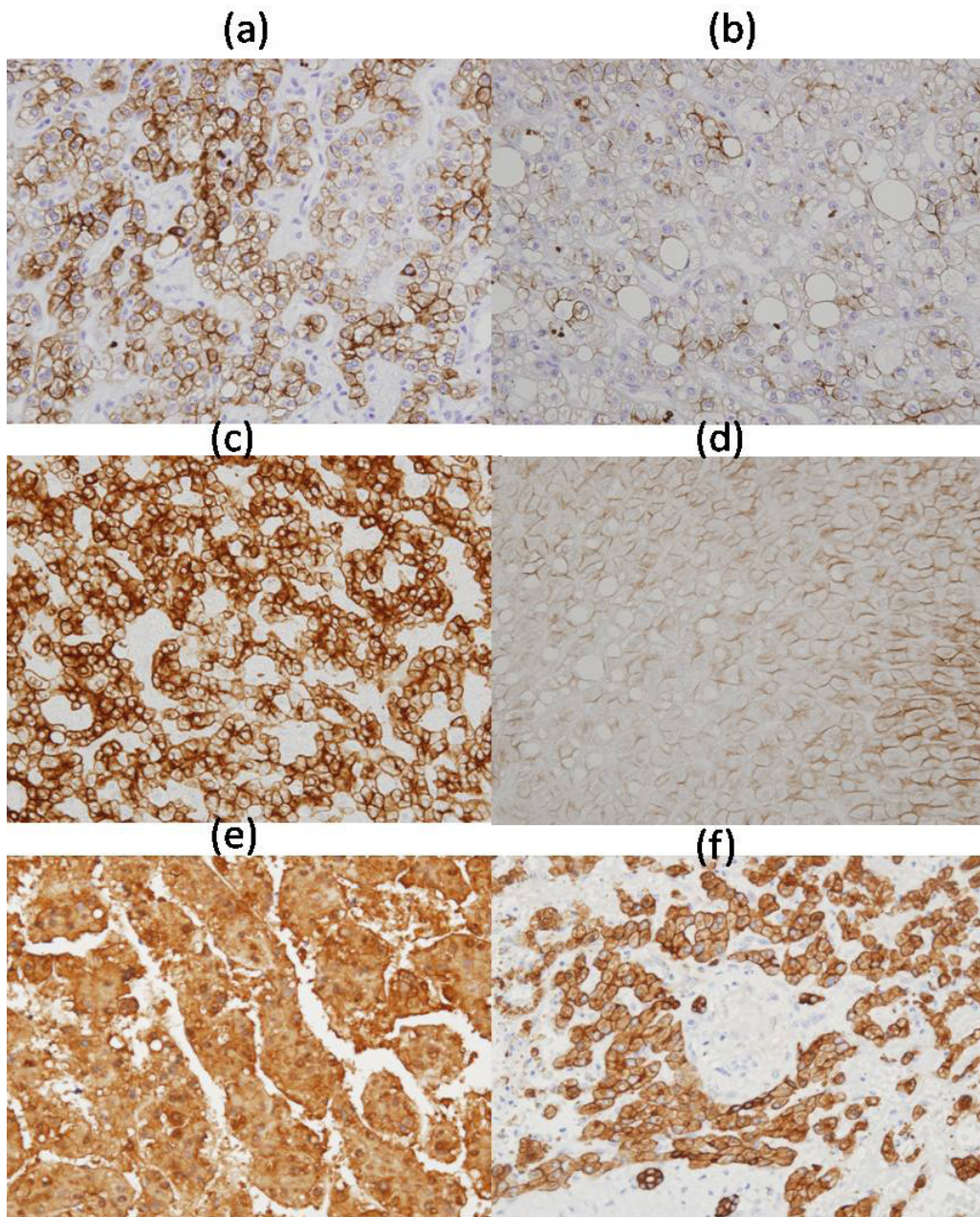


Figure 1: CK8/18 expression in hepatocellular carcinoma (a; case 1, c; case 2, e; control) and background liver (b; case1, d; case 2, f; control), demonstrated with mouse monoclonal antibodies B22.1/B23.1 (Cell Marque, USA) and visualized using the Envision method (Dako) (a-f, $\times 400$). Control (a 79-year-old male, moderately differentiated adenocarcinoma in background of nearly normal liver)

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Conflicts of interest

There are no conflicts of interest.

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Detecting hepatic nodules and identifying feeding arteries of hepatocellular carcinoma: efficacy of cone-beam computed tomography in transcatheter arterial chemoembolization

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ABSTRACT

Aim: To evaluate the effectiveness of using cone-beam computed tomography (CBCT) in transcatheter arterial chemoembolization (TACE) to detect hepatocellular carcinoma (HCC) nodules and their feeding arteries. **Methods:** Twenty-four patients with HCCs who underwent TACE using CBCT in addition to conventional digital subtraction angiography (DSA) were enrolled. After both conventional DSA and CBCT through the hepatic artery were acquired, TACE were performed. The nodules were defined as an HCC when dense accumulation of iodized oil was found within the nodule on CT obtained 2 weeks after the TACE. The number of detected nodules and identified feeding arteries, and their correlations with anatomical locations were assessed. **Results:** A total of 39 HCC nodules (tumor diameter, 7-40 mm; mean, 17.4 ± 7.9 mm) were detected. Thirty-one nodules were detected by DSA alone but 8 nodules were additionally detected by adding CBCT to DSA. There were 53 feeding arteries associated with the 39 HCC nodules. Among these arteries, 21 were identified by DSA alone; however, 47 were identified by combining CBCT with DSA. Additional feeding arteries, especially for the nodules located at the right and caudate lobes, were identified by CBCT. On the other hand, there was no difference in detection of nodules between the anatomical locations by CBCT. **Conclusion:** The use of CBCT in addition to DSA offers potential for increasing the number of detected nodules, and the number of their feeding arteries at the right and caudate lobes. CBCT might improve the quality of TACE procedure for HCC than DSA alone.

Key words: Hepatocellular carcinoma; transcatheter arterial chemoembolization; cone-beam computed tomography; interventional procedure

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INTRODUCTION

Transcatheter arterial chemoembolization (TACE), an endovascular intervention for hepatocellular carcinoma (HCC), is an established treatment procedure performed worldwide along with other therapeutic techniques such as surgical resection and percutaneous treatment. It has many advantages such as less invasiveness, the ability to act on

multiple lesions, and the ability to be utilized on lesions in areas where percutaneous treatment is anatomically difficult to perform. In recent years, treatment with superselective catheterization has become easier, thanks to the advances in angiography devices such as the microcatheter and micro-guide wire. Recent TACE treatment results, such as

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the local suppression rate and survival rate, are reported to have improved compared with previous results.^[1-3]

Previously, endovascular intervention mainly employed conventional cut-film angiography; however, the usage of digital subtraction angiography (DSA) and the interventional radiology features (IVR)-CT system, combining conventional angiography with CT, has now become more prevalent. The IVR-CT system can obtain tomographic images when performing IVR, providing much useful additional information. CT hepatic arteriography and CT arteriportography are recognized as techniques with the highest detection rates in HCC diagnosis.^[4-7] However, because the IVR-CT system obtains information from two separate X-ray imaging devices (DSA and CT equipments), difficulties are often experienced while correlating the two sets of data obtained from the two devices.

Angiography devices equipped with a flat panel detector-based cone-beam CT (CBCT) imaging system can provide three-dimensional and tomographic images on a single X-ray device through rotational CBCT imaging in addition to a conventional two-dimensional DSA image. Furthermore, because of no change in the conventional angiography equipment, no additional space is required for the IVR-CT system.^[8,9]

We hypothesized that a three-dimensional understanding of hepatic artery anatomy and multiple planar reconstruction (MPR) images obtained from arbitrary cross-section may contribute to nodule detection and feeding artery identification by combining CBCT imaging with conventional DSA imaging in endovascular intervention for HCC. In this study, we retrospectively examined the HCC nodule detection and feeding artery identification capabilities of CBCT imaging.

METHODS

Subjects

The subjects were 24 patients (12 males and 12 females) from our facility with clinically suspected HCC who underwent TACE using CBCT in addition to conventional digital subtraction angiography (DSA). The trial period was from October 2006 to January 2008. The patients were aged between 52 and 84 years (average age, 71.2 years). All patients had underlying chronic hepatitis or cirrhosis. In all cases, a dynamic study using multi-row detector computed tomography (MDCT; Aquilion-16 or Aquilion-64; Toshiba, Tokyo, Japan) was conducted within one month before TACE. All patients were clinically diagnosed with HCC by dynamic CT and/or the elevation of tumor markers.

Imaging device

The angiography equipment used in endovascular intervention was AXIOM Artis dBA (Siemens). In addition to conventional two-dimensional DSA imaging, rotation of the detector with the C-arm helped in three-dimensional rotational imaging.

The protocol used for the three-dimensional rotating image was as follows: detector, 30 cm × 38 cm × 154 μm; FOV, LR 22.5 cm × AP 22.5 cm × HF 18 cm; matrix, 1,024 × 1,024; projection, 30 projection/s for 5 s, rotation 200°; dosage, 1.2 μ Gy/pulse; contrast agent, iopamidol solution (150 mg I/mL) (Iopamiron 150; Bayer; Osaka, Japan); infusion rate and duration, 1-2.5 mL/s, 8 s; and delay time, 3 s. Both upper limbs were raised, and imaging was performed with the patients holding their breath. The data obtained were transferred to an X-Leonard workstation (Siemens) and maximum intensity projection (MIP), volume-rendering (VR), and MPR (axial, coronal, and sagittal thickness, 3 mm) images were generated.

Endovascular intervention (TACE)

First, a 3F or 4F sheath was inserted by the percutaneous approach from the groin into the femoral artery, and two-dimensional DSA imaging of the celiac and superior mesenteric arteries was performed using diagnostic catheters. Next, a two-dimensional DSA image of the hepatic artery (any one of the common, proper or replaced hepatic arteries) was obtained, and maintaining the catheter in the same location, CBCT three-dimensional rotating imaging was performed. As mentioned above, the CBCT volume data were processed in the workstation. Using this information, we performed superselective catheterization of the subsegmental branches of the hepatic artery. After confirming tumor staining on the two-dimensional DSA image, a suspension of the chemotherapeutic agent epirubicin (farmorubicin, 10-40 mg; Kyowa Hakko, Tokyo, Japan) and iodized oil (lipiodol, 1-6 mL; Andre Guerbet, Aulnay-sous-Bois, France) was infused arterially. Embolization was performed using a gelatin sponge (Gelpart, 1-10 mg; Nippon Kayaku/Astellas, Tokyo, Japan) in patients with preserved hepatic function (Child-Pugh classification A). In addition, for diagnostic purposes, small amounts of iodized oil were introduced into the subsegmental arterial branches, including those supplying the densely stained tumors. After two weeks of treatment, CT imaging was performed to confirm the presence or absence of iodized oil deposition.

Evaluation

HCC was defined as vascular enhancement on DSA imaging after superselective catheterization and nodular deposition of Lipiodol on CT imaging after treatment. Retrospectively, the study coordinator (Y.U.) reviewed all DSA, CBCT and CT imaging after treatment and recorded the size and location of each HCC on a subsegmental basis. The gold standard of a feeding artery was also based on tumor staining on DSA imaging after superselective catheterization. First, two radiologists engaged in interventional radiology (M.H. and D.K.) evaluated the presence of HCC on DSA from a common, proper or replaced hepatic artery with or without CBCT in a consensus fashion. When a focal vascular enhancement was seen, they diagnosed it as HCC. Next, the identification of a feeding artery was also attempted in subsegmental branch unit. A feeding artery was defined as a vessel continuing with tumor stain and visualized separately

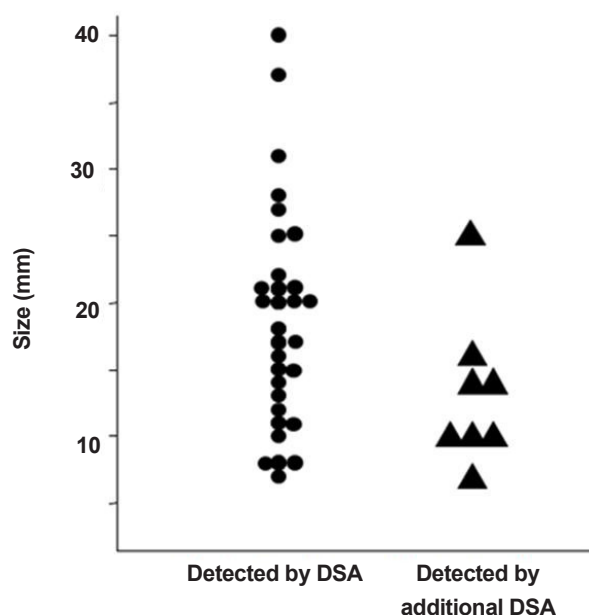


Figure 1: The correlation between detectoin of nodule and its size by DSA and additional CBCT. DSA: digital subtraction angiography; CBCT: cone-beam computed tomography

from other vessels. The number, size and location of HCC nodules detected, and the number and location of feeding arteries identified were compared between DSA images with and without CBCT. Comparison between mean sizes of nodules detected on DSA with and without CBCT was statistically performed using a Student's *t*-test. A *P* value of < 0.05 indicated a statistically significant difference.

RESULTS

A total of 39 HCCs were confirmed by the study coordinator. The size of these nodules on CT ranged from a diameter of 7-40 mm (17.4 ± 7.9 mm), and they were located at S1 ($n = 5$), S2 ($n = 1$), S3 ($n = 3$), S4 ($n = 5$), S5 ($n = 2$), S6 ($n = 5$), S7 ($n = 7$), and S8 ($n = 11$). DSA imaging alone detected 31 nodules, but the additional eight nodules, which were difficult to identify with DSA imaging alone, were detected by combining DSA imaging with CBCT imaging. The diameter of the 31 nodules was 18.4 ± 1.4 mm and that of the additional eight nodules was 13.3 ± 2.3 mm. The *P*-value of Student's *t*-test was 0.09 (> 0.05). No significant difference was observed between the two methods [Figure 1]. The sites of the detected nodules are displayed in Figure 2. A maximum of two extra nodules were detected in any given subsegment with the addition of CBCT imaging, and no bias by location was observed in nodule detection.

Fifty-three feeding arteries were associated with the 39 HCC nodules. Among them, 21 arteries were identified by DSA imaging (angiography from the proximal hepatic artery), however, 26 additional feeding arteries were identified by combined application of DSA and CBCT imaging. The relationship between feeding arteries and sites are displayed in Figure 3. Nodules with an additional number of feeding

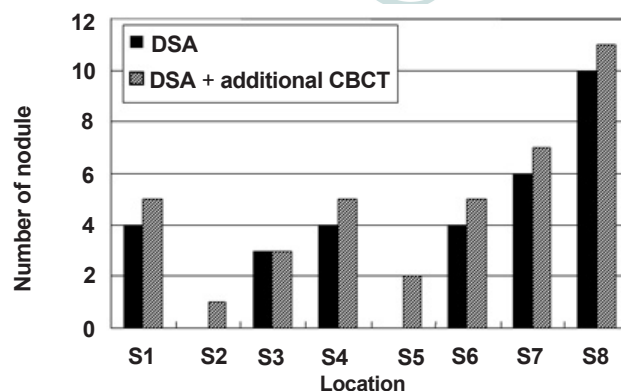


Figure 2: The correlation between detection of nodule and its location by DSA and DSA + CBCT. In any subsegments, extra nodules detected by additional CBCT were less than two. No bias by location was observed in nodule detection. DSA: digital subtraction angiography; CBCT: cone-beam computed tomography

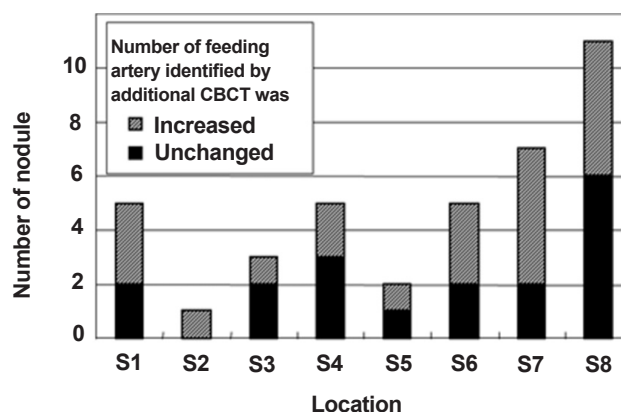


Figure 3: The correlation between identification of feeding artery and its location by additional CBCT. Number of nodules, in which number of their feeding arteries identified by additional CBCT was increased, was more than three in S1, S6, S7 and S8. On the other hand, the number of nodules was one or two in S2, S3, S4 and S5. CBCT: cone-beam computed tomography

arteries were observed in each subsegment, although an obvious increase in feeding arteries was observed in nodules located in the right and caudate hepatic lobes.

DISCUSSION

The CBCT imaging system is a device in which an X-ray radiation beam and a two-dimensional detector are rotated around the subject, and a three-dimensional image is reconstructed from the two-dimensional data. Because a two-dimensional detector is used, axial scanning of the body is not required. Thus, compared to images with conventional X-ray CT, images with a greater axial spatial resolution of the body can be acquired with CBCT imaging. Although the density resolution is inferior to CT, arbitrary tomographic images can be obtained in addition to the three-dimensional images.^[10] In recent years, various IVR procedures using these techniques were reported.^[11-14] In this study, we examined the HCC detection and feeding artery identification capabilities of CBCT imaging in order to clarify its utility in endovascular intervention.

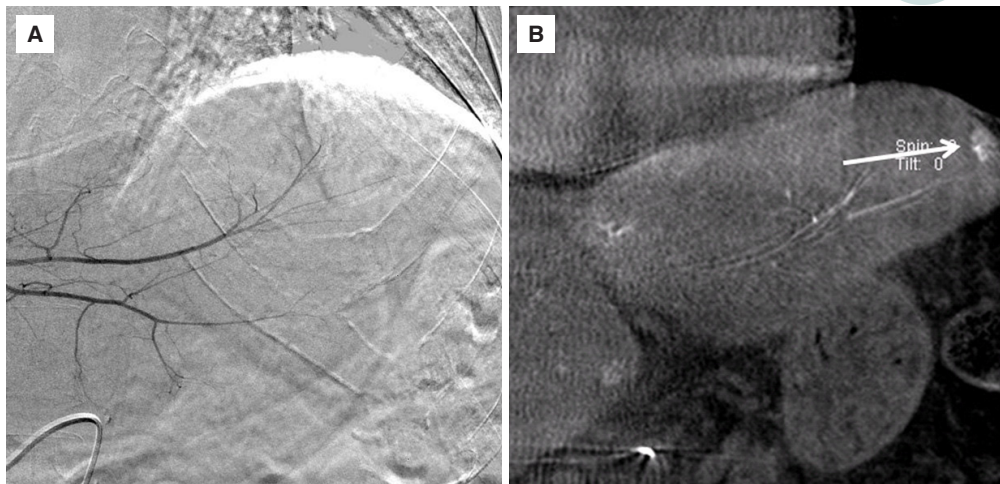


Figure 4: A 64-year-old man with hepatocellular carcinoma in the S2 of the liver. (A) Conventional digital subtraction angiography didn't show tumor stain, affected by heart pulsation; (B) coronal multiple planar reconstruction image of cone-beam computed tomography showed a hypervascular tumor with a diameter of 14 mm in the liver (arrow)

With regard to nodule detection ability, DSA imaging combined with CBCT imaging detected all 39 hepatic nodules, including the eight nodules that were difficult to identify with DSA imaging. Miyayama *et al.*^[15] previously reported that small HCC nodules approximately 1 cm in size, undetected by DSA imaging, were detected with CBCT imaging, thus making TACE possible for such cases. The average nodule diameter in our study was similar to that in the study by Miyayama *et al.*^[15] at 13 mm. In contrast, Kakeda *et al.*^[16] reported that CBCT imaging did not detect tumors but detected feeding arteries. This is believed to be because their evaluation was based only on MIP and VR images, with small or weakly enhanced nodules not being revealed during three-dimensional reconstruction. In this study, evaluation was made using three-dimensional MPR images as well as MIP and VR images. Evaluation with MPR images is considered necessary for the identification of small nodules and nodules with weak enhancement. The advances in diagnostic imaging technology in recent years have made fine dynamic imaging with MDCT and liver-specific MRI contrast agents possible, and the small hepatic nodules identified by these methods are becoming increasingly subjective to endovascular intervention.^[17] It is believed that CBCT imaging could become a useful tool for identification of such small nodules.

No bias by site was observed in nodule detection with CBCT imaging, although Figure 4 clearly shows that CBCT imaging is considered useful in areas where DSA imaging alone is insufficient because of cardiac pulsations and respiratory movements.

With regard to feeding artery identification capabilities, CBCT imaging was superior in identifying feeding arteries associated with nodules located in the right hepatic lobe. This lobe is deep anteroposteriorly, and the right anterior and posterior branches of the hepatic artery often overlap, leading to poor isolation in two-dimensional images of the anteroposterior direction.^[18,19] The addition of CBCT

three-dimensional imaging (MIP and VR images) facilitates the easy isolation of anteroposterior overlapping vessels. A detailed search for feeding arteries associated with the nodules may be conducted if tomographic images (MPR) are used after understanding the general vessel anatomy with three-dimensional imaging [Figure 5]. Another advantage of CBCT imaging is that it helps in freely determining the most favorable working angle to isolate the feeding arteries and nodules that are the target of treatment, using three-dimensional images as a reference. This also makes three-dimensional CBCT images useful in superselective catheterization.

The number of feeding arteries identified by CBCT imaging in caudate lobe nodules has also increased. Endovascular intervention for HCC in the caudate lobe is generally considered technically difficult.^[20] Caudate branches are high in number and variation. They branch off from the proximal side of the right or left hepatic arterial trunks, as well as from the distal branches to the right lobe.^[21,22] These thin branches arising from thick vessels are difficult to identify with two-dimensional DSA imaging, further making selective catheterization difficult. In addition, multiple caudate branches become feeding arteries. With the addition of CBCT imaging in such cases, we can appreciate the detailed anatomical relationship between nodules and caudate branches with three-dimensional and tomographic imaging of the vessel, which is considered useful in selecting an appropriate working angle.

In endovascular intervention for HCC, we place a catheter in the common hepatic artery (proper or replaced hepatic artery) and obtain mapping CBCT images with the purpose of establishing therapeutic objectives. CBCT imaging with selective catheterization is believed to be useful in confirming the territory of the tumor that was stained before arterial infusion and embolization,^[21] although it is not routinely performed at our facility because its utility is unclear. In addition, frequent imaging takes time and effort.

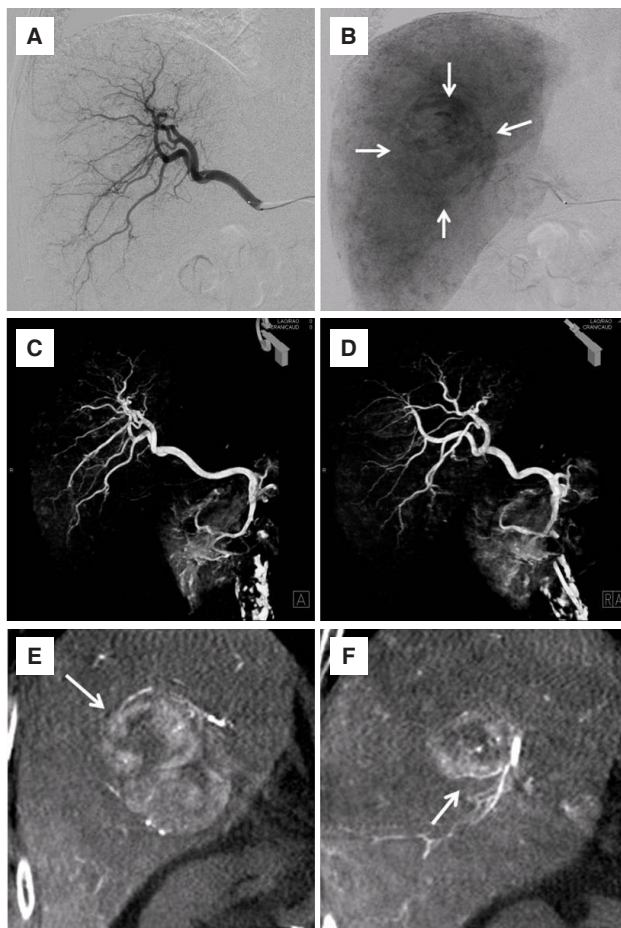


Figure 5: An 84-year-old man with hepatocellular carcinoma in the right lobe of the liver. Conventional digital subtraction angiography of the arterial phase (A) and parenchymal phase (B) showed right hepatic artery branches and tumor stain with a diameter of 40 mm in the right lobe of the liver (arrow). Separation of the right hepatic artery branches was poor. It was difficult to identify the feeding vessel of the tumor. Volume-rendering image of the CBCT in frontal (C) and right oblique (D) view obtained the three-dimensional information in the right hepatic artery and the branches could be separated. Coronal multiple planar reconstruction image of the CBCT (E,F) showed the hypervascular tumor and A7 branches of the right hepatic artery (arrow) as feeding arteries. CBCT: cone-beam computed tomography

CBCT imaging is a technique that provides information useful in endovascular intervention for HCC, although there is adequate scope for debate regarding when and how it should be used. Many cases of endovascular intervention for HCC have been effectively performed solely based on conventional two-dimensional DSA imaging. In addition, the raising of both upper extremities, positioning, and reducing the concentration of contrast medium takes time when performing CBCT imaging, as does processing the three-dimensional images. Even if CBCT imaging is performed, conventional two-dimensional DSA imaging cannot be completely excluded, with the amount of radiation exposure greater than conventional two-dimensional DSA imaging. When these factors are taken into consideration, performing routine CBCT imaging may not yet be suitable for all cases of HCC requiring endovascular intervention.

In our examination, there was increased identification of feeding arteries associated with HCC in the right and caudate hepatic lobes. CBCT imaging is also reported to be useful for detecting HCC of approximately 1 cm in size that were unidentified following DSA imaging. CBCT imaging is considered desirable in such cases.

Advances in technology will simplify CBCT imaging, reduce radiation exposure, and process images faster. If all three-dimensional imaging becomes possible, then three-dimensional treatment using CBCT imaging is expected to become routine practice, replacing the traditional two-dimensional treatment procedure in endovascular intervention for HCC.^[23]

There are some limitations in this study. First, the field of view by CBCT was narrower than DSA. Therefore, CBCT is unable to obtain a complete liver image in some cases. We should set the field of view around the region of interest in the liver. Next, we defined HCC as vascular enhancement on DSA imaging after superselective catheterization and nodular deposition of Lipiodol on CT imaging after treatment. There were no residual HCCs on CT performed two-weeks after treatment. However, we cannot exclude the possibility of very small residual HCCs. Moreover, we did not evaluate the specificity of detection of HCC nodules and their feeding arteries.

In this study, adding CBCT imaging to conventional two-dimensional DSA imaging increased the HCC detection and feeding artery identification capabilities, especially in the right and caudate hepatic lobes. CBCT may be a useful, complementary modality for the endovascular intervention of HCC.

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Conflicts of interest
There are no conflicts of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors. The retrospective design of the study was approved by the Institutional Ethics Committee and the requirement for informed written consent was waived.

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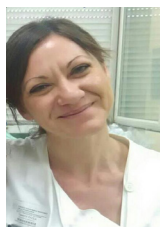
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Comment on “Preliminary outcome of microwave ablation of hepatocellular carcinoma: breaking the 3-cm barrier?”

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Thamtorawat S, Hicks R, Yu J, Siripongsakun S, Lin WC, Raman S, McWilliams JP, Douek M, Bahrami S, Lu DSK. Preliminary outcome of Microwave ablation of hepatocellular carcinoma: breaking the 3-cm barrier? *J Vasc Interv Radiol* 2016;27:623-30.

Nowadays, surgical resection represents the gold standard for the treatment of hepatocellular carcinoma (HCC) in eligible patients, and liver transplantation is considered the best option for selected patients with HCC. However, in the last years the role of thermal ablation therapies is becoming more and more relevant. Their effectiveness and safety have widely been proven, and they play a key role in the treatment of HCC patients who are not eligible or poor candidates for surgery, or who refuse surgery.^[1-4] Moreover, they can also be used as a bridge to liver transplantation.

In the Barcelona Clinic Liver Cancer (BCLC) guidelines for treatment of HCC, tumors up to 3 cm in diameter are considered eligible for radiofrequency ablation (RFA) with curative intent in non-surgical candidates.^[5] Moreover, recent studies showed that RFA of very early HCC is as effective as surgical resection in terms of overall survival and recurrence-free survival rates.^[6,7] On the basis of these reports and their own experience, most skilled interventional oncologists and radiologists are advocating an update of the current guidelines, as it is time to consider RFA at least equivalent to surgical resection in the treatment of HCC up to 2 cm, in particular when the liver tumor is centrally located.

RFA represents the “historical”, best established and

experienced thermal ablation technique, but its efficacy is well known to decrease in presence of tumors larger than 2-3 cm. Last generation microwave ablation (MWA) systems offer some advantages compared with RFA, such as greater intratumoral temperature, deeper penetration of energy, propagation across the poorly conductive tissues, less sensitivity to the heat-sink effect, and larger ablation volume. These peculiarities could enable to treat larger tumors than RFA with adequate safety margin. So to date the question is: is it time to break the 3-cm barrier for thermal ablation?

To the best of our knowledge, no previous studies compared the efficacy of MWA in nodules up to 5 cm with respect to nodules up to 3 cm. Thamtorawat *et al.*^[8] recently published an interesting retrospective study including 129 patients with 173 HCCs up to 5 cm treated with MWA: 118 nodules were ≤ 3 cm in size, whereas 55 nodules were from 3.1 to 5 cm in size. The reported overall technical success rate of MWA was 96.5%. Local tumor progression occurred in 20/173 tumors (11.6%), and recurrences were successfully retreated by additional thermal ablation session. The mean follow-up period was 11.8 ± 9.8 months. The 1-year and 2-year overall survival rates for nodules ≤ 3 cm and for nodules from 3.1 to 5 cm were 91.3% and 81.7%, respectively. Eighteen patients out of 129 (13.9%) were bridged to liver transplantation.

Interestingly, there was no statistically significant difference in local progression rates between the two groups of HCC, with a 2-year local tumor control of 83.9% and 82.1%, respectively.

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As reported by the authors, the study has some limitations. First, it is a retrospective non-randomized study. Second, all the treatments were performed by using MWA. Therefore, a comparison with other thermal techniques is not possible. Finally, long-term outcome would also require longer follow-up times. However, as stated by the authors, this study was intended to be a pilot report on the treatment of larger HCC by using MWA.

Surgical resection and RFA can actually achieve the same good results in the treatment of very-early HCC (≤ 2 cm). Surgical resection remains the gold standard for the treatment of early (< 3 cm) HCC, although RFA represents an effective alternative in patients who are not eligible for surgery. Based on the BCLC guidelines, single nodules from 3 to 5 cm are classified as intermediate HCC, and transarterial chemoembolisation (TACE) is recommended as the best treatment option.^[5] Nevertheless, most experts consider surgery the very best option for the treatment of resectable large nodules with curative intent. However, most patients with intermediate HCC are not eligible for surgery because of inadequate liver function, anatomic limitations, multifocal disease, or medical comorbidities. This group of patients can benefit from TACE, or combined treatments including RFA plus TACE. RFA alone is frequently unable to obtain an adequate safety margin in nodules > 3 cm, particularly when the tumor is strictly close to large vessels, because thermal energy is partially shunted away by the cooler blood (the so-called heat-sink effect).^[9,10] Moreover, the treatment of large nodules require multiple overlapping insertions of the needle electrode, and it is known that the insertions following the first or second ones can be inaccurate owing to the steam generated during the procedure.^[11] As a consequence of these limitations, at present the use of RFA alone with curative intent is limited to nodules up to 3 cm.

Several studies demonstrated that last generation MWA systems enable to achieve larger ablation volumes than RFA, with comparable safety and survival rates.^[12-16] A randomized prospective comparison of MWA and RFA in the treatment of HCC did not demonstrate any difference in the rates of residual or untreated disease,^[17] and the capability of MWA to produce larger coagulation areas could result particularly useful in the treatment of tumors ≥ 3 cm. Reported mortality and major complication rates using the most recent MWA devices are similar to RFA.^[18] Complication rates reported by Thamtorawat *et al.*^[8] agree with the data reported by other authors, despite the larger size of the treated nodules. Moreover, although MWA appears less feasible than RFA in the treatment of high-risk located and subcapsular nodules, no difference in local tumor progression rate was found for subcapsular nodules in the study of Thamtorawat *et al.*^[8]

In conclusion, in our opinion this article could be considered the starting point for breaking the 3-cm barrier in the treatment of non surgical HCC. Our preliminary experience in the treatment of large nodules supports the efficacy of MWA for HCC up to 5 cm (unpublished data), and we hopefully expect further studies with longer follow-up aimed at extending the dimensional barrier of thermal ablation with curative intent.

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Conflicts of interest

There are no conflicts of interest.

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Introduction of the special issue: “Advances in Minimally Invasive Cirrhotic Surgery”

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Hepatocellular carcinoma (HCC) is the sixth most common type of cancer worldwide and the third leading cause of cancer-related death. It is the most common primary liver cancer and its incidence increases when associated with the development of cirrhosis. Liver resection is a curative therapy, when liver transplantation due to the patient age and alcohol abuse, associated diseases, and shortage of donors, is not feasible. During the last decades, progress in preoperative patient assessment, refinement of the indications for resection, improved surgical technique, and the development of new surgical devices have greatly enhanced the safety of open hepatectomy in normal and even in cirrhotic liver. After the first laparoscopic cholecystectomy performed about 30 years ago, laparoscopic approach has been applied more and more frequently to the full spectrum of abdominal

surgery, becoming the gold standard in the surgical treatment of much pathology such as biliary lithiasis and gastro-esophageal reflux. Since the first laparoscopic hepatectomy reported in 1991, laparoscopic liver surgery developed more slowly. There are many reasons for the slow diffusion of the laparoscopic hepatic surgery, such as the presumed technical difficulties, the complicated management of the bleeding during parenchymal transection, the lack of dedicated tools and the presumed risk of gas embolism. However, despite this initial slow development, laparoscopic liver surgery is now performed worldwide, even in cirrhotic patients. After the 2 Consensus Conferences (Louisville-USA, 2008 and Morioka-Japan, 2014) the advantages connected with the minimally invasive approach are evident, important and significant, especially in the treatment of HCC in cirrhotic liver.



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In fact, the role of the minimally invasive approach in liver surgery continues to increase, and many types of liver resections, even in cirrhotic liver, including major hepatectomies, are now performed laparoscopically in specialized centers. It is now clear that laparoscopic liver resection for HCC on cirrhotic liver has better short results when compared to an open approach. In fact, despite its technical challenges, reduced operative blood loss, fewer early postoperative complications, such as postoperative ascites, lower analgesic drug requirements, and shorter hospital stay are the clear advantages of the laparoscopic approach. Therefore, laparoscopic resection of HCC in cirrhotic liver is not only feasible and safe in selected patients with excellent short-term results, but achieves not inferior long-term survival and recurrence rates compared with open surgery when stratified for tumor characteristics known to be related to survival outcome.

In this special issue of *Hepatoma Research* focusing on “Advances in Minimally Invasive Cirrhotic Surgery”, the challenges of this rapidly developing field are addressed. Each of the contributors has referred specific aspects of their experienced area, discussing its limits but also its advantages. They have also

discussed their technique and results.

The authors of this issue have demonstrated that minimally invasive liver surgery in cirrhotic liver is feasible, safe and reproducible. It has been performed in highly specialised centres by surgeons using new technologies. In addition, all the surgeons are expert in both liver and advanced laparoscopic surgery.

I am very pleased and sincerely grateful to all of the authors for their outstanding effort in contributing to this issue.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Not involved.

Ethics approval

Not involved.

Indications and technique for laparoscopic liver resection in patients with hepatocellular carcinoma and liver cirrhosis

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ABSTRACT

Liver resection is the preferred initial treatment option for solitary or limited multifocal hepatocellular carcinoma (HCC). However, because of the characteristics of HCC, including its high recurrence rate and the frequent presence of chronic hepatitis and cirrhosis, both curability and invasiveness must be considered when selecting a treatment for HCC. Laparoscopic liver resection (LLR) is minimally invasive and increasingly performed worldwide as a curative surgical option for treatment of liver tumors. The 2014 International Consensus Conference on LLR concluded that minor LLRs are now standard practice. Meta-analyses suggest that, as compared with open hepatectomy, LLR for patients with HCC, including those with cirrhosis, resulted in less blood loss, lower postoperative hospitalization rates, and similar oncological outcomes. Although candidates for this procedure should be carefully evaluated, LLR appears to be a feasible option for treatment of HCC with liver cirrhosis. This review describes the indications for LLR in this patient subgroup and offers guidance on appropriate surgical technique.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common neoplasm and the third most frequent cause of cancer death in the world.^[1] Because of the specific characteristics of HCC, including its high recurrence rate and the frequent presence of chronic hepatitis and cirrhosis, HCC treatment should focus on both curability and invasiveness.

Liver resection is the preferable initial treatment option for solitary or limited multifocal HCCs with no extrahepatic

spread.^[2-6] The mortality and morbidity of liver resection have significantly decreased in the last two decades because of improvements in patient evaluation, surgical technique, and perioperative care. Resection is the ideal treatment, as it allows for complete removal and pathological confirmation of lesions. However, it is more invasive than other locoregional therapies such as transarterial chemoembolization, tumor ablation therapy, and radiation therapy.^[7-10]

Laparoscopy has been used extensively and continues to improve as a surgical option. Laparoscopic liver resection



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(LLR), a minimally invasive treatment for liver cancer, is now increasingly performed worldwide.^[11] However, few studies have investigated LLR for HCC patients with liver cirrhosis, and its applicability for this population is thus unclear. This review describes the indications for LLR in this patient subgroup and offers guidance on appropriate surgical technique.

CURRENT STATUS OF LLR IN THE TREATMENT OF HCC

LLR was initially described by Reich *et al.*^[12] Subsequent studies showed that it offered minimal invasiveness with no reduction in safety or disease curability for primary and metastatic liver tumors in selected patients.^[13,14] However, because of the technical difficulty of this procedure, it was not performed until the 1990s. Development of surgical devices and technical refinements in the early 2000s increased surgical interest. In the First International Consensus Conference (Louisville Consensus), convened in 2008, LLR was described as a safe and effective surgical approach for management of liver disease when performed by trained surgeons with experience in both hepatobiliary and laparoscopic surgery.^[15] In addition, a small number of studies reported that LLR was useful for cirrhotic patients.^[16,17] With the subsequent uptake of LLR, the Second International Consensus Conference on LLR, held in 2014, concluded that minor LLRs, which were performed for left lateral sectionectomies or resections of anterior and lateral segments (Couinaud's segments II, III, IVb, V, and VI), had become standard practice.^[11] Despite encouraging findings from high-volume centers,^[18,19] the efficacy of LLR for patients with cirrhosis remains inconclusive because of the low sample sizes of published studies. The most recent meta-analysis indicated that the benefits of LLR would lead to expansion of its indications to include HCC with chronic liver disease.^[20]

SURGICAL INDICATIONS

Resection of HCC in patient with liver cirrhosis

In patients with HCC with liver cirrhosis, careful selection of surgical candidates is essential in order to avoid treatment-related complications, e.g. liver failure. Because of differences in the characteristics of cirrhosis between Asian and Western countries, there is considerable variability regarding the indications for HCC resection. Therefore, surgical indications for HCC associated with portal hypertension remain controversial. Surgery is contraindicated for patients with encephalopathy, uncontrollable ascites, or jaundice (serum total bilirubin level > 2.0 mg/dL).^[21]

Asian centers have been more aggressive than Western

centers in resecting HCC. In Western countries, treatment is driven by the Barcelona Clinic Liver Cancer algorithm,^[22,23] in which evidence of portal hypertension is a contraindication for surgical resection. Clinically relevant portal hypertension is defined as the presence of esophageal varices or splenomegaly associated with a platelet count lower than $100 \times 10^9/L$.^[22]

In East Asian countries, the best candidates for resection are identified by using indocyanine green retention rate as part of a detailed assessment of preoperative hepatic functional reserve.^[24,25] Additionally, use of volumetric computed tomography for assessment of remnant liver volume after resection is as important as estimation of hepatic functional reserve.^[26] Therefore, patients with signs of portal hypertension can be candidates for resection if they receive adequate perioperative management, e.g. endoscopic treatment of esophageal varices to minimize risk of rupture and pre-hepatectomy or concomitant splenectomy to improve hypersplenism.^[21] Anatomic resection, which can remove the tumor-bearing portal territory, is preferred from an oncological perspective for radical treatment of HCC.^[24] Outcomes of liver resection for patients with HCC and cirrhosis has been dramatically improved with parenchyma-preserving technique.^[21]

Percutaneous ablation therapies are another treatment of choice for small nodular HCC in patients with cirrhosis located deep inside the liver; however, such treatment is not suitable for superficially located HCC, because of the increased risk of bleeding,^[27] tumor seeding,^[28] and thermal injury to adjacent organs.^[29] Therefore, surgical resection might be the ideal option for superficial small HCCs.

Patient selection for LLR

The selection of candidates for LLR is the most important consideration in safely performing LLR. With respect to host factors, an LLR candidate should have liver function sufficient for the same procedure performed as open liver resection. With respect to tumor factors, the classical indications for LLR are that the tumor should have a diameter less than 5 cm and be located in areas with easy technical access to laparoscopy, i.e. in the left lateral section (Couinaud's segments II and III) or on the surface of the inferior region of the liver (Couinaud's segments IVb, V, and VI).

Partial liver resection or left lateral sectionectomy are the typical procedures for such tumors. With accumulating experience and technical advancement, LLR has been performed for tumors larger than 5 cm and for lesions located in the posterior-to-superior region of the liver (Couinaud's segments VII, VIII, and IVa), including advanced non-anatomical and anatomical LLR such as major hepatectomy (involving the abovementioned

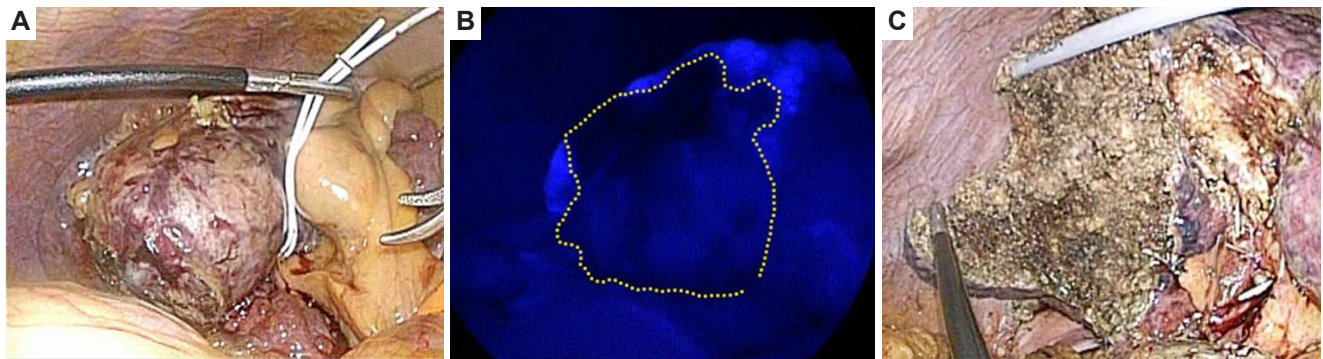


Figure 1: Laparoscopic limited anatomical resection in segment 6 in a patient with hepatocellular carcinoma (HCC) and Child-Pugh class B cirrhosis. (A) HCC and liver cirrhosis in segment 6; (B) illumination by indocyanine green fluorescence imaging shows the inflow preserved area after occlusion of the Glissonean pedicle of segment 6. Dotted line shows liver transection line; (C) liver resection plane

three Couinaud's segments), at high-volume centers.

In patients with cirrhosis, parenchyma-preserving limited anatomical resection along a demarcation line on the liver surface (formed by division of the Glissonean sheath at the hepatic hilus) is now achievable using laparoscopic technique. A recent modality, laparoscopic near-infrared fluorescence imaging,^[30] allows for precise anatomic resection [Figure 1].

LLR has a steep learning curve; therefore, the technical ability of a particular surgical team should be considered when assessing the applicability of LLR. A recent study proposed a difficulty scoring system for stepwise application of LLR, which was based on experience at high-volume Japanese centers.^[31] The proposed system predicts surgical difficulty by considering tumor location, extent of liver resection, tumor size, proximity to major vessels, and existing chronic liver damage.

SURGICAL TECHNIQUE FOR LLR

Patient position and setting

Under general anesthesia, the patient is positioned depending on the location of the tumor. In general, resection of the left hemi-liver or right anterior region of the liver is performed with the patient in conventional supine position. Resection of the right posterosuperior region of the liver is performed with the patient in a left hemilateral decubitus position, especially for resections requiring mobilization of the right liver from the retroperitoneum. In the left hemilateral position, the patient's body is fixed using a negative pressure bag packed with plastic beads, which is placed under the patient, and several support arms to prevent slipping during abrupt position changes.

The laparoscopic tower contains the light source, camera, and insufflators and is positioned to the right of the patient. Monopolar and bipolar generators for electrocautery devices, a microwave tissue coagulator,

and ultrasonically activated devices are placed on the right or left. An ultrasound diagnostic system is also positioned to the left of the patient.

Trocar placement

After pneumoperitoneum is achieved by means of an umbilical incision, the laparoscope is inserted. For operative manipulation in partial hepatectomy, three or four trocars are placed in a concentric circle radiating from the tumor. In left lateral sectionectomy, three trocars are placed at the right hypochondrium and bilateral abdomen. For anatomical hepatectomies other than left lateral sectionectomy, four trocars are usually necessary: at the epigastrium, right hypochondrium, and bilateral abdomen. Intercostally inserted trocars are useful for manipulation during resection of the superior region of the liver. A 5-mm trocar is placed in the upper abdomen for Pringle's maneuver, when it is needed.

During trocar insertion, surgeons should attempt to preserve collaterals on the abdominal wall in patients with liver cirrhosis. This may require use of ultrasonography to identify collateral vessels.^[32]

Hepatic parenchymal transection

For this procedure, laparoscopic ultrasound is performed using a flexible-angle ultrasound probe, to confirm the location of the tumor in relation to the vascular anatomy and identify other lesions in the liver. Although surface roughness from cirrhosis may impede ultrasound inspection, use of a water drip around the ultrasound probe can improve penetration of the ultrasound signal into the liver parenchyma.

Because of the risk of CO₂ gas embolism caused by pneumoperitoneum,^[33,34] intra-abdominal pressure should be maintained below 8-12 mmHg during the procedure.^[35]

Chronic liver disease is characterized by significant alterations in the intra- and extrahepatic vasculature. Division of collaterals within the falciform ligament or

hepatoduodenal ligament, which develop as a result of portal hypertension, should be minimized in the cirrhotic liver.

Parenchymal transection in cirrhotic liver is more hemorrhagic than in non-cirrhotic liver, because of loss of elasticity due to fibrosis and regeneration of liver tissue, the weakness of the altered intrahepatic vasculature, difficulty in identifying intraparenchymal structures, coagulative disorders caused by liver dysfunction, portal hypertension, and hypersplenism. Therefore, reduction of blood loss is a key to successful LLR. Although controversial in laparoscopic surgery, temporary or intermittent application of Pringle's maneuver, use of a vessel tape tourniquet or vessel clamp, can help reduce blood loss during liver parenchymal transection. While performing Pringle's maneuver, surgeons should be careful not to injure collaterals around the hepatoduodenal ligament.

Pre-coagulation technique, in which the resection line is diathermically coagulated using a microwave tissue coagulator or monopolar electrocautery before liver parenchymal transection, can help reduce blood loss in cirrhotic liver. In anatomical hepatectomy, hepatic inflow vessels are isolated with tape traction and occluded before liver parenchymal transection, to identify optimal segmental territory before liver transection. In liver parenchymal transection, laparoscopic coagulating shears are used to divide the superficial layer of the liver. Deeper transection should be performed by meticulously exposing intraparenchymal structures with an ultrasonic surgical aspirator or clamp-crushing technique. Vessels with a diameter of 3-7 mm are divided with vessel-sealing devices or clips. Then, vessels with a diameter of 2 mm or less are diathermically sealed using bipolar sealing devices and then divided. Hemostasis of the resection plane is achieved with monopolar or bipolar cautery. A laparoscopic stapler is used to divide major hepatic vessels and for simple transection of liver parenchyma with a thickness of 1-1.5 cm.^[36]

LLR is usually performed by pure laparoscopic procedure; however, there are options for a minimally invasive approach. Hand-assisted and laparoscopy-assisted procedures are also occasionally used in technically challenging cases. A hand-assisted procedure is suitable for resection of tumors located in the posterosuperior regions of the liver, to verify tumor margins in the limited operative field and control bleeding. The laparoscopy-assisted procedure divides the liver attachment by laparoscopy and transects the liver parenchyma through a small upper abdominal incision under direct vision. It can be used for major hepatectomy or LLR when dense adhesion is present in the abdomen.^[37] These approaches may serve as a bridge to pure laparoscopic procedure.

Robot-assisted technique in LLR has been attempted, although the population of patients with liver cirrhosis is quite limited.^[38] A recent report suggested that the augmented dexterity and greater range of motion provided by endowristed instruments are helpful, especially in LLR of posterosuperior segments of the liver.^[39]

Specimen retrieval

After liver resection is completed, the removed specimen should be placed in a plastic bag, to avoid seeding and implantation of tumor cells in the operative field. Small specimens can be retrieved from a trocar wound made at the umbilical site. Larger specimens are retrieved from an extended umbilical incision, suprapubic incision, or an incision made at an incision site for a previous surgery.

OPERATIVE OUTCOMES OF LLR FOR HCC WITH LIVER CIRRHOSIS

Short-term outcomes

Liver resection for HCC can be performed in some patients with advanced liver disease. Post-hepatectomy morbidity is reported to be high, and long-term prognosis is poor in patients with portal hypertension.^[40-42] Such patients might be better served by liver transplantation or ablation.^[43] However, some recent studies reported encouraging liver resection outcomes, even in patients with portal hypertension.^[21,44,45] Therefore, hepatic resection may be regarded as the primary treatment option for patients with mild portal hypertension, if liver transplantation is not possible.

Systematic reviews and meta-analyses of nonrandomized comparative or case-control studies of HCC suggest that LLR results in less blood loss and shorter postoperative hospital stays^[46-49] as compared with open hepatectomy.^[50-54] With respect to technical considerations, the reported conversion rate to open surgery for LLR is 0-19.4%.^[49,53,54] Hemorrhage during hepatic parenchymal transection is the most frequent reason for conversion.^[49,53,54] To control hemorrhage during liver parenchymal transection, it is essential to select the appropriate surgical devices, including diathermy pre-coagulation of the resection plane before liver transection.^[55]

A clear benefit of minimally invasive surgery is that it minimizes abdominal wall trauma. LLR preserves collateral formation in the abdominal wall and thus results in lower incidences of ascites accumulation and postoperative liver failure, as compared with open surgery.^[18,51,54] Less-incisional procedures, such as single-port endoscopic surgery, are likely to be less destructive when performed for carefully selected patients.^[56]

Additionally, repeat hepatectomy for recurrent HCC

may be indicated, with or without prior local ablation therapy, if abdominal adhesion is limited and tumor location and characteristics are suitable for a laparoscopic approach.^[57]

Long-term outcomes

With respect to oncological considerations, evidence from meta-analyses suggests that surgical tumor margins are adequately maintained during LLR.^[46,47,50-54] In addition, an analysis of long-term outcomes showed no oncological disadvantage as compared with open liver resection in relation to disease-free or overall survival in meta-analyses of LLR for HCC, especially in well compensated (Child-Pugh class A) liver cirrhosis.^[49,51,53]

The rate of HCC recurrence is positively associated with cirrhosis progression; therefore, long-term outcomes of HCC patients with substantial liver cirrhosis (Child-Pugh class B or C) remain poor. Liver transplantation is the most effective treatment for end-stage liver disease and localized malignancy.^[58] Current evidence indicates that liver transplantation is the only suitable treatment for HCC in patients with Child-Pugh class C disease.^[59] Unfortunately, this optimal procedure is rarely performed because of the severe shortage of donor organs. A study comparing liver resection and liver transplantation for HCC with liver cirrhosis found that liver resection resulted in comparable overall survival in patients with solitary HCC less than 3 cm and cirrhosis.^[58] Interestingly, LLR may be better than open liver resection as a bridge to liver transplantation in patients with superficial HCC accompanied by liver cirrhosis, because of the ability of LLR to control disease while limiting postoperative abdominal adhesion.^[60] Because of the necessity of careful maintenance of liver function and oncological control, LLR could play an important role in treating small peripherally located HCC in patients with advanced liver cirrhosis.^[51-53] However, the effects of liver resection for HCC on subsequent suitability for liver transplantation are not well clarified.

CONCLUSION

Existing evidence suggests that LLR is an important alternative treatment option for HCC in patients with substantial liver cirrhosis, although this hypothesis will require further evaluation in future studies. Candidates for this procedure should be carefully evaluated. Use of proper technique would maximize the benefits of LLR and make it an ideal treatment option for this patient subgroup.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Not involved.

Ethics approval

Not involved.

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Laparoscopic liver resection in the cirrhotic patient

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ABSTRACT

Aim: The adoption of laparoscopic liver resection has been expansive in the last 2 decades with the exception of cirrhotic patients. The current study examines the outcomes of our cirrhotic resections to determine the potential limitations of this technique. **Methods:** Retrospective analysis of 114 cirrhotic patients. Seventy-five (65.8%) laparoscopic resections were compared to 39 open resections. Seventy-six (66.7%) resections in the series were minor resections (less than 3 segments). Surgical approach and extent of resection were analyzed using student's *t* test and regression multivariate analysis with SAS. **Results:** The laparoscopic group had lower operative times (2.4 vs. 4.8 h; $P < 0.001$), blood loss (250 vs. 609 mL; $P < 0.001$), length of stay (4.4 vs. 10.1 days; $P = 0.013$) and complications (28% vs. 48%; $P = 0.028$). Subset analysis by technique and extent of resection identified the laparoscopic group lost the advantage in blood loss and lengths of stay when utilized in major resections. Multivariate regression analysis for blood loss further confirmed open resection ($P = 0.014$) and major resection ($P = 0.026$) as significant indicators of bleeding and transfusion. **Conclusion:** Laparoscopic liver resection in cirrhotic patients is safe and efficacious. However, the significant variability in outcomes for major resections in cirrhotics leads us to recommend further examination of the learning curve and significant caution in the selection of cirrhotics requiring major hepatic resections.

INTRODUCTION

Liver resection has dramatically evolved over the last four decades. Initial series incurred high morbidity and mortality rates.^[1,2] However, with the introduction of modern anesthesia and improved knowledge of the surgical anatomical segments the mortality decreased to acceptable levels leading to the proliferation of resection programs.^[3-5] In the last decade we have witnessed a second proliferation of hepatic resections

attributed to the introduction of the laparoscopic technique.^[6,7] However, the greatest challenge in the resection of hepatic tumors remains their management in the setting of cirrhotic liver.^[8,9]

Despite the introduction of effective antivirals for the treatment of hepatitis C, the incidence of cirrhosis is expected to continually rise worldwide most frequently attributed to the ever increasing prevalence of obesity, fatty liver disease and non alcoholic steato hepatitis.^[10] A



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Table 1: Patient demographics

| | Open | Laparoscopic | P-value |
|--------------------------|--------------|--------------|----------|
| Number | 39 | 75 | |
| Age (years) | 58.1 ± 12.8 | 61.3 ± 9.86 | 0.152 |
| Male gender (%) | 89.7 ± 30.7 | 66.7 ± 47.5 | 0.007* |
| Government insurance (%) | 25.6 ± 44.2 | 32.0 ± 47.0 | 0.486 |
| BMI | 28.2 ± 6.0 | 27.8 ± 4.9 | 0.713 |
| HTN (%) | 74.4 ± 44.2 | 80.0 ± 40.3 | 0.494 |
| DM (%) | 33.3 ± 47.8 | 30.7 ± 40.2 | 0.664 |
| INR | 1.1 ± 0.2 | 1.1 ± 0.1 | 0.638 |
| Bilirubin | 0.83 ± 0.42 | 0.691 ± 0.36 | 0.071 |
| Creatinine | 1.05 ± 0.58 | 0.86 ± 0.24 | 0.013* |
| PLT | 187.5 ± 87.2 | 173.4 ± 73.7 | 0.357 |
| Varicies (%) | 33.3 ± 47.7 | 17.3 ± 38.1 | 0.054 |
| Ascites (%) | 2.6 ± 47.7 | 10.7 ± 31.1 | 0.130 |
| ASA score | 3.3 ± 0.5 | 3.1 ± 0.3 | 0.020* |
| Tumor size (cm) | 6.56 ± 4.26 | 3.20 ± 2.01 | < 0.001* |

*Statistically significant. BMI: body mass index; HTN: hypertension; DM: diabetes mellitus; INR: international normalized ratio; PLT: platelet; ASA: American Society of Anesthesiologists

significant proportion of these cirrhotic patients will present for the management of hepatocellular cancer.^[11] However, with the incidence of cirrhosis ever rising in the general population, other pathologic lesions will be presented for diagnosis and surgical management including symptomatic benign tumors, colorectal metastases and in the era of increasing resolution imaging indeterminate lesions.

This study examines our experience with laparoscopic liver resection in cirrhotic patients for multiple pathologies. The aim of the current study was to elucidate the potential benefits of laparoscopic liver resection over open hepatic resection in the management of surgically resectable liver lesions in cirrhotic patients.

METHODS

This is a retrospective study analyzing the effect of a laparoscopic approach on the resection of liver tumors in cirrhotic patients. The current study was submitted and approved by an institutional review board at our institution. One hundred and fourteen cirrhotic liver resections were identified in a surgical database performed by a single surgeon. The cohort of cirrhotic resections was evaluated for patient demographics, operative outcomes, morbidity, mortality, and long-term patient survival. The impact of laparoscopic liver resection was then compared to the open liver resection group. Further examination was performed using a subset analysis to evaluate the extent of resection. Major resections were defined as in prior studies as removal of three or greater segments.

The surgical evaluation and resection techniques used during this study were identical throughout the series. All resection candidates were evaluated with an established criterion including: preoperative

imaging with triphasic computed tomography scan or contrasted magnetic resonance imaging, estimation of functional liver remnant, confirmation of platelet count, and selective measurement of transjugular wedge pressures. In the setting of high risk patients such as platelet counts less than 100 K or presence of significant varicies, transhepatic wedge pressures and extent of resection dictated the decision to proceed with resection and in marginal cases portal vein embolization was employed. Both open and laparoscopic resections were performed using a parenchymal sparing intent with the aid of low central venous pressures, and parenchymal division with an ultrasonic dissector and stapler hepatectomy.

Patient demographics, tumor characteristics, operative and postoperative outcome data were collected and analyzed. Data was reported with means and standard deviations. Statistical comparisons were calculated and analyzed using SAS software. Significant differences were identified at a *P*-value of < 0.05. Multivariate regression analysis was then applied to evaluate the effects of laparoscopic liver resection on patient morbidity, mortality and readmission.

RESULTS

The study cohort of cirrhotic resection patients was composed of 114 cirrhotic patients. The laparoscopic liver resection group was comprised of 75 patients (65.8%) and the open liver resection group 39 patients. Age, gender, race, and demographics were all similar between the 2 groups [Table 1]. The tumor size was significantly larger in the open resection group while the preoperative diagnosis and etiology of cirrhosis were similar.

The operative outcomes were noted to have significant

Table 2: Patient outcomes by technique of resection

| | Open | Laparoscopic | P-value |
|-------------------------|---------------|---------------|----------|
| Number | 39 | 75 | |
| Major resections (%) | 66.7 ± 47.7 | 16.3 ± 36.9 | < 0.001* |
| OR time (h) | 4.8 ± 2.0 | 2.4 ± 1.1 | < 0.001* |
| EBL (mL) | 609.0 ± 603.8 | 250.7 ± 344.6 | < 0.001* |
| Transfusion (%) | 38.5 ± 49.3 | 17.3 ± 38.1 | 0.012* |
| Margin (cm) | 1.05 ± 0.8 | 0.90 ± 0.6 | 0.269 |
| ICU admission (%) | 89.7 ± 30.7 | 32.0 ± 49.8 | < 0.001* |
| Complications (%) | 48.7 ± 50.5 | 28.0 ± 45.2 | 0.028* |
| LOS (days) | 10.1 ± 18.3 | 4.4 ± 3.8 | 0.013* |
| 90-day readmissions (%) | 15.4 ± 36.6 | 14.6 ± 35.7 | 0.926 |
| 90-day mortality (%) | 5.1 ± 22.4 | 2.7 ± 16.2 | 0.502 |

*Statistically significant. OR: operating room; EBL: estimated blood loss; ICU: intensive care unit; LOS: length of stay

Table 3: Patient outcomes analyzed by extent and technique of resection

| | Minor resections (n = 76) | | | Major resections (n = 38) | | |
|---------------------|---------------------------|--------------|----------|---------------------------|--------------|----------|
| | Open | Laparoscopic | P-value | Open | Laparoscopic | P-value |
| Number | 13 | 63 | | 26 | 12 | |
| Age (years) | 60.1 | 60.4 | 0.913 | 57.1 | 65.8 | 0.058 |
| BMI | 29.8 | 27.5 | 0.171 | 27.4 | 28.7 | 0.533 |
| INR | 1.1 | 1.1 | 0.846 | 1.1 | 1.1 | 0.599 |
| Bilirubin | 0.8 | 0.7 | 0.355 | 0.8 | 0.6 | 0.184 |
| Creatinine | 1.3 | 0.9 | 0.002* | 0.9 | 0.9 | 0.427 |
| ASA score | 3.4 | 3.1 | 0.040* | 3.3 | 3.1 | 0.201 |
| Tumor size (cm) | 6.0 | 3.1 | < 0.001* | 6.8 | 3.7 | 0.023* |
| EBL (mL) | 438.5 | 215.9 | 0.033* | 694.2 | 433.3 | 0.225 |
| OR time (h) | 4.8 | 2.2 | < 0.001* | 4.9 | 3.0 | < 0.001* |
| Transfuse (%) | 42.9 | 18.5 | 0.023* | 33.0 | 0 | 0.193 |
| ICU utilization (%) | 84.6 | 31.7 | 0.006* | 92.3 | 33.3 | < 0.001* |
| LOS (days) | 16.1 | 4.1 | 0.004* | 7.0 | 6.0 | 0.510 |
| Complications (%) | 46.2 | 23.3 | 0.103 | 46.2 | 50.0 | 0.831 |

*Statistically significant. BMI: body mass index; INR: international normalized ratio; ASA: American Society of Anesthesiologists; EBL: estimated blood loss; OR: operating room; ICU: intensive care unit; LOS: length of stay

differences in resection extent, bleeding, transfusions, and operative times [Table 2]. Length of stay, and complications were significantly different while the readmission and mortality rates were not dramatically different [Table 2]. Seventy-six (66.7%) resections were minor in extent with 63 (82.9%) of them performed through the laparoscope. Thirty-eight resections in this series were major as defined by removal of 3 or more segments with 12 (31.6%) removed through the laparoscope. Minor and major resections witnessed a reduction in operative times, ICU utilization and length of stay. Blood loss and complications were significantly less in the laparoscopy group only in minor resection. The previously described advantages were not identified in the major resection subgroup [Table 3]. Multivariate analysis for bleeding identified open resection ($P = 0.014$) and major resection ($P = 0.026$) as significant risk factors for blood loss. In subset analysis only international normalized ratio ($P = 0.018$) was significant in the major resection group. Multivariate analysis identified tumor size ($P = 0.023$) as a risk for complications. In subset analysis this persisted while in major resections this effect was lost. Multivariate analysis for death identified creatinine ($P = 0.016$), bilirubin ($P = 0.019$), and obesity defined by

body mass index (BMI) > 35 ($P = 0.043$). Creatinine ($P < 0.001$) and BMI ($P = 0.019$) persisted in significance in minor resection but was lost in major resections.

DISCUSSION

Liver resection in the cirrhotic patient is significantly more complex than in the non cirrhotic patient.^[12-14] Cirrhotic patients are frequently metabolically compromised, coagulopathic and may suffer from a degree of portal hypertension. However, the most dreaded complication of hepatic resection in the cirrhotic patient is post-operative liver failure resulting from an inadequate functional liver remnant. Decades of efforts in preoperative assessment including metabolic challenge of the liver with indomethacin green and calculated functional liver remnant have been critical in reducing operative mortality.^[15,16]

Since the initial Louisville Consensus Conference, there have been over 500 cases of laparoscopic resection for hepatocellular carcinoma reported in the literature.^[17-21] Most patients in this group are cirrhotic, but a considerable percentage were non-cirrhotic or pre-cirrhotic arising in the setting of chronic

hepatitis. Multiple studies have confirmed the benefits of laparoscopic liver resection in decreasing operative times, bleeding, complications and length of stay in non cirrhotic patients.^[22-25] Most cirrhotic resection data has been included into larger hepatocellular cancer reports making assessment of this data questionable at best. However, a recent meta analysis of laparoscopic resection of hepatocellular cancer in cirrhotic patients confirmed this approach was associated with a reduced risk of transfusion, decreased length of stay and wider surgical margins but failed to identify a difference in operative times, and morbidity.^[26] This is in contrast to a smaller French case-controlled study that identified laparoscopic resection resulted in shorter operative times, hospital stays and lower morbidity rates.^[27]

Our current data presented in this study confirms laparoscopic resection in cirrhotics provide shorter operative times, blood loss, transfusion, intensive care utilization, length of stay, and post operative complications. However, when operative outcomes were analyzed in regard to the extent of resection the laparoscopic group persisted in shorter operative times with less intensive care utilization while reduction of blood loss and shorter lengths of stay were not realized in the major resection group.

Multiple studies have attributed the advantages of laparoscopic liver resections to a less aggressive approach, minimizing peritoneal dissection, and bleeding leading to lower incidence of ascites and post-hepatectomy liver failure.^[19-22] Two authors have even suggested laparoscopic liver resection may extend the indication of liver resection into selected Child B patients.^[20,28] Our experience with would support this supposition in well selected Child B patients. An additional advantage of laparoscopic liver resection of was reduction in postoperative adhesions facilitating subsequent liver transplantation with decreased morbidity. This observation was advanced in an article on salvage transplantation after laparoscopic liver resection for hepatocellular cancer.^[29] Alternatively, the results reported from the meta analysis indicate all groups have not witnessed such a clear and dramatic advantage with laparoscopic liver resection.^[26]

Our data would support these general suppositions but identified significant differences in outcome related complications after major laparoscopic resections. This may arise from the significantly increased need for dissection, bleeding and transfusion. Alternatively, this may reflect a steeper and longer learning curve required in the performance of laparoscopic cirrhotic liver resections and most importantly major laparoscopic liver resections in cirrhotics. As observed in early

open resection of cirrhotic tumors, these challenges have resulted in greater difficulty in achieving wide resection margins and performing formal anatomical resections, as well as increasing the difficulties in mobilization and in particular parenchymal transection, with risk of massive bleeding. These concerns and potential issues have been the major obstacles to the widespread adoption of laparoscopic liver resection in the management of liver tumors in cirrhotics.

In conclusion, laparoscopic liver resection in cirrhotic patients appears safe and efficacious in experienced centers resulting in overall significantly shorter operative times, lower blood loss, and shorter hospital stays and few complications. In subset analysis several advantages of the laparoscopic approach are lost including lower blood loss and few complications. Our current experience in laparoscopic major resections in cirrhotics has lead us to reconsider the learning curve and temper our enthusiasm for major resection in cirrhotics. Perhaps with increasing experience these benefits will be realized but currently our group advocates a tempered and highly selective approach to the laparoscopic approach to major cirrhotic resections.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Not involved.

Ethics approval

Approved by an institutional review board at authors' institution.

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Case report of the fourth laparoscopic liver resection and review of repeat laparoscopic resection for recurrent hepatocellular carcinoma in cirrhotic liver

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ABSTRACT

A 73-year-old woman with liver cirrhosis caused by hepatitis C virus (HCV) underwent treatment of three hepatocellular carcinomas (HCCs) in liver segment 4, following three previous laparoscopic liver resections (LLRs) over 73 months. Contrast-enhanced computed tomography showed three 0.5-1.2 cm HCCs deep within the portal territories of subsegments 4a and 4b. The patient underwent laparoscopic resection of 4a and 4b, with the preservation of the portal branch to 4c, after minimal adhesiolysis around segment 4. The operation lasted 284 min, there was 50 mL of intra-operative bleeding and her recovery was uneventful. She was well, had experienced no recurrence and was HCV-negative, after taking oral anti-HCV therapy, 21 months later. LLR is associated with fewer adhesions after surgery and requires less adhesiolysis, because the laparoscope and forceps can be used in the small spaces between adhesions. The present patient underwent four LLRs over 6 years without severe deterioration of liver functional reserve. LLR is a useful localized therapy, which can be performed repeatedly and may prolong the survival of patients with multicentric metachronous HCCs.

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INTRODUCTION

Since the first successful report of laparoscopic liver wedge resection in 1991,^[1] laparoscopic liver resection (LLR) has been thought to be a “less invasive” procedure than open liver resection. Use of this technique is especially beneficial for patients with concurrent hepatocellular carcinoma (HCC) and chronic liver disease (CLD).^[2-4] However, accumulated experience of this technique and technological developments have facilitated the expansion of the indications for LLR.^[5-7] It is becoming clear that the magnified caudal view offered by laparoscopy allows improved visualization, especially for the hilar and dorsal area of the liver, and is thus beneficial for the dissection of hilar Glissonian pedicles and the inferior vena cava (IVC).^[7-9] LLRs of major hepatectomy and, even, with combined resection of major hepatic veins are now increasingly reported.^[10-12] despite the latter previously being a contraindication. Reports of repeated LLR procedures^[13-16] are also increasing. However, these reports have generally included both cases of HCC with CLD and of metastatic disease without background liver disease.^[17-21] The indication and efficacy of repeated LLR for HCC in a setting of CLD alone has yet to be fully determined. Here we present a case report of a fourth LLR for recurrent HCCs in cirrhotic liver and review the previously reported cases

of repeat LLR for the treatment of HCC.^[22,23]

CASE REPORT

A 73-year-old woman with hepatitis C virus (HCV)-related liver cirrhosis (LC) was admitted to our department for treatment of three lesions in liver segment 4. These were revealed by contrast-enhanced computed tomography (CT) examination undertaken during the follow up to three LLRs that were performed 73, 45, 23 months previously [Figure 1]. The patient had no history of hepatic encephalopathy, ascites (except immediately postoperatively) and no specific treatment history except that of the liver disease.

The laboratory data showed decreased white blood cell and platelet counts (1,800 and 68,000/ μ L, respectively) and plasma albumin (3.5 g/dL) and mild elevations in plasma aspartate transaminase (AST, 76 IU/L) and alanine transaminase (ALT, 71 IU/L). The prothrombin time (78%), plasma levels of total bilirubin (0.6 mg/dL) and prothrombin induced by vitamin K absence-II (PIVKA-II, 9 mAU/mL) were within their normal ranges, but alpha-fetoprotein (AFP) showed a mild elevation (to 67.5 ng/mL). The 15-min value during the clearance rate of indocyanine green loading test (ICG-R15) was 24.1%; this had not deteriorated over the 73 months since the first LLR [Table 1].

Table 1: Perioperative clinical variables associated with each LLR

| | 1st | 2nd | 3rd | 4th |
|----------------------|------|------|------|------|
| ICG-R15 | 20.9 | 27.5 | 27.0 | 24.1 |
| Bleeding (mL) | 35 | 30 | NC | 50 |
| Operating time (min) | 288 | 168 | 216 | 274 |
| POHS (days) | 11 | 9 | 9 | 8 |

LLR: laparoscopic liver resection; ICG-R15: 15 min value during the clearance rate of indocyanine green loading test; 1st: ICG-R15 and perioperative course of first LLR; 2nd: ICG-R15 and perioperative course of second LLR; 3rd: ICG-R15 and perioperative course of third LLR; 4th: ICG-R15 and perioperative course of fourth LLR; NC: low, unquantifiable; POHS: postoperative hospital stay



Figure 1: Contrast-enhanced computed tomography (CT) examination at the first (A), second (B) and third (C) laparoscopic liver resection. (A): The patient's first laparoscopic liver resection [LLR, extended segment 3 (S3) segmentectomy] was performed for two hepatocellular carcinomas (HCCs, 18 mm and 12 mm in size) in S3 and at the border of S2-3, 73 months before the fourth LLR. Contrast-enhanced CT examination (venous phase) shows two lesions (arrowheads). (B): The patient's second LLR (partial resection of S5-6) was performed for HCC (30 mm in size) on the edge of the border of S5-6, 45 months before the fourth LLR. Contrast-enhanced CT examination (portal phase) shows the lesion (arrowhead). (C): The patient's third LLR (partial resection of S7-1) was performed for a HCC (8 mm) next to the inferior vena cava, 23 months before the fourth LLR. Contrast-enhanced CT examination (portal phase) shows the lesion with lipiodol accumulation (arrowhead); this had been previously treated by trans-arterial chemo-embolization

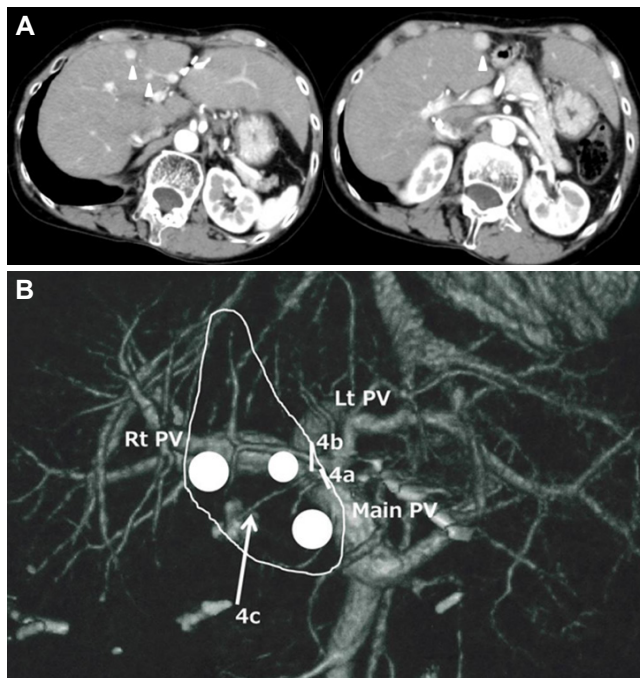


Figure 2: Contrast-enhanced computed tomography (CT) examination at the fourth laparoscopic liver resection (A) and schema of the surgical resection plan (B). (A): A contrast-enhanced CT examination demonstrated three (12, 7 and 5 mm) lesions (arrowheads) in the deep area of liver segment 4, inside the portal territories of subsegments 4a and 4b. (B): A laparoscopic anatomical liver resection of subsegments 4a and 4b was planned for the removal of possible disseminated tumor cells in the portal territories and the preservation of maximum liver volume. Glissonian branches to subsegments 4a and 4b were divided at their roots (bars), while 4c was preserved on the bottom of the resection plane (arrow). White circles indicate tumors

CT demonstrated three 0.5-1.2-cm-sized low-density lesions in the deeper region of liver segment 4, within the portal territories of subsegments 4a and 4b. The lesions were enhanced with contrast during the arterial phase and washout of the enhancement was observed in the portal-venous phase [Figure 2]. Laparoscopic anatomical resection of subsegments 4a and 4b were planned, with the preservation of the portal branch to 4c on the bottom of the resection plane. This procedure would ensure a surgical margin appropriate to the diagnosis of multiple HCCs in cirrhotic liver, given the possibility for the removal of tumor cell dissemination in the portal territory, but also preserve the maximum possible liver volume [Figure 2].

During the surgery, the patient was placed in a supine position. The first trocar port was introduced by mini-laparotomy on the umbilicus; CO₂-pneumoperitoneum (8-12 mmHg) was established through this port and it was also used for laparoscopy. Three other 12-mm ports and one 8-mm port were placed in the left upper abdomen and used for introducing surgeons' forceps, electrical devices (SonoSurg®, BiClamp® bipolar forceps and irrigation monopolar electrical cautery using soft-mode coagulation), clips and a Cavitron ultrasonic surgical

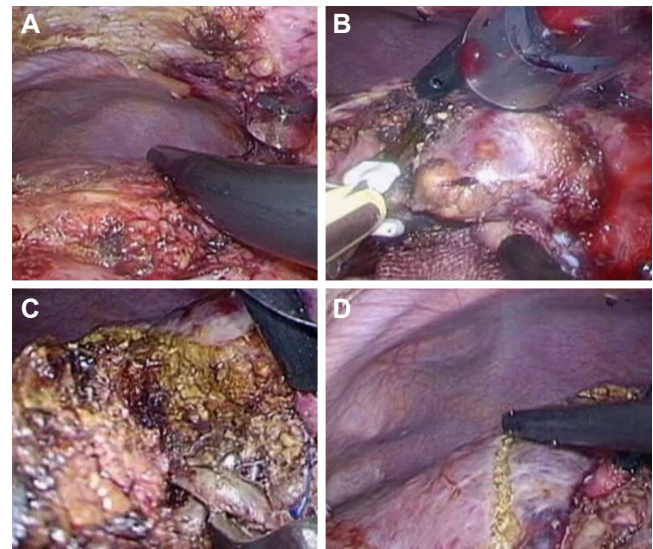


Figure 3: Intraoperative findings. (A): Before the liver transection, minimum adhesiolysis was performed around the area of segment 4 of the liver. Intraoperative ultrasonography was used to demonstrate the locations of the tumors and the line of the umbilical plate, which were marked. (B): The liver parenchymal transection was commenced along a line to the right side of the umbilical plate. (C): During transection along this line, the Glissonian branches to subsegment 4a, and subsequently, 4b, were encircled and divided. (D): After dividing the branches to subsegments 4a and 4b, the area containing the hepatocellular carcinomas was clearly recognized as an ischemic area, prior to resection

aspirator. The Pringle maneuver was not applied to this patient. After minimum adhesiolysis around segment 4, intraoperative ultrasonography was performed and the locations of the tumors and the line of the umbilical plate were marked [Figure 3A].

Transection of the liver parenchyma was commenced to the right of the line of the umbilical plate [Figure 3B]. During the transection, the Glissonian branches supplying subsegments 4a, and subsequently 4b, were encircled and divided [Figure 3C]. After dividing the branches to 4a and 4b, the area containing the HCCs was clearly recognized as an ischemic area, in advance of resection [Figure 3D]. The ischemic area was resected laparoscopically, leaving the Glissonian branch to subsegment 4c exposed deep to the transection plane [Figure 4A]. The operation took 284 min and 50 mL of blood was lost intra-operatively.

Pathological examination of the three tumors identified them to be well-differentiated HCCs with fibrous capsules, but without vessel invasion, surrounded by grade F4 liver cirrhosis [Figure 4].

The patient recovered uneventfully and she was well, without recurrence, 21 months after surgery. Furthermore, she was then HCV-negative, having been taking a newly developed oral anti-HCV therapy (Daclatasvir/Asunaprevir).

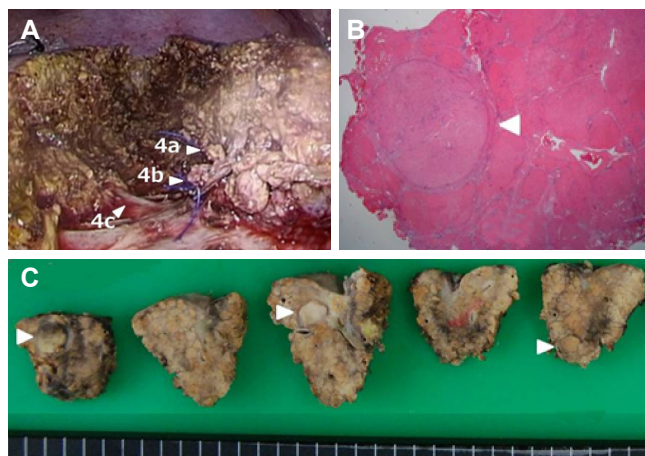


Figure 4: Intra-operative findings after resection (A), pathological findings (B), and examination of the resected specimen (C). (A): The area was resected laparoscopically, with the Glissonian branch of subsegment 4c being exposed on the bottom of the transection plane. The sites labelled 4a and 4b indicate the stumps of the Glissonian pedicles of subsegments 4a and 4b. The site labelled 4c indicates the Glissonian branch supplying subsegment 4c, exposed on the bottom of the transection plane. (B): Pathologically, the three tumors were well-differentiated hepatocellular carcinomas with fibrous capsules but without vessel invasion, surrounded by stage F4 tissue (liver cirrhosis)

DISCUSSION

The development of post-operative adhesion is known to increase the surgical time in subsequent surgeries, as a result of the need for adhesiolysis, the risk of intraoperative complications^[24] and the possibility of conversion from laparoscopic procedure to laparotomy.^[25] Although a history of abdominal surgery had been considered a contraindication for laparoscopic surgery in the early days of the procedure, improvements in technique and instrumentation have more recently permitted many laparoscopic procedures to be safely applied to such patients.^[24,26-29] However, LLR remains a technically demanding procedure and the indications for and efficacy of repeat LLRs are still under discussion. Successful liver resection requires adequate adhesiolysis and mobilization of the involved liver area. Adhesions can be obstacles to the visualization and dissection of the hepatoduodenal ligament and hilar area, which are often crucial steps in LLR. Liver capsule bleeds easily during adhesiolysis and mobilization, creating a suboptimal surgical field, in addition to the increase in blood loss.^[30]

The outcomes of repeated LLRs have been reported in several small case series.^[13-16] However, these studies often included both HCC/CLD and metastatic patients,^[17-21] while the clinical settings for repeated LLR are quite different in HCC/CLD and metastatic patients. Patients with metastasis sometimes undergo major liver resection involving the handling of Glissonian pedicles in soft, congested and/or fatty parenchyma. Conversely,

HCC/CLD patients often undergo minor resection of the hard, fibrotic liver, which has a poor functional reserve and is surrounded by blood or lymphatic collateral vessels, which should be preserved. The number of reported repeat LLR cases for HCC/LLR patients is very small, and these are summarized in [Table 2](#).

There are three previous reports of repeat LLR focused for HCC/CLD patients. Belli *et al.*,^[13] Hu *et al.*,^[15] and Kanazawa *et al.*^[22] reported 12, 6, and 20 cases, respectively. They all concluded that repeat LLR for recurrent HCC in cirrhotic patients is a safe and feasible procedure. Belli *et al.*^[13] reported that the surgical time for repeat LLR was shorter and the adhesiolysis was easier for patients previously treated using LLR compared to open LR (OLR), and also detailed the advantages of the minimally invasive approach for managing the chronic oncologic sequelae of cirrhosis. Kanazawa *et al.*^[22] compared repeat LLR to repeat OLR in $n = 20$ groups of patients and concluded that postoperative morbidity and the duration of postoperative hospitalization have been decreased by the introduction of LLR for patients with recurrent HCC.

We previously reported that LLR is useful for patients with severe liver dysfunction, as it minimizes disturbance of the collateral blood/lymphatic flow caused by laparotomy and liver mobilization, and the mesenchymal injury caused by compression of the liver.^[31,32] Thus, LLR limits the occurrence of complications, such as massive ascites, which can lead to postoperative liver failure.^[3] We also reported that the smaller working space required for LLR necessitated less adhesiolysis, with a direct approach to the region affected by the tumor being possible in repeat LLR.^[20] This also meant that patients undergoing repeat LLR had similar perioperative results to patients without a history of surgery, especially in the case of minor resections for HCC/CLD patients. The majority of the patients described in previous reports of repeat LLR for HCC/CLD underwent minor resection as a repeat LLR. Therefore the influences of alterations to hilar and intrahepatic anatomy from the first hepatectomy should have been relatively small. Since alterations in hilar and intrahepatic vascular supply would greatly impact on the second hepatectomy, further consideration of a role for major or anatomical repeat LLR is needed. However, results to date suggest that a clear advantage of LLR for minor repeat resections of impaired liver is that it only requires minimal adhesiolysis.

In the case reported here, the patient underwent four LLRs over six years without severe deterioration of liver functional reserve, represented by the

Table 2: Summary of previous reports of repeat laparoscopic hepatectomy that included cases of hepatocellular carcinoma

| Authors | n | Age (year) | Disease | First Hx (open:lap) | Procedure | Bleeding (mL) | Operating time (min) | Con. (n) | POHS (days) | Morbidity | Mortality |
|---|----|--------------|--|----------------------------|---|--------------------------|-----------------------------|-------------|------------------------|-----------|-----------|
| Belli <i>et al.</i> ^{[13]*} (2009) | 12 | 69 (58-75) | HCC | 4:8 | LLS (n = 5), Pt (n = 4), Seg (n = 3) | 297 ± 134 272.2 ± 120 | 114.4 ± 11.0 63.9 ± 13.3 | 1 | 7.4 ± 2.5 6.2 ± 3.0 | 26.6% | 0% |
| Hu <i>et al.</i> ^[17] (2011) | 6 | 49 (46-61) | HCC | 3:3 (Lap RFA, n = 2) | LLS (n = 2), Pt (n = 4) | 283.3 ± 256.3 | 140.8 ± 35.7 | 0 | 5.67 ± 1.63 | 16.7% | 0% |
| Shafae <i>et al.</i> ^[16] (2011) | 76 | 61 (29-82) | Met (n = 63), HCC (n = 3), others (n = 10) | 28:44 | LLS (n = 4), Pt, seg (n = 53), above-seg (n = 19) | 300 (0-5000) | 180 (80-570) | 8 | 6 (2-42) | 26% | 0% |
| Ahn <i>et al.</i> ^[15] (2011) | 4 | 57 (54-60) | HCC (n = 3), Met (n = 1) | 0:4 | LLS (n = 1), Pt (n = 3) | 481.7 ± 449.5 | 312.3 ± 158.4 | 1 | 10.6 ± 7.4 | 23.4% | 0% |
| Tsuchiya <i>et al.</i> ^[19] (2012) | 3 | 73 (52-79) | HCC | 0:3 | | 281.3 (mean) | 264.6 (mean) | 0 | 8.6 (mean) | | 0% |
| Kanazawa <i>et al.</i> ^[20] (2013) | 20 | 70 (46-83) | HCC | 15:5 | Pt | 78 (1-1500) | 239 (69-658) | 2 (HALS) | 9 (5-22) | 5% | 0% |
| Shelat <i>et al.</i> ^[23] (2014) | 20 | 57.5 (23-79) | HCC (n = 2), Met (n = 16), others (n = 2) | 0:20 | Minor (n = 14) Major (n = 6) | 400 (IQR 150-200) | 285 (IQR 195-360) | 3 | 4 (1-57) | 10% | 0% |
| Isetani <i>et al.</i> ^[22] (2015) | 12 | 70 (57-81) | HCC (n = 8), Met (n = 2), others (n = 2) | 8:4 | Pt (n = 9), Subseg (n = 3) | 50 (NC-840) | 301 (104-570) | 0 | 12 (9-30) | 0% | 0% |

Data are expressed as median (range) or mean ± standard deviation, unless stated otherwise. *In the paper by Belli *et al.*,^[13] operating time, bleeding and POHS are described separately for patients whose previous hepatectomy was open (upper) or laparoscopic (lower). Con: conversion to laparotomy; HALS: hand-assisted laparoscopic surgery; HCC: hepatocellular carcinoma; IQR: interquartile range; LLS: left lateral sectorectomy; Met: metastasis; Minor: resection of 2 segments or less; Major: resection of more than 2 segments; NC: low, unquantifiable; POHS: postoperative hospital stay; Pt: partial resection; RFA: radiofrequency ablation; Seg: segmentectomy; Subseg: subsegmentectomy

ICG-R15, and became HCV-negative, after taking a newly developed oral anti-HCV therapy. The patient remained in compensated LC throughout the period in which the four LLRs were performed. As a result, and because of the shortage of cadaver donors in Japan, liver transplantation was not undertaken. During both the first and fourth LLRs, minor anatomical resections (extended segment 3 segmentectomy and 4ab subsegmentectomy, respectively) were undertaken to remove multiple tumors in the same portal territories, because the patient's liver functional reserve (estimated by ICG-R15) was insufficient to support sectionectomy or more extended resection. Furthermore, ablation therapy was not performed for the protuberant tumors necessitating the first and second LLRs and for the tumor adjacent to the IVC at the time of third LLR, owing to the technical challenges associated. Trans-arterial chemo-embolization (TACE) was used prior to the third LLR, but the target tumor had regrown six months after TACE; therefore, LLR was selected for the follow-up treatment.

LLR is highly suitable for repeated laparoscopic partial or local anatomical LR for the treatment of multicentric

metachronous HCCs within impaired liver and for surface HCC in severe LC.^[31,32] The deterioration of liver function should be minimized with the reduced adhesiolysis and dissection required during a laparoscopic approach. In addition, LLR better prepared patients both physically and psychologically for a subsequent repeat LR, illustrated by a shortened hospital stay for the patient reported here. Thus, LLR is a powerful localized therapy which can be applied repeatedly and may prolong the survival of patients with multicentric metachronous HCCs/CLD.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Obtained.

Ethics approval

The patient was treated within the standards of our institute and the report was approved.

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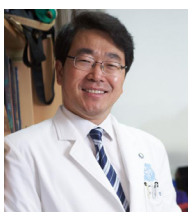
Laparoscopic liver resection for hepatocellular carcinoma in patients with cirrhosis

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ABSTRACT

Hepatocellular carcinoma (HCC) is a common malignant tumor and many cases occur in patients with liver cirrhosis. Although liver transplantation is the most effective treatment option, hepatectomy is still the first curative treatment option because liver transplantation is limited by the donors and high cost. In recent years, laparoscopic liver resection (LLR) has increasingly been performed in patients with liver cirrhosis, and has several advantages over open liver resection. Besides less pain and shorter hospital stay, LLR in patients with liver cirrhosis is also associated with lower incidences of postoperative liver failure and ascites because of greater preservation of collateral veins and less liver manipulation. With increasing experience, LLR for HCC located in segments 7 or 8 is now feasible, and anatomic LLR could be performed in patients with cirrhosis. Many comparative studies have shown that LLR is better than open liver resection in patients with liver cirrhosis in terms of a lower incidence of postoperative liver failure and similar patient survival. In conclusion, LLR is a promising treatment modality for HCC in patients with liver cirrhosis.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignant tumor, the most common primary

liver cancer,^[1] and the third most common cause of cancer-related death worldwide.^[2] Most HCCs are found in patients with liver cirrhosis, although HCC occurs in 60-90% of all patients with liver cirrhosis.^[3]



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Asian countries, especially, have a disproportionately high prevalence of HCC, mainly because chronic hepatitis B and C viruses are endemic in these countries,^[4] and are associated with high risks of liver cirrhosis and HCC.^[5]

Liver transplantation (LT) appears to be the most attractive treatment option because it treats both the cancer and the underlying disease. However, LT is limited by its high cost and the burden of lifelong immunosuppression.^[6,7] Furthermore, the scarcity of donors does not permit LT in all patients with early HCC.^[2] With recent technical advances and improvements in postoperative patient management, liver resection for HCC is now considered to be a safer procedure than it was in the past.^[8-11] Therefore, liver resection is currently regarded as the first-line treatment in many centers for HCC, especially in patients with compensated cirrhosis.^[12]

Since the first report of laparoscopic liver wedge resection, steadily increasing numbers of small case-series have demonstrated the feasibility, adequacy, and safety of laparoscopic liver resection (LLR).^[13-16] Now, LLR is commonly performed in patients with HCC and chronic liver disease.

The aim of this review was to assess the current indications, advantages, and limitations of LLR for HCC in patients with cirrhosis. We also discuss the feasibility of LLR and its oncologic outcomes relative to open surgery.

INDICATIONS

The indications for LLR have changed substantially since its introduction. In the early stages of LLR, it was limited to benign diseases. With increasing knowledge and experience of this procedure, its indications have expanded to malignant diseases, including HCC and colorectal liver metastasis.^[17] However, unlike laparoscopic cholecystectomy, laparoscopy has been limitedly used for liver resection due to the risk of air embolism and the difficulty of parenchymal dissection and bleeding control.^[18] Therefore, LLR has been frequently performed for tumors superficially located in the anterolateral segments.^[19]

For HCC located in segment 7, right posterior sectionectomy is choice of type of resection because it can preserve more functional volume of the liver than right hepatectomy. However, right posterior sectionectomy is technically more difficult and considered as major hepatectomy because it requires parenchymal dissection along the intersectional plane.^[20-23] For HCC located in segments 7 or 8

in patients with very poor hepatic reserve, non-anatomical minor liver resection such as tumorectomy is usually performed. However, it is sometimes very difficult and unexpected huge bleeding from hepatic vein could occur because the operative field is poor, intra-abdominal free space is narrow for manipulation of many instruments, and the transection line can be curved or angled.^[24] LLR for HCC in the posterosuperior segments in selected patients was reported to be as safe and feasible, and offered comparable oncologic outcomes to open liver resection. Moreover, LLR has other benefits, including reduced blood loss, fewer complications, and shorter postoperative hospital stay than open liver resection.^[25]

SELECTION OF SUITABLE PATIENTS

When considering liver resection in patients with cirrhosis, both surgical stress and the oncologic outcomes should be considered.^[13] Similar to open surgery, uncompensated cirrhosis is generally considered to be a contraindication for liver resection and hence LLR.^[26] Uncontrolled portal hypertension, including esophageal varices and low platelet count, is usually considered as a contraindication for LLR.^[27] Because patients with HCC usually have associated chronic liver disease or cirrhosis, these patients may be predisposed to hepatic failure after surgery. Therefore, it is important to preoperatively predict the patient's liver remnant volume and liver function after surgery before selecting the type and extent of liver resection. The hepatic reserve functional capacity is estimated before liver resection to facilitate patient selection and predict the safety margin of parenchymal resection in individual patients.

The Child-Turcotte-Pugh (CTP) score is a simple and the most widely used system for scoring hepatic function before liver resection. It is based on 5 easily measurable variables and, for more than 4 decades, has been considered the gold standard for selecting candidates for liver resection.^[28] However, even CTP class A patients may develop liver failure after LLR.^[29]

The model for end-stage liver disease (MELD) score was made to predict the survival of patients with severe portal hypertension and variceal bleeding who underwent transjugular intrahepatic portosystemic shunt procedure,^[30] and then has been further developed for the selection of patients who are waiting for LT.^[31] Several studies showed that the application of MELD score to predict mortality in patients who underwent liver resection, not LT worked well, and it may outperform the CTP classification in terms of predicting operative risk before liver resection.^[32] However, because MELD score was developed in

non-surgical setting, it is necessary to validate MELD score in patients undergoing liver resection.

The indocyanine green (ICG) test is one of the most commonly used liver reserved function test in Asia-Pacific region. The cut-off value of ICG retention rate at 15 min for safe major liver resection is less than 14%.^[33] However, it is unclear whether this cut-off value is also applicable to patients with liver cirrhosis.

LLR IN PATIENTS WITH CTP CLASS B OR C

Liver cirrhosis is one of risk factors for developing postoperative morbidities after hepatectomy.^[34] Severe blood loss or prolonged ascites after major hepatectomy, especially by open surgery, can occur by interruption of collateral circulation in the parietal wall and surrounding ligaments in patients with liver cirrhosis.^[35] These complications may prolong the postoperative hospital stay or cause hepatic failure in some patients. However, LLR may minimize the reduction in collateral and lymphatic flow caused by laparotomy and mobilization, and may reduce compressive mesenchymal injury, as demonstrated in previous studies of patients undergoing LLR of HCC.^[36,37] The benefits of LLR in this setting include earlier ambulation, less postoperative pain, earlier feeding, and a less postoperative complications. Other important advantages of LLR in patients with liver cirrhosis are the lower incidences of postoperative liver failure and ascites due to minimal invasiveness of LLR, which helps to preserve collateral circulation.^[13] Therefore, laparoscopic hepatectomy may be a good option in patients with cirrhosis.^[38]

Most studies consider CTP class B or C cirrhosis to contraindicate liver resection, and surgeons face a considerable challenge in treating patients with uncompensated cirrhosis. There have been a few reports describing the oncological outcomes of patients with CTP class B or C cirrhosis.^[39] A recent retrospective study of 16 patients with CTP class B or C cirrhosis who underwent LLR showed that LLR did not compromise the oncological outcomes of patients with HCC and clinically significant cirrhosis.^[40] Recently, precoagulation technique before parenchymal transection, intermittent Pringle maneuver during resection, and hybrid technique using hand port were proposed to decrease the technical difficulty of LLR in cirrhotic liver.^[41]

ANATOMICAL VERSUS NON-ANATOMICAL RESECTION

There are still many controversies, but many surgeons believe that anatomical liver resection has some

advantages compared to non-anatomical liver resection for HCC in terms of patient survival and recurrence.^[42,43] HCC recurs after resection mostly in the liver because HCC can spread along the portal branches by microscopic vascular invasion, which contributes to the poor prognosis of HCC.^[44] On this basis, anatomic resection including the whole segment according to the portal tributaries could remove small microscopic metastasis and prolong patient survival and disease free survival.^[45] Anatomical monosegmentectomy of segments 6 or 7 is extremely difficult even in open surgery.^[46] For deep seated large tumor in segments 6 or 7, laparoscopic right posterior sectionectomy will be chosen for more resection margin because segmentectomy or tumorectomy could be insufficient. For deep seated tumor near to right hepatic vein, laparoscopic extended right posterior sectionectomy (resection of right posterior section together with right hepatic vein) can be alternative treatment instead of right hemihepatectomy.^[47]

ONCOLOGIC OUTCOMES OF LLR IN PATIENTS WITH LIVER CIRRHOSIS AND ITS CHALLENGES

Several recent studies have compared the oncologic outcomes between LLR and open liver resection. These studies showed that LLR was associated with lower morbidity and mortality rates, but not 5-year overall and disease-free survival rates.^[48-50] In addition, the most up-to-date and comprehensive systematic review and meta-analysis prepared at the second international consensus conference on LLR highlighted a reduction in the rates of postoperative ascites and liver failure following LLR in cirrhotic liver.^[51,52]

Radiofrequency ablation is a compelling alternative to liver resection in patients with liver cirrhosis, especially in terms of the overall morbidities. In patients with peripherally located lesions, percutaneous ablation may carry a high risk of tumor seeding while LLR can be safely performed and may permit pathological assessment of tumor biology and of the surrounding liver parenchyma.^[53] One propensity score matching analysis showed that liver resection offered a consistent survival benefit and did not increase the incidence of major complications compared with radiofrequency ablation in patients with hepatitis B virus-related HCC and portal hypertension.^[54]

CONCLUSION

LLR has a vital role to play in the first-line treatment of HCC in selected patients with compensated cirrhosis and portal hypertension.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

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Ethics approval

Not involved.

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Laparoscopic resection of hepatocellular carcinoma in patients with and without cirrhosis: the Brisbane experience

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ABSTRACT

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Aim: Laparoscopic liver resection for hepatocellular carcinoma (HCC) is increasingly common around the world. There may be significant advantages over open resections. However, due to technical difficulties, they are performed in few centers with expertise in liver and advanced laparoscopic surgery. In this study the authors summarize the experience to date. **Methods:** A retrospective analysis of consecutive patients undergoing laparoscopic liver resection for HCC in 2 tertiary academic hepatobiliary units in Brisbane, Australia, between 1999 and 2015 was performed. Operative characteristics, perioperative morbidity, and pathological data were described. Patients with and without cirrhosis were analyzed and compared. **Results:** Fifty-two patients underwent resection of 79 HCCs. Sixty-five percent of patients had cirrhosis. Fourteen percent of patients underwent a major hepatectomy. There was a trend towards more parenchyma-sparing resections for cirrhotic patients. Blood loss was higher in cirrhotics. Conversion to an open procedure occurred in 9%. There was one 90-day mortality due to liver failure (1.9%), and 7 patients (13%) experienced a complication. R0 resection was achieved in 92%. Overall survival at 1, 3, and 5 years was 88%, 81%, and 61%, respectively. **Conclusion:** Laparoscopic liver resection for HCC, particularly in cirrhotic patients, is technically challenging. It can be performed with acceptable morbidity and adequate surgical margins.



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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cause of cancer and the third most common cause of cancer-related death worldwide. Furthermore, it is the leading cause of death for patients with cirrhosis.^[1,2] In North America and Europe, the incidence of HCC has been rising, likely due to changing risk factors for cirrhosis, including hepatitis C infection in baby-boomers, alcohol use, obesity, and non-alcoholic fatty liver disease.^[3,4] At presentation, only 10-15% of patients are candidates for surgical resection. Other treatment options include liver transplantation, ablation, transcatheter arterial chemoembolisation, and systemic chemotherapy. Liver resection, more commonly reported as an open procedure, is a well-established, potentially curative treatment for patients with HCC, and is the procedure of choice in many patients with preserved liver function.^[5,6]

Minimally invasive management of HCC is increasing in frequency, including patients with underlying cirrhosis.^[2,7-11] There may be a number of benefits of laparoscopy over laparotomy for HCC, which have been widely reported. Potential benefits include decreased blood loss and need for blood transfusion, decreased complications (i.e. less postoperative ascites, wound infection), decreased length of stay, and reduced technical difficulty with subsequent surgery, including transplantation.^[2,9,10,12,13] Oncological principles can be maintained with laparoscopy and outcomes are comparable, if not better, with laparoscopy compared to open HCC resection.^[13-17] The benefits may derive from the pathophysiological changes that occur with laparoscopy compared to laparotomy, which may be accentuated in the presence of cirrhosis. These include less disruption of the abdominal wall, reduced immune response, and the tamponade effect of pneumoperitoneum.

Laparoscopic resection of HCC is technically challenging. It requires both laparoscopic skills and advanced liver surgical skills. The limited viewing angles, fulcrum effect of laparoscopic ports, instrument clash, reduced tactile feedback, and reduced operating dexterity pose significant challenges in complex surgery. Liver exploration and mobilization, hemorrhage control during parenchymal transection, the use of laparoscopic ultrasound, ensuring adequate oncological margins, and suturing can be more difficult with laparoscopy, especially in the presence of cirrhosis.^[18] The operative time is generally longer than open surgery. Skilled assistance is essential, and for long cases surgeon and assistant fatigue is common. The cirrhotic poses additional complexity with potentially altered vasculature

in the abdominal wall, hilum and retroperitoneum, and a stiffened liver that resists laparoscopic handling. However, laparoscopic resection of HCC in both non-cirrhotic and cirrhotic patients can provide good clinical outcomes, act as bridge-to-transplant and deliver acceptable survival rates.^[19]

The worldwide experience in laparoscopic liver resection for HCC is increasing, although major resections are still limited to few centers. The current study describes our experience in Brisbane, Australia, with focus on perioperative outcomes.

METHODS

Data acquired from a prospectively collected laparoscopic liver surgery database from multiple centers in Brisbane, Australia, were examined and retrospectively reviewed. Ethics approval was obtained prior to the commencement of the study. Consecutive patients who underwent laparoscopic resection of HCC between January, 1999 and September, 2015 were selected. All patients underwent high quality preoperative imaging with contrast-enhanced computer tomography and magnetic resonance imaging. Selection for laparoscopic resection took into consideration tumor size and location, the degree of underlying liver disease and portal hypertension, and the patient's fitness and ability to tolerate a prolonged pneumoperitoneum. Patients with Child-Pugh B and C cirrhosis were generally excluded. All patients were discussed and management was agreed upon at a multi-disciplinary team meeting.

Collected intraoperative data included details of the surgical procedure (minor vs. major; anatomic vs. non-anatomic), operation duration, blood loss and conversion to laparotomy. A wide range of clinicopathological factors were collected regarding underlying liver disease (METAVIR score), etiology, number and size of tumors, lymphatic or vascular invasion, tumor differentiation, presence of satellite nodules, and pathological margins. A microscopic margin of ≥ 1 mm was defined as R0.

Surgical technique had been described previously.^[20,21] In brief, pneumoperitoneum was established via an open access technique and maintained at 12-15 mmHg. Four to 6 working ports were used. The Pringle maneuver was used selectively. In selected patients with dome lesions, additional intercostal and transthoracic trocars were used. For major hepatectomies, inflow vascular structures were controlled with clips or vascular staplers and the hepatic veins were controlled extrahepatically. Parenchymal transection was performed using LigaSure (Covidien, Mansfield, MA, USA), harmonic

Table 1: Baseline characteristics

| Baseline characteristics | Median (range) or n (%) |
|--------------------------------|-------------------------|
| Age, years | 58 (44-81) |
| Female | 26 |
| α -fetoprotein, ng/mL | 8.5 (1.7-59,000)* |
| No cirrhosis | 18 (35) |
| Cirrhosis | 34 (65) |
| Child-Pugh class | |
| A | 33 |
| B | 1 |
| C | 0 |
| Known underlying liver disease | 37 (71) |
| HBV | 2 (4) |
| HCV | 23 (46) |
| Alcohol | 3 (6) |
| NASH | 5 (10) |
| Unknown (with cirrhosis) | 4 (8) |

*14.6% of patients had an α -fetoprotein level ≥ 200 ng/mL. HBV: hepatitis B virus; HCV: hepatitis C virus; NASH: non-alcoholic steatohepatitis

shears, or the 10 mm laparoscopic Cavitron Ultrasonic Surgical Aspirator (Integra Life sciences Corporation, NJ, USA), combined with use of locking clips (Hem-o-lok, Teleflex Medical, Durham, NC, USA) and staplers. Drains were reserved for patients deemed to be high risk for bile leak. Postoperatively, patients who underwent major resections (> 3 segments) were admitted to the intensive care unit (ICU). Patients with minor resections (< 3 segments) were admitted to the general surgical ward. Chemical and mechanical thromboprophylaxis were used routinely. Early mobilization and return to a normal diet was encouraged.

Postoperative parameters examined included duration of ICU and total hospital stay, postoperative morbidity (stratified according to the Clavien-Dindo system of classification), and 30-day and 90-day mortality. One-, 3- and 5-year survival and disease-free survival status was recorded and actuarial survivals were estimated using the Kaplan-Meier method. Mid and long-term follow up included clinical, biological and radiological assessment 1-month post surgery and every subsequent 6-month. Survival follow-up was achieved through updated medical records and phone calls.

Statistical analyses were performed using R 3.2.0 (R Core Team, 2015). Categorical data were compared using Fisher's exact test; median values from continuous data were compared using the 2-sample rank test; P -values of < 0.05 were considered statistically significant.

RESULTS

Fifty patients underwent a total of 52 operations. In total, 79 HCCs were resected. The annual frequency of laparoscopic resection of HCC in our centers gradually

Table 2: Operative characteristics

| Operative characteristics | Total | Non-cirrhotic | Cirrhotic |
|--|----------------|---------------|----------------|
| Laparoscopic liver resection | 52 | 18 | 34 |
| Major hepatectomy, n (%) | | | |
| Right hepatectomy | 4 (8) | 4 (22) | 0 (0) |
| Left hepatectomy | 1 (2) | 1 (6) | 0 (0) |
| Extended right hepatectomy | 2 (4) | 0 (0) | 2 (6) |
| Left lateral sectionectomy, n (%) | 11 (21) | 4 (22) | 6 (18) |
| Segmentectomy, n (%) | 24 (46) | 7 (39) | 17 (50) |
| Subsegmental resection, n (%) | 10 (19) | 2 (11) | 9 (26) |
| Posterosuperior (segments 1, 4a, 7, 8) resections, n (%) | 8 (15) | 3 (17) | 5 (15) |
| Conversion to laparotomy, n (%) | 5 (9) | 2 (11) | 3 (9) |
| Operating time, min, median (range) | 120 (75-300) | 117 (75-240) | 120 (90-300) |
| Blood loss, mL, median (range) | 300 (20-1,600) | 150 (20-600) | 350 (30-1,600) |

increased, from 7 patients during the early period (1999-2004), to 18 in the middle period (2005-2010), to 25 in the later period (2011-2015) [Figure 1]. The percentage of patients having cirrhosis also increased, from 57%, to 67%, to 72% respectively. Overall in this study, 34 patients (65%) had cirrhosis (33 Child-Pugh A, 1 Child-Pugh B). Of the patients with cirrhosis, 56% had evidence of portal hypertension. The most common etiology of cirrhosis was hepatitis C (65%) and non-alcoholic steatohepatitis (non-alcoholic steatohepatitis, 14%). The baseline characteristics of the patients are summarized in Table 1.

Operative characteristics are summarized in Table 2. There were 7 (14%) major hepatectomies, including 2 extended hemihepatectomies. Seventy-six percent of cirrhotic patients underwent a segmentectomy or subsegmentectomy, compared with 50% of non-cirrhotic patients ($P = 0.068$).

There were 52 operations and of these, 51 were pure laparoscopic (including 2 involving the addition of intercostal or transthoracic trocars) and 1 was hand-assisted. There were 5 conversions (9%), 3 of which (pure laparoscopic intent) were due an inability to progress as a result of difficult dissections secondary to intra-abdominal adhesions. One conversion occurred due to uncontrollable hemorrhage in a non-cirrhotic patient. The final conversion occurred in the hand-assisted case of a posterior dome lesion (segment 7/8). This patient had recognised positive margin due to the awkward angle and was opened for wider resection.

Table 3: Postoperative and histopathological data

| Postoperative and histopathological data | All patients | Non-cirrhotic | Cirrhotic |
|---|--------------|---------------|------------|
| Length of hospital stay, days, median (range) | 5 (1-72) | 5 (3-13) | 5 (1-72) |
| Mortality, 90-day, <i>n</i> (%) | 1 (1.9) | 0 (0) | 1 (3) |
| Overall morbidity, <i>n</i> (%) | 7 (13) | 2 (11) | 5 (15) |
| Infection | 3 (6) | 1 (6) | 2 (6) |
| Ascites | 3 (6) | 0 (0) | 3 (9) |
| Bile leak | 1 (2) | 1 (6) | 0 (0) |
| Tumor margin, mm, median | 9 | 5 | 15 |
| Margin status, <i>n</i> (%) | | | |
| R0 | 48 (92) | 17 (94) | 31 (91) |
| R1 | 4 (8) | 1 (6) | 3 (9) |
| Tumor size, mm, median (range) | 33 (5-220) | 40 (20-150) | 28 (5-220) |
| Number of tumors, median (range) | 1 (1-5) | 1 (1-2) | 1 (1-5) |
| Multifocality, <i>n</i> (%) | 17 (33) | 2 (11) | 15 (44) |
| Tumor differentiation, <i>n</i> (%) | | | |
| Well differentiated | 13 (25) | 6 (33) | 9 (26) |
| Moderately differentiated | 36 (70) | 11 (61) | 23 (67) |
| Poorly differentiated | 3 (5) | 1 (6) | 2 (6) |
| Vascular invasion, <i>n</i> (%) | 17 (33) | 8 (44) | 9 (26) |
| Lymphatic invasion, <i>n</i> (%) | 4 (9) | 1 (6) | 3 (9) |

Subsequent cases involving dome lesions were preferentially performed with a combined transthoracic and transabdominal approach.

Median operative time was 120 min and median blood loss was 300 mL. Patients with cirrhosis experienced more bleeding than those without (median 350 mL in cirrhotics vs. 150 mL in non-cirrhotics, $P = 0.049$). The median length of stay was 5 days and was not different between the cirrhotic and non-cirrhotic groups.

Postoperative and pathological data are summarized in Table 3. There was 1 mortality, which occurred early in the series. The patient had Child-Pugh B cirrhosis and underwent a left lateral sectionectomy. Blood loss was 1,600 mL and this patient died due to decompensated liver failure. Overall 7 patients (13%) developed a postoperative complication. Morbidity rates were not

different between patients with and without cirrhosis. Apart from the single post-operative mortality, all complications were Clavien-Dindo grade 1 or 2.

Multifocal HCC was far more prevalent in cirrhotics compared with non-cirrhotics (44% vs. 11%, $P = 0.008$). Multifocality was generally diagnosed incidentally on pathology of the resected specimen as patients with multifocal disease were excluded from resection. Microvascular invasion was present in 17 patients (33%). Equivalent R0 resection rates were achieved for cirrhotics and non-cirrhotics (94% vs. 91%). Despite the higher rate of subsegmental resections, the median margin in cirrhotics was 15 mm compared with 5 mm in the non-cirrhotics ($P = 0.067$).

Median follow-up was 41 months. Median overall survival was 89 months. Overall survival for the entire cohort at 1, 3, and 5 years was 88%, 81%, and 61%, respectively [Figure 2]. The corresponding survival for non-cirrhosis patients was 93%, 77%, and 67%, and for patients with cirrhosis was 86%, 83%, and 50%. Disease-free survival at 5 years was 57% for all patients, 43% for non-cirrhosis, and 71% for patients with cirrhosis.

DISCUSSION

Laparoscopic liver resection is a technically demanding operation. The presence of cirrhosis increases the operative difficulty, and is considered a relative contraindication to laparoscopic resection in some centers. To our knowledge, there are no data suggesting poorer outcomes for patients who undergo

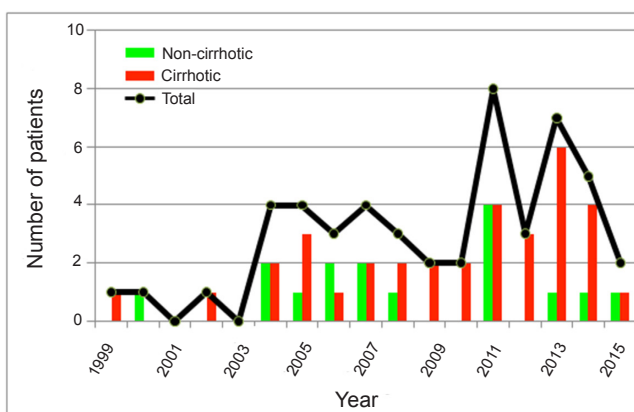


Figure 1: Annual frequency of laparoscopic resection of HCC. HCC: hepatocellular carcinoma

laparoscopic compared to open liver resection in the setting of cirrhosis. In the current study, 65% of patients had cirrhosis (33 Child-Pugh A, 1 Child-Pugh B). There was a trend toward more segmental and subsegmental resections in cirrhotics compared to those without cirrhosis. This reflects the desire to spare parenchyma to reduce post-operative liver insufficiency, but this needs to be balanced against obtaining adequate margins, resecting the “oncological territory” of the tumor, and minimizing blood loss and bile leak. Recent publications have suggested that anatomic resection should be the norm due to the proclivity of HCC to invade the vasculature and metastasize within the liver. However, the heterogeneity with regards to the presence of cirrhosis may be a confounding factor.^[22-24]

One patient with Child-Pugh B cirrhosis underwent a laparoscopic left lateral sectionectomy. This patient died within 30 postoperative days due to postoperative liver failure. This case occurred early in the series and as a result, Child-Pugh B status remains a relative contraindication to surgical resection in our center. However, other authors have demonstrated good short and long-term outcomes with reasonable safety in well-selected individuals.^[25,26]

Compared to open resection, laparoscopy may have a number of benefits in the setting of cirrhosis. Laparoscopy allows for smaller incisions, which may lead to less disruption of the abdominal wall collateral circulation and cause less fluid shifts from exposure of the peritoneal cavity. In those series, 3 patients (9%) with cirrhosis developed postoperative ascites. Post-operative ascites is common after liver resection, even when a relatively small amount of parenchyma is resected. Some studies have demonstrated less postoperative ascites after laparoscopic liver resection compared to laparotomy.^[27-30]

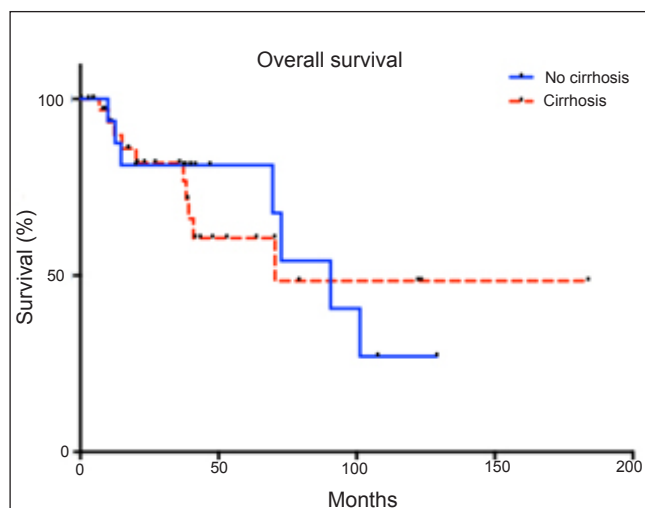


Figure 2: Kaplan-Meier survival analysis

Laparoscopy is also associated with less blood loss and subsequent need for blood transfusion compared to open surgery,^[28,29,31] possibly due to the tamponade effect of pneumoperitoneum on the exposed veins and intra-abdominal varices. To reduce blood loss, pneumoperitoneum can be transiently increased to pressures of 16-20 mmHg during parenchymal transection. Despite concerns over the risk of CO₂ embolism and respiratory compromise during high-pneumoperitoneum, this was not a feature in our series. Laparoscopic ultrasound guidance assists in identifying major vascular structures during transection, but the sensitivity of intraoperative ultrasound in localizing small tumors is reduced in cirrhosis.

For parenchymal transection, we favor the use of the LigaSure which combines the sealing ability of bipolar coagulation forceps and recapitulates crush-clamping technique. Laparoscopic staplers were used mainly for pedicle control and avoided for parenchymal transection due to their tendency to tear the cirrhotic liver.

Ensuring adequate margins is fundamental to the overall outcome of the surgery and subsequent patient prognosis. Whilst the benefits of digital palpation in open surgery may be overstated (especially in cirrhosis), laparoscopy eliminates this capability.^[7,32] We found the use of laparoscopic ultrasound essential in order to determine a precise transection line in relation to the tumor margin and locate important vascular structures.^[32,33]

Straight resection planes are preferred whenever possible. This is relatively easy to achieve for a lateral sectionectomy or a major hepatectomy, dividing the liver along well defined scissura. However, in cases of laparoscopic non-anatomical subsegmentectomies, there is a significant risk of undermining the tumor leading to a positive margin. This is especially true for tumors with a wider circumference deep to the liver surface. Starting the dissection 2 cm wider, particularly on the side of the tumor nearest to the surgeon, helps achieve clearance of the deep margin. Angling the transection away from the tumor may reduce this risk and we frequently employ metal clips as ultrasound visible “markers” which are re-checked through the transection.

Laparoscopic management of liver tumors has been reported more commonly for lesions located within the anterolateral segments of the liver. Some centers consider posterosuperior lesions (segments 1, 4a, 7, 8), particularly dome lesions adjacent to the hepatic veins a contraindication for laparoscopic surgery. This is due to limited visualization, difficult angle of attack, and reduced capability to control the vena cava in the event

of haemorrhage.^[34-36] Such difficulties can be partially overcome by performing a formal hepatectomy, but this is often not ideal as it may be important to preserve hepatic volume in the setting of cirrhosis. In our series, we had 8 dome lesions. During our early experience, we approached a segment 8 dome lesion with a hand-assisted technique. Adequate margins could not be obtained and the case required conversion. We have subsequently modified our technique to use intercostal and transthoracic trocars (ITT) for such lesions. We found that the ITT approach offered better visualization, access for resection and ability to control hemorrhage compared with the hand-assisted technique.

This study is limited by its retrospective nature. We showed that laparoscopic resection is feasible and safe, but without an open comparison group, the true perioperative benefits are unclear. The long-term recurrence and survival outcomes in our cohort need to be further investigated in order to further define the oncological equivalence of laparoscopic resection compared with open.

In conclusion, laparoscopic liver resection for hepatocellular carcinoma can be performed with acceptable morbidity and adequate surgical margins. The technical challenges of liver resection are often magnified with laparoscopy, particularly in patients with cirrhosis. However, such difficulties can be overcome with increasing experience. We believe that the benefits of a minimally invasive approach are also more pronounced in cirrhotics, due to the potential to reduce morbidity compared to an open approach. Future studies comparing laparoscopic to open resection with long-term follow-up should be performed to further define its role.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Necessary consent was obtained.

Ethics approval

Approved by an institutional board at Royal Brisbane Hospital.

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Liver resection for hepatocellular carcinoma within a fast-track management: a propensity-score matched analysis between open and laparoscopic approach

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ABSTRACT

Aim: The study was designed to assess the implications of enhanced recovery after surgery (ERAS) approach in patients submitted to open liver resection for hepatocellular carcinoma (HCC) comparing their short term outcome with patients treated by laparoscopic approach, in a case-matched design. **Methods:** The open-group ($n = 60$) was matched in a ratio of 1:1 with patients undergoing laparoscopic liver resection for HCC (Lap-group, $n = 60$), with a matching achieved on a basis of propensity scores including 6 covariates representing patients characteristics and severity of the disease. Primary outcome analysis was performed in terms of ERAS-specific items and postoperative morbidity and mortality. **Results:** Overall morbidity and mortality were comparable between groups. Incidence of ascites was slightly higher in the open- compared with the Lap-group (respectively 11.7% and 13.3%), without statistical significance. The need for introduction or increase of chronic diuretic therapy was significantly higher in the open-compared with the Lap-group (16.7% vs. 11.7%, $P = 0.046$). Furthermore, ascites more frequently required percutaneous drainage in the open-compared with the Lap-group (5% vs. 1.7% respectively, $P = 0.041$). **Conclusion:** In patients who can't benefit from minimally-invasive approach because of disease characteristics, ERAS management seems to be associated with an improved postoperative functional recovery and postoperative outcomes, comparable to those of the minimally invasive approach.

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enhanced recovery after surgery,
liver failure

INTRODUCTION

Thanks to the widespread diffusion of laparoscopic surgery of the liver, surgical technique has experienced

a significant improvement that was widen to encompass even patients management.^[1-3] Indeed, this innovative trend included the application of multimodal perioperative care protocols, called fast track or enhanced recovery



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programmes (enhanced recovery after surgery, ERAS) which allowed to achieve a significant gain in terms of postoperative outcome in many abdominal surgical procedures.^[4] Many factors have a recognized impact on delayed postoperative recovery (pain, gut dysfunction and immobility): to reduce peri-operative stress and organ dysfunction, fast-track programmes were developed with the rationale of targeting these factors and with the aim of accelerating postoperative recovery and reduce length of stay, even lowering the rate of postoperative complications.^[5] Furthermore, it is reported that the establishment and adoption of evidence-based practice guidelines improves surgical outcomes:^[6] with this aim, a dedicated and specific program with well-defined recovery and discharge criteria was developed and applied into daily clinical practice of centres with a strong commitment in minimally-invasive approach. Thanks to encouraging results, many items of ERAS program have been more extensively implemented and their application was extended even to conventional open surgery.^[7,8]

The preservation of wall portosystemic shunts is one of the advantages of laparoscopic approach when performed in patients with hepatocellular carcinoma (HCC), with a favourable impact on postoperative outcome leading to a reduced rate of hepatic decompensation.^[9,10] Many reports, including a meta-analysis from Zhou *et al.*^[11] concluded that laparoscopic liver resection (LLR) for HCC allows to obtain more favourable outcomes compared with open liver resection (OLR) in terms of its perioperative results, although it does not negatively affect the oncological outcomes. However, while most studies evaluating the results of LLR and OLR for HCC are retrospective series collected out of the fast-track perspective,^[12] patients affected by HCC, requiring liver resection but unsuitable for laparoscopy, might benefit from ERAS management since they have a baseline higher risk of postoperative complications due to peri-operative stress. To our knowledge, no specific report exists to prospectively evaluate this topic. The present study was designed to assess the implications of ERAS approach in patients submitted to open liver resection for HCC comparing their short term outcome with patients treated by laparoscopic approach, in a case-matched design using propensity scores.

METHODS

Study population

In total 2,058 liver resections were performed at the Hepatobiliary Surgery Division of San Raffaele Hospital, Milano in the period between January 2004 and April 2016. Of these, 469 (22.8%) were performed for HCC. Fast-track principles were systematically

applied to LLR and in 2011 these principles were broadened to encompass even OLR. From 2012 on, a dedicated, “ERAS items-based” database was used to prospectively collect data from these procedures and to improve their reproducibility and comparability. During the study period (2012-2016), 203 resections for HCC were performed. Procedures with any of the following characteristics were identified and excluded: re-resections, need for associated vascular or biliary reconstruction, major vascular involvement or thrombosis, extra-hepatic disease. A group of 156 eligible resections was obtained. Among these, 81 had been operated on by an open approach.

With a ratio of 1:1 patients undergoing open liver resection were matched with those who had undergone LLR for HCC, to constitute the open-group ($n = 60$, study group) and the Lap-group, ($n = 60$, control group). Propensity scores were used to achieved the matching, with the following 6 covariates included: age, American Society of Anesthesiology score, Child-Pugh class, tumor size, nodularity, and extent of hepatectomy.

Preoperative workup

Liver function tests (to assess Child-Pugh classification) and serum tumor markers, abdominal ultrasonography, thoracoabdominal imaging were used as a standard preoperative assessment. Weekly multidisciplinary meetings, including liver surgeons, radiologists and medical oncologist were systematically performed, discussing patients who were potential candidates for LLR to define the final indication for the surgical procedure and both the type and the resection technique.

Surgical technique

A right subcostal extended to midline incision was performed in open cases. The “French” position was used to place patients submitted to laparoscopic resections, with the first surgeon standing between the patient’s legs and one assistant on each side. A 4-trocar configuration was generally used with a 15 mm port to house the 30° laparoscope. The SonoSurg system (Olympus, Tokyo, Japan) integrating both the ultrasonic coagulating cutter and the conventional ultrasonic dissector was used to perform the hepatic transaction.^[13] Pringle maneuver was used to control intraoperative bleeding.

Perioperative management

The ERAS multimodal protocol was adapted from the initial model to elective liver surgery,^[7] with the main goal to enhance functional recovery [Table 1]. A specific anaesthesiological management protocol was also developed to guide both intraoperative monitoring

Table 1: Fast-track management protocol

| | |
|----------------------------|--|
| Before surgery | |
| | Preoperative counselling (surgeon, anaesthesiologist, nurse) |
| | Normal oral nutrition until midnight |
| | No preanaesthetic medication |
| | No bowel preparation |
| Day of surgery | |
| | Carbohydrate drinks up to 2 h before surgery |
| | Local analgesia* |
| | Short-acting i.v. anaesthetic agent |
| | Nasogastric drainage remove immediately after surgery |
| | Warm i.v. fluids and lower body air-warming |
| | Avoidance of excessive i.v. fluids (intraoperative SVV > 12%)* |
| | No routine drainage of the peritoneal cavity |
| | Allowed intake of water/nutrition after surgery |
| | Patient sent to surgical ward |
| Postoperative day 1 | |
| | Patient mobilizes with physiotherapist |
| | Patient drinks at least 1.5 L |
| | Normal diet |
| | Continue portable local analgesia |
| | 1,000 mg paracetamol every 8 h |
| | Laboratory tests |
| Postoperative day 2 | |
| | Continue portable local analgesia |
| | Discontinuation of ev fluids |
| | Remove urinary catheter |
| | Continue mobilization |
| | 1,000 mg paracetamol every 8 h |
| | Normal diet |
| Postoperative day 3 | |
| | Start tapentadol |
| | Stop local analgesia |
| | Continue mobilization |
| | Normal diet |
| | Laboratory tests |
| | Check discharge criteria |
| Postoperative day 4 | |
| | Check discharge criteria |
| | Patient receives telephone number of case manager nurse |
| | Discharge |
| Discharge criteria | |
| | Adequate oral feeding |
| | Adequate pain control with oral analgesics |
| | Normal deambulation and self-care autonomy |
| | No complications |
| | Bowel recovery |
| | Patient agreement |

*See Table 2 for anaesthesiological management protocols. SVV: stroke volume variation

of patients volemic status and postoperative pain management [Table 2].

Definition of functional recovery was based on the following criteria (the patient is considered functional recovered when all the criteria are met):

Table 2: Intra- and postoperative management of volemic status and pain

| | Minor open | Major open | Laparoscopic |
|-------------|------------------------------------|------------------------------------|------------------------------------|
| CVC | No | No | No |
| Vigileo | Yes | Yes | Yes |
| Anaesthesia | Gen + Peri or Gen + Spin & TAP | Gen + PVT | Gen + Spin & TAP |
| Paracetamol | 1 g × 3 | 1 g × 3 | 1 g × 3 |
| Tapentadol | 50 mg × 2 (if spinal) | 50 mg × 2 | 50 mg × 2 |
| NSAID | Ketorolac 30 mg ab (max 90 mg die) | Ketorolac 30 mg ab (max 90 mg die) | Ketorolac 30 mg ab (max 90 mg die) |

CVC: central venous catheter; Gen: general; Peri: peridural; Spin: spinal; TAP: transversus abdominis pain block; PVT: paravertebral; NSAID: nonsteroidal anti-inflammatory drug

- (1) Pain adequately controlled with oral analgesics;
- (2) Independently mobile (mobile at preoperative level);
- (3) Tolerance of solid food: fluid and solid food intake is monitored and must be returned to normal tolerance level, i.e. when oral intake of water and normal food is resumed and continued for at least 24 h. Since postoperative nausea and vomiting obviously influences the intake of fluid and solid food, a specific prophylaxis is always performed;
- (4) Normal or decreasing serum bilirubin;
- (5) No intravenous fluids.

Outcome evaluation

Data regarding general characteristics of patients and disease were recorded. Intraoperative and postoperative outcome were evaluated, including morbidity and mortality. Postoperative complications were reviewed for 90 days following liver resection and were graded according to Dindo-Clavien classification of surgical complications.^[14] Ascites was defined as an output > 500 mL per day from abdominal drainage (when positioned) or a clinically relevant abdominal distension requiring diuretics and/or iv albumin. Postoperative mortality was defined as any death during postoperative hospitalization or within 90 days after resection.

Specific issue regarding ERAS management (nasogastric tube and drainage placement, oral feeding, mobilization, bowel canalization, adequate pain control with oral analgesics, time for functional recovery, agreement for discharge, rate of readmission, length of stay) were specifically collected and analyzed.

Statistical analysis

Matching control patients undergoing laparoscopic surgery were selected according to propensity scores based on 6 covariates in a ratio of 1:1 with the open-group: this study design was chosen to adjust for the

Table 3: Preoperative characteristics of patients among groups

| Variables | Lap-group (n = 60) | Open-group (n = 60) | P |
|------------------------------------|--------------------|---------------------|-------|
| Age, mean \pm SD* | 66 \pm 7 | 69 \pm 6 | NS |
| Gender, M/F, n (%) | 35/25 (58.3/41.7) | 29/31 (48.3/51.7) | NS |
| ASA, 2/3, n (%)* | 31/29 (51.6/48.4) | 31/29 (51.6/48.4) | NS |
| Comorbidities, n (%) | 36 (60) | 38 (63.3) | NS |
| Underlying liver impairment, n (%) | | | NS |
| Healthy liver | 10 (16.7) | 10 (16.6) | |
| Mild impairment | 18 (30) | 31 (51.7) | |
| Cirrhosis | 32 (53.3) | 19 (31.7) | |
| Child class, n (%)* | | | NS |
| A | 53 (88.3) | 57 (95) | |
| B | 7 (11.7) | 3 (5) | |
| C | 0 (0) | 0 (0) | |
| Tumor size, cm, mean \pm SD* | 3.6 \pm 1.2 | 4.1 \pm 1.6 | NS |
| Tumor location, n (%) | | | 0.039 |
| Laparoscopic Sg | 48 (80) | 1 (1.7) | |
| Non laparoscopic Sg | 12 (20) | 59 (98.3) | |
| Nodularity, n (%)* | | | NS |
| Single | 53 (88.3) | 53 (88.3) | |
| Multiple | 7 (11.6) | 7 (11.6) | |

*Covariate used for propensity score matching. M: male; F: female; ASA: American Society of Anesthesiology; Sg: segment; NS: not significant

different covariate distributions of the 2 groups. After matching, all variables were compared using the χ^2 or Fisher's exact test for categorical data, the Mann-Whitney U test for non-normally distributed continuous data, and Student's *t*-test for normally distributed continuous variables. All data are expressed as mean plus or minus the standard deviation or median and range, as appropriate. Significance was defined as $P < 0.05$. All analyses were performed using the Statistical Package SPSS 18.0 (SPSS, Chicago, IL, USA).

RESULTS

Patients and disease characteristics

Patients and disease characteristics are summarized in Table 3. A minority of patients had impaired liver function, classified as Child B (respectively 11.7% in the Lap-group and 5% in the open-group). A different distribution of lesions within liver segments was recorded comparing the 2 groups: in particular lesions in the so called non-laparoscopic segments (1, 7, 8) were 20% in the Lap-group and 98.3% in the open-group ($P = 0.039$).

Surgical procedures and intraoperative outcome

The procedures are reported in details in Table 4. In particular, major hepatectomies were performed in 18.3% of patients in the Lap-group and in 15% of patients in the open-group. Mean intraoperative blood loss was higher in the open-compared with the Lap-group (respectively 300 ± 250 mL and 200 ± 100 mL), even though this difference was not statistically significant. Thirteen point 3% of patients belonging to Lap-group required conversion to open approach:

most frequent reasons for conversion were bleeding (3 patients) and oncological adequacy (5 patients). A R0 resection margin was obtained in 59 patients (98.3%) in the Lap-group and 58 patients (96.7%) in the open-group, without significant differences.

Nasogastric tube was routinely removed after surgery in all the patients, following ERAS principles; only one patient with known swallowing disorder (in the open-group) had the tube removed in the second postoperative day. Patients who required surgical drainage were those with intraoperative evidence of bile leakage from the surface of the transected liver or with lesions located in areas unsuitable for an eventual percutaneous drainage (11 patients in the Lap-group and 13 patients in the open-group). Four patients in the Lap-group and 6 in the open-group underwent central venous catheter placement during surgery, while volemic status was intraoperatively monitored by the means of stroke volume variation measure.

Postoperative outcome

Table 5 reports postoperative outcome. Overall morbidity and mortality were comparable between groups. A detailed analysis of the rate of postoperative liver failure in terms of hepatic decompensation was performed: incidence of ascites was slightly higher in the open-compared with the Lap-group (respectively 11.7% and 13.3%), without statistical significance. Despite this, the need for introduction or increase of chronic diuretic therapy (both for ascites or peripheral edema) was significantly higher in the open-compared with the Lap-group (16.7% vs. 11.7%, $P = 0.046$). Furthermore, ascites more frequently required percutaneous drainage in the open-compared with the Lap-group (5% vs.

Table 4: Intraoperative outcome among groups

| Variables | Lap-group (n = 60) | Open-group (n = 60) | P |
|---|--------------------|---------------------|----|
| Procedure, n (%) | | | NS |
| Wedge resection | 19 (31.7) | 16 (26.7) | |
| Segmentectomy | 16 (26.7) | 21 (35.0) | |
| Left lateral sectionectomy | 8 (13.3) | 3 (5) | |
| Bisegmentectomy | 6 (10) | 11 (18.3) | |
| Right hepatectomy | 5 (8.3) | 5 (8.3) | |
| Left hepatectomy | 6 (10) | 4 (6.7) | |
| Resection extent, n (%) [*] | | | NS |
| Minor | 49 (81.7) | 51 (85) | |
| Major | 11 (18.3) | 9 (15.0) | |
| Associated procedures, n (%) | | | NS |
| Cholecistectomy | 31 (51.7) | 33 (55) | |
| RF ablation | 4 (6.7) | 3 (5) | |
| Operative time, min, mean ± SD | 190 ± 55 | 140 ± 45 | NS |
| Blood loss, mL, mean ± SD | 200 ± 100 | 300 ± 250 | NS |
| Conversion to laparotomy, n (%) | 8 (13.3) | NA | |
| Pringle maneuver, n (%) | 50 (83.3) | 48 (80) | NS |
| Resection margin, n (%) | | | |
| R0 | 59 (98.3) | 58 (96.7) | NS |
| R1 | 1 (1.7) | 2 (3.3) | NS |
| Total PRBC transfusion, n (%) | 6 (10) | 7 (11.7) | NS |
| Nasogastric tube removed in OR, n (%) | 60 (100) | 59 (98.3) | NS |
| Drainage placement, n (%) | 11 (18.3) | 13 (21.7) | NS |
| CVC placement, n (%) | 4 (6.7) | 6 (10) | |
| Epidural/paravertebral analgesia, n (%) | 52 (86.7) | 51 (85) | NS |
| Need for ICU, n (%) | 1 (1.7) | 1 (1.7) | NS |

^{*}Covariate used for propensity score matching. PRBC: packed red blood cells; OR: operating room; ICU: intensive care unit; RF: radiofrequency; CVC: central venous catheter; NA: not available; NS: not significant

Table 5: Postoperative outcome among groups

| Variables | Lap-group (n = 60) | Open-group (n = 60) | P |
|--|--------------------|---------------------|----|
| Postoperative mortality, n (%) | 0 (0) | 0 (0) | NS |
| Postoperative morbidity, n (%) | 9 (15) | 10 (16.7) | NS |
| Minor (grade I-II) | 6 (10) | 8 (13.3) | NS |
| Major (grade III-V) | 3 (5) | 2 (3.3) | NS |
| Postoperative liver failure, n (%) | 1 (1.7) | 2 (3.3) | NS |
| Ascites, n (%) | 7 (11.7) | 8 (13.3) | NS |
| Hemorrhage, n (%) | 1 (1.7) | 1 (1.7) | NS |
| Biliary fistula, n (%) | 1 (1.7) | 2 (3.3) | NS |
| Pleural effusion, n (%) | 2 (3.3) | 5 (8.3) | NS |
| Oral feeding, median (range) | 1 (0-1) | 1 (0-2) | NS |
| Mobilization, median (range) | 1 (1-2) | 1 (1-3) | NS |
| Bowel canalization, median (range) | 2 (1-4) | 3 (2-5) | NS |
| Adequate pain control orally, median (range) | 3 (2-4) | 4 (2-5) | NS |
| Time for functional recovery, median (range) | 3 (2-5) | 3 (3-5) | NS |
| Agreement for discharge, median (range) | 3 (2-8) | 4 (3-6) | NS |
| Hospital stay, median (range) | 4 (3-9) | 5 (4-10) | NS |
| Rate of readmission, n (%) | 2 (3.3) | 3 (5) | NS |

NS: not significant

1.7%, $P = 0.041$) and finally, in patients who required intraoperative placement of the surgical drainage, daily output was higher in the open compared with the Lap-

group (respectively $1,000 \pm 200$ mL and 600 ± 100 mL, $P = 0.05$), so that the drainage was left in place longer. The analysis of the series after exclusion of converted patients confirmed the same findings.

Overall, median length of postoperative stay was comparable between groups, being respectively 4 days (range: 3-9 days) in the Lap-group and 5 days (range: 4-10 days) in the open-group. Median time for functional recovery was 3 days in the Lap-, as well as in the open-group. The rate of readmission was 3.3% in the Lap-group (2 patients were re-admitted: 1 due to fever and 1 for refractory ascites) and 5% in the open-group (3 patients: 1 biliary fistula, 1 pleural effusion and 1 fever).

DISCUSSION

Liver surgery for HCC, in patients managed within a fast-track approach, seems to be feasible and safe both when performed by minimally-invasive and by open approach. This is, to our knowledge, the first series that compares the two techniques in an ERAS perspective, specifically in patients with HCC, for which laparoscopic approach was proved to be associated with improved outcomes in terms of intraoperative bleeding and postoperative complications. Outside

of randomization, a case match design was chosen as the most suitable to address this bias of a possibly higher severity of disease or of a different liver function in laparoscopic patients.

The present study reports how the application of fast-track management in the field of liver surgery for HCC allows to improve the results of open approach and to obtain a short term outcome similar to that of the laparoscopic technique. Despite this, laparoscopy confirms its advantage, as already reported in most series and meta-analyses available until now in the literature.^[9-12] Indeed, in spite of a comparable incidence of postoperative hepatic decompensation (ascites) between the Lap- and the open-group, patients in the open-group more frequently required the introduction or the increase of diuretic therapy in the period after surgery. In cirrhotic patients indeed, the advantages of laparoscopy include the preservation of wall portosystemic shunts and the round ligament, consequently no increases in portal pressure are recorder: this is the physiopathological basis for the increased risk of bleeding and ascites.^[9,11,12] Moreover, the impact of laparoscopy on postoperative outcome, due to negative effects related to inflammatory profile and coagulation homeostasis alterations, are reduced compared to conventional surgery,^[9] thanks to the conceptual change in perioperative management protocols, that was recently applied even in open surgery. Factors that delay postoperative recovery (pain, gut dysfunction and immobility) were targeted, resulting in a reduction of the peri-operative stress and organ dysfunction.

As widely reported in the literature,^[4-8] ERAS approach is based on several different items, with a different range of penetration and application among centers implementing fast-track programs. Furthermore, Wong-Lun-Hing *et al.*^[8] demonstrated that the advantage associated with this perioperative management significantly correlates with compliance with the ERAS program, so that there is further need to further optimize the ERAS strategy within a multidisciplinary effort. In our center, the implementation of fast-track was wide since the beginning of the experience: then, after the first period of application, the protocol was revised by the multidisciplinary team to allow the use of a protocol tailored on the characteristics of both the institution and the series. Due to the relatively statistically limited power of a comparison between the first and the subsequent experience related to a still reduced pool of patients, the effective improvement of results along with the reappraisal of the protocol was not analyzed in the present series and was beyond study aims.

The figure of the “case-manager nurse” was introduced with the aim of being a contact-person during patients hospital stay and to monitor the early period following discharge: indeed, thanks to the frequent contact, the family and the patient himself have the feeling of a “protected-discharge” regimen and any complication occurring at home is not misinterpreted or misdiagnosed. This even allows to lower the rate of unnecessary or inappropriate accesses in the Emergency Department.

The issue of the impact of prophylactic drainage in patients with underlying liver impairment was analysed in a specifically designed randomized controlled trial,^[15] which reported a detrimental effect of abdominal drainage on morbidity, without really be adequate in detection of bile leakages and bleedings. A meta-analysis by Petrowsky *et al.*,^[16] including all randomized trials^[15,17,18] focused on the issue of drainages in liver surgery, concluded that there is a slight outcome advantage for nondrained patients. While in our first experience, the abdominal drainage was systematically avoided both in the laparoscopic and in the open approach. In the current clinical practice we recommend the avoidance of drainage unless there is any concern in terms of biliostasis or if the transection surface can't be easily drained by the means of an eventual percutaneous approach. Indeed, the avoidance of postoperative drainage as prescribed by ERAS protocols (unless necessary to specifically monitor the risk of biliary fistula), may confer an advantage to patients with impaired liver function.

The role of intraoperative volemic control was a flagship issue in the ERAS protocol: indeed, maintenance of patient's hypovolemia and avoidance of water overload seem to favourably affect the intraoperative outcome of candidates to hepatic resection reducing blood loss and transfusion rate.^[19,20] In laparoscopic liver surgery, the positive effect of hypovolemia is increased since it allows to reduce bleeding from hepatic veins: indeed, this kind of bleeding can't be controlled by portal triad clamping and it is frequently responsible for conversion to open approach.^[21,22] Cardiac preload has been traditionally monitored by central venous pressure, while recently, haemodynamic changes during surgery have been successfully assessed using minimally-invasive devices like Flotrac/Vigileo that is proved to be safe and reliable.^[23] Since in cirrhotics baseline systemic vascular resistance is lower and less sensitive to hemodynamic changes, these patients have altered capability to respond to portal clamping so that intraoperative administration of vasopressors (norepinephrine and dopamine) might be required. Crystalloid administration was generally suspended

the second day after surgery unless specifically required by clinical conditions of the patient. In the setting of patients with liver impairment, total body water expansion and renal sodium retention may lead to excessive loss of water across the splanchnic capillaries into the peritoneum, causing expansion of extravascular compartment, worsening ascites decompensation and contributing to hyperdynamic circulatory syndrome.^[24] In patients with clinically evident ascites and without drainage, paracentesis is usually not recommended, while administration of albumin and diuretics has to be preferred (unless ascites infection is suspected).^[25]

The task of faster postoperative functional recovery could be addressed even thanks to a better management of postoperative pain allowing earlier mobilization and return to a good quality of life. In patients with cirrhosis and alterations of coagulation profile and platelet count, placement of epidural catheter (recommended in fast track programs) should be avoided,^[26] right paravertebral thoracic block^[27] and spinal block^[28] are available alternatives, allowing to avoid side effects of opioids.

In conclusion, in patients who can't benefit from minimally-invasive approach because of disease characteristics (i.e. tumor location within the liver), ERAS management seems to be associated with an improved postoperative functional recovery and postoperative outcomes comparable to those of the minimally-invasive approach. So, any further effort to optimize and implement fast-track programs in the daily clinical practice for these patients has to be strongly recommended.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Consents from subjects were waived.

Ethics approval

Approved by the internal review board of IRCCS San Raffaele Hospital.

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Is transarterial embolization a valuable treatment option for spontaneous rupture of hepatocellular carcinoma: experience from a tertiary care hospital of South-Asia

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ABSTRACT

Aim: Transarterial embolization (TAE) has been found beneficial in treatment of ruptured Hepatocellular carcinoma (HCC) in earlier studies. So far no data is available from Pakistan. The aim of this study was to evaluate clinicopathological characteristics, outcomes of patients presented with spontaneously ruptured, unresectable HCC treated with or without TAE and to evaluate the factors associated with 30-day mortality. **Methods:** This was a cross sectional study. Patients ≥ 18 years old, presented with spontaneous rupture of unresectable HCC, were evaluated. The outcome measures were control of bleeding, in-hospital mortality, 30-day mortality and factors associated with 30-days mortality. **Results:** Out of 850 patients, 24 patients were diagnosed with spontaneously ruptured HCC. Mean age was 58.29 ± 15.26 years. A total of 11 (45.8%) patients were treated conservatively and 13 (54.2%) underwent TAE. Control of bleeding due to ruptured HCC was significantly higher for those treated via TAE as compared to those who were treated conservatively (92.3% vs. 36.4%, $P = 0.008$). Overall median duration for which the patients remained alive after HCC rupture was longer for TAE group (39 days vs. 5 days, $P = 0.03$). In-hospital mortality (30.8% vs. 72.7%, $P = 0.04$) and 30-day mortality was also lower in TAE group (38.5% vs. 90.9%, $P = 0.01$). Those who underwent TAE had lower risk of mortality then conservative group [odds ratio (OR) 0.25, 95% confidence interval (CI) 0.07-0.90, $P = 0.03$]. Failure to control bleeding was associated with higher 30-day mortality (OR 2.14, 95% CI 1.24-3.68, $P = 0.009$). **Conclusion:** Ruptured HCC is a life threatening complication requiring early diagnosis and treatment. TAE is an effective and well-tolerated treatment in the management of ruptured HCC.



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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer related mortality worldwide.^[1] Relatively higher incidence rates have been reported from South Eastern Asia and sub-Saharan Africa.^[2] The incidence rate of HCC in Pakistan is equivalent to 2.5 per 100,000 persons per year which is higher than the Sub-continent and Western countries.^[3] Moreover, hepatitis C and B virus infection have been reported to be the major attributable factors responsible for HCC in Pakistan.^[4]

While, most of the patients remain asymptomatic, HCC can manifest with right hypochondrial pain, weight loss, new onset jaundice and ascites.^[5] Hemoperitoneum caused by spontaneous rupture of HCC is a rare but fatal complication associated with mortality ranging between 25-75%.^[6,7] The incidence of spontaneous rupture of HCC ranges 3-15% in South-East Asian countries, which is higher as compared to the reported incidence of < 3% in Western countries.^[6,8,9] Spontaneous rupture of HCC is associated with poor liver functional reserve, advanced stage of tumor and high mortality rates ranging 32-62% as seen in various studies.^[10-12]

It is not only difficult to anticipate the HCC rupture; there are few therapeutic options available to treat such patients. The treatment modalities that have been employed include emergency liver resection in case of preserved liver function and resectable tumor, transarterial embolization (TAE) or transarterial chemoembolization (TACE) in case of advanced disease.^[12,13] TAE has been found beneficial in the treatment of ruptured HCC in earlier studies by allowing control of bleeding and the selection of suitable patients for later liver resection. However, the utility of available treatment options is limited due to the patient's clinical condition and disease stage.^[9,13,14] Moreover, most of the data available consists of studies with non-homogenous study population with variable disease stages, small sample size and limited results related to prognostic factors. No data is available from Pakistan so far.

Hence, in the current study, we report five years' experience with patients who presented with spontaneous rupture of unresectable HCC treated with or without TAE. The aim is to evaluate clinicopathological characteristics and outcomes of patients presenting with spontaneously ruptured, unresectable HCC treated with or without TAE and to evaluate the factors associated with 30-day mortality.

METHODS

Study population and duration

This was a retrospective cross sectional study. Patients ≥ 18 years of age, already diagnosed to have HCC and admitted to Gastroenterology ward of Aga Khan University Hospital (AKUH) during 2006-2015 were identified from our data base by using ICD code 1550. AKUH is a 563 bed, large tertiary care hospital in the metropolitan city of Karachi with a population of 18 million.^[15] The medical record coders at AKUH assign numerical codes for diseases and procedures to all records in accordance with standards outlined in the International Classification of Diseases code book. Those HCC patients who presented with spontaneous rupture of unresectable HCC were studied and analyzed. However, patients with hemorrhagic ascites without HCC or where the required information was incomplete were excluded.

The information about patient's demographics, etiology of underlying cirrhosis, clinical, radiological characteristics, laboratory parameters, stage of HCC, treatment provided and follow up in days were recorded. Child-Pugh score and Model for End Stage Liver Disease (MELD) score were used to define the severity of liver disease.

The main outcome measure was control of bleeding. The other outcome measures were in-hospital mortality, 30-day mortality, overall duration of survival and factors associated with 30-day mortality.

Diagnosis and staging of ruptured of HCC and cirrhosis

The diagnosis of HCC was made by combination of elevated alfa fetoprotein (AFP) (> 20 ng/mL) and characteristic features of HCC on triple-phase computerized tomography (CT) scan/magnetic resonance imaging (MRI); or in the absence of elevated AFP when the concurrent results were found on CT scan/MRI along with presence of background chronic liver disease, with or without histological verification. The diagnosis of cirrhosis was made either on liver biopsy or in the absence of liver biopsy by clinical and laboratory features of portal hypertension i.e. varices on upper gastrointestinal endoscopy, radiological features suggestive of cirrhosis including irregular liver margins, dilated portal vein, splenomegaly and ascites.^[16]

"Spontaneous HCC rupture" was defined when it happened without a history of recent procedure or trauma and the "diagnosis" was established by using contrast CT of the abdomen.

Modality of ruptured HCC diagnosis was defined as: (1) incidental when an asymptomatic HCC was discovered on imaging done during diagnostic procedures performed for some other disease; or (2) symptomatic when diagnosed during workup after symptom appearance. The HCC was considered as “non-advanced” if the lesion was solitary ≤ 5 cm or paucifocal ≤ 3 lesions, with the largest diameter ≤ 3 cm, in the absence of vascular invasion and distant metastases or “advanced,” when the tumor exceeded these limits. Moreover, the HCC was also classified for macroscopic types as: (1) solitary; (2) paucifocal (≤ 3 nodules); (3) multifocal (> 3 nodules); (4) infiltrative (infiltrating pattern of HCC); or (5) massive (huge mass with a diameter of > 10 cm and an undefined boundaries).^[17] In the presence of ≥ 2 lesions, the largest tumor was considered as representative of HCC and the diameter of the representative tumor measured in its greatest dimension was recorded as tumor size. Furthermore, information was recorded regarding hepatic lobes involved, presence of portal vein thrombosis and extra hepatic spread.

The patients were treated “conservatively” when liver reserves were poor defined by a Child class C or they were severely ill due to other comorbid conditions. TAE was performed in a well-equipped interventional radiological suite by a team of experienced interventional radiologists and Gel foam was used as embolizing agent. The study was conducted by maintaining compliance with the Helsinki Declaration and was approved by the Ethical review committee of Aga Khan University Hospital, Karachi.

Statistical analysis

Data was entered and analyzed in SPSS version 17.0. Mean \pm SD and ranges were calculated for continuous variables and proportions for categorical variables. To see the difference between two groups independent student *t*-test, Chi square or Fisher exact was used where appropriate. A univariate logistic regression analysis was conducted to assess the (crude) association of the prognostic factors for 30-day mortality. Biological significance and a value of $P \leq 0.1$ were considered as criteria for a variable to be significant at univariate analysis. Biological plausible interactions among variables and confounding were also checked. Multivariable logistic regression was done and results are expressed as odds ratio (OR), along with 95% confidence interval (CI).

RESULTS

Clinical characteristics of patients

The medical records of 850 patients with HCC who had visited our center during the study period were

reviewed. A total of 24 patients were diagnosed to have spontaneously ruptured, unresectable HCC and were analyzed. The mean age was 58.29 ± 15.25 years (range 17-93 years) and most of them 21 (87.5%) were males. Hepatitis C was the most common cause of cirrhosis (79.2% cases). The mean Child-Pugh score was 9.96 ± 2.85 (range 7-15) and mean MELD score was 17.92 ± 6.38 (range 9-32). On presentation 62.5% had decompensated cirrhosis and many of them had prior history of hospitalization with spontaneous bacterial peritonitis (16.7%), portosystemic encephalopathy (20.8%), variceal bleed (12.5%) or hepatorenal syndrome (4.2%). The most common clinical manifestations of ruptured HCC on presentation were sudden abdominal pain (83.3%), hemoperitoneum (54.2%), symptoms of anemia (83.3%) and hypovolemic shock (25.0%). Diagnosis of ruptured HCC was confirmed on CT scan of abdomen in all cases. The mean tumor size was 7.76 ± 4.22 cm (range 1.7-17.7 cm). Almost two-third of patients had multifocal (50.0%) or massive/infiltrative (25.0%) HCC. Moreover, advanced HCC was found in 87.5% cases on presentation [Table 1].

A total of 11 (45.8%) patients were treated conservatively who either had poor general condition, impaired hepatic reserves, multiple lesions, or when patient had declined any intervention. TAE was performed in 13 (54.8%) cases of ruptured HCC. None of them underwent for emergency resection.

Comparison of patients treated conservatively vs. those treated with TAE

There was no statistically significant difference in age, gender, etiology of underlying cirrhosis or symptoms and signs at presentation among those treated conservatively as compared to those who underwent TAE. The tumor size, macroscopic types, location and stage of HCC were also comparable among both groups [Table 2]. Although the prior hepatic decompensations, MELD and Child score were comparable in both groups, most of the patients in conservative group had patients with Child class C as compared to TAE group (54.5% vs. 15.4%, $P = 0.08$). Likewise, serum total bilirubin level (5.14 ± 3.50 vs. 2.15 ± 1.04 , $P = 0.008$) was higher and albumin was lower (2.04 ± 0.41 vs. 2.63 ± 0.49 , $P = 0.004$) in conservative treatment group as compared to TAE group.

The control of HCC bleeding was achieved in 66.7% cases which was significantly higher for those who were treated via TAE as compared to those who were treated conservatively (92.3% vs. 36.4%, $P = 0.008$). Overall median duration for which the patients remained alive after HCC rupture was longer for

Table 1: Demographic and clinic-pathological characteristics of all HCC patients at baseline (n = 24)

| Characteristics | Data, mean \pm SD or n (%) |
|--|---------------------------------|
| Age (years) | 58.29 \pm 15.26 (range 17-93) |
| Etiology of CLD | |
| HCV | 19 (79.2) |
| HBV | 3 (12.5) |
| NBNC | 2 (8.3) |
| Child class | |
| A | 0 (0) |
| B | 16 (66.7) |
| C | 8 (33.3) |
| Abdominal pain | |
| Yes | 20 (83.3) |
| No | 4 (16.7) |
| Abdominal distension | |
| Yes | 16 (66.7) |
| No | 8 (33.3) |
| Anemia | |
| Yes | 20 (83.3) |
| No | 4 (16.7) |
| Hypovolemic shock | |
| Yes | 6 (25) |
| No | 18 (75) |
| Hemoperitonium | |
| Yes | 13 (54.2) |
| No | 11 (45.8) |
| Mean hemoglobin (g/dL) | 8.4 \pm 3.0 |
| Platelet count (10^9 /L) | 202.58 \pm 176.50 |
| Total leucocyte count (10^9 /L) | 10.96 \pm 4.17 |
| Prothrombin time (s) | 17.38 \pm 5.64 |
| Mean creatinine (mg/dL) | 1.35 \pm 0.57 |
| Serum total bilirubin (mg/dL) | 3.52 \pm 2.87 |
| Alanine transaminase (IU/L) (median) | 50.00 (range 13-768) |
| Alkaline phosphatase (IU/L) | 210.13 \pm 158.07 |
| Albumin (g/dL) | 2.36 \pm 0.54 |
| Tumor size (size of largest lesion in cm) | 7.76 \pm 4.22 (1.7-17.7) |
| AFP (IU/mL) (median) | 52.00 (range 1.00-100000) |
| Macroscopic types | |
| Solitary | 3 (12.5) |
| Paucifocal (\leq 3 nodules) | 3 (12.5) |
| Multifocal ($>$ 3 nodules) | 12 (50.0) |
| Massive (huge diameter $>$ 10 cm, undefined boundaries)/infiltrative | 6 (25.0) |
| Hepatic lobes (location of rupture) | |
| Right | 12 (50.0) |
| Left | 1 (4.2) |
| Both | 11 (45.8) |
| Stage of HCC | |
| Non-advanced | 3 (12.5) |
| Advanced | 21 (87.5) |
| PVT | |
| Yes | 10 (41.7) |
| No | 14 (58.3) |
| Extra hepatic spread | |
| Yes | 9 (37.5) |
| No | 15 (62.5) |

HCC: hepatocellular carcinoma; CLD: chronic liver disease; HCV: hepatitis C virus; HBV: hepatitis B virus; NBNC: non-B, non-C; MELD: Model for End Stage Liver Disease; AFP: alfa fetoprotein; PVT: portal vein thrombosis

TAE group (39 days, interquartile range 88 days) as compared to conservatively treated group (5 days, interquartile range 10 days) ($P = 0.03$). In addition, in-

hospital mortality was significantly lower in TAE group as compared to patients treated conservatively (30.8% vs. 72.7%, $P = 0.04$). Moreover, 30-day mortality was also lower in patients treated with TAE (38.5% vs. 90.9%, $P = 0.01$) [Table 3].

Predicting factors for 30-day mortality

To find out the predicting factors for 30-day mortality, biologically plausible variables were tested on univariate analysis [Table 4]. The only factors which were found significant on univariate and multivariate analysis were TAE to control HCC bleed and control of bleeding. Those who underwent TAE had lower risk of mortality than conservatively treated group (OR 0.25, 95% CI 0.07-0.90, $P = 0.03$). Failure to control bleeding was associated with higher 30-day mortality (OR 2.14, 95% CI 1.24-3.68, $P = 0.009$).

DISCUSSION

In this study, we have evaluated the clinicopathological characteristics, treatment outcomes and survival of patients presenting with spontaneously ruptured HCC who were treated conservatively or with TAE. Success rate for control of bleeding via TAE was higher than with conservative treatment. Overall median duration of survival after HCC rupture was longer for patients treated with TAE. In-hospital and 30-day mortality were significantly lower in TAE group.

The reported prevalence of spontaneously ruptured HCC ranges 5-15%.^[18] The exact mechanism and risk factors for spontaneous rupture are not well known. However, subcapsular localization, rapid growth with tumor necrosis, portal hypertension and regional increase of venous pressure due to tumor thrombi or direct invasion could be responsible for HCC rupture.^[19]

Sudden abdominal pain, hemoperitoneum and hypovolemic shock have been reported as the typical clinical features of ruptured HCC.^[6,9,20] Moreover, hemoperitoneum ascertained by performing abdominal paracentesis has been considered a reliable test to confirm the diagnosis in up to 86% of clinically suspected HCC rupture.^[21] Consistent with the results of other studies most of our patients were male, presented with abdominal pain and distention, hemoperitoneum and shock.

Doppler ultrasound and CT are useful modalities for the diagnosis of HCC rupture.^[22] The CT scan demonstrate HCC rupture by showing the vascular tumor, extent of the bleed and by showing serial density changes with the age of the hematoma.^[23,24] Triphasic contrast enhanced CT scan was done for all

Table 2: Comparison of baseline characteristics of patients treated conservatively vs. those treated with TAE

| Characteristics | Conservative treatment, mean \pm SD or <i>n</i> (%), <i>n</i> = 11 | TAE, mean \pm SD or <i>n</i> (%), <i>n</i> = 13 | <i>P</i> value |
|-------------------------------------|---|--|----------------|
| Age (years) | 61.36 \pm 16.13 | 55.69 \pm 14.60 | 0.37 |
| Gender | | | 0.45 |
| Male | 9 (81.8) | 12 (92.3) | |
| Female | 2 (18.2) | 1 (7.7) | |
| Etiology of cirrhosis | | | 0.72 |
| HCV | 8 (72.7) | 11 (84.6) | |
| HBV | 2 (18.2) | 1 (7.7) | |
| NBNC | 1 (9.1) | 1 (7.7) | |
| Decompensated cirrhosis | 6 (54.5) | 9 (62.5) | 0.67 |
| Prior history of SBP | 3 (27.3) | 1 (7.7) | 0.30 |
| Prior history of PSE | 3 (27.3) | 2 (15.4) | 0.63 |
| Prior history of variceal bleed | 2 (18.2) | 1 (7.7) | 0.57 |
| Prior history of HRS | 0 (0) | 1 (7.7) | 1.0 |
| Abdominal pain | 8 (72.7) | 12 (92.3) | 0.22 |
| Anemia | 9 (81.8) | 11 (84.6) | 0.85 |
| Hypovolemic shock | 2 (18.2) | 4 (30.8) | 0.64 |
| Ascites | 10 (90.9) | 9 (69.2) | 0.32 |
| Hemoperitonium | 7 (63.6) | 6 (46.2) | 0.39 |
| Child class | | | 0.08 |
| A | 0 (0) | 0 (0) | |
| B | 5 (45.5) | 11 (84.6) | |
| C | 6 (54.5) | 2 (15.4) | |
| Child score | 11.0 \pm 2.90 | 9.08 \pm 2.60 | 0.105 |
| MELD score | 19.27 \pm 7.17 | 16.77 \pm 5.67 | 0.361 |
| Prothrombin time (s) | 19.83 \pm 5.50 | 15.32 \pm 5.07 | 0.51 |
| Serum total bilirubin (mg/dL) | 5.14 \pm 3.50 | 2.15 \pm 1.04 | 0.008 |
| Albumin (g/dL) | 2.04 \pm 0.41 | 2.63 \pm 0.49 | 0.004 |
| AFP (IU/mL) | | | 0.99 |
| \leq 20 | 4 (36.4) | 5 (38.5) | |
| $>$ 20 | 7 (63.6) | 8 (61.5) | |
| Tumor size (cm) | 7.64 \pm 4.14 | 7.88 \pm 4.45 | 0.892 |
| Macroscopic type | | | 0.84 |
| Solitary | 2 (18.2) | 1 (7.7) | |
| Paucifocal | 1 (9.1) | 2 (15.4) | |
| Multifocal | 5 (45.5) | 7 (53.8) | |
| Infiltrative | 3 (27.3) | 3 (23.1) | |
| Stage of HCC | | | 0.99 |
| Non-advanced | 1 (9.1) | 2 (15.4) | |
| Advanced | 10 (90.9) | 11 (84.6) | |
| Hepatic lobes (location of rupture) | | | 0.53 |
| Right | 5 (45.5) | 7 (53.8) | |
| Left | 0 (0) | 1 (7.7) | |
| Both | 6 (54.5) | 5 (38.5) | |
| PVT | | | 0.69 |
| Yes | 4 (36.4) | 6 (46.2) | |
| No | 7 (63.6) | 7 (53.8) | |
| Extra hepatic spread | | | 0.42 |
| Yes | 3 (27.3) | 6 (46.2) | |
| No | 8 (72.7) | 7 (53.8) | |

TAE: transarterial embolization; SBP: spontaneous bacterial peritonitis; PSE: porto systemic encephalopathy; HRS: hepatorenal syndrome; HCV: hepatitis C virus; HBV: hepatitis B virus; NBNC: non-B, non-C; MELD: Model for End Stage Liver Disease; AFP: alfa fetoprotein; HCC: hepatocellular carcinoma; PVT: portal vein thrombosis

of our patients and was found very useful in our study to confirm HCC rupture in all cases.

For spontaneously ruptured HCC, emergency hepatic resection with hepatic artery ligation has been used as preferred method of treatment in past. However, the procedure was found to be associated with high mortality of 44-73%. Moreover, it is technically

difficult to perform in decompensated liver disease and in palliative setting for advance disease where it could be associated with high likelihood of peritoneal seeding and poor outcome after resection.^[6,12,18,25] The majority of our patients had advanced HCC, with large tumor size (mean diameter 7.76 cm) and multifocal disease. Hence, none of our patients had emergency hepatic resection.

Table 3: Comparison of outcome among patients treated conservatively vs. those treated with TAE

| Outcomes | Overall | Conservative treatment, <i>n</i> (%) or median \pm range | TAE, <i>n</i> (%) or median \pm range | <i>P</i> value |
|------------------------|------------------------------------|---|--|----------------|
| Control of bleeding | | | | 0.008 |
| Yes | 16 (66.7) | 4 (36.4) | 12 (92.3) | |
| No | 8 (33.3) | 7 (63.6) | 1 (7.7) | |
| In hospital mortality | | | | 0.04 |
| No | 12 (50.0) | 3 (27.3) | 9 (69.2) | |
| Yes | 12 (50.0) | 8 (72.7) | 4 (30.8) | |
| 30-day mortality | | | | 0.01 |
| No | 9 (37.5) | 1 (9.1) | 8 (61.5) | |
| Yes | 13 (54.2) | 10 (90.9) | 5 (38.5) | |
| Median survival (days) | 11.5 (interquartile range 53 days) | 5 (interquartile range 10 days) | 39 (interquartile range 87.5 days) | 0.03 |

TAE: transarterial embolization

Table 4: Univariate analysis for predicting factors for 30-day mortality

| Factors | OR (95% CI) | <i>P</i> value |
|--|-------------------|----------------|
| Age (years) | 1.1 (0.95-1.06) | 0.76 |
| Gender | 1.23 (0.09-15.87) | 0.87 |
| Abdominal pain | 2.0 (0.17-22.79) | 0.57 |
| Hypovolemic shock | 0.20 (0.30-13.06) | 0.46 |
| Child score | 1.04 (0.80-1.47) | 0.58 |
| Child's class | 2.33 (0.35-15.30) | 0.37 |
| MELD score | 1.01 (0.87-1.14) | 0.98 |
| INR | 2.2 (0.30-16.16) | 0.43 |
| Serum total bilirubin (mg/dL) | 1.42 (0.85-2.38) | 0.17 |
| Albumin (g/dL) | 0.43 (0.08-2.23) | 0.31 |
| AFP (IU/mL) | | |
| ≤ 20 | 1 | |
| > 20 | 1.33 (0.23-7.51) | 0.74 |
| Tumor size (cm) | 0.90 (0.73-1.11) | 0.34 |
| Stage of HCC | | |
| Non-advanced | 1 | |
| Advanced | 1.23 (0.09-15.87) | 0.87 |
| PVT | | |
| No | 1 | |
| Yes | 2.5 (0.45-13.64) | 0.29 |
| Extra hepatic spread | | |
| No | 1 | |
| Yes | 0.75 (0.13-4.22) | 0.74 |
| Intervention for control of bleeding (TAE) | | |
| No | 1 | |
| Yes | 0.25 (0.07-0.90) | 0.03 |
| Control of bleeding | | |
| Yes | 1 | |
| No | 2.14 (1.24-3.68) | 0.009 |

OR: odds ratio; CI: confidence interval; MELD: Model for End Stage Liver Disease; INR: international normalized Ratio; AFP: alfa fetoprotein; HCC: hepatocellular carcinoma; PVT: portal vein thrombosis; TAE: transarterial embolization

TAE has been found to be associated with many complications including bleeding, post-embolization syndrome, implanted peritoneal metastases and mortality rate up to 30%. However, considering TAE as minimally invasive and effective in achieving immediate hemostasis in patients with ruptured HCC as compared to resection, TAE could be a procedure of choice to achieve hemostasis without surgery for ruptured HCC.^[26,27] In the past it has been suggested that TAE should only be administered only in the

presence of a patent portal vein.^[28] However, in our study we did not find any significant difference in the control of bleeding and 30-day mortality between patients having a patent or a thrombosed portal vein, its success in both conditions is comparable.

In a series of 62 patients with ruptured HCC, control of bleeding was achieved in 91% (57/62) cases after TAE. Moreover, 30-day mortality was 38% and overall median survival time was 39 days.^[29] In another study, 3 out of 4 patients treated with TAE died within 30 days but most of them had Child's class C cirrhosis.^[30] Likewise, TAE was found effective for control of bleeding in all 14 patients with HCC rupture, without significant impairment in liver function or treatment related deaths. However, only 3 patients survived for more than 6 months.^[31] A success rate of 83% has been reported in series from Hong Kong.^[9] Contrary to that conservative treatment has been reported to carry 100% mortality.^[32] Our results are consistent to the existing evidence. We found higher rates for control of bleeding after TAE as compared to conservative treatment (92.3% vs. 36.4%, $P = 0.008$). In hospital mortality was 72.7% for those treated conservatively as compared to 30.8% after TAE. Moreover, our 30-day mortality rate was lower among our patients after TAE (38.5%) as compared to what has been reported in previous studies.^[6,9,33] None of our patients had procedure related complications.

Severity of underlying cirrhosis, tumor size, vascular and extrahepatic spread, serum creatinine and hypovolemic shock have been reported as prognostic factors influencing survival after spontaneously ruptured HCC.^[13,14,29,34] Although majority of patients who underwent TAE had Child's class A or B and the conservative group had many patients with Child's class C; no significant difference was found in Child's class or MELD score between the two groups. In our study, the only variables that were found to be associated with 30-day mortality were TAE and control of HCC bleed. This might be due to small sample size in our study.

Our study had certain limitations; this is a retrospective, single-center study and our sample size was small. Moreover, none of our study patients had further loco-regional therapy, or chemotherapy later on that could improve their life expectancy. However, considering ruptured HCC is an uncommon, life threatening complication, our study could provide some information about its manifestations and treatment options from this part of the world. The primary aim of managing patients with ruptured HCC is control of bleeding which could be an important factor in determining early mortality. Considering high success rate in control of bleeding, lower mortality rates and improvement in survival as well as quality of life, TAE could be used as procedure of choice to achieve hemostasis at presentation for ruptured HCC. Larger studies would be required to support currently available evidence in favor of TAE.

In conclusion, ruptured HCC is a life threatening complication requiring early diagnosis and treatment. TAE is an effective and well-tolerated treatment in the management of unresectable, ruptured HCC in patients with liver cirrhosis.

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None.

Conflicts of interest

There are no conflicts of interest.

Patient consent

Consent forms were obtained from the patients.

Ethics approval

The study was conducted by maintaining compliance with the Helsinki Declaration and was approved by the Ethical review committee of Aga Khan University Hospital, Karachi.

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Epstein-Barr virus associated secondary hemophagocytic lymphohistiocytosis with an unusual presentation of abdominal compartment syndrome

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ABSTRACT

Hemophagocytic lymphohistiocytosis (HLH) is a cytokine storm syndrome caused by an overactive but ineffective immune reaction. Without prompt diagnosis and treatment, HLH is life-threatening. However, presenting symptoms are often nonspecific, with fatigue and fever being the most common. A high index of suspicion is therefore critical for early diagnosis and timely management. A previously healthy, 65-year-old female who initially presented with fever and abdominal pain developed abdominal compartment syndrome (ACS) requiring decompressive laparotomy on hospital day 6. Intraoperative frozen sections of biopsied liver showed intense portal lymphohistiocytic infiltrates. Epstein-Barr virus DNA copy numbers escalated from 600 copies/ mL after admission to 134,000 copies/mL before death. The diagnostic criteria of HLH-2004 were met. Patient expired on hospital day 12. It is important to raise awareness of ACS being an unusual presentation of HLH. Recent changes in diagnostic criteria tailored to adult HLH cases are reviewed.

INTRODUCTION

Hemophagocytic lymphohistiocytosis (HLH), as its name implies, is featured by the hallmark finding of hemophagocytosis in addition to uncontrolled lymphohistiocytic proliferation. The exact etiology remains unknown. A widely accepted explanation is cytokine storm due to an overactive but ineffective immune reaction.

HLH is classified into primary and secondary forms

according to the World Health Organization classification. Primary HLH is typically seen in children, and caused by mutations inherited in an autosomal recessive pattern. In contrast, adults tend to have the secondary form, which is often triggered by malignancy, infection or autoimmune disorders, with T-cell lymphoma being the most common malignancy and Epstein-Barr virus (EBV) being the most common infection.^[1] A genetic predisposition has been recognized in some but not all of the adult cases, even with targeted high-



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throughput sequencing.^[2] The mutations in adult HLH, when present, are less likely to be bi-allelic.^[3] From the genetic point of view, adult or secondary HLH cases are intrinsically different.^[4] Because HLH-2004 diagnostic guidelines were established for pediatric cases, it has always been a question whether or not HLH-2004 can be readily applied to adult patients.

It is important yet challenging to recognize HLH in a timely manner because HLH can be quickly fatal without prompt diagnosis and treatment, but the presenting symptoms are often nonspecific. We herein present a fulminant fatal case in an elderly female with an unusual presentation of abdominal compartment syndrome (ACS), and review recent advances in diagnosing adult HLH.

CASE REPORT

The patient was a previously healthy, 65-year-old female who presented with fever and chills for 4 days, and mild right upper quadrant abdominal pain for 1 day. Complete blood count (CBC) showed neutropenia ($1.4 \times 10^9/L$) and thrombocytopenia ($72 \times 10^9/L$), which progressed to pancytopenia with hemoglobin level of 7.2 g/dL in 3 days. EBV DNA copy numbers by quantitative real-time polymerase chain reaction (PCR) were 600 copies/mL on hospital day 2. Other viral tests were negative, including cytomegalovirus, herpes simplex virus, human immunodeficiency virus, and hepatitis B and C.

Ultrasonography at admission showed marked nonspecific gallbladder wall thickening in the setting of positive Murphy's sign. Computed tomography (CT) next day suggested severe acute cholecystitis and hepatosplenomegaly, with the liver enlarged from 17.2 cm at admission to 22.3 cm within 21 h, and the spleen from 10.9 cm to 14.2 cm. Other minor findings include prominent portahepatic and periaortic lymph nodes measuring up to 1.0 cm in short axis, pyloric and duodenal wall edema, and the 12.3 cm uterus enlarged by a 9.5 cm fibroid. Subsequent endoscopic retrograde cholangiopancreatography showed gastric ulcers and large circumferential duodenal ulcers. Cholecystostomy was performed. Bacterial and fungal cultures of the biliary drainage were negative.

The patient progressively developed ACS, with abdominal pressures ranging from 15-26 mmHg. An emergent decompressive laparotomy was performed on hospital day 6. Because of worsening hepatic dysfunction and a diffusely enlarged firm liver, a liver biopsy was sent for intraoperative rapid frozen sections. The histologic sections showed large

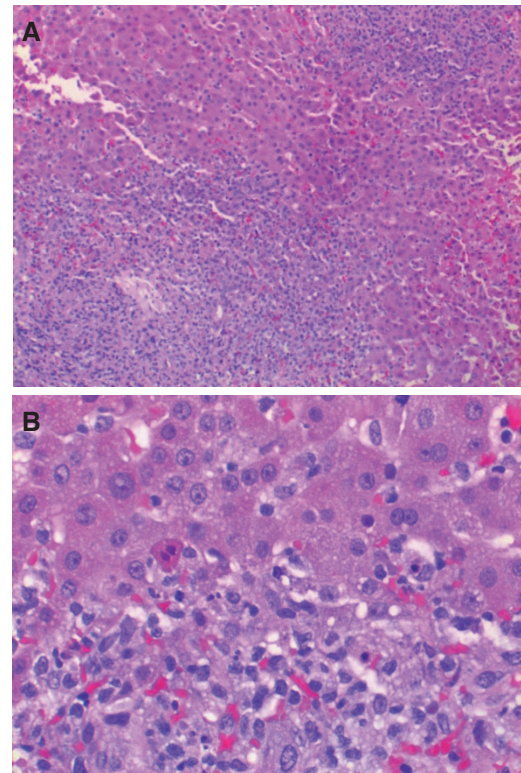


Figure 1: Low-power view (A) shows moderate-sized portal lymphohistiocytic infiltrates (HE, $\times 100$); high-power view (B) shows lymphohistiocytic infiltrates, periportal karyorrhexis and background reactive hepatocytes (HE, $\times 400$)

portal lymphohistiocytic infiltrates [Figure 1A], in a background of reactive hepatocytes and periportal karyorrhexis [Figure 1B]. No hemophagocytosis was identified. Hepatic parenchymal cells appeared to be uninvolved, with only mild limiting plate changes.

The portal lymphocytes were predominantly CD3 positive T cells [Figure 2A], with admixed rare CD20 positive B cells in the background [Figure 2B]. The T cells showed an inverted CD4: CD8 ratio of approximately 1:2 [Figure 2C and 2D], partial loss of CD7 [Figure 2E] and CD45 but appropriate expression of CD5 and CD43. Immunostaining for CD68 highlighted Kupffer cells as well as portal aggregates of histiocytes [Figure 2F].

The paraffin block was sent to integrated oncology for Epstein-Barr virus-encoded small RNAs (EBER) by in situ hybridization and T-cell receptor (TCR) gene rearrangements analysis by multiplex PCR. The portal lymphohistiocytic infiltrate was negative for EBER, with adequate control. Clonalities were detected with primers targeting the conserved variable and joining regions in the TCR gamma and beta genes including TRG V1-8, 9 + J1/2, TRG alternate V + J1/2 and TRB V + J2.

Other relevant laboratory findings included hyperferritinemia

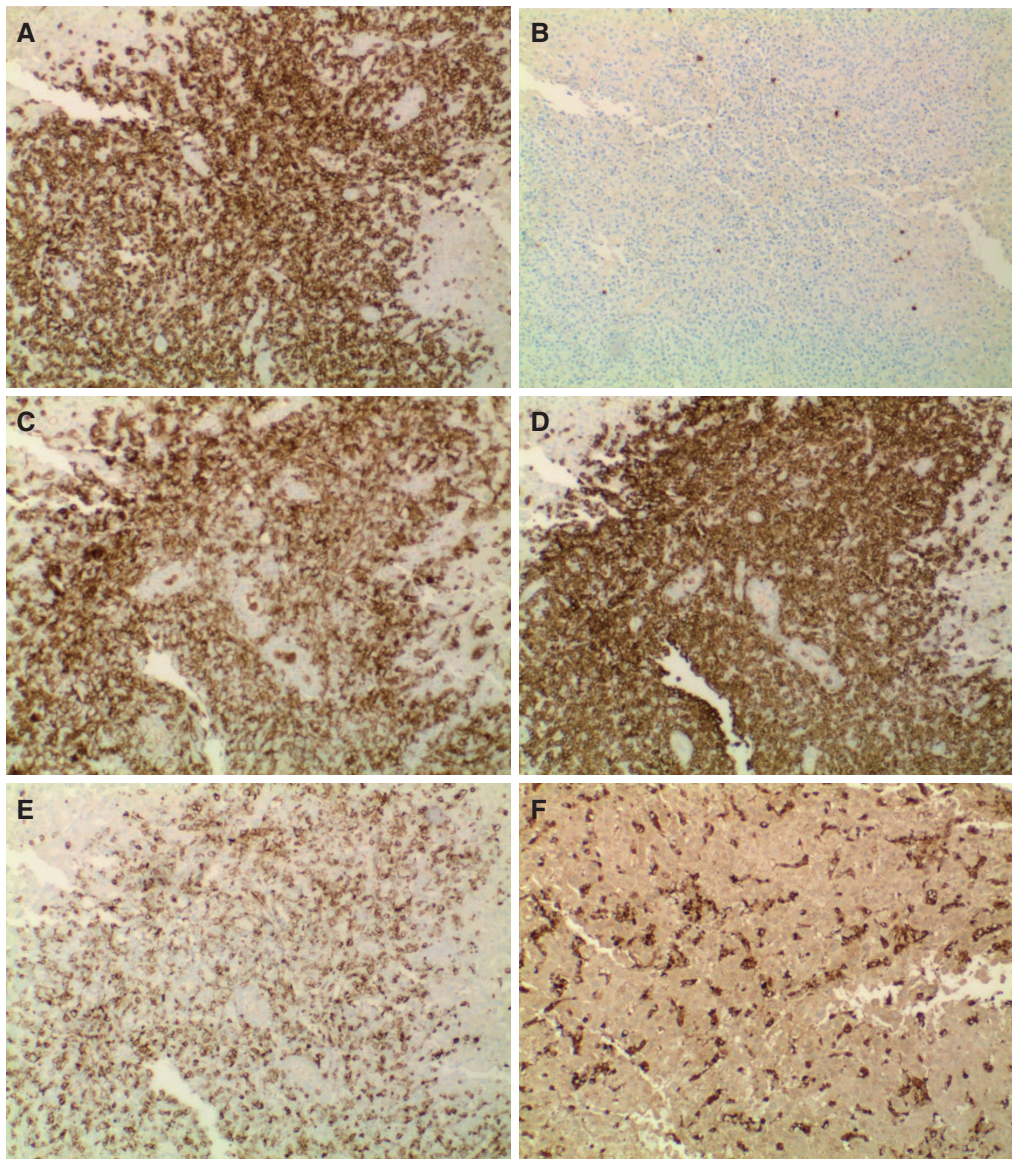


Figure 2: The lymphocytes were predominantly CD3 positive T cells (A), with admixed rare CD20 positive B cells in the background (B); the T cells showed an inverted CD4:CD8 ratio of approximately 1:2 (C, CD4 and D, CD8), partial loss of CD7 (E) and CD45; immunostaining for CD68 highlighted sinusoidal and portal aggregates of histiocytes (F). (IHC, $\times 100$)

(1012 ng/mL), hypertriglyceridemia (662 mg/dL), hypofibrinogenemia (nadir < 50 mg/dL), hyperbilirubinemia (1.3 mg/dL), hyponatremia (126 mmol/L), elevated lactate dehydrogenase (LDH 711 U/L), elevated liver enzymes including aspartate aminotransferase (AST 189 U/L), alanine aminotransferase (143 U/L) and alkaline phosphatase (196 U/L), increased prothrombin time (14.7 s, international normalized ratio 1.5) and activated partial thromboplastin time (51.1 s). C-reactive protein (CRP) was within normal range (0.5 mg/dL). EBV DNA copy numbers escalated to 134,000 copies/mL on hospital day 11. A diagnosis of EBV associated HLH was made.

Patient's clinical condition deteriorated rapidly, despite aggressive attempts at lowering intra-abdominal

pressure via decompressive laparotomy, correcting severe acidosis, improving acute liver failure and acute renal failure, supporting acute respiratory failure with pressure control ventilation, and supporting cardiac failure with epinephrine and other vasopressors. A bone marrow biopsy and cytogenetic testing were therefore not performed. The patient expired on hospital day 12.

DISCUSSION

Our patient initially presented with fever and mild right upper quadrant abdominal pain. Imaging at admission showed marked acalculous gallbladder wall thickening, which is most commonly seen in cholecystitis but can be encountered in a variety of conditions unrelated to intrinsic gallbladder disease. In

a case series reviewing the diseases associated with gallbladder wall thickening, HLH accounted for 6.0%.^[5] Though hepatosplenomegaly noted on CT next day is a common finding in HLH, rapid progression to ACS is unusual. To the best of the authors' knowledge, this has never been reported in the PubMed listed literature.

With our single case report of ACS associated with HLH, it is difficult to ascertain the underlying mechanism(s) responsible for ACS in this clinical setting. However, the fulminant course of hepatosplenomegaly seen in our case would not allow compensative stretch of the abdominal wall, and is therefore expected to cause rapid elevation of intra-abdominal pressure and consequent ACS. In addition, prominent lymphadenopathy, gallbladder and duodenal wall edema, large uterine fibroid may also contribute more or less to the development of ACS in our patient. We postulate that, ACS is less likely to be encountered in HLH cases of relatively chronic clinical course; at the other extreme of HLH, ACS may not be fully developed or recognized promptly before the patients expire. Raising awareness of ACS as an unusual presentation of HLH would facilitate timely treatment and improve survival rate.

The HLH-2004 diagnostic guidelines proposed by the Histiocyte Society include a molecular diagnosis consistent with HLH or fulfillment of five out of the following eight criteria: fever, splenomegaly, cytopenia affecting two or more lineages (hemoglobin < 9 g/dL, platelets < $100 \times 10^9/L$ and/or neutrophils < $1.0 \times 10^9/L$), hypertriglyceridemia (≥ 265 mg/dL) and/or hypofibrinogenemia (≤ 150 mg/dL), hemophagocytosis in bone marrow, spleen or lymph node, impaired natural killer (NK) cell function, hyperferritinemia (≥ 500 $\mu g/L$), and elevated soluble CD25/sIL-2R ($\geq 2,400$ U/mL).^[6,7] Our patient met the HLH-2004 diagnostic criteria based on fever, splenomegaly, pancytopenia, hyperferritinemia, hypertriglyceridemia and hypofibrinogenemia. Other features that have been documented in adult HLH cases but not listed in the HLH-2004 guideline include hyponatremia, hyperbilirubinemia, elevated AST, LDH and CRP.^[8] Except elevated CRP, all other ancillary features were observed in the present case.

Because HLH-2004 guidelines were established for primary HLH in pediatric patients, whether or not it can be readily applied to secondary HLH in adults has been questioned. For instance, significantly elevated ferritin is considered specific for HLH in the pediatric population but not in adults.^[3,7] To define the diagnostic guidelines for secondary HLH, an international consensus survey was recently conducted.^[9] Major revisions made

to HLH-2004 for adult HLH are summarized as follows. First of all, unilineage cytopenia is emphasized as an absolutely required criterion, in contrast to bilineage involvement as a dispensable criterion in HLH-2004. Secondly, a known predisposing underlying disease is considered of major importance in diagnosing adult HLH, but not mentioned in HLH-2004. Thirdly, high LDH is included, which is not part of HLH-2004 either. Fourthly, NK cell activity and soluble CD25 are considered of limited use due to the poor availability of these tests. Molecular diagnosis, which is adequate by itself to diagnose primary HLH, is disregarded in the consensus survey for the adult HLH. Lastly, the value of hypertriglyceridemia and hypofibrinogenemia for diagnosing adult HLH fails to reach consensus among experts.

A scoring system, available online at <http://saintantoine.aphp.fr/score/>, has recently been proposed to estimate an individual's risk of having reactive HLH.^[10] Additional differences reflected in this system include hepatomegaly and elevated AST. Degree of fever also contributes to the final score (HScore). An HScore ≥ 169 has been chosen as the cut-off value for confirming the diagnosis of HLH, with a reported sensitivity of 93%, specificity of 86% and correct classification rate of 90%.^[10,11] Using the scoring system, our patient has an HScore of 203, and her probability of having HLH is estimated to be 90%.

Though hemophagocytosis documented in bone marrow, spleen or lymph nodes is one of the diagnostic criteria and a hallmark of HLH, it should be noted that hemophagocytosis per se is neither sensitive nor specific for HLH. The reported incidence of hemophagocytosis on bone marrow examination of patients with HLH ranges from 25% to 100%.^[1,8,12] On the other hand, hemophagocytosis may be encountered in conditions other than HLH, including sepsis, post transfusion or cytotoxic therapies, and critically ill patients who fall short of diagnostic criteria of HLH.^[8,13] Because hemophagocytosis is a systemic event, it can be observed in many other organs, such as liver and brain.^[14,15] However, on liver biopsy, hemophagocytic histiocytes are present in variable numbers, and therefore not always seen.^[16] A more common but less specific finding is portal, periportal and intrasinusoidal infiltrates of T lymphocytes and histiocytes,^[17-19] as seen in the present case. Interestingly, destruction of interlobular bile ducts has been described as an important feature of hepatic involvement by primary HLH,^[18] but not in cases of secondary HLH for reasons that are poorly understood.

The trigger of HLH in our patient is most likely EBV infection, as evidenced by the dramatic increase of EBV

DNA copies to 1.3×10^5 copies/mL. Teramura *et al.*^[20] reported that the median EBV genome copy number at diagnosis was 3.0×10^3 (range: undetectable to 5.5×10^7) copies/mL in EBV associated HLH, in contrast to 6.6×10^1 (range: undetectable to 1.0×10^3) copies/mL in infectious mononucleosis. EBER negativity may be explained by the relatively early stage of the clinical course when the liver biopsy was performed, or simply technical difficulties encountered in some cases.^[21] In addition, the liver biopsy specimen of our patient had previously been frozen for intraoperative consultation. The freeze-and-thaw process may have damaged the EBV RNA to cause a falsely negative test.

Since our patient had no prior history of immunodeficiency, it is intriguing what initiated the defect of her cellular immunity. According to two independent large cohort studies,^[8,22] concomitant hematologic malignancy and active infection were found in 2.9-3.7% of adult HLH cases. Given the predominant T-lymphocytic infiltrate on the liver biopsy, a T-cell lymphoma is high in our differential diagnoses. However, the possibility of an underlying T-cell lymphoma is difficult to confirm or exclude in our case due to the fulminant clinical course. Flow cytometry and cytogenetics might have aided in the diagnosis if the patient had been able to tolerate additional biopsies, particularly a bone marrow study. A sIL-2R/ferritin ratio of ≥ 2.0 has been proposed as a useful marker for lymphoma associated HLH.^[23,24] Serum beta2 microglobulin level was also reported to be significantly higher in lymphoma associated HLH than benign disease-associated HLH.^[25] Unfortunately, serum sIL-2R and beta2 microglobulin were not evaluated in a timely fashion in this case.

Monoclonal TCR gene rearrangements support the diagnosis of T-cell lymphoma in the proper clinical scenario. However, TCR clonality is not uncommon in EBV associated HLH.^[26-28] The clonality is likely due to monoclonal proliferation of EBV-infected T cells,^[29] and can become polyclonal after eradication of EBV-infected T cells using immunochemotherapy.^[28] Though some of these patients may eventually progress to lymphoma should they survive, clonality assay does not help identify patients with underlying lymphoma in the context of EBV associated HLH. Indeed, EBV associated HLH and systemic EBV-positive lymphoproliferative disease may represent a biologic continuum rather than discrete entities.^[21] A clear-cut distinction is not always possible.

Despite the progress in the management of HLH, one-month mortality rate is 20-44% for secondary HLH, much worse than primary HLH.^[1,8,30] Among all the clinical features and laboratory findings, underlying malignancy,

particularly T-cell lymphoma, is most consistently associated with worse prognosis,^[1,8,31] followed by older age^[11,22,32] and abnormal karyotype.^[21,26] Other factors found to correlate with poor prognosis include high EBV viral load ($\geq 1,000$ copies/mL),^[27] organ failure at admission,^[11] hyperferritinemia,^[31] hypoalbuminemia,^[11] male, splenomegaly and thrombocytopenia.^[22] TCR clonality does not appear to be of prognostic value.^[26,27]

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None.

Conflicts of interest

There are no conflicts of interest.

Patient consent

A Notice of Privacy Practices signed by the patient for approval of use of patient information for research purposes is available for review upon request.

Ethics approval

Case report is automatically waived and does not require further approval by Institutional Review Board at Loma Linda University Medical Center.

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Hepatocarcinoma with metastasis to the anterior mediastinum

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ABSTRACT

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Liver malignancies are the sixth leading cause of cancer worldwide, whereas hepatocellular carcinoma (HCC) is the most frequent histological type of liver cancer. Extrahepatic metastasis, which rarely involves the mediastinum, is associated with poor prognosis. An 80-year-old male presenting with mild diffuse abdominal pain for 4 months, associated with hyporexia, increased abdominal volume, dry cough, and loss of 4 kg in 1 month, sought medical assistance due to hemoptysis and chest pain. Tomographic study revealed HCC with mediastinal metastasis, after which sorafenib therapy was started. Disease progressed to death 4 months after the start of the treatment.

INTRODUCTION

Hepatocellular carcinoma (HCC) has high incidence and mortality rates, being the most common primary liver cancer^[1] and the third leading cause of cancer mortality. It usually starts with a solitary encapsulated lesion that often shows slow growth and is asymptomatic for a long time.^[2] HCC is usually associated with liver cirrhosis, which may impair treatment tolerability and thus increase the risk of complications in cases of advanced cirrhosis. Although there are great differences in the global incidence and frequency of coexisting HCC and cirrhosis that vary according to

ethnicity, the coexistence of these two conditions has the same basic clinical characteristics and leads to poor prognosis, regardless of race and location.^[2]

Liver cirrhosis of any etiology and chronic infection by hepatitis B are the main risk factors for the development of HCC. All patients with these two conditions benefit from biannual screening for HCC with abdominal ultrasound and measurement of alpha-fetoprotein (AFP) levels, although the latter has been shown to have questionable efficacy in population surveillance.^[3,4]



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Non-invasive diagnosis is made by imaging techniques, such as computed tomography and/or magnetic resonance imaging,^[4] based on the vascular findings for these tumors, which exhibit a hypervascular pattern during the arterial phase and a washout pattern during the portal venous or delayed phase. Such radiological characteristic occurs in a small number of 1-2 cm tumors. In these cases, biopsy and tissue biomarkers, such as AFP, are used to confirm the diagnosis. Disease staging should be established at this point, in order to plan treatment and assess prognosis.^[3,4]

The most frequent location of metastatic HCC is the lungs, due to possible hematogenous dissemination through their capillary network, followed by bones and abdominal lymph nodes. Conversely, mediastinal metastasis is an uncommon manifestation of HCC and shows poor prognosis.^[5,6] We present a case of an elderly patient admitted to our institution after being diagnosed with HCC and mediastinal metastasis.

CASE REPORT

This is the case report of an 80-year-old retired widower coming from São Paulo, southeastern Brazil. His past medical history included high blood pressure, dyslipidemia, diabetes mellitus, benign prostatic hypertrophy, and osteopenia. He was a former smoker of 90 packs a year and denied alcohol abuse. Four months before admission, the patient started to present with mild diffuse abdominal pain, hyporexia, increased abdominal volume, dry cough, anterior chest pain, and loss of 4 kg in the last month. He sought medical assistance after two episodes of hemoptysis.

A tomographic study revealed a contrast-enhanced expansive heterogeneous mass measuring 9.7 cm × 5.1 cm × 5.6 cm at this largest diameter and located on the left midline of the anterior mediastinum, with no clear interface between mediastinum and pericardium. No mediastinal lymphadenomegaly was detected. There were hepatic lesions showing a washout pattern in segments V, VI, VII, VIII and IV and protruding toward the hepatic hilum. Evidence of splenic vein thrombus was found [Figure 1].

Laboratory findings were as follows: hemoglobin = 10.5 g/dL; hematocrit = 34%; leukocytes = 7,500.000/mm³ (segmented: 64%, band cells: 0%, lymphocytes: 28%, eosinophils: 5%, monocytes: 3%, basophils: 1%); total bilirubin = 0.7 mg/dL; alanine aminotransferase = 48 U/L; aspartate aminotransferase = 40 U/L; AFP = 14,000 ng/mL; gamma-glutamyl transferase = 350 U/L; alkaline

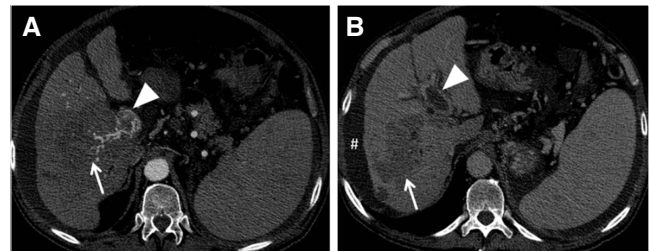


Figure 1: Axial contrast-enhanced computed tomography slices at arterial (A) and portal (B) phases. Note tumor infiltration (arrow) extending from part of the right hepatic lobe to the hilar region and tumor infiltration of the portal vein (arrow head)

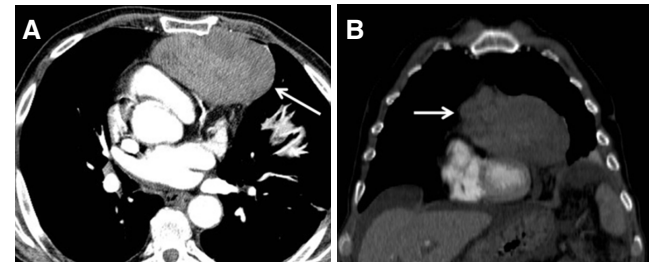


Figure 2: Axial contrast-enhanced computed tomography (CT) slice (A) and coronal reconstruction of CT scan (B). Note large heterogeneous hypervascular anterior mediastinal mass (arrow) posteriorly compressing the right atrium and pulmonary artery

phosphatase = 148 U/L; international normalized ratio = 1.75; creatinine = 0.77 mg/dL.

The patient and his family decided to start sorafenib therapy, and he did not want to undergo invasive procedures, such as chemoembolization, for the treatment of mediastinal metastasis. Drug therapy was maintained for 3 months, when the patient was readmitted due to clinical worsening, with the development of jaundice and severe ascites. Patient staging was reassessed, showing evidence of growth of the mediastinal mass, which measured 11.0 cm × 6.3 cm × 7.7 cm at that time and was compressing the right atrium and the pulmonary artery [Figure 2]. There was also an increase in the size of hepatic solid lesions and in the extension of thrombosis. AFP levels reached 45,000 ng/mL. It was decided to discontinue chemotherapy with sorafenib, and the patient died 1 month after readmission.

DISCUSSION

HCC is one of the most common primary tumors worldwide, and its prognosis has improved over the past few decades with the assessment of tumor vascular pattern by imaging methods and the emergence of therapeutic procedures. A considerable amount of literature has been published on the different presentations of HCC and on extrahepatic metastases, which occur in 30-50% of HCC cases. The most common metastatic sites are lungs, bones,

Table 1: Cases of mediastinal metastases reported in the literature

| Ref. | Case report | Treatment |
|--|---|--|
| Chiou <i>et al.</i> ^[9] | Patient with HCC of 8.6 cm in segment 4 liver, underwent transcatheter arterial embolization and following the appearance of mediastinal injury of 4 cm in the right paratracheal region | Surgical resection |
| Shinya <i>et al.</i> ^[10] | 3 lesions of HCC, 2 years after liver transplant tumor recurrence in the upper mediastinum | Surgical resection |
| Huang <i>et al.</i> ^[13] | 2 patients with hepatitis-C related HCC, after several courses of TACE developed mediastinal and pericardial neoplastic growth | Radiotherapy |
| Chen <i>et al.</i> ^[14] | HCC 3 cm treated with TACE; 2 years after presents hoarseness, a chest CT scan revealed a 5-cm tumor over the aortopulmonary window of the mediastinum | TACE |
| Oncale <i>et al.</i> ^[15] | HCC with liver mass 11 cm × 13 cm with vena cava invasion and extension to the right atrium (4 cm × 4 cm) | Sorafenib |
| Sung <i>et al.</i> ^[16] | HCC with associated thrombus was found to extend from the liver through the inferior vena cava into the right atrium | Surgical resection |
| Masci <i>et al.</i> ^[17] | Right intraventricular metastasis from HCC in a patient who had undergone a partial hepatectomy for HCC more than two years earlier | Systemic chemotherapy with cisplatin and doxorubicin |
| Ulus <i>et al.</i> ^[18] | HCC who was incidentally found to have an intracavitary mass completely occupying the right atrium | Surgical resection |
| Tastekin <i>et al.</i> ^[19] | HCC with hepatectomy; 1 year after patient started to present dyspnea, hoarseness, palpitation, chest CT scan showed a mass of 4 cm in the left heart ventricle and myocardial invasion | Surgical resection |
| Lei <i>et al.</i> ^[20] | The first patient was noted to have a large RV tumor mass with intracavitary growth and myocardial invasion; the second had massive pulmonary and LA metastasis; and the third patient had a right atrial tumor mass with concomitant RV and LA involvement | |
| Fukuoka <i>et al.</i> ^[21] | Patient with pulmonary metastases from HCC, who presented with a tumor in the left lung, extending to the left atrium through the left pulmonary vein | Sorafenib |

HCC: hepatocellular carcinoma; TACE: transcatheter arterial chemoembolization; RV: right ventricular; LA: left atrial

and loco-regional lymph nodes, presenting commonly with dyspnea and bone pain.^[7] A study in autopsy files showed that unusual extrahepatic metastatic sites include diaphragm, pancreas, gall bladder, stomach, colon, adrenal gland, pleura, peritoneum, cervical lymph nodes, brain, skin, and oral cavity.^[8]

It is important to emphasize that the diagnosis of our patient was based on the presence of mediastinal mass and hepatic lesions. We decided not to perform liver biopsy because an imaging study revealed that the tumor had a washout vascular pattern and the patient showed AFP levels of 14,000 ng/mL.

The involvement of mediastinal lymph nodes occurs in 4% to 5% of the cases of HCC. In patients with mediastinal metastasis, lesions were mostly diagnosed simultaneously with the viable intrahepatic tumor.^[9] Mediastinal metastases are unusual, and mediastinal involvement usually leads to dissemination to lymph nodes, which occurs by three routes of hepatic lymphatic drainage. The first route is from the left hepatic lobe via anterior phrenic lymph nodes to the parasternal or subcarinal lymph nodes; the second, from the liver through the hepatic falciform ligament to the parasternal or paratracheal lymph nodes; and the third, from the right hepatic lobe through the right triangular ligament to the paratracheal lymph nodes.^[9,10]

The predictors of the presence of extrahepatic metastases are: size and number of HCC nodules, presence of tumor vascular invasion or tumor biomarkers.^[2]

Patients with initial HCC, i.e. with no distant metastases, may undergo partial liver resection, which is potentially curative, as well as liver transplantation or percutaneous ablation. More advanced cases, such as the one presented in this study, are eligible to palliative treatment with sorafenib.^[4] However, research has shown that treatment of intrahepatic lesions should not be contraindicated in the presence of extrahepatic metastasis. Moreover, radical treatments for extrahepatic metastases should be considered when hepatic lesions are under reasonable control or if metastasis is accompanied by severe symptoms.^[11,12] In the case of mediastinal metastasis, transarterial chemoembolization has shown good response and adequate symptom control, in addition to increasing survival.^[13,14] In Table 1, we detail HCC cases of mediastinal metastases reported in the literature.

The present study showed that, despite therapeutic advances and the use of target therapy, survival is very limited when tumor is advanced, diagnosis is made at a later stage, and there are distant metastases.

Financial support and sponsorship

None.

Conflicts of interest

There are no conflicts of interest.

Patient consent

Consent form was obtained from the patient family.

Ethics approval

The study was approved by the Institutional Review Board of IAMSPE.

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An extra-adrenal pheochromocytoma mimicking a primary liver cancer

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ABSTRACT

Despite doctors' every effort to be vigilant when diagnosing, sometimes a preoperative diagnosis is disproved by postoperative pathological examination. A patient was diagnosed with hepatocellular carcinoma and received surgery as treatment. On operation, a solitary retroperitoneal mass rather than a liver lesion was seen. On histopathological examination, the retroperitoneal mass was found to be an extra-adrenal pheochromocytoma.

INTRODUCTION

Despite doctors' every effort to be vigilant when diagnosing, sometimes a preoperative diagnosis is disproved by postoperative pathological examination. Below is a case of a patient having surgery as treatment for hepatocellular carcinoma (HCC) but the patient's disease was actually not HCC.

CASE REPORT

A 57-year-old Chinese man presented to a private surgeon for epigastric pain and significant weight loss of 20 pounds in a few weeks. He was not a heavy drinker (having a can of beer weekly) and

had been generally healthy. Physical examination found nothing significant and his blood pressure was normal. Results of initial blood tests showed a normal complete blood picture and normal hepatic and renal functions. He had no chronic hepatitis B or C. His α -fetoprotein and carcinoembryonic antigen levels were within normal ranges.

Computed tomography of the abdomen showed a 5.8 cm \times 4.8 cm \times 7.7 cm heterogeneous arterial enhancing mass with portovenous washout occupying the whole caudate lobe of liver. It was abutting on the inferior vena cava (IVC). There was suspected tumor thrombosis invading the hepatic and infrahepatic portion of the IVC with heterogeneous enhancement



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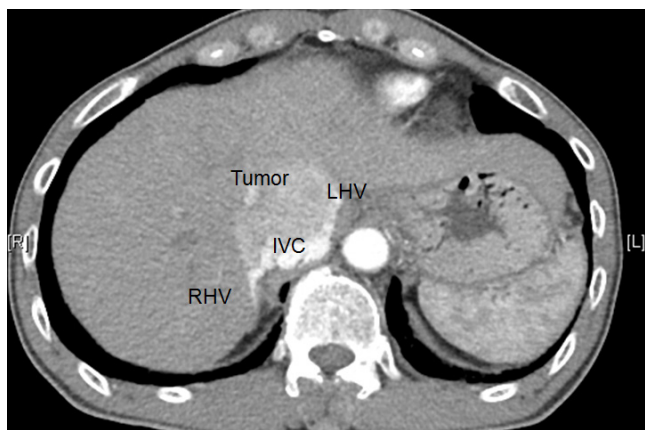


Figure 1: Arterial enhancing mass at the central part of the liver abuts on the inferior vena cava (IVC) and splay the right hepatic vein (RHV) and left hepatic vein (LHV), with suspected invasion of the IVC. The middle hepatic vein is not visible

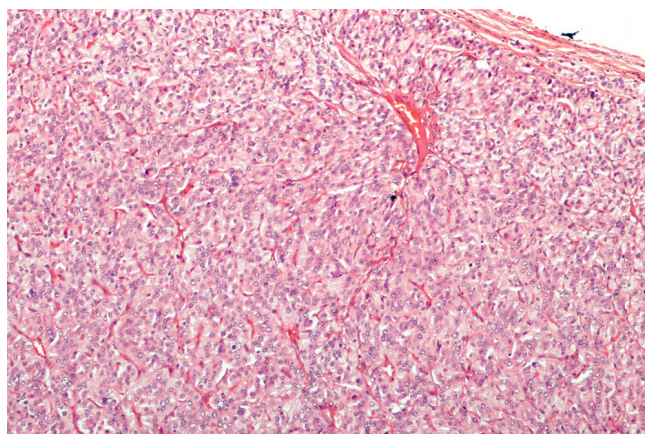


Figure 3: Tumor cells with round nuclei containing dispersed chromatin and granular amphophilic cytoplasm (high power, HE, $\times 40$)

[Figure 1]. The mass was splaying the main portal vein but the vein was still patent [Figure 2]. What would be the diagnosis?

Differential diagnosis considered primary liver tumor (such as HCC), focal nodular hyperplasia and hepatic adenoma. Also possible were secondary liver tumors like renal cell carcinoma, neuroendocrine tumor and thyroid carcinoma which also show arterial enhancing and portovenous washout on computed tomography. A radiological diagnosis of HCC was made.

The patient was referred to our center for treatment. Curative resection was decided. The planned operation was right hepatectomy + caudate lobectomy + IVC resection with immediate reconstruction. Preoperative biopsy was considered but not performed because the tumor was highly vascular and access would be difficult. The patient's indocyanine green retention rate was 8.6% at 15 min.

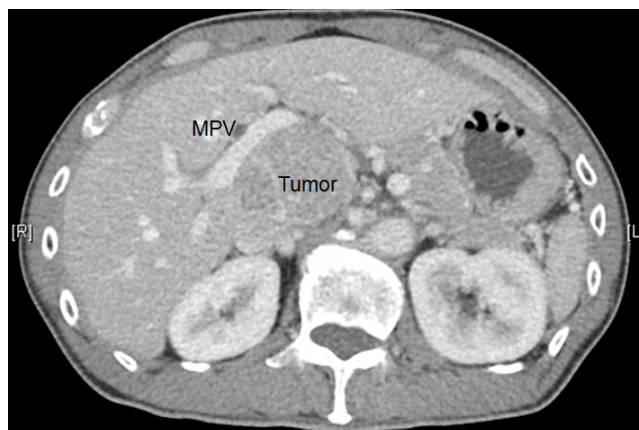


Figure 2: The mass, showing portovenous washout, extends to the head of pancreas. The main portal vein (MPV) is splayed

Surgery was performed on 26 January 2015. In the operation, a solitary 8-cm retroperitoneal mass with very vascular blood supply from surrounding structures and the aorta was found. Complete resection was done. The intraoperative blood loss was 400 mL and the operation time was 3 h and 43 min.

On histopathological examination, the retroperitoneal mass was in fact an extra-adrenal pheochromocytoma [Figure 3], not HCC. The patient had a smooth recovery and was discharged on postoperative day 6.

DISCUSSION

HCC is the third leading cause of cancer-related deaths in Hong Kong,^[1] early referral to expert center is definitely beneficial. At our center, we treat HCC with surgery whenever possible since it is the only chance of cure. In the city, 8% of the population have chronic hepatitis B and hepatitis-B-related HCC is common. However, the patient in the present case did not have any chronic hepatitis. His α -fetoprotein level was normal too. The diagnosis of HCC was made based on radiological findings and on the consideration that HCC is prevalent in the population and not all HCC patients have hepatitis. In fact, 5% of the HCC patients at our center have no chronic hepatitis.

This patient might be regarded as inoperable elsewhere. But our center, with vast experience in liver resection and transplantation, has the expertise as major vascular resection with immediate reconstruction is a routine here. Therefore surgery was decided. Dynamic imaging was not performed for him since he would be offered surgery anyway. At our center, diagnostic dynamic imaging is performed for patients with cirrhosis or regressed cirrhosis,^[2] or patients without fully developed cirrhosis but with chronic hepatitis.

To our surprise, no liver lesion was found. Instead, there was a solitary 8-cm retroperitoneal mass with very vascular blood supply. Complete resection was done. Further hormonal and genetic workup confirmed that the mass was a rare occurrence of extra-adrenal pheochromocytoma rather than a multiple endocrine neoplasia type 2.

In the literature, there are a few reports of extra-adrenal pheochromocytoma.^[3-5] It is rare and therefore easily overlooked, and it could easily be misdiagnosed as primary liver cancer. The lesson to learn from the present case is that extra-adrenal pheochromocytoma, a potentially life-threatening disease, should be included on the list of candidate conditions for differential diagnosis.

Financial support and sponsorship

None.

Conflicts of interest

There are no conflicts of interest.

Patient consent

Consent form was obtained from the patient.

Ethics approval

According to regulation of Queen Mary Hospital, no approval is needed for this kind of studies as long as patients' identity is not disclosed.

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Aggressive primary hepatic histiocytic sarcoma: case report and literature review

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ABSTRACT

Histiocytic sarcoma is an uncommon non-Langerhans histiocyte disorder of mature tissue histiocytes. The authors presented an example of this rare tumor in a 14-year-old girl who presented with left upper quadrant pain, loss of appetite, and weight loss. A large 18 cm × 10 cm heterogeneous solid and cystic enhancing mass was found in the left lobe of the liver. Based on the histomorphology and positivity for histiocyte-specific markers in a needle biopsy, a diagnosis of histiocytic sarcoma was made. Chemotherapy was initiated, but the tumor did not respond well, and she died about 7 weeks following initial diagnosis with multi-organ failure. At autopsy, the tumor showed extensive necrosis, with no evidence of metastatic spread. In conclusion, the diagnosis of histiocytic sarcoma is challenging, and requires a high index of suspicion, with an appropriate panel of confirmatory immunohistochemical stains. Recognition of this rare tumor is important because of its poor response to chemotherapy and high mortality.

INTRODUCTION

Histiocytic sarcoma (HS) is an extremely uncommon neoplasm with morphologic and immunophenotypic characteristics of mature histiocytes. It is believed that HS originates from monocytes/macrophages, which are critical in the processing and presentation of antigens to T cells or B cells.^[1]

Before the application of immunohistochemical techniques and the availability of molecular genetic tools, HS was occasionally diagnosed. It is now generally recognized however that the majority of the previously diagnosed HS cases are actually examples of non-Hodgkin lymphomas, most of which are diffuse

large B-cell lymphoma and anaplastic large cell lymphoma.^[2]

HS is an extremely aggressive neoplasm and responds poorly to regular therapy; most patients die of progressive disease, in part related to the high clinical stage (stage III/IV) at presentation in the majority of patients.^[3] The most common affected site of HS is in lymph nodes, followed by different extranodal locations such as the gastrointestinal tract, spleen, soft tissue and skin.

Herein, we describe a child with an aggressive primary hepatic histiocytic sarcoma who responded poorly to



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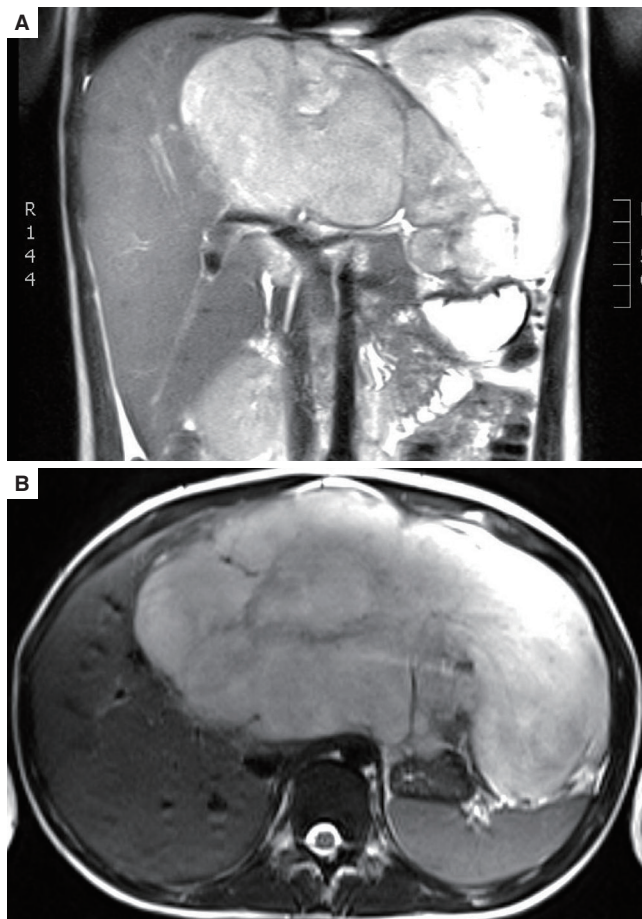


Figure 1: Abdominal magnetic resonance imaging (A: coronal; B: axial) revealed an 18.3 cm large heterogeneous enhancing mass with cystic components arising from the left hepatic lobe with associated mass effect

multiple rounds of chemotherapy and passed away 7 weeks after initial diagnosis.

To the best of our knowledge, only three primary hepatic HS cases have been previously reported in the English literature.^[4-6] Recognition of this type of rare tumor is important, due to its limited responsiveness to conventional chemotherapy and high mortality.

CASE REPORT

A 14-year-old previously healthy young girl presented with left upper quadrant pain, loss of appetite and weight loss for one month. An abdominal ultrasound showed a large heterogeneous solid and cystic appearing mass in the mid abdomen, measuring 18.3 cm × 10 cm, arising from the liver with associated mass effect and surrounding increased vascularity. Computed tomography (CT) scan of the abdomen and pelvis and magnetic resonance imaging revealed similar findings, with marked regional mass effect, including on the hepatic inferior vena cava and stomach, with extracapsular extension into the left

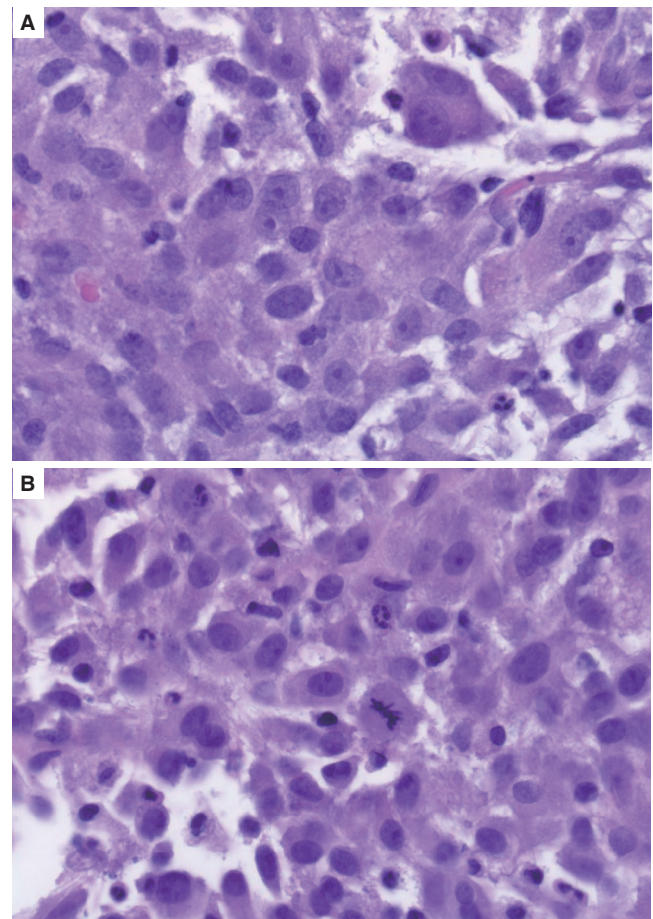


Figure 2: (A) Tumor cells comprising of primarily epithelioid cells with abundant eosinophilic cytoplasm, pleomorphic nuclei with open vesicular chromatin and prominent nucleoli; (B) Mitotic figures were easily found in the tumor. HE, ×40

upper quadrant [Figure 1].

Laparoscopic liver needle biopsy was performed and showed a tumor of primarily epithelioid cells with areas of spindling also present. The lesional cells contained abundant eosinophilic cytoplasm and nuclei with open vesicular chromatin and prominent nucleoli. Prominent nuclear pleomorphism and elevated mitotic index were also identified [Figure 2]. A subsequent perihepatic fine needle aspiration biopsy and cell block of the tumor similarly showed a poorly differentiated neoplasm with epithelioid and spindle cell features in a background of fibrin and blood [Figure 3].

Immunohistochemical studies showed no staining with markers for epithelial, lymphomatous, germ cell, rhabdomyosarcoma, melanoma, gastrointestinal stromal or neuroendocrine tumors, or for Langerhans cell histiocytosis, including CK cocktail, CAM5.2, EMA, CD15, PAX-5, CD45, CD43, CD3, CD5, ALK1, Factor XIIIa, CD31, CD23, CD35, SALL-4, CD117, desmin, myogenin, SMA, melanoma cocktail, S-100,

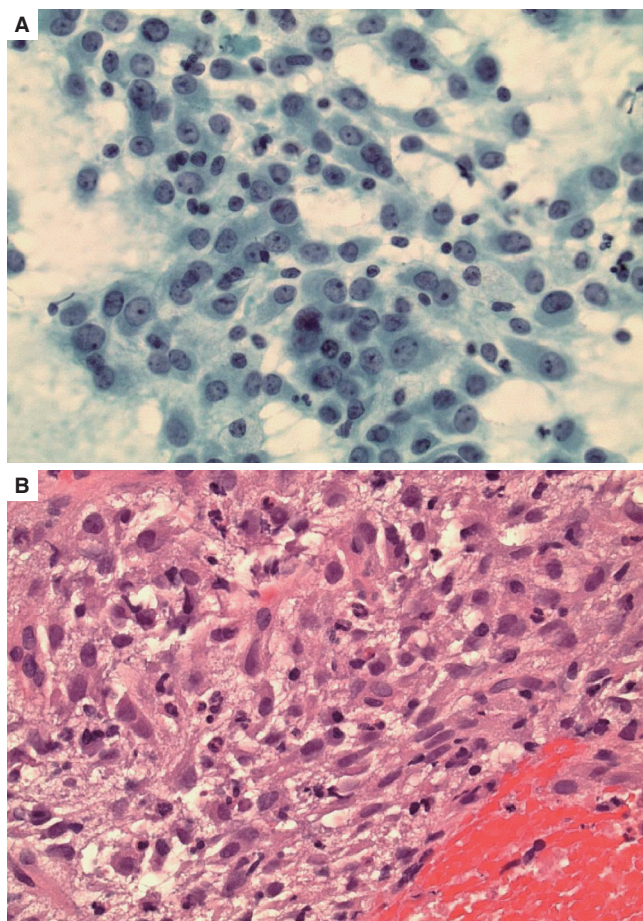


Figure 3: (A) Fine needle aspiration showed pleomorphic nuclei with prominent nucleoli (Papanicolaou, $\times 40$); (B) Cell block of the tumor showed a poorly differentiated neoplasm with epithelioid and spindle cell features in a background of fibrin and blood (HE, $\times 40$)

vimentin, CD34, chromogranin, synaptophysin, CD56, inhibin, and CD1a. Many lesional cells were strongly positive for CD163, with less distinct staining for CD68 [Figure 4]. Her bone marrow biopsy showed a mildly hypercellular marrow for age, with trilineage hematopoiesis, a left shift of the granulocytes, a moderate erythroid hyperplasia, and plasmacytosis. No morphologic or immunophenotypic evidence of malignancy was identified. A diagnosis of HS was made based on the combination of morphology and immunophenotype, and this diagnosis was supported by outside expert consultation.

Clinical course

Because of the large size of this tumor and the non-availability of liver donor at that time, complete tumor resection and liver transplantation were not performed. The patient received two rounds of chemotherapy (1st round ifosfamide and doxorubicin, and 2nd round thalidomide) and intensive supportive measures. However, she gradually developed worsening multi-organ failure requiring mechanical ventilation and

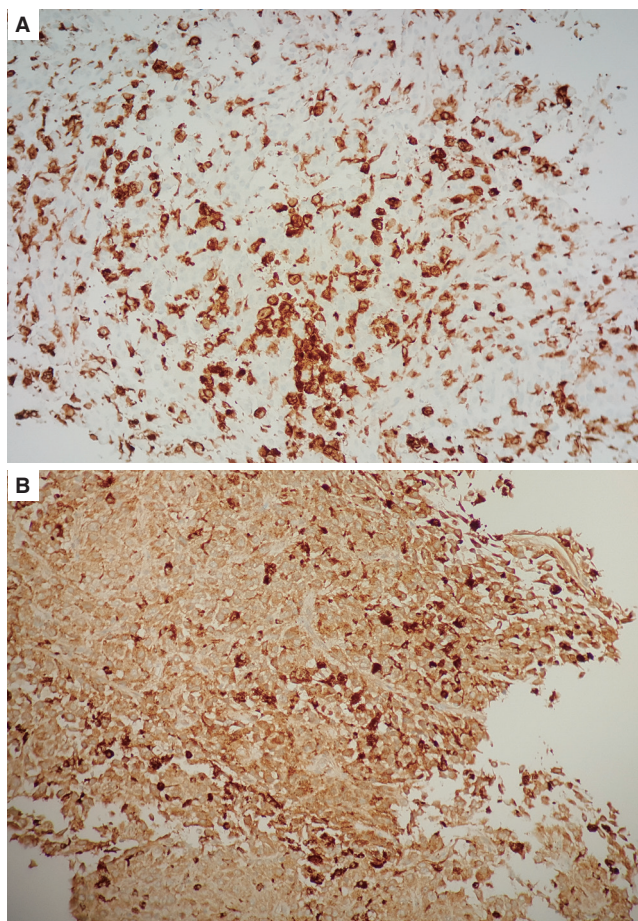


Figure 4: (A) CD163 immunostain; (B) CD68 immunostain. Tumor cells are strongly positive for CD163, with less distinct staining for CD68 ($\times 10$)

hemodialysis. Positron emission tomography CT scan suggested development of possible metastases at multiple sites. She developed abdominal distension and multi-organ failure, and passed away 7 weeks following initial diagnosis.

Autopsy findings

At autopsy, there was single large (20 cm \times 17 cm \times 9 cm) tumor arising from the left lobe of the liver, with extensive necrosis. No evidence of metastatic tumor was identified at any of the other suspected sites. Microscopic examination of the tumor was complicated by moderate autolysis with extensive necrosis, but otherwise showed similar morphology to the original biopsies.

DISCUSSION

HS is a malignant proliferation of cells showing morphologic and immunophenotypic features of mature tissue histiocytes.^[7] In 1970, "histiocytic sarcoma" was introduced for the first time to describe a collection of malignant tumor cells with histologic

Table 1: Clinical features of primary hepatic histiocytic sarcoma cases

| Case | Age/gender | Initial presentation | Tumor location/size | Clinical course | Ref. |
|------|-----------------|--|---|--|---------------|
| 1 | 55 years/male | Right shoulder and right upper quadrant pain for about 3 months | Right lobe 15 cm × 11 cm × 9 cm | Right hepatic lobectomy and partial diaphragmectomy | [9] |
| 2 | 68 years/male | Cutaneous langerhans cell histiocytosis and rosai-dorfman disease and splenic marginal zone lymphoma. Patient was stable for a few years; then suddenly developed remarkable enlargement of spleen and liver without lymphadenopathy or skin lesions | Remarkable enlargement of spleen (24 cm, compared to 14 cm from 2 years earlier) and liver (size not available) | Died 24 h after presentation with sudden hepatosplenomegaly, despite treatment with systemic chemotherapy combined with prednisone | [6] |
| 3 | Unknown | Unknown | Liver, not further specified | Unknown | [8] |
| 4 | 14 years/female | Left upper quadrant pain, anorexia and weight loss for 1 month | Left lobe 20 cm × 17 cm × 9 cm | Died 7 weeks following initial diagnosis despite aggressive systemic chemotherapy without tumor resection | Current study |

characteristics of large macrophages with abundant eosinophilic cytoplasm.^[8] Now HS is defined as a malignant proliferation of cells showing morphologic and immunophenotypic characteristics of mature tissue histiocytes. Immunohistochemical studies are critical for the correct diagnosis of HS because it doesn't have definitive morphologic features. As a matter of fact, many cases that were diagnosed as malignant histiocytosis and histiocytic medullary reticulosis in the past have been shown to be different subtypes of non-Hodgkin lymphoma.^[9]

HS is extremely rare and accounts for less than 1% of all hematolymphoid neoplasms. It can occur over a wide range of ages (0.5-89 years, median age 46 years), showing bimodal age distribution with a small peak at 0-29 years and a larger peak at 50-69 years. HS is slightly more common in males than females.^[1]

Although the etiology of HS remains unknown, some cases have occurred in patients with mediastinal germ cell tumor, raising the consideration that HS may arise from pluripotential germ cells. Associations between HS and follicular lymphoma, myelodysplastic syndrome, and acute lymphoblastic leukemia have also been made. Moreover, a study has reported trans-differentiation in patients with HS and follicular lymphoma and reported the presence of t(14;18) and immunoglobulin heavy chain (*IGH*) gene rearrangements in all of the patients, suggesting a common clonal origin of follicular lymphoma and HS. Another study reported that 2 patients with HS had a clonal immunoglobulin rearrangement, suggesting a clonal evolution of HS from chronic lymphocytic leukemia/small lymphocytic leukemia. Further research is needed to confirm these findings.^[10]

Clinically, HS has been found to involve lymph nodes, skin, and at many extranodal locations, especially the gastrointestinal tract, often with the presentation of clinically advanced disease and aggressive clinical

course. Cases arising primarily at extranodal sites often appear to go unsuspected and unrecognized.^[2] Both localized and disseminated forms of HS exist. Systemic symptoms are relatively common and include fever, fatigue, night sweats, weight loss and weakness. Additionally, depending on the sites of involvement, HS can also present as skin rash, intestinal obstruction, hepatosplenomegaly, lytic bone lesions, and pancytopenia.^[1] Although the liver is the most common site of murine HS,^[11] human primary hepatic HS is very rare. To the best of our knowledge, the present case is the fourth HS primarily arising from the liver. The clinical features of these four reported cases are presented in Table 1.

Morphologically, HS tumor consists of diffuse sheets of medium to large epithelioid cells with abundant, pale eosinophilic or foamy cytoplasm. The nuclei are generally irregular, vesicular with prominent nucleoli; binucleated or large multinucleated forms are commonly seen. Mitotic activity is usually high and cellular pleomorphism can occasionally be seen. Necrosis is common and an admixed inflammatory infiltrate of small lymphocytes and neutrophils may be seen. In some cases, focal areas of spindle cell morphology may be found.^[12]

In terms of immunohistochemical studies, HS tumor cells are typically positive for one or more histiocytic markers, such as CD163, CD68 and lysozyme, with typical absence of markers for lymphocytes, Langerhans cells, follicular dendritic cells, epithelial cells, melanocytes and myeloid cells. The Ki-67 index is variable. S-100 and CD1a can occasionally be positive but usually only with weak and patchy staining. However, none of the antibodies are specific for histiocytic differentiation; therefore, it is important to evaluate with a panel of antibodies.^[11] BRAF exon 15 mutational analysis shows that 62% of HS cases have BRAF V600E mutations. Clonal antigen receptor gene rearrangement for T-cell receptor gamma, T-cell

receptor beta, and *IGH* genes may be seen and do not exclude the diagnosis of HS. However, when HS is associated with lymphoma, identical clonal gene rearrangements may be present.^[13]

HS most commonly presents at an advanced clinical stage, with poor response to chemotherapy and a high mortality rate. Although some patients may respond to chemotherapy with or without radiotherapy, the majority of the patients die of progressive disease within two years. Important prognostic factors include stage at presentation and tumor size. There are no accepted staging or treatment guidelines due to the rarity of the disease.^[1]

In conclusion, we present a rare case of primary hepatic HS. The diagnosis is challenging, and requires a high index of suspicion. The diagnosis should be based on a combination of compatible histomorphology and positivity for histiocyte-specific markers, and also requires exclusion of more common neoplasms by extensive immunophenotypic studies.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

The patient's parent(s) signed consents and documents are available for review upon request.

Ethics approval

Case report is waived and does not require further

approval by Institutional Review Board at Loma Linda University Medical Center.

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Evaluating normalization approaches for the better identification of aberrant microRNAs associated with hepatocellular carcinoma

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ABSTRACT

Aim: Dysregulated microRNAs (miRNAs) have been identified in hepatocellular carcinoma (HCC), but only a small proportion have been confirmed. An appropriate normalizer is crucial to determining the accuracy and reliability of data from miRNA studies. **Methods:** Different normalization strategies were used to validate genome-wide miRNA profiles in HCC tumor and non-tumor tissues, and to determine the consistency and discrepancy of data on dysregulated miRNAs. **Results:** Two sets of stable miRNAs (miR-30c/miR-30b and miR-30c/miR-126) were identified in HCC tissues by geNorm and NormFinder tools, respectively. The mean of global miRNAs also showed good stability for ranking the top 1-2 miRNAs, but the stabilities of the manufacturer-recommended ncRNAs controls were poor. Four panels of miRNAs were significantly associated with HCC by separately using various normalizers, and 14 miRNAs were consistently identified by three normalization strategies. Although fewer miRNAs (17-26) were dysregulated in HCC using the global mean or the 2 stable miRNAs as normalizers, perfect clustering of tissues was also obtained with only 1 to 2 misclassifications, suggesting the efficiency of the miRNA panels. Using global mean as the normalizer, the authors identified 7 miRNAs, including 2 novel (miR-324-5p and miR-550) significantly upregulated in HCC that were omitted when using 3 endogenous controls as the normalizer. **Conclusion:** An optimal normalization strategy to identify biologically important miRNAs in HCC tissue studies of miRNA may be the combination of global mean and 2 stable miRNAs. Selection of appropriate normalization strategies to adjust miRNAs levels is particularly important for epidemiological studies dealing with large data sets and covering multiple experimental batches.

INTRODUCTION

MicroRNAs (miRNAs) have important functions in negatively regulating coding genes' expression and

controlling multiple biological processes (DNA damage/repair, apoptosis, proliferation, differentiation, etc.) involved in tumorigenesis and progression.^[1,2] Genome-wide and candidate gene approaches have



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been broadly used to identify miRNA biomarkers in order to better understand the effects of carcinogenic exposure, pathogenesis, and cancer risk, as well as for early diagnosis and prognostic prediction. Currently, over 100 mature miRNAs have been found to be dysregulated in hepatocellular carcinoma (HCC) tissues or blood.^[3,4] Many are also associated with various HCC risk factors, such as hepatitis B/C virus infection,^[5,6] aflatoxin B1 exposure,^[7] alcohol drinking, non-alcoholic fatty liver disease or non-alcoholic steatohepatitis.^[8-10] However, only a small proportion of miRNAs (miR-1, miR-9, miR-16, miR-18a, miR-21, miR-92a, miR-101, miR-122, miR-199a, miR-221, miR-222/223/224, miR-375, miR-483-5p)^[11,12] were consistently confirmed by different studies for their role in hepatocarcinogenesis.^[13,14] These discrepant results may be attributed to a variety of factors that potentially impact miRNA patterns but differ by studies. These factors include the difference in study design (cross-sectional, retrospective or prospective); heterogeneity of cancer patients (tumor types, stages, progression, treatment, hepatitis B, C or mixed viral etiologies); comparison groups (healthy or hepatitis infection controls or non-tumor tissues); types of biospecimens (fresh, frozen or formalin-fixed, paraffin-embedded tissue, serum, plasma, or exosome); and variations in sample collection, preservation and processing. Differences of RNA isolation assays, the input RNA quantity/quality and detection methods can also impact miRNA expression levels.

Even if a careful study design is used and consistent implementation is applied to pre-analytical and analytical procedures, different methods and “housekeeping” transcripts used to normalize miRNA expression levels may also bias the results and lead to misinterpretation of the biological role of miRNAs in tumorigenesis. The purpose of normalization is to remove as much non-biological variations as possible to ensure accurate miRNA results within or between experiments.^[15] Therefore, how to select an appropriate normalizer to adjust miRNA expression profiles are crucial to obtaining comparable results. This is particularly important for epidemiological studies dealing with large data sets usually covering multiple experimental batches.

The most common methods to quantitate miRNA levels are quantitative real-time polymerase chain reaction (qPCR) and hybridization microarrays. The methods apply stem-loop reverse transcription and TaqMan probes (TaqMan low density arrays, TLDA, Life Technologies) or locked nucleic acid (LNA) primers (miRCURY LNA™ miRNA arrays, miRCURY, Exiqon) or poly (A)-tailed primers (miScript miRNA

PCR arrays, miScript, QIAGEN) and SYBR Green detection. The normalization for miRNA levels usually uses “housekeeping” transcripts, i.e. a reference-gene-based method. Because no universal references have been accepted by all researches, a variety of endogenous or exogenous transcripts have been selected as references by different microarray and qPCR assays for data normalization. TLDA includes a total of 5 endogenous controls (U6 snRNA, RNU44, RNU48, RNU24 and MammU6) and miScript uses spike in cel-miR-39 and 6 references (SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A and RNU6B/RNU6-2) as normalizers, while miRCURY recommends 5 most stable miRNAs (hsa-let-7i-5p, hsa-miR-222-3p, hsa-miR-425-5p, hsa-miR-93-5p, hsa-miR-152) as endogenous references, rather than small RNA species (snoRNA and snRNA). Even using the same microarray, different studies may artificially select various numbers of references to normalize their results. One study applied miScript array to profile expression of 84 miRNAs in hepatitis B virus-related HCC and controls, but only used 2 (SNORD61, RNU6-2) out of 6 snRNAs and spiked in cel-miR-39 as the normalizer to standardize miRNAs expression.^[16] Another study examined miRNAs in HCC patients and matched controls by the miRCURY assay using the median of 50% quantile intensity to normalize data.^[12] Seven published studies including ours have screened miRNA profiles by TLDA in either HCC tissue or serum/plasma. Two used four endogenous controls (U6 snRNA, RNU24, RNU44 and RNU48) to normalize target miRNA expression in HCC tissues;^[17,18] four studies only used one reference (U6 snRNA^[11,19,20] or RNU48);^[21] and one study did not indicate the reference.^[22] More importantly, whether those endogenous normalizers are stable among tested samples is unknown.^[11,17-22]

Global normalization is another strategy which uses either the mean or median of detectable miRNAs in each sample as the calibrator to adjust miRNA expression profiles; this method is adopted from mRNA microarray data normalization protocols.^[23] It is assumed that the mean or the median level of global or most miRNAs is constant across different tissues or conditions.^[24] Although many studies have demonstrated the advantages of using global normalization,^[23,25] the total number of detectable miRNAs are much less than mRNAs, which makes it susceptible to extreme values and may bias miRNAs expression patterns.^[15,26] In addition, large epidemiological studies usually require independent validation for a limited number of miRNAs identified in a discovery set. Practically, it is also not feasible to use global miRNA profiles to normalize expression of

candidate miRNAs.

Measurement of miRNAs by qPCR is considered the gold standard for specific and sensitive detection of interesting miRNAs that may be present at very low levels. This approach is usually used to validate the findings from previous large-scale microarray profiling. Although commercial ready-to-use kits are available for almost all human mature miRNAs, how to select an appropriate endogenous control to normalize miRNA expression is still a challenge. A number of endogenous miRNAs, snRNA/snoRNA, and synthesized exogenous RNAs have been used as controls to normalize target miRNA expression in different studies because there is no widely accepted endogenous control. Most are based on previous literature or because a low standard deviation (SD) was observed in the microarray data. Several small RNAs (U6 snRNA,^[16,19,27] RNU44,^[18] cel-miR-39,^[16,20] cel-miR-54)^[22] have been frequently used as calibrators in previous HCC studies. One study also used a standard curve approach for absolute quantitation by spiking in an artificial reference (ath-miR-156a).^[12] Therefore, it is not surprising that many previously identified miRNA panels are quite different between studies.

Here, we utilized genome-wide miRNA expression data derived from HCC tumor and non-tumor tissues to compare the miRNA panels identified as differentially expressed by using different normalization strategies. We sought to identify an optimal strategy to select stable references for miRNA normalization that can generate the most concordant miRNA panel deregulated in HCC. This strategy should also be feasible for large epidemiological studies, and more likely to reproducibly identify HCC-associated miRNAs in different studies.

METHODS

Participants come from a previous HCC tissue study^[28-30] conducted at Columbia University Medical Center (CUMC) and approved by the Institutional Review Board of CUMC. A waiver of consent was given in the study because the majority of patients died before the research was carried out.

A total of 16 paired frozen tumor and adjacent non-tumor tissues were screened for miRNA profiling. Tissue samples were collected and stored in the Molecular Pathology Shared Resource of the Herbert Irving Comprehensive Cancer Center. Tumor samples were microdissected to ensure > 80% purity of tumor. Tumor stage was determined according to

the American Joint Committee on Cancer criteria.^[31] To insure adjacent non-tumor tissue did not contain any tumor cells, tissue sections were cut from frozen tissues, and hematoxylin and eosin stained. The stained sections were carefully observed under a microscope by the study pathologist (HR) to ensure no tumor tissues or cells were present in the whole sections. Frozen tissue blocks of adjacent tissue were also evaluated with respect to the presence (Batts-Ludwig stage of 4) or absence of cirrhosis (Batts-Ludwig stage < 4).

The demographic and clinic pathological data were collected from medical and pathological records including age, gender, ethnicity, viral infection (hepatitis B, hepatitis C), α -fetoprotein, tumor size and number, tumor grade, presence of vascular invasion, and capsular infiltration. Hepatitis B virus surface antigen and antibody against hepatitis C virus determined by immunoassay were also obtained [Supplementary Table 1].

Total RNA, including miRNAs was isolated from 32 tissues by RNeasy Microarray Tissue Mini Kits (Qiagen, Frederick, MA) according to the manufacturer's protocols. TaqMan Low Density Arrays (TLDA, Applied Biosystems, Foster City, CA), covering 733 miRNAs (670 unique human mature miRNAs), were used to quantify genome-wide miRNAs levels using a 7900HT Fast Real-Time PCR System. The same amount (750 ng) of total RNA was used for each array measurement. The means of RNA integrity number and A260/A280 ratio were respectively 5.9 and 2.1. The quantification cycle (Cq) defined as the cycle number when fluorescence passes the detectable threshold was obtained and raw Cq values ≥ 40 were excluded. These data have been deposited in NCBI's Gene Expression Omnibus database (accession number GSE54751).^[29,30]

The first strategy used the endogenous controls recommended by the TLDA array manufacturer as the normalizer. Three (U6 snRNA, RNU44 and RNU48), detected in all tissue samples, were selected as normalizers in order to obtain reliable results. The second strategy used the mean of global miRNAs obtained from miRNAs detectable in all tested samples as the normalizer. There were 157 miRNAs/ncRNAs detected in 100% of HCC tissues [Supplementary Table 2]; means of all miRNAs were separately calculated for each sample and then used as the normalizer. The third strategy evaluated the stabilities of those 100% detectable miRNAs/ncRNAs by statistical algorithms. The most stable 2 miRNAs were selected as normalizers.

Two statistical algorithms (geNorm^[32] and NormFinder^[33]) were used to estimate the stabilities of the miRNAs profiles. The algorithm of geNorm is to calculate the average pairwise variation (V) for each miRNA with all others across the samples, and estimate a stability score (M) defined as the average V of a miRNA with all others. The less stable miRNA with the highest M is gradually removed until the 2 most stable normalizers are obtained. The algorithm of NormFinder is to calculate the inter- and intra-group variances of the log-transformed miRNAs expression data, and integrate it into a stability value to represent the systematic error of each miRNA. A lower value of systematic error indicates a more stable miRNA, and the combination of the most stable miRNAs is selected as the normalizer. Different types of normalizers (array recommended ncRNAs, mean of global miRNAs and the most stable miRNAs combination) were separately used to generate miRNA expression profiles from HCC tissue samples for future statistical data analysis.

Before performing any statistical analysis, the genome-wide miRNA profiles were checked to ensure the reliability and abundance of miRNAs. If the missing data ($C_q \geq 40$) for any miRNA exceeded 50% of samples, this miRNA was excluded from further data analyses. Paired *t*-test was used to identify miRNAs that were significantly different by the univariate test ($P < 0.001$) with at least a 2-fold expression change between paired HCC tumor/non-tumor tissues or HCC cases and matched controls. Volcano plots were generated to describe the distribution of significant miRNAs with over 2-fold changes. Hierarchical clustering and heat maps were produced with average linkage and Pearson correlations to examine the classification of samples based on significant miRNAs. All statistical analyses were performed using BRB-ArrayTools (version 4.4) developed by Dr. Richard Simon and the BRB-ArrayTools Development Team (<http://linus.nci.nih.gov/BRB-ArrayTools.html>)^[34] and Statistical Analysis System 9.0 (SAS Institute). The panels of significant miRNAs identified by different normalizers were compared using a web-based InteractiVenn tool (<http://www.interactivenn.net/>)^[35] to determine the consistent and discrepant miRNAs.

RESULTS

The stabilities of global miRNAs/ncRNAs

Using geNorm and NormFinder tools, we separately examined the stabilities of global miRNA profiles in HCC tissues. Among 157 miRNAs/ncRNAs tested in HCC tissues, the combination of miR-30c/miR-30b had the smallest M score of 0.024 by geNorm [Supplementary Figure 1], and miR-30c/miR-126

together had the lowest systematic error (stability value) of 0.133 by NormFinder [Supplementary Table 3], suggesting their good stabilities for normalization. If we included the means of global miRNAs in the stability analyses, the M score and stability value for miR-30c/mean of miRNAs combination were, respectively 0.022 and 0.088, lower than the combination of miR-30c with miR-30b or miR-126 [Supplementary Table 3]. This suggests that the mean of global miRNAs may be a good normalizer due to its high stability among samples. The stabilities of the manufacturer-recommended normalizer ncRNAs (U6 snRNA, RNU44 and RNU48) ranked much lower in the 17th to 140th range out of a total 157 candidates, indicating their poor stability in HCC tissue.

Aberrant miRNA panels identified by using varied normalizers

After excluding non-abundant miRNAs and those with missing data in over 50% of samples, a total of 361 miRNAs were finally analyzed. Using 3 endogenous controls (U6 snRNA, RNU44 and RNU48) as the normalizer, we found 46 miRNAs significantly dysregulated ($P < 0.001$) in HCC tumor tissues with at least 2-fold changes in expression [Supplementary Table 4, Figure 1A]. Most miRNAs (43) were significantly down-regulated in HCC tumor tissue (from 2 to 10-fold), and only 3 miRNAs were significantly up-regulated with fold changes of 5 to 9.

Using the mean of all miRNAs as a normalizer, a total of 26 miRNAs were significantly different between HCC tumor and non-tumor tissues with over 2-fold changes [Table 1, Figure 1B]. The aberrant expression pattern was quite different from that identified by using 3 endogenous controls. More miRNAs (17) were upregulated 2- to 16-fold compared with 9 significantly downregulated miRNAs (2-5 fold) in HCC tumor tissues. The expression levels of endogenous controls (U6 snRNA and RNU44) were increased while RNU48 was reduced in tumor tissue when using the mean of all miRNAs as a normalizer, but no significant difference was obtained (data not shown). The fold-changes between tumor and non-tumor tissues were varied from -1.28 to 4.09 times.

Supplementary Table 5 and Figure 1C display a panel of 17 miRNAs aberrantly expressed in HCC tumor tissue using 2 stable miRNAs (miR-30c and miR-30b) identified by the geNorm tool as the normalizer. Six were significantly upregulated 2- to 15-fold; 11 were downregulation 3- to 6-fold in HCC tumor tissue. Similarly, using 2 stable miRNAs (miR-30c and miR-126) identified by the NormFinder tool as the normalizer, we found 20 significantly deregulated

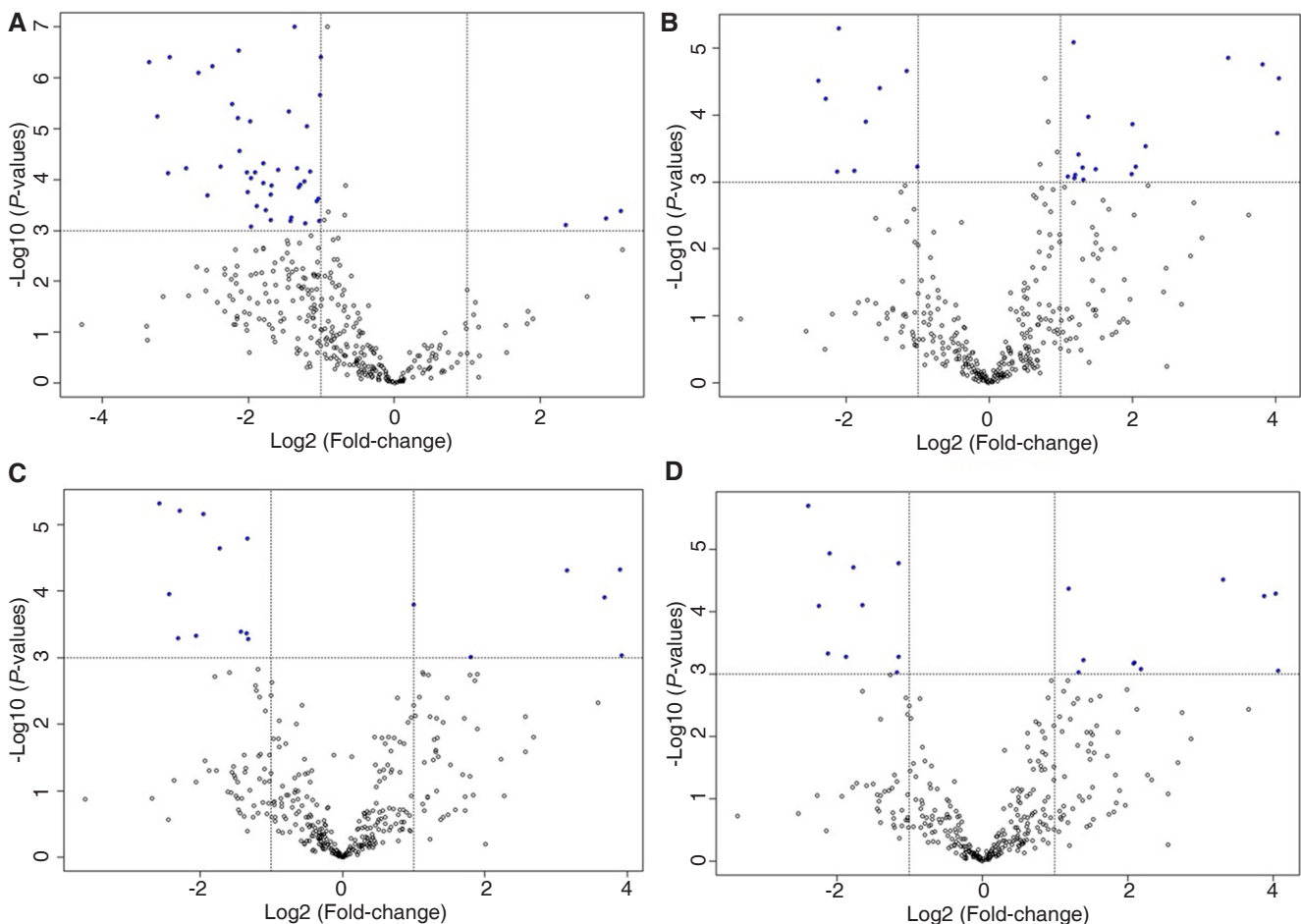


Figure 1: Volcano plots of the microRNAs (miRNAs) with significant ($P < 0.001$) over 2-fold expression changes in hepatocellular carcinoma (HCC) tumor compared to nontumor tissues. (A) There were more downregulated miRNAs aberrantly expressed in HCC tumor tissue with only 3 miRNAs significantly up-regulated if using 3 endogenous controls (U6 snRNA, RNU44 and RNU48) as the normalizer; (B) Expression of 26 miRNAs (17 upregulated and 9 downregulated) significantly differed between tumor and non-tumor tissues; (C) Seventeen miRNAs were aberrantly expressed in HCC tumor tissue using 2 stable miRNAs (miR-30c and miR-30b) identified by geNorm tool as the normalizer; (D) Twenty miRNAs were significantly deregulated in HCC tumor tissues using 2 stable miRNAs (miR-30c and miR-126) identified by NormFinder tool as the normalizer. The overall expression patterns of miRNAs in (B, C and D) were similar but different from (A) with many more down-regulated miRNAs

miRNAs with over 2-fold changes between HCC tumor and non-tumor tissues [Supplementary Table 6, Figure 1D]. Half were upregulated 2- to 17-fold, and the others were downregulated 2- to 5-fold in HCC tumor tissue. Thus, the identified miRNA panels varied with the different normalization strategies.

Comparisons of different miRNA panels in classification of HCC status

The hierarchical clustering and heat map showed that a panel of 46 significant miRNAs could well distinguish HCC tumor from non-tumor tissues using 3 endogenous controls as the normalizer [Figure 2]. Only 1 tumor and 1 non-tumor tissue were misclassified. Similarly, the other 3 panels of miRNAs identified using as normalizers the mean of all miRNAs or the 2 most stable miRNAs also well classified HCC tumor from non-tumor tissues with

only 1 or 2 misclassifications. The later three panels consisting of 17 to 26 aberrant miRNAs contained many fewer miRNAs than that using 3 endogenous controls as the normalizer, suggesting a more efficient panel of HCC classification.

Consistence of identified miRNAs by different normalizers

Using InteractiVenn to compare different panels of miRNAs dysregulated in HCC, we found that most miRNAs identified using the mean of miRNAs overlapped with those identified when using 2 stable miRNAs as the normalizer [Supplementary Figure 2A]. There were, respectively 14 (54%) and 18 (69%) miRNAs consistent with the 26 significant miRNAs identified from the global mean analysis. A total of 14 miRNAs (miR-196b, miR-183, miR-182, miR-10b#, miR-18a, miR-106a, miR-139-5p, miR-144#, miR-214, miR-

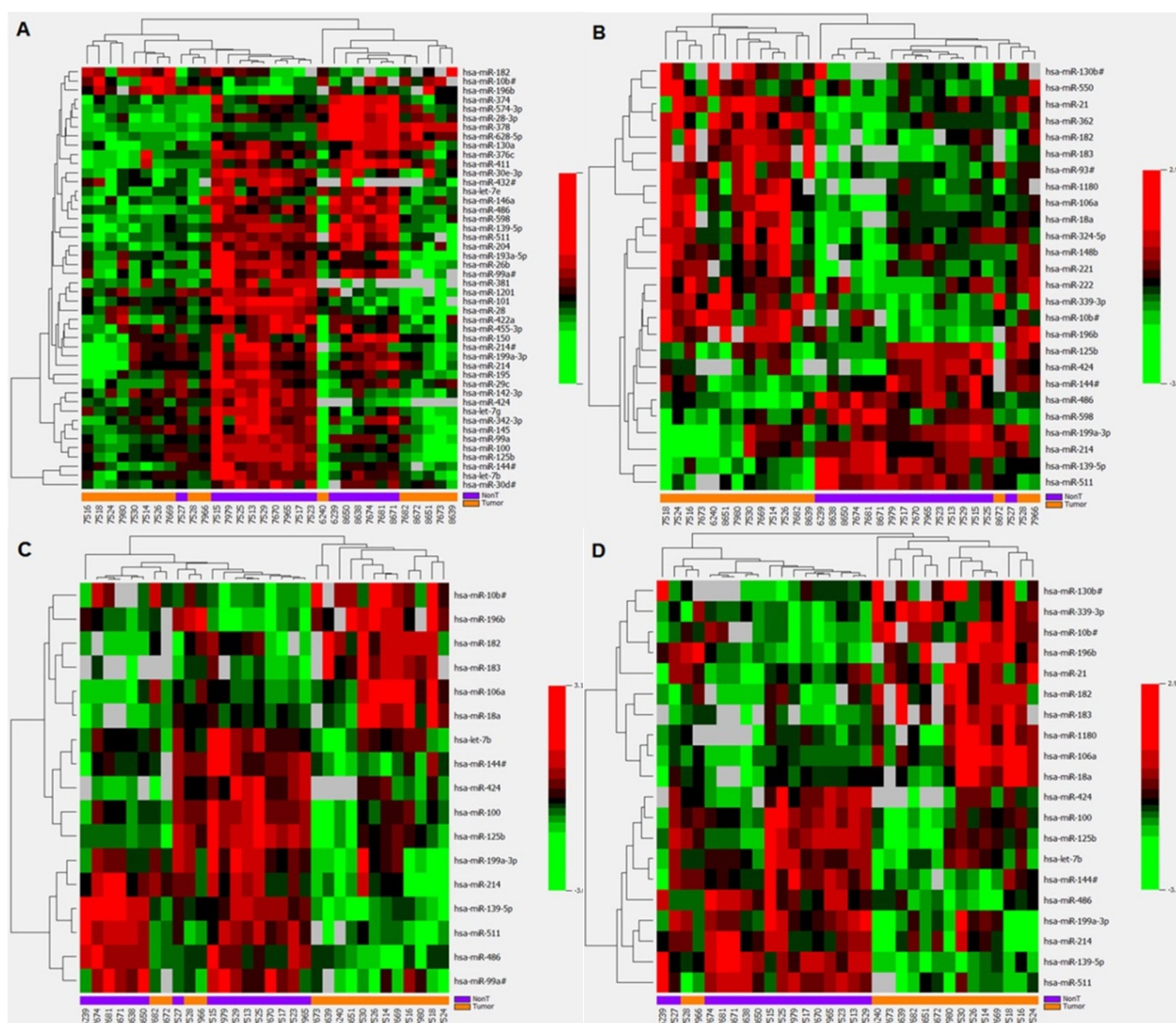


Figure 2: Comparisons of different microRNAs (miRNAs) panels identified with varied normalization strategies in distinguishing hepatocellular carcinoma (HCC) tumor from non-tumor tissues. (A) A panel of 46 miRNAs identified using 3 endogenous controls as the normalizer can distinguish HCC tumor from non-tumor tissues with 2 misclassifications. The other 3 panels of miRNAs identified by using as normalizers of the mean of all miRNAs (B), miR-30c/miR-30b (C) and miR-30c/miR-126 (D) also classified HCC tumor from non-tumor tissues with only 1 or 2 misclassifications

486, miR-199a-3p, miR-511, miR-424 and miR-125b) were consistently dysregulated by using the mean of miRNAs or 2 stable miRNAs as normalizers [Figure 3A], suggesting a high consistency for these normalization strategies. Using the mean of miRNAs as the normalizer, we found 8 additional dysregulated miRNAs (miR-221, miR-222, miR-324-5p, miR-550, miR-362, miR-148b, miR-93# and miR-598) in HCC tissue that were not significant when using 2 stable miRNAs as the normalizer [Supplementary Table 7]. Seven dysregulated miRNAs (miR-183, miR-1180, miR-18a, miR-130b#, miR-339-3p, miR-21 and miR-106a) were identified in HCC tumor tissues using either the mean of global miRNAs or miR-30c/miR-

126 (selected as the most stable miRNAs by using NormFinder tool) as normalizers, including functionally important oncogenic miR-21, miR-18a, miR-106a and miR-183 [Supplementary Figure 3A]. Using either the mean of global miRNAs or miR-30c/miR-30b (selected as the most stable miRNAs by using geNorm tool) as normalizers, the same oncogenic miR-183, miR-18a and miR-106a were identified [Supplementary Figure 3B]. These miRNAs would not have been identified if using 3 endogenous ncRNAs as the normalizer. These data suggest that the combination of using the mean of miRNAs and 2 stable miRNAs identified by NormFinder as normalizers may be a good option to pinpoint biologically important miRNA.

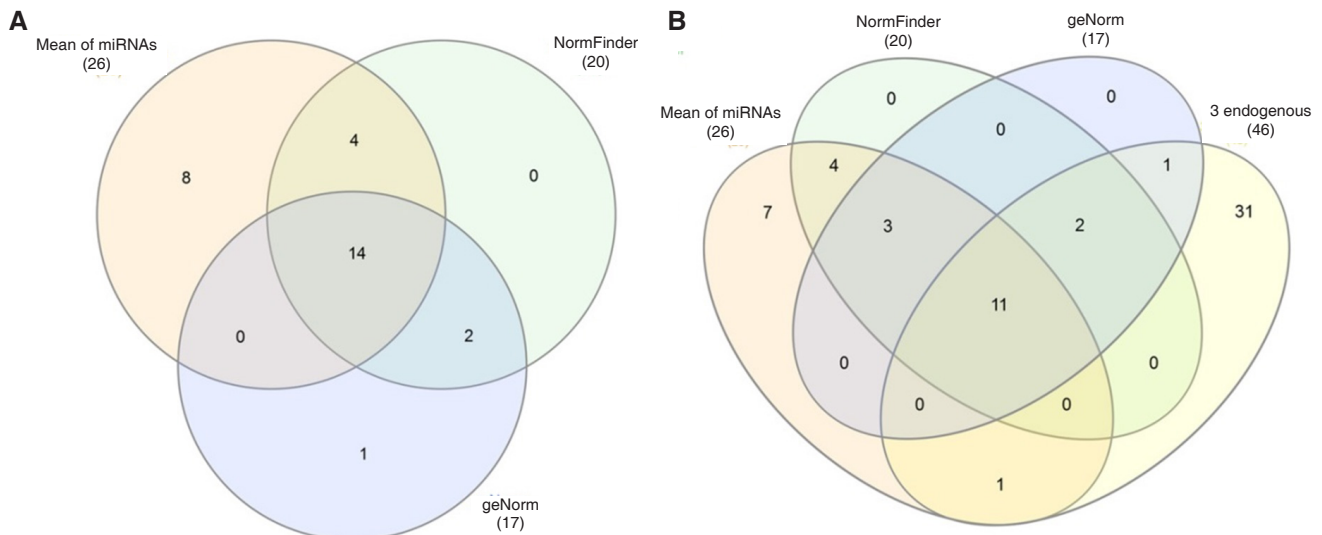


Figure 3: InteractiVenn determination of the consistent and discrepant microRNA (miRNA) panels identified using different normalization strategies. Using the mean of miRNAs and 2 stable miRNAs as normalizers (A), 14 miRNAs (miR-196b, miR-183, miR-182, miR-10b#, miR-18a, miR-106a, miR-139-5p, miR-144#, miR-214, miR-486, miR-199a-3p, miR-511, miR-424 and miR-125b) were consistently identified as dysregulated in hepatocellular carcinoma tumor tissue. (B) Compared to the panel identified using endogenous controls as the normalizer, a total of 11 miRNAs (miR-196b, miR-182, miR-10b#, miR-139-5p, miR-144#, miR-214, miR-486, miR-199a-3p, miR-511, miR-424 and miR-125b) were consistently identified by all normalization strategies

Comparing these miRNA panels with that identified by using 3 endogenous controls as the normalizer, less than one third (12-14) out of a total 46 miRNAs overlapped [Supplementary Figure 2B]. A total of 11 miRNAs (miR-196b, miR-182, miR-10b#, miR-139-5p, miR-144#, miR-214, miR-486, miR-199a-3p, miR-511, miR-424 and miR-125b) were consistently identified by all normalization strategies [Figure 3B, Supplementary Table 8], suggesting the importance of these miRNAs. Thirty-one miRNAs were only downregulated in HCC when using 3 ncRNAs (U6 snRNA, RNU44 and RNU48) as the normalizer, which may be due to the fact that the expression levels of the 3 ncRNAs were significantly enhanced in HCC tumor (data not shown). The mean Cq of ncRNAs in HCC tumor was significantly higher than in non-tumor tissues (22.2 vs. 23.0, $P = 4.77\text{E-}07$). Therefore, using the unstable and upregulated ncRNAs as the normalizer, we may falsely identify miRNAs downregulated in target tissue. In contrast, using the more stable global mean of miRNAs as the normalizer, an additional 7 miRNAs (miR-221, miR-222, miR-324-5p, miR-550, miR-362, miR-148b, miR-93#), including 2 novel (miR-324-5p, miR-550) were identified as significantly upregulated in HCC tumor tissues, which would not have been identified when using 3 endogenous controls as the normalizer [Supplementary Table 8].

DISCUSSION

Our study for the first time, demonstrated that using different normalizers identifies diverse aberrant

miRNA patterns in HCC tumors [Figures 1 and 2], and a combination of global mean and the top stable miRNAs as normalizer might be an optimal strategy to identify biologically meaningful miRNAs [Table 1 and Figure 3]. We derive this conclusion based on the assumption that overall miRNA expression levels are invariable because all up- and down-regulated miRNAs are similarly distributed^[15,26] and only a small proportion of specific miRNAs significantly vary across samples due to different biological conditions,^[23,36] such as hepatocarcinogenesis. Therefore, selection of the most stable candidate as the normalizer is the key principle to adjust for variations from sample and technical differences during miRNA measurements. We found that both global mean and 2 sets of miRNAs as normalizers in the current study ranked high in terms of stability, while the endogenous controls recommended by the manufacturer were not stable and usually up-regulated in HCC tumors [Supplementary Figure 1]. If using the endogenous controls as normalizer, we would obtain more miRNAs that were significantly down-regulated in HCC tumor tissue, but many of them might be false positive findings due to using an inappropriate normalizer to adjust miRNA expression [Figures 1-3]. In contrast, using the global mean of miRNAs and miR-30c/miR-126 as normalizers, several functionally important oncogenic miRNAs (miR-21,^[37-40] miR-18a,^[41] miR-106a^[41,42] and miR-183^[37,43-45]) were identified as dysregulated in HCC tumor tissues [Supplementary Figure 3]. Several well-known oncogenic miRNAs (miR-221^[46-49], miR-

Table 1: The 26 miRNAs significantly aberrantly expressed in HCC tumor compared to nontumor tissues using the mean of all miRNAs as the normalizer

| miRNAs | Geometric mean in tumor tissue | Geometric mean in non-tumor tissue | Fold-change | P-value | FDR |
|-------------|--------------------------------|------------------------------------|-------------|----------|----------|
| miR-196b | 1.10E-01 | 7.30E-03 | 16.56 | 2.88E-05 | 1.41E-03 |
| miR-183 | 1.90E-02 | 1.40E-03 | 16.29 | 1.86E-04 | 4.47E-03 |
| miR-182 | 4.10E-02 | 3.20E-03 | 14.11 | 1.77E-05 | 1.41E-03 |
| miR-10b# | 8.10E-02 | 8.60E-03 | 10.13 | 1.41E-05 | 1.41E-03 |
| miR-1180 | 2.50E-02 | 9.10E-03 | 4.56 | 2.90E-04 | 6.55E-03 |
| miR-221 | 3.10E-01 | 7.40E-02 | 4.13 | 5.87E-04 | 1.00E-02 |
| miR-18a | 1.90E-01 | 5.70E-02 | 4.00 | 1.39E-04 | 3.58E-03 |
| miR-130b# | 1.10E-02 | 3.20E-03 | 3.97 | 7.69E-04 | 1.06E-02 |
| miR-222 | 1.81E+01 | 6.42E+00 | 2.81 | 6.48E-04 | 1.02E-02 |
| miR-339-3p | 1.70E-01 | 6.50E-02 | 2.62 | 1.08E-04 | 3.54E-03 |
| miR-21 | 1.30E+01 | 5.20E+00 | 2.49 | 9.27E-04 | 1.12E-02 |
| miR-324-5p | 8.30E-02 | 3.40E-02 | 2.47 | 6.11E-04 | 1.00E-02 |
| miR-550 | 3.30E-02 | 1.40E-02 | 2.38 | 3.87E-04 | 7.76E-03 |
| miR-362 | 1.10E-01 | 4.90E-02 | 2.30 | 7.96E-04 | 1.06E-02 |
| miR-148b | 6.60E-02 | 3.00E-02 | 2.28 | 8.78E-04 | 1.09E-02 |
| miR-106a | 5.19E+01 | 2.29E+01 | 2.27 | 8.20E-06 | 1.41E-03 |
| miR-93# | 1.30E+00 | 6.10E-01 | 2.14 | 8.28E-04 | 1.07E-02 |
| miR-139-5p | 4.70E-01 | 2.47E+00 | -5.26 | 3.13E-05 | 1.41E-03 |
| miR-144# | 4.60E-02 | 2.10E-01 | -5.00 | 5.78E-05 | 2.09E-03 |
| miR-214 | 5.20E-01 | 2.29E+00 | -4.35 | 7.09E-04 | 1.02E-02 |
| miR-486 | 1.90E-01 | 8.20E-01 | -4.35 | 5.10E-06 | 1.41E-03 |
| miR-199a-3p | 1.61E+00 | 5.95E+00 | -3.70 | 6.84E-04 | 1.02E-02 |
| miR-511 | 3.00E-02 | 1.10E-01 | -3.33 | 1.28E-04 | 3.54E-03 |
| miR-424 | 1.40E-02 | 2.70E-02 | -2.94 | 4.02E-05 | 1.61E-03 |
| miR-125b | 1.23E+00 | 2.74E+00 | -2.22 | 2.24E-05 | 1.41E-03 |
| miR-598 | 4.30E-02 | 8.50E-02 | -2.00 | 5.96E-04 | 1.00E-02 |

miRNA: microRNA; HCC: hepatocellular carcinoma; FDR: false discovery rate

222^[46,48,49], miR-362^[50,51]) and two miRNAs (miR-324-5p and miR-550) first identified in HCC tumor tissue were significantly over-expressed by using miRNA global mean as the normalizer [Supplementary Table 7]. These miRNAs would not have been discovered using the 3 endogenous controls as normalizer. Our results were strongly supported by the evidence obtained from previous studies that using global expression mean as normalizer significantly reduces technical variation (standard deviations) across samples and faithfully represents the input amount of total RNA.^[23,52] More importantly, this approach also showed maximum separation for biologically different samples and significantly reduces false positive findings of down-regulated miRNAs.^[23,52] It suggests that the combination of global mean and

the top stable miRNAs as the normalizer may be a good option to identify biologically important miRNA in hepatocarcinogenesis.

Although using this strategy may raise concern that measuring global miRNA profiles for all participants in a large epidemiological study is not feasible, we strongly recommended running at least a subset of representative samples or samples mixed from all subjects to select the most stable candidates among detectable miRNAs for normalization. This additional step is necessary to ensure a proper normalization strategy for miRNA quantification and comparison. Mestdagh *et al.*^[23] proposed a similar strategy that first obtained miRNA expression levels by using global mean of miRNAs as normalizer, and then identified

the most stable miRNAs by comparing the normalized miRNAs, which may be influenced by the extreme values of specific miRNAs that were used to estimate the global mean.^[26] We simplified the procedure by directly evaluating the stabilities of fully detectable miRNAs using unadjusted raw Cq value to exclude this potential impact of extreme data. Most aberrant miRNAs (54-69%) identified by using global mean and 2 stable miRNAs as normalizers overlapped [Supplementary Figure 2A] in the current study, which is consistent with a previous study that showed over 65% of miRNAs displaying significant correlation coefficients of above 0.9 using global mean and stable miRNAs as normalizers.^[23] Therefore, this normalization strategy outperforms other available approaches and is also straightforward to be performed in future large epidemiological studies.

Three miRNAs (miR-30c, miR-30b and miR-126) were identified as normalizers in the current study, indicating their expression levels remained stable in liver tissue regardless of HCC status. In contrast, several previous studies found significant dysregulation of miR-30c,^[21,53] miR-30b^[21] and miR-126^[54,55] in HCC tumor tissue and blood samples, suggesting their potential etiologic or diagnostic roles. However, none of those previous studies used global mean of miRNAs as the normalizer and did not evaluate miRNAs expression stabilities that might lead to false positive findings. Several studies also identified these 3 miRNAs as dysregulated in other types of human cancers.^[56-58] Because miRNAs have a characteristic of tissue type specificity,^[24] the expression stabilities of miR-30c, miR-30b and miR-126 should be separately validated in relevant tissues before drawing conclusions on their role in carcinogenesis.

Overall, the optimal normalization strategy described here can help identify the most concordant miRNA panel differentiating HCC tumor from non-tumor tissues that are feasible to be used for future validation in large epidemiological studies. This strategy can also prevent potential false positive findings, largely down-regulated miRNAs. Of course, this strategy may improve the identification of novel miRNAs. Two novel miRNAs (miR-324-5p and miR-550) were identified as significantly over-expressed in HCC tumor tissue using the optimal normalization strategy. However, the weaknesses of current study need to be recognized, such as a small sample size, no data for different arrays and lack of validation for identified miRNAs in larger and independent patients.

In summary, normalization methods impact on miRNAs that are differentially expressed in tumors; often many

studies do not consider how normalization methods impact their findings. We first ascertained two sets of stable miRNAs in liver tissue that are independent of HCC status, and emphasize the importance of using a proper normalization strategy to identify aberrant miRNAs associated with HCC. In combination with the global mean of miRNA profiles as the normalizer, we finally identified a panel of miRNAs dysregulated in HCC, and were able to exclude potential false positive findings in hepatocarcinogenesis. Our results need to be further validated in other independent studies to ensure distinguishing of biologically meaningful miRNAs in HCC. Studies using different approaches (TLDA, miScript, miRCURY, etc.) but the same set of stable miRNAs to validate our findings are also warranted to strengthen the significance.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

A waiver of consent was given in the study because the majority of patients died before the research was carried out.

Ethics approval

The study was approved by the Institutional Review Board of Columbia University Medical Center.

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Role of contrast-enhanced ultrasound in the evaluation of vascularization of hepatocellular carcinoma

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ABSTRACT

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Key words:

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Aim: Early individualization of hepatocellular carcinoma is crucial to obtain good therapeutic results, thanks to several options such as percutaneous therapies, surgical resections and transplant. Aim of this study is to evaluate the vascularization of hepatocarcinoma using contrast enhanced ultrasound (CEUS) in comparison with multislice computed tomography (MSCT). **Methods:** Between January 2009 and May 2014, 67 patients affected by hepatocarcinoma, who presented an overall of 92 nodules, were examined and enrolled in the study. **Results:** There was a significant difference in the percentage of comparison of the vascularization between the nodules situated at a depth not greater than 9 cm, compared to those studied at a greater depth. In reference to the size of the lesion, the percentage of vascularization to the CEUS in arterial phase, compared with the MSCT, was 84% in lesions with dimensions equal or less than 1 cm, 91% in lesions with dimensions included between 1 and 2 cm, and 96% in the lesions greater than 2 cm. **Conclusion:** CEUS is a method capable of documenting with very reliable accuracy the intralesional vascularization of hepatic carcinoma, in a superimposable manner to the MSCT. However, CEUS also presents some limitations, mainly in relation to the site of lesions.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the primitive, most common malignant tumor of the liver accounting for 70-84% of hepatic tumors. The early identification of HCC is very important in obtaining good therapeutic outcomes, thanks to several

options such as percutaneous therapies, surgical resections and transplant. Ultrasound examination and measurements of the levels of alpha-fetus protein in serum are the main screening options. Several methods have been used to evaluate the intralesional vascularization of hepatic focal lesions.^[1-5] Computed tomography (CT) is the second most common



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screening option.

The development of second-generation ultrasound contrast media and dedicated software has improved the diagnostic capabilities of ultrasound in the individualization and characterization of focal hepatic lesions,^[6-10] as it allows clinicians to study intralésional vascular architecture in real time in all contrastographic phases.^[11-15]

Each contrastographic phase has its own specificity, useful in diagnosis; in particular, in the arterial phase, it is fundamental to evaluate the pattern and the grade of vascularization, while the portal and late phase are also useful for the correct diagnosis.

METHODS

Between January 2009 and May 2014, 67 patients affected by hepatocarcinoma, who presented an overall of 92 nodules, were examined and enrolled in the study. There were 23 females and 44 males with an average age of 68 years, of whom 62 presented a chronic liver disease, while 5 did not present any hepatic symptoms. The diagnosis of HCC was established by confirming the presence of a lesion, which assumed enhancement in the arterial phase with wash-out in portal and late phases. The same parameters were utilized in both contrast-enhanced ultrasound (CEUS) and in multislice CT (MSCT).

The three vascular phases were evaluated: arterial (0-35 s from the injection of the MdC), portal (35-90 s) and sinusoidal (from 90 s to approximately 6 min). Increases in serum levels of alpha-fetoprotein were also evaluated.

CEUS

The examinations used GE LOGIQ 5 EXPERT and ESAOTE MY LAB equipment with a specific incorporated software designed to work at low mechanical index, with 3.5 MHz convex transducers. The contrast medium in all cases was the SonoVue (Bracco, Italy), consisting of micro bubbles of stabilized phospholipids containing sulphur hexafluoride. This was injected as a bolus in an antecubital vein, followed by an injection of 10 mL of physiological solution. In no case was a second injection of MdC given. Before the intravenous injection, a basal echography of the liver was done in order to evaluate the most suitable ultrasound window to study the lesion. The identified lesion was studied "in real time" up to approximately 6 min from the injection and the enhancement was always compared with the surrounding parenchyma. All phases of the tests were registered on a compact

disc (CD) to be evaluated again. In no case were complications manifested.

MSCT

The examinations used a CT multidetector scanner of GE light-speed (16 and 64 canals). All examinations were done in basal conditions and after intravenous injection of approximately 90-120 mL of MdC, at a 4 mL/s speed. Smart prep was always used for acquisition of the arterial phase.

Analysis of the images

The vascularization of the single lesion, using both CEUS and CT, was classified as hyper-, iso- and hypovascular in each one of the evaluated phases, always in relation to the enhancement of the condition of surrounding parenchyma.

Statistical analysis

Fisher's test was used to compare the results of CEUS with MSCT. Furthermore, the results of the vascularization comparing CEUS and MSCT were evaluated in relation to the site and the size of the lesions. A *P*-value of < 0.05 was considered statistically significant.

RESULTS

In the arterial phase [Table 1], 75 of 92 nodules were hyperdense in MSCT; of these in using CEUS, 66 (88%) were hypervascular [Figure 1] and 9 (12%) were isovascular [Figure 2].

Eleven of 92 nodules were isodense in MSCT; of these using CEUS, 3 (27%) were hypervascular [Figure 3], 8 (73%) were isovascular. Six of 92 nodules were hyperdense in MSCT, using CEUS, 2 (33%) were hypervascular, 4 (67%) isovascular. Seventy-six of 92 nodules were localized at a depth not greater than 9 cm from the abdominal wall, 16 of 92 were localized at a greater depth [Table 2]. Of the 64 out of 76 nodules localized at a depth not greater than 9 cm that appeared hypervascular using MSCT, 61 (95%) appeared hypervascular using CEUS. Of the

Table 1: Comparison between arterial phase seen with MSCT and early vascular phase seen with CEUS

| Arterial phase in MSCT | Early vascular phase seen in CEUS | | |
|------------------------|-----------------------------------|----------|-------|
| | Hyper | Iso | Total |
| Hypervascular | 66 (88%) | 9 (12%) | 75 |
| Isovascular | 3 (27%) | 8 (73%) | 11 |
| Hypovascular | 2 (33%) | 4 (67%) | 6 |
| Total | 71 (77%) | 21 (23%) | 92 |

MSCT: multislice computed tomography; CEUS: contrast enhanced ultrasound

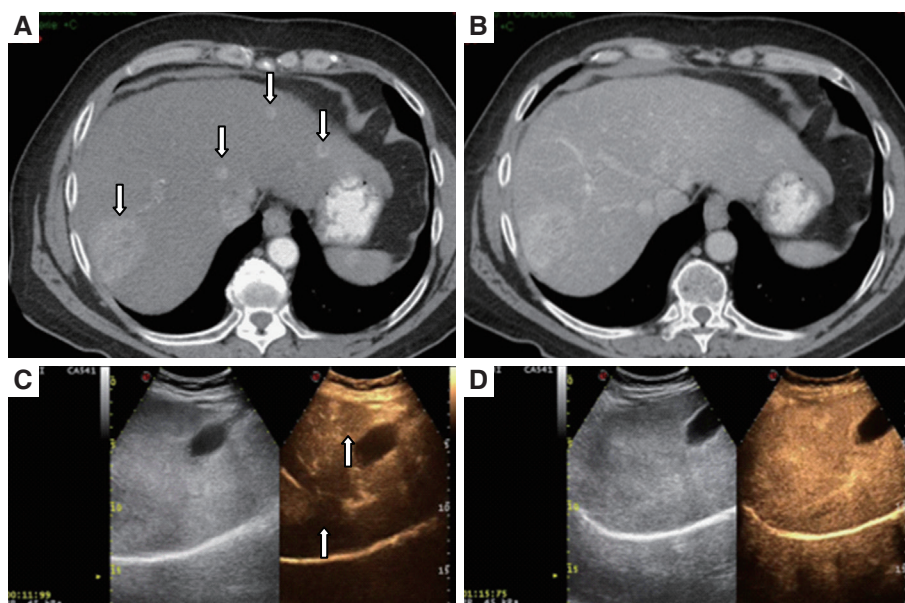


Figure 1: Arterial phase (A, C) and portal phase (B, D) seen in MSCT and CEUS, in the same patient with multifocal hepatocarcinoma. CEUS (C, D) appears in a double image: B mode on the left and contrast enhancement on the right. Both modalities allow a good evaluation of the hypervascularization of the lesions in arterial phase (arrows) and the wash-out in the portal phase. MSCT allows the same scan in the arterial phase (A) to show 4 nodules. CEUS, in its windows in the same phase (C) shows only 2 nodules. CEUS allows an extremely rapid scan of the arterial phase (C) with enhancement of lesions after 11 s since the administration of MdC. MSCT: multislice computed tomography; CEUS: contrast enhanced ultrasound

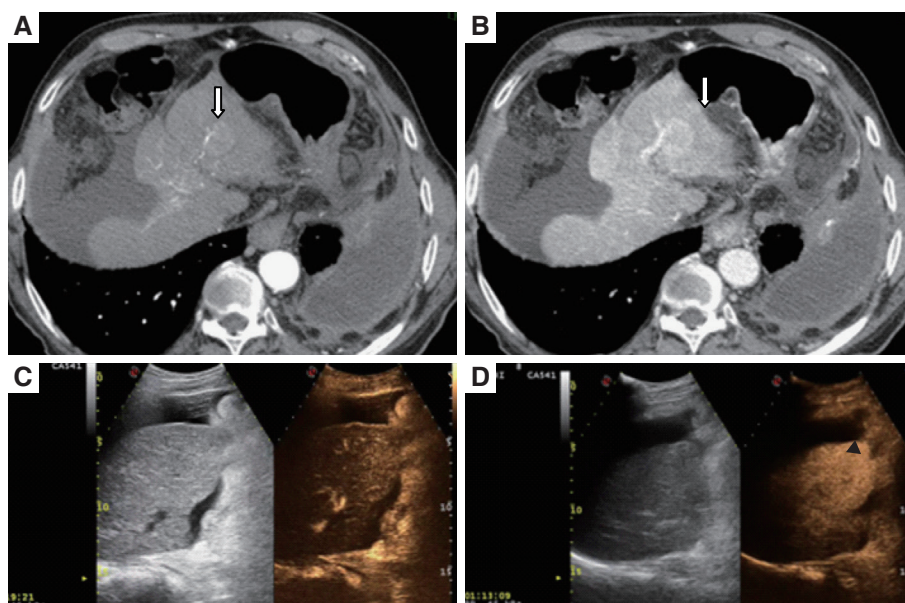


Figure 2: Arterial phase (A, C) and portal phase (B, D) seen in MSCT and CEUS in the same patient with hepatocarcinoma. MSCT (A, B) allows a good documentation of the nodular lesion (arrows) slightly hypervascular in the arterial phase with wash-out in the portal phase. In CEUS the lesion, not detected in B mode, is isovascular in the arterial phase (C); in the portal phase (D) is slightly hypovascular (black arrow head). MSCT: multislice computed tomography; CEUS: contrast enhanced ultrasound

Table 2: Determination of vascularization using CEUS, related to the depth of nodules

| Arterial phase in MSCT | Depth (cm) | Early vascular phase seen in CEUS | | | |
|------------------------|------------|-----------------------------------|----------|-------|---------|
| | | Hyper | Iso | Total | P-value |
| Hypervascular | ≤ 9 | 61 (95%) | 3 (5%) | 64 | 0.0007 |
| | > 9 | 7 (58%) | 5 (42%) | 12 | |
| Isovascular | ≤ 9 | 3 (43%) | 4 (57%) | 7 | NS |
| | > 9 | 1 (25%) | 2 (75%) | 3 | |
| Hypovascular | ≤ 9 | 2 (40%) | 3 (60%) | 5 | NS |
| | > 9 | 0 | 1 (100%) | 1 | |

MSCT: multislice computed tomography; CEUS: contrast enhanced ultrasound; NS: not significant

12 of 76 nodules localized at a depth greater than 9 cm that appeared hypervascular using MSCT, 7 (58%) appeared hypervascular using CEUS ($P = 0.0007$).

These results showed that there was a significant difference in the percentage of comparison of the vascularization between the nodules situated at a depth not greater than 9 cm, compared to those studied at a greater depth.

In reference to the size of the lesion, the percentage of vascularization using CEUS in the arterial phase comparing with MSCT was 84% in lesions with dimensions equal or less than 1 cm, 91% in lesions with dimensions between 1 and 2 cm and 96% in lesions greater than 2 cm [Table 3].

Therefore there was no significant difference in comparing the dimensions of lesions and their vascularization in the arterial phase.

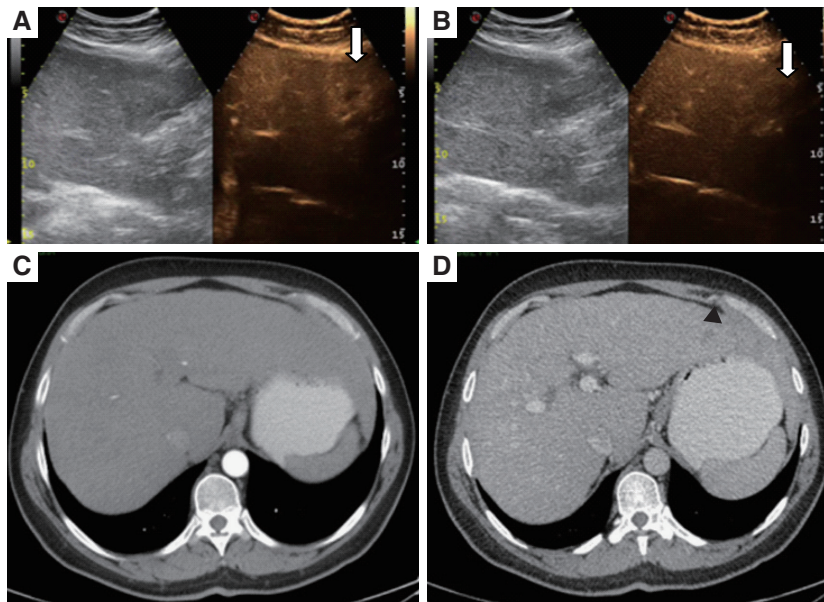


Figure 3: Arterial phase (A, C) and portal phase (B, D) seen in MSCT and CEUS in the same patient with hepatocarcinoma. In CEUS the nodule (arrows) is slightly hypervascular in the arterial phase (A) with wash out in the portal phase (B). The enhancement is disomogeneous because of the presence of an avascular area in the cranial portion of the lesion. In MSCT the lesion is isovascular in the arterial phase (C). In the portal phase (D) it is hypovascular (black arrow head). MSCT: multislice computed tomography; CEUS: contrast enhanced ultrasound

Table 3: Determination of vascularization seen in CEUS related to sizes of lesions

| Arterial phase in MSCT | Sizes (cm) | Early vascular phase seen in CEUS | | | |
|------------------------|------------|-----------------------------------|----------|-------|---------|
| | | Hyper | Iso | Total | P-value |
| Hypervascular | ≤ 1 | 10 (84%) | 2 (16%) | 12 | NS |
| | 1-2 | 34 (91%) | 3 (9%) | 37 | |
| | > 2 | 24 (96%) | 1 (4%) | 25 | |
| Isovascular | ≤ 1 | 3 (60%) | 2 (40%) | 5 | |
| | 1-2 | 0 | 6 (100%) | 6 | |
| | > 2 | 0 | 0 | 0 | |
| Hypovascular | ≤ 1 | 1 (100%) | 0 | 1 | |
| | 1-2 | 0 | 3 (100%) | 3 | |
| | > 2 | 1 (33%) | 2 (67%) | 3 | |

MSCT: multislice computed tomography; CEUS: contrast enhanced ultrasound; NS: not significant

Similar results were shown for the portal and sinusoidal phase [Table 4]: 85 of 92 nodules appeared hypodense using MSCT; using CEUS, 70 nodules (82%) were hypovascular, the other 15 (18%) being isovascular. Seven of 92 nodules appeared isodense using MSCT; using CEUS, 6 (86%) were hypovascular, 1 (14%) was isovascular.

The basic pathology, a chronic liver disease displayed by almost all the examined patients, did not limit significantly the study of intralesional vascularization using CEUS as compared with using CT, particularly in the arterial phase. In the portal phase, the parenchymal enhancement found using CEUS was less intense and more delayed when compared to the enhancement found using CT.

DISCUSSION

The advent of second-generation echographic contrast

Table 4: Lesions detected in portal phase using CEUS, related to MSCT

| Portal phase MSCT | Portal phase seen in CEUS | | |
|-------------------|---------------------------|----------|-------|
| | Iso | Hypo | Total |
| Isodense | 1 (14%) | 6 (86%) | 7 |
| Hypodense | 15 (18%) | 70 (82%) | 85 |
| Total | 16 (17%) | 76 (83%) | 92 |

MSCT: multislice computed tomography; CEUS: contrast enhanced ultrasound

media and the generation and development of dedicated software allow clinicians, working at a low mechanical index, to study perfusion in real time and also makes easier the study of small-dimension lesions by considerably increasing the diagnostic capabilities of ultrasound.^[16-20]

In the guidelines for management of HCC provided in 2005 by the American Association for the Study of Liver Disease (AASLD), CEUS has been considered among the non-invasive methods able to detect the typical enhancement of HCC. This condition is characterized by hypervascularization in the arterial phase with progressive wash-out of the MdC in the portal and late phases.^[21,22] Such contrastographic characteristics demonstrated high diagnostic validity, in various case studies being characterized by a 92-94% sensitiveness and by specificity of 87-96%.

The feedback from typical enhancement with the use of CT or magnetic resonance (MR), methods can be considered conclusive for correct diagnosis. Also, MR, if performed with hepatospecific MdC (BOPTA and/or EOB), can also allow in late sequences (colangiographic) the demonstration of hepatocyte alteration characterized by hypointensity of signal

within the lesion, giving further diagnostic evidence of malignancy.

In a multicentric study (DEGUM) with 1,349 patients with focal hepatic lesions identified with basal ultrasound, CEUS was compared with biopsy in 75% of the cases and in the remaining 25% with spiral CT or MR. The diagnostic accuracy of CEUS was 90.3%.^[23,24]

Two other, more recent prospective studies (DEGUM) have evaluated the potential of CEUS in the characterization of focal hepatic lesions by comparing CEUS with CT and with MR; in both studies it was concluded that there are not statistically significant differences.^[25,26]

In the first study the authors concluded that CEUS must be used first, before using CT; they have also documented that CEUS utilization can considerably reduce the number of diagnostic biopsies.^[25]

The second study demonstrated a substantial overlap between the vascularization documented using CEUS when compared with that documented using MR.^[26]

Gaiani *et al.*^[16] have found that 91% of hypervascular hepatocarcinoma using MSCT presented hypervascularization in arterial phase with CEUS as well, and that 75% of hypervascular hepatocarcinoma showed hypovascularization in portal or late phase.

Xu *et al.*^[19] reported in their series that 87% of hepatocarcinoma, all with dimensions equal to or less than 2 cm, appeared hypovascular in the portal phase, while 46% were isovascular in the portal phase.

In this study, a high comparability was demonstrated between CEUS and MSCT, with 88% of nodules appearing hypervascular in the arterial phase using both methods, independently of lesion dimensions.

Two studies, however, have demonstrated that the sensitivity of CEUS diagnosing HCC is in direct proportion to lesion dimensions. For the nodules with dimensions equal to or less than 2 cm, Gaiani *et al.*^[16] and Giorgio *et al.*^[20] reported a 83.3% and 56.3% sensitivity for CEUS, respectively. Conversely, in nodules with dimensions > 2 cm, sensitivity was significantly increased by 94% and 91%, for CEUS, in the respective studies.

In this study, there were no statistically significant differences in individualization of vascularization in lesions, in relation to dimensions in the arterial phase. Conversely using CEUS, the evaluation of

vascularization in relation to the lesion depth was statistically significant. In particular, only 58% of the lesions situated at a depth greater than 9 cm from the abdominal wall presented in arterial phase CEUS, the same vascularization as with the corresponding phase in MSCT; this contrasts with 95% of the lesions situated more superficially.

In this study, the homogeneity of the enhancement was not evaluated because this element can be extremely variable due to a number of factors. Particularly in the arterial phase, inhomogeneity of enhancement is frequently present due to the presence of adipose degeneration or intratumoral necrosis. In the portal phase, a “mosaic” aspect is often noticed, particularly in the larger lesions.^[27]

The use of CEUS also allows clinicians to differentiate HCC from other benign or malignant focal hepatic lesions.^[28-32]

The intrinsic limitations of CEUS vary in relation to various patient characteristics (cooperation, obesity), various characteristics of lesions (site-dimensions-depth), and the CEUS operator.^[33]

Another important CEUS limitation is that the technique focuses study on a single lesion, mainly in the arterial phase, because it can often be particularly challenging to evaluate the enhancement of the entire hepatic parenchyma in a short period of time. By contrast, the panoramic views of CT and MR allow scans to evaluate the entire hepatic parenchyma.

In the 2010 AASLD guidelines for the management of HCC, CEUS was removed from the protocol because it can give false positives in patients with intrahepatic cholangiocarcinoma.^[34] However, CEUS is the only method that allows the study of the vascularization of a single lesion “in real time”. Such a possibility provides the advantage of accurately documenting the neoangiogenesis typical of hepatocarcinoma, characterized by the formation of neoarterioles at the periphery and the inside of the lesion that can be enhanced at a very early stage. Furthermore, in some cases (mainly in small-dimension lesions) such precocity can be transitory and thus assessable only in a continuous view.

Some studies have demonstrated that a certain number of lesions, varying between 5% and 25%, remain undetermined after a CEUS study, because they do not present a characteristic enhancement.^[28-30] This number can be reduced, even if not in a significant manner, if a second method of CT or MR is added to

CEUS for the study of vascularization.

In our study the sensitiveness, specificity and accuracy of CEUS in the diagnosis of HCC were not evaluated. The intralesional vascularization documented with CEUS compared with that documented with MSCT was respectively compared in a series of hepatic carcinoma. The MR was not documented for comparison because it is not utilized in all cases.

In the event, when using various imaging techniques, when it is not possible to obtain a differential diagnosis between a benign or malignant lesion, it is essential to perform biopsy or monitoring of the patients, depending on the dimensions of the lesion.

In conclusion, this study demonstrates that CEUS is a reliable and accurate method for documenting the intralesional vascularization of hepatic carcinoma, in particular when combined with MSCT. However, CEUS presents some limitations, mainly in relation to the site of lesions.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Each patient was informed of the study and gave their consent.

Ethics approval

This study was approved by the Corporate Ethics Committee.

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Effect of obesity on perioperative outcomes after laparoscopic hepatectomy

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ABSTRACT

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Aim: Laparoscopic hepatectomy is increasing in utilization, however the procedure has not been adequately examined in the obese patient. This study aims to analyze the effect of obesity on perioperative outcomes after laparoscopic hepatectomy. **Methods:** Retrospective analysis of 396 laparoscopic hepatectomies in normal [body mass index (BMI) < 25], overweight (BMI ≥ 25), obese (BMI ≥ 30), and severely obese (BMI ≥ 35) patients using multivariate regression models to determine the risk factors for post-operative complications. **Results:** Normal BMI ($n = 78$; 20%), overweight ($n = 209$; 52%), obese ($n = 86$; 22%), and severely obese ($n = 23$; 6%). Demographics were similar except for a higher American Society of Anesthesiologists (ASA) score in the obese group. Estimated blood loss and operating time were greatest in the overweight group, while length of stay and complications were statistically similar between groups. Univariate analysis identified that complications were associated with weight class, ASA score, blood loss, and resection; multivariate analysis revealed ASA and transfusion were best correlated with complications. **Conclusion:** Obese and overweight patients have similar complication profiles to normal BMI patients while severely obese patients have a higher incidence of complications that are primarily limited to Clavien-Dindo class I and II.

INTRODUCTION

Obese patients have frequently been perceived as challenging operative candidates often believed to incur increases in complications. Obesity is not only an increasing problem in the United States, but worldwide. Over 35% of the American population is

now obese, and with the introduction of the Western diet these figures are climbing in both Europe and Asia.^[1,2] Worldwide, the prevalence of obesity has nearly doubled between 1980 and 2008.^[3] Despite the dramatic rise in obesity, few studies have carefully examined the impact of a laparoscopic approach on the surgical outcomes of liver resection in these patients.



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As early as 1972, a Veterans Affairs study demonstrated that obese patients have a significantly higher incidence of pre-operative co-morbidities, specifically hypertension and diabetes.^[4] Lending to this fact obese patients subsequently had a higher incidence of post-operative complications, including atelectasis and wound infection. Not surprisingly, the incidence of wound infections were most pronounced in obese diabetics, placing them at three fold increasing risk of post-operative mortality. The power of this study was limited because only 5% of the study cohort meets criteria for obesity at that time. Unfortunately, in the subsequent three decades, the landscape of mean body mass index and the incidence of obesity has dramatically changed.

Dindo *et al.*^[5] first presented a classification system designed to identify and define post-operative complications. Their study examined and analyzed the outcomes of over six thousand open general surgery patients. This analysis identified the only increased complication in the obese patient was the rate of wound infections, and failed to identify an increased rate of any additional complications. This observation was not only true for the obese patient, but also for the severely obese patients with body mass index (BMI) greater than 35. In their final analysis they concluded and advocated for surgical intervention in the “rapidly expanding obese population”.

Ironically this is in sharp contrast with the early National Institutes of Health guidelines for laparoscopy cholecystectomy that excluded the morbidly obese patients.^[6] Despite this consensus guideline, surgeons quickly identified laparoscopy was an ideal approach for obese patients. Theoretically, laparoscopy lends to improved visualization, smaller incisions, and less physiologic impact. These advantages result in: (1) shorter hospital stays; (2) rapid return to normal diet; and (3) fewer complications.^[7] However, it would be decades before Tsinberg first examined the effect of obesity in a small cohort of minor laparoscopic hepatic resections.^[8] This, and subsequent studies, have confirmed the benefits of laparoscopy but only at the expense of increased operative times with the occurrence of Clavien-Dindo class I and II complications.^[9,10] Our current study seeks to evaluate the effect of laparoscopy on a large group of open and laparoscopic minor and major resections.

METHODS

From January 2001 to September 2015, 640 patients underwent liver resection by a single surgeon. Of those, 396 patients underwent a laparoscopic hepatectomy. All patients were included in this study.

Patients were evaluated in 4 weight groups: normal (BMI < 25), overweight (BMI ≥ 25), obese (BMI ≥ 30), and severely obese (BMI ≥ 35). Based on World Health Organization (WHO) classifications of obesity, there were 78 (20%) normal weight patients, 209 (52%) overweight patients, 86 (22%) obese, referred by the WHO as class I obesity, and 23 (6%) severely obese, referred by WHO as class II and III obesity.^[11]

Patient demographics, clinical status, tumor characteristics, operative and postoperative outcomes, as well as clinicopathologic data were analyzed among each weight class against the normal BMI group. The surgical technique of laparoscopic hepatectomy utilized by this surgeon has been well reported in the literature.^[12] Laparoscopic hepatectomy was selectively performed with hand port assistance based on tumor location, accessibility and condition of the underlying liver parenchyma.

Statistical analysis

Continuous variables were compared between groups using Student's *t*-test; categorical variables were compared using chi-squared test. Serial values were compared using analysis of variance. A univariate model was used to identify all variables significantly associated with post-operative complications. To examine the effect of obesity on the laparoscopic approach a full cohort analysis of all resections was performed while a second analysis of only laparoscopic resections was carried out. A multivariate regression model was then developed to identify the independent variables that maintained significance in multivariate analysis. A *P*-value of < 0.05 was considered statistically significant.

RESULTS

Several demographic differences were noted between the study groups. Mean age was similar, while severe obesity and malignant disease had a higher association with male gender. The incidence of co-morbidities including hypertension and diabetes increased with increasing weight class. American Society of Anesthesiologists (ASA) score increased concordantly with patients' body weight. However, the incidence of cirrhosis, the number of segments resected and the percentage of major resections were similar across all weight groups [Table 1].

Outcome data identified an increase of blood loss, transfusions, and complications in patients moving from normal, overweight, obese and severely obese patients. Surgical margins were similar across groups. Clavien-Dindo I-V complications were significantly different between the severely obese and the other

Table 1: Patient demographics and tumor characteristics

| | Normal (BMI < 25) | Overweight (BMI 25-30) | Obese (BMI 30-35) | Severely obese (BMI ≥ 35) | ANOVA <i>P</i> -value |
|--------------------|-------------------|------------------------|-------------------|---------------------------|-----------------------|
| Number of patients | 78 | 209 | 86 | 23 | |
| Median BMI | 23 | 27 | 30 | 40.5 | |
| Age, years | 53.6 | 53.8 (0.92) | 56.3 (0.26) | 61.1 (0.69) | |
| Male gender | 28% | 36% (0.19) | 42% (0.07) | 65% (< 0.01) | 0.03 |
| ASA | 2.76 | 2.68 (0.34) | 2.92 (0.11) | 3.00 (0.14) | < 0.01 |
| Hypertension | 42% | 40% (0.35) | 63% (< 0.01) | 78% (< 0.01) | < 0.01 |
| Type II DM | 12% | 10% (0.62) | 37% (< 0.01) | 70% (< 0.01) | < 0.01 |
| Cirrhosis | 22% | 12% (0.03) | 19% (0.61) | 13% (0.35) | 0.98 |
| Segments | 2.3 | 2.5 (0.22) | 2.3 (0.89) | 2.5 (0.39) | 0.92 |
| Major | 24% | 23% (0.96) | 20% (0.62) | 17% (0.53) | 0.93 |
| Malignancy | 47% | 40% | 50% | 57% | |

Data in brackets represent *P*-values compared to normal. BMI: body mass index; ASA: American Society of Anesthesiologists; DM: diabetes mellitus; ANOVA: analysis of variance

Table 2: Intraoperative and postoperative data

| | Normal (BMI < 25) | Overweight (BMI 25-30) | Obese (BMI 30-35) | Severely obese (BMI ≥ 35) |
|--------------------|-------------------|------------------------|-------------------|---------------------------|
| Number of patients | 78 | 209 | 86 | 23 |
| OR time (h) | 2.2 | 2.5 (0.02) | 2.4 (0.18) | 2.5 (0.13) |
| EBL (mL) | 177 | 234 (0.05) | 165 (0.95) | 254 (0.22) |
| Transfusion | 6.4% | 7.1% (0.05) | 7.0% (0.08) | 8.7% (0.08) |
| Margin (cm) | 1.1 | 1.0 (0.38) | 1.0 (0.40) | 1.2 (0.65) |
| LOS (days) | 3.5 | 3.2 (0.53) | 3.3 (0.73) | 4.6 (0.08) |
| Complication | 15.3% | 17.2% (0.71) | 18.6% (0.58) | 47.8% (0.01) |
| Deaths | 2.5% | 0.4% (0.12) | 1.1% (0.54) | 4.3% (0.66) |

Data in brackets represent *P*-values compared to normal. BMI: body mass index; OR: operating room; EBL: estimated blood loss; LOS: length of stay

Table 3: Distribution of complications

| | Normal (<i>n</i> = 78) | Overweight (<i>n</i> = 209) | Obese (<i>n</i> = 86) | Severely obese (<i>n</i> = 23) |
|---|-------------------------|------------------------------|------------------------|---------------------------------|
| Overall incidence | 12 (15%) | 36 (17%) | 16 (19%) | 11 (48%) |
| Cardiac | 1 (1.3%) | 2 (0.9%) | 1 (1.2%) | 1 (4.3%) |
| Pulmonary | 2 (2.6%) | 6 (2.9%) | 2 (2.3%) | 2 (8.7%) |
| PE | 0 (0%) | 0 (0%) | 2 (2.3%) | 2 (8.7%) |
| Gastrointestinal | 6 (7.7%) | 10 (4.8%) | 7 (8.1%) | 4 (17.4%) |
| Bile leak | 4 (5.1%) | 5 (2.3%) | 4 (4.7%) | 3 (13%) |
| Wound | 0 (0%) | 4 (1.9%) | 2 (2.3%) | 1 (4.3%) |
| Other | 1 (1.3%) | 15 (7.1%) | 4 (4.7%) | 1 (4.3%) |
| Percentage of Clavian-Dindo complications | | | | |
| 5 | 2 (17%) | 1 (3%) | 1 (6.3%) | 1 (9%) |
| 4 | 1 (8%) | 6 (17%) | 4 (25%) | 1 (9%) |
| 3 | 2 (17%) | 17 (47%) | 4 (25%) | 1 (9%) |
| 2 | 2 (17%) | 1 (3%) | 1 (6.3%) | 3 (27%) |
| 1 | 5 (42%) | 7 (19%) | 6 (38%) | 5 (45%) |

Data are shown as *n* (%). PE: pulmonary embolism

BMI groups [Tables 2 and 3]. In the first regression analysis, we analyzed all hepatectomies (*n* = 640), both open and laparoscopic. Initial univariate analysis of factors associated with complications included open surgery, race, BMI, ASA, transfusion, operating room time, and major resection; however, the multivariate regression analysis resulted in only two significant factors: ASA (*P* < 0.001) and transfusion requirement (*P* < 0.001). A subsequent analysis was performed on just the laparoscopic hepatectomy group (*n* = 396); where univariate analysis identified BMI, ASA, diabetes,

transfusion, and major hepatectomy were associated with complications. After multivariate regression modeling, only ASA (*P* < 0.001) and transfusion requirement (*P* < 0.002) were again significantly associated with postoperative complications [Table 4].

DISCUSSION

Historically, obese patients have been perceived to incur poorer post-operative outcomes compared to normal body mass index patients. Initially this assumption was

Table 4: Final regression models

| Variables | Univariate analysis | | Multivariate regression | |
|--|---------------------|---------|-------------------------|---------|
| | t-stat | P-value | t-stat | P-value |
| All open and laparoscopic liver resections (n = 640) | | | | |
| Open surgery | -5.60 | < 0.001 | | |
| Gender | 1.98 | 0.048 | | |
| Race | 3.47 | < 0.001 | | |
| ASA | 6.29 | < 0.001 | 5.07 | < 0.001 |
| Obesity | 4.11 | < 0.001 | | |
| Hypertension | 0.24 | 0.810 | | |
| Diabetes | 2.14 | 0.033 | | |
| INR | 1.57 | 0.117 | | |
| Cirrhosis | 1.71 | 0.242 | | |
| Major resection | 3.50 | < 0.001 | | |
| OR time | 5.96 | < 0.001 | | |
| EBL | 4.20 | < 0.001 | | |
| Transfusion | 7.24 | < 0.001 | 6.02 | < 0.001 |
| Laparoscopic liver resections (n = 396) | | | | |
| Gender | 1.45 | 0.148 | | |
| Race | -0.20 | 0.842 | | |
| ASA | 3.11 | < 0.001 | 3.45 | < 0.001 |
| BMI | 2.18 | 0.029 | | |
| Hypertension | 0.32 | 0.749 | | |
| Diabetes | 2.24 | 0.026 | | |
| INR | 1.32 | 0.188 | | |
| Cirrhosis | 1.32 | 0.188 | | |
| Major resection | 2.17 | 0.031 | | |
| OR time | 0.81 | 0.418 | | |
| EBL | 2.30 | 0.022 | | |
| Transfusion | 2.75 | 0.006 | 3.10 | < 0.002 |

BMI: body mass index; ASA: American Society of Anesthesiologists; OR: operating room; EBL: estimated blood loss; INR: international normalized ratio

not only applied to open surgery but also laparoscopic surgery, considering the inherent technical challenges in the obese patient. However, laparoscopy has quickly become the favorable or even preferred approach for general surgery in obese patients including cholecystectomy, and colectomy.^[13] The current study examines a single surgeon's experience with laparoscopic and open hepatectomy in a broad group of obese and non-obese patients. Our hypothesis was to affirm the laparoscopic approach's viability as an alternative to open hepatectomy with respect to operative outcomes, including length of stay and the incidence and severity of complications.

In our analysis of the laparoscopic group, a higher incidence of complications was not identified until patients reached severe obesity. Univariate analysis of the entire group did identify obesity measured by BMI as a predictor of complications. However, under multivariate regression analysis, BMI lost significance and was no longer a predictor of complications in patients undergoing laparoscopic hepatectomy.

As a baseline the overweight, obese and severely obese patients in our study had a significantly higher incidence of co-morbidities including hypertension and type II diabetes resulting in higher ASA scores. This same increased incidence in co-morbidities and ASA scores did not result in longer operative times when comparing obese and severely obese patients to normal BMI patients.

Our patient cohort had several interesting trends that may have led to some bias including a higher incidence of male patients undergoing laparoscopic hepatectomy and the use of laparoscopic resection in males with malignant disease. This selection bias for males and malignant disease may have contributed to the severely obese patients incurring a higher incidence of complications. The distribution of complications defined by the Clavien-Dindo classification was similar across all BMI groups except the severe obese. In this group of severely obese there was a higher incidence of pulmonary complications. However, the low incidence of class III and IV complications was observed in the severe obese population, which may reflect the small study population or a selection bias.

Our final regression model identified ASA score and transfusion as the best associations with the occurrence of complications. The statistical model for complications increased with rising ASA scores. This positive predictor underscores the power and utility of ASA in clinical decision-making. Despite the presence of transfusion in the final model, its impact on complications may require further evaluation. Transfusion may be more complex variable than a measure of blood loss, the need for blood or blood products. Transfusion may serve also as a surrogate marker for a complex surgical patient with a multitude of inherent and underlying variables such as liver steatosis, functional hepatic reserve or even case complexity due to obesity.

In our experience, laparoscopic hepatectomy is a safe, effective procedure with complications rates and Clavien-Dindo severity scores comparable to open hepatectomy for most obese patients. The caveat to this statement is that in severely obese patients (BMI > 35) there was significant rise in complications. This may reflect the effect of obesity or is a direct result of increasing patient ASA or even selection bias. This lends to the last question of does the underlying liver quality, most notably steatosis, contribute the incidence and severity of complications? This study however, reaffirms the belief that the benefits of laparoscopic hepatectomy apply to the overweight

and obese patients, while identifying areas that warrant further studies.

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There are no conflicts of interest.

Patient consent

All patients gave their consent forms before treatment.

Ethics approval

The study was approved by the ethical review board of Tulane University.

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Comments on “The severity of non-alcoholic fatty liver disease is associated with gut dysbiosis and shift in the metabolic function of the gut microbiota”

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Non-alcoholic fatty liver disease (NAFLD) has a rising prevalence worldwide. It is characterized with lipid deposition in hepatocytes that is unrelated to alcohol consumption. Insulin resistance and oxidative damage plays a key role in its pathogenesis.^[1] NAFLD is a complex disease, classified in simple steatosis (SS) and non-alcoholic steatohepatitis (NASH). Lifestyle changes and treatment of hyperinsulinaemia could reverse SS. However, 20-30% of NAFLD patients develop to NASH, which could lead to liver fibrosis, cirrhosis and cancer.^[2]

Recently, the intestinal microbial flora has gained great attention in various diseases, such as obesity,

metabolic syndrome, diabetes, and cardiovascular diseases.^[3] Gut dysbiosis, especially the microbial translocation and their products such as endotoxin (lipopolysaccharides) across the intestinal gut barrier is highly investigated in patients with chronic liver diseases.^[4] Besides that, gut microbiota may influence the pathogenesis of NAFLD by increased production and absorption of gut short-chain fatty acids; changes in dietary choline metabolism; altered bile acid pools; increased production of microbiota-derived endogenous ethanol; and interaction between dietary factors and microbiota.^[5] Nowadays there is no evidence-based, effective therapy of NAFLD. Current therapy for NAFLD includes lifestyle interventions, medical treatment (e.g. antioxidants, oral hypoglycaemic agents, and lipid-lowering agents), and bariatric surgery. Lately, probiotics have been discussed as a potential treatment of NAFLD.^[6]

Boursier *et al.*^[7] present a remarkable study about the analysis of composition of gut microbiota of stool samples from patients affected by this disease.



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They used 16S ribosomal RNA gene sequencing to determine the microbial flora of biopsy-proven NASH patients. Here, we highlight the importance of this work, because of its novelty in the demonstration of the association between gut dysbiosis and severity of the disease. Another great advantage of this study is the well phenotyped liver lesions of the studied group with diagnostic liver biopsy. In so far, the histological assessment of liver samples is the gold standard of chronic liver diseases, which is an invasive intervention. Not only NAFLD, but NASH can be hardly diagnosed with clinical, laboratory parameters. For this reason there is a great claim worldwide for easy, reproducible, cheap, safe, non-invasive scores or imaging modalities to identify NAFLD or NASH.^[8-11]

In particular, the authors reported an increased abundance of *Bacteroides* genus was independently associated with NASH and *Ruminococcus* abundance was independently associated with fibrosis severity (> F2). Besides, patients with NASH had lower abundance of *Prevotella*.^[7] The exact mechanism of the liver injury is not yet clear. However, metabolites of the members of the *Bacteroides* family directly stimulate the farnesoid X receptor (FXR), a ligand-activated transcription factor, which plays an important role in the pathogenesis of liver damage. They improve the obesity phenotype including body weight gain, liver damage, lipid metabolism in a mouse model.^[12] Despite the relatively small number of cases ($n = 57$), these results show that analysis of gut microbiota is a good tool to gain information about NAFLD severity. According to this work, dysbiosis may have a significant role in the pathogenesis of human NAFLD/NASH. Moreover gut microbiota analysis adds prognostic information to NAFLD severity.^[7]

Recent studies show, that probiotic/prebiotic supplementation may be useful in both animal and human models. *Bifidobacterium* and *Lactobacillus* strains are the most widely used bacteria.^[13] Control of the bacterial flora lowers proinflammatory cytokine production (tumor necrosis factor- α , interleukin-6, interferon- γ) via down-regulation of the nuclear factor kappa B, and decreases the oxidative stress. Probiotic can reduce the urease activity of bacterial microflora, decreases fecal pH value and reduces amino-acid fermentation and ammonia adsorption. They may reduce aminotransferases (alanine aminotransferase, aspartate transaminase), improve the lipid status (total cholesterol, low-density lipoprotein) in NAFLD patients.^[6,14-17] In fact, probiotic was reported to improve liver histology, and reduce hepatic total fatty acid content in an animal model of NAFLD. In addition probiotic therapy with lifestyle modification

significantly decreased fibrosis scores, as determined by transient elastography compared with placebo.^[18] In fact, it can improve disease severity. Probiotics are consumed in various forms, such as fermented foods, like yogurt, cheese and other fermented milk products. The oral intake of probiotics is recommendable for the prevention and treatment of obesity, insulin resistance, type 2 diabetes and NAFLD. As a co-adjuvant therapy, probiotic combination with metformin can lower liver aminotransferases better than metformin alone in patients with NASH.^[19,20] The combination of cholesterol-lowering probiotics and anthraquinone increase the therapeutic effect on NAFLD by affecting the process of fat metabolism in rats (up-regulation of CYP7A1, LDL-R, FXR mRNA, PPAR- α protein and down-regulation of HMGCR, PPAR- γ and SREBP-1c).^[21]

These findings confirm that dietary interventions can affect the composition and diversity of gut microbiota.

Finally, association of gut dysbiosis with histological subtypes of NAFLD may be a prognostic factor of liver-related morbidity and mortality. Analysis of microbial composition may be a useful predictor of disease stage. However additional analysis will have to explain how metabolic functions of the gut microbiota might have role in NASH pathogenesis and progression. Further studies are required to understand the precise mechanism of how gut microbiota affect the pathomechanism of NAFLD/NASH and the role of probiotics in the therapy of the disease. Since there is no available treatment for NAFLD/NASH yet, probiotic therapy, as a safe, inexpensive and non-invasive strategy, may be a good alternative to reduce pathophysiological symptoms and improve different types of liver diseases without side effects.

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There are no conflicts of interest.

Patient consent

There is no patient involved.

Ethics approval

Ethics approval is waived for this kind of article.

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Novel predictive and prognostic strategies of hepatitis B virus related hepatocellular carcinoma

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ABSTRACT

Hepatocellular carcinoma (HCC) is a common malignancy and an important cause of cancer death worldwide. Chronic hepatitis B virus (HBV) infection is the major cause of HCC. Recent studies of HBV-induced carcinogenesis not only discovered many new biomarkers but also developed a novel theory: Cancer Evolution-Development (*Cancer Evo-Dev*). *Cancer Evo-Dev* provides an evolutionary insight of developing more reasonable predictive and prognostic strategies. Characterizing chronic inflammatory microenvironment of cancer evolution, genetic polymorphisms of inflammatory factors, and HCC-related HBV mutations that negatively selected by host immunity may help greatly in identifying HBV-infected individuals who are more likely to develop HCC or benefit from HCC prophylactic options. Gene expression signatures and somatic mutation profiles reflect the different patterns of signaling pathway networks underlying tumor heterogeneity and can be applied to improve the molecular classification and prognostic stratification of HCC patients. Mutant cells that survive the selection can retro-differentiate into tumor initial cells and aggressive sub-clones. Detection of mutants or their hallmarks in cell-free DNA in peripheral blood potentially improve the early diagnosis, prognosis prediction, and personalized treatment of HBV-caused HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequently diagnosed cancers and an important cause of cancer death worldwide. Annually, there are 782,500 HCC incident cases and 745,500 HCC-caused deaths worldwide.^[1] Developing countries in East Asia and Sub-Saharan Africa contribute 80% of new HCC cases

and related deaths.^[2] Chronic infection of hepatitis B virus (HBV) is the major etiological reason for HCC in these areas, which contributes 80-90% of HCC patients.^[3,4] According to a cohort study conducted in Taiwan, the cumulative lifetime (age 30 to 75 years) incidences of HCC for men and women that positive for hepatitis B surface antigen (HBsAg) were 27.38% and 7.99%, far more than those of men and women negative



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for HBsAg and anti-hepatitis C virus (1.55% and 1.30%).^[5] Besides, HBV infection is also responsible for the increasing trend of HCC in western countries because of the travel and immigration of HBV infected populations.^[6] Most HCC patients are diagnosed at advanced stage and cannot accept resection operation or liver transplantation.^[7] Approximately 70% patients that have curative hepatectomy will relapse within 5 years.^[8] Both the narrow therapeutic window and the high recurrence rate highlight the importance of developing more rigorous surveillance and more active prevention for chronic HBV infected subjects with high HCC risk, and tailoring more suitable treatment options for HCC patients, which depend on continuously discovering promising biomarkers as well as developing carcinogenesis theory for the specific prophylaxis.

Cancer Evo-Dev is a novel scientific theory describing the mechanism of HBV-induced hepatocarcinogenesis.^[9] The central aspects of its framework are as follows. Carcinogenesis is an evolutionary process under the microenvironment of chronic non-resolving inflammation. This microenvironment is characterized by immune imbalance due to the interaction between the genetic predisposition of immune/proinflammatory molecules and HBV infection. Cytidine deaminases and their analogous are persistently activated by proinflammatory factors and subsequently induced mutations both in host and viral genomes. Mutant cells are mostly eliminated by selective pressures. Only a small proportion can survive in the inflammatory microenvironment because the somatic mutations alter signaling pathways. Those surviving clones usually share some characteristics of stem cells and gradually retro-differentiate into cancer initiating cells.

This theory was presented based on recent outcomes of HBV-related carcinogenesis researches, mainly including molecular epidemiological studies, cancer genomic mutation analyses, and signaling transduction researches.^[10-20] Those breakthroughs not only improved the understanding of cancer evolution from different aspects but also discovered many novel biomarkers and therapeutic targets. Therefore, this theory can provide an evolutionary insight of predicting HCC risk and developing more reasonable predictive and prognostic biomarkers and therapeutic targets. Here, we summarize the important novel viral, inflammatory, genetic, and protein biomarkers of HCC occurrence and prognosis and evaluate them through the lens of *Evo-Dev* theory.

EVALUATING THE MICROENVIRONMENT OF CANCER EVOLUTION

In the evolution process of HBV-induced hepatocarcino-

genesis, inflammatory microenvironment plays an important role via facilitating the generation of viral and host genetic mutation and also providing selective pressure. Therefore, the characteristics of the microenvironment in different evolutionary phases and in different populations can be used to stratify HBV-infected individuals with different risk of developing HCC. Although inflammatory microenvironment is a complex system, it can be elucidated in two aspects: HBV itself and immune imbalance.

HBV

Despite the high incidence of HCC in HBV-infected population, only small percentages of chronic hepatitis B (CHB) patients develop HCC. HBV variables can serve as clues to identify distinctive outcomes of HBV-infected populations, and to guide the personalized preventive medication accordingly.

HBV replication

The level of HBV replication directly reflects the selective stress from the inflammatory environment, which can influence the evolution of HCC as well. Currently, HBV DNA load is regularly applied in clinic as an indicator of initiating antiviral treatment. It has been demonstrated by various studies that HBV DNA load increases the risk of HCC in CHB patients.^[21-23] High level of HBV DNA load either in serum or liver tissue can also predict poor postoperative prognosis in HCC.^[24] Hepatitis B e antigen (HBeAg), encoded by HBV precore region, is another marker for active replication of HBV. HBeAg positivity has been proved to be associated with an increased risk of HCC.^[25] However, due to HBeAg seroconversion during the natural course of HBV infection, HBeAg expression is not usually high in HCC patients, explaining the reasons that HBeAg positivity is not significantly associated with an increased risk of HCC in some case-control studies.^[14] Thus, HBV DNA load should be a more reliable indicator in the prediction of HCC.

HBV genotypes

According to a sequence divergence of no less than 8% in whole viral genome, HBV can be classified into eight genotypes A to H, which can be further classified into sub-genotypes if the sequence divergence is between 4% and 8%.^[26] Variant genotypes are distributed unevenly around the world, and the predominant one in mainland China is genotype C (68.3%), followed by genotype B (25.5%).^[27] Under selection pressure from inflammatory microenvironment, the fates of different genotypes/sub-genotypes are distinct in a given population. Genotype C HBV infection is an independent risk factor for HCC development.^[16,21,28,29] Meanwhile, genotype B HBV infection was associated

with the development of HCC in young patients (< 50 years old).^[30] Our study further revealed that genotype B2 HBV infection was related to HCC recurrence, and that HBV genotype C2 HBV was predominant in HCC patients, which was related to its high prevalence.^[31] As the HBV genotype is usually identified through a complex procedure that includes extracting HBV DNA, polymerase chain reaction, sequencing, and phylogenetic analysis, the wide application of HBV genotype/subgenotype for preliminary screening in community is limited.

HBV mutations

In the process of HBV-HCC evolution, one of the most prominent molecular events is the generation of HBV mutation, especially mutations in the preS region and basic core promoter (BCP) region of HBV genome. Due to lack of proof reading capacity, HBV genome has a higher mutation rate than other DNA viruses. Moreover, inflammatory factors induced by HBV infection can activate the expression of apolipoprotein B mRNA editing enzyme catalytic polypeptides (APOBECs). HBV genome can be degraded and edited by APOBECs.^[32] Most HBV mutants are cleared by host immune system, and only those that gained the ability to escape immune eradication survived. The mutant viruses, in return, keep on stimulating the immune system and maintain the inflammatory microenvironment. The HBV mutations reflect, to some extent, the selection pressure of host immune system and serve as risk factors of HCC.

Our recent study of HBV mother-to-child transmission revealed that mutated viruses lost their advantages in infecting infants, whereas the wild-type HBV had advantage of infecting newborn's hepatocytes, interestingly, the HCC-risk HBV mutations was being gradually selected since the establishment of chronic infection.^[10] Mutations in HBV the preS region (including A2962G, A2964C, C3116T, C7A, T105C, and preS start codon mutation) and mutations in the BCP region (including C1653T, T1753V, and A1762T/G1764A) were independently associated with an increased risk of HCC.^[11,15,21,33] Mutations in combination (combo mutations) can enhance the validity of predicting the occurrence of HCC.^[21,33,34] HBV combo mutations of C1653T, T1753V, and A1762T/G1764A increase the validity of HCC prediction compared with single HBV mutation.^[21] The HBV mutations can improve the sensitivity and specificity of HCC prediction model based on age, gender, cirrhosis and HBV DNA loads.^[21,25,35]

The carcinogenic effects of HBV can be blocked by antiviral treatments. In our prospective hospital-based cohort study, antiviral treatment against HBV

using interferon and nucleoside analogues (NAs) significantly reduced HCC occurrence (13.90/1,000 vs. 7.70/1,000 person-years, $P = 0.005$).^[36] Furthermore, proved by a cohort study and randomized clinical trial, treatment with NAs can also significantly reduce the risk of early recurrence (hazard ratios, 0.41; $P < 0.001$).^[13] However, levels of those protective effects are distinct among HBV-infected subjects with different viral mutations. Antiviral treatment with NAs cannot reduce HCC risk in patients without A1762T/G1764A or C1653T and in those with T1753V.^[36] The protective function of antiviral treatments for postoperative recurrence cannot be observed in the HCC patients expressing carboxylic acid-terminal truncated HBV X protein (Ct-HBx) in their liver remnants.^[13]

Immune imbalance

Immune imbalance is responsible for the maintenance of chronic non-resolving inflammation and subsequently provides a fertile microenvironment for cancer evolution. Immune imbalance can be reflected by the proportion shift of immune cells, abnormal activation of inflammatory pathways, and genetic predisposition of inflammatory molecules, which can serve as biomarkers for HCC prediction and prognosis.

Immune cells

The liver is enriched with innate immune cells such as macrophages and natural killer (NK) cells, as well as adaptive immune cells such as CD8⁺ cytotoxic T cells, CD4⁺ T helper cells and B cells, playing an important role not only in host defenses against invading microorganisms and tumor transformation, but also in liver injury and repair. Their presence or enrichment can be seen as predictive or prognostic factors for HCC. CD8⁺ T in liver tissues, for example, is the protective factor, while the enrichment of M2 macrophages and T helper 17 cells (Th17) as well as the imbalance between CD8⁺ T cells and regulatory T (Treg) cells or between Th1 and Th2 are the risk factors of HCC.^[37] Immune cells that infiltrated into HCC tissues function distinctly on HCC prognosis. Intratumoral natural killer cells and CD8⁺ T cells indicate good prognosis, while intratumoral Treg cells, neutrophils, and M2 macrophages indicate poor prognosis.^[37]

Inflammatory pathways

The abnormal alteration of inflammatory pathways can be reflected by hallmark cytokines. Biomarkers indicating the abnormal activation of inflammatory pathways can also predict the occurrence and recurrence of HCC.^[38,39] For example, Wnt/ β -catenin signaling pathway plays an important role in inflammation-induced carcinogenesis via regulating the expression of cytokine-induced human inducible nitric

oxide synthase.^[40] Activation of Wnt/ β -catenin pathway contributes to HCC development. The hallmarks of Wnt/ β -catenin pathway, Wnt-1 and Wnt3a, have both predictive and prognostic value.^[37,41,42] Likewise, signaling pathways such as phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway, and insulin-like growth factor pathway also play an important role in hepatocarcinogenesis.^[43]

Genetic polymorphisms of immune/inflammatory molecules

Genetic polymorphisms of immune/inflammatory molecules can also serve as predictive biomarkers for HCC development. For example, genetic polymorphisms of signal transducer and activator of transcription 3 (*STAT3*), class II human leukocyte antigen DP (*HLA-DP*), *HLA-DQ*, miRNA-122-binding site, pre-miR-218, nuclear factor-kappaB (*NF- κ B*), and its inhibitor I κ B α are significantly associated with HCC risk.^[12,17,18,44-47]

IDENTIFYING SIGNATURES OF SIGNALING PATHWAY ALTERATION

Gene signatures

The alteration of signaling pathways confers stemness characteristics and competitive advantages to cancer cells. These alterations usually affect complex signaling networks that cannot be represented by a signal gene. More than 300 published microarray studies of human HCC samples provide sufficient information regarding tumor gene expression profiles.^[48] The accumulation of data regarding differentially expressing genes makes it possible to conduct meta-analysis and subsequently determine gene signatures. Recent gene signature studies are summarized in Table 1.^[49-66] Gene signatures developed in those studies were used to separate patients into 2 or more subgroups with different clinical outcomes, phenotypes, and altered signaling pathways. The methods of developing gene signatures fall into two major groups. The first group of gene signatures was generated in case-control studies with the data of training cohort or published gene expression data. Most of the gene signature studies belong to this group.^[50,52,53,55,57,59,61-65] The second group of gene signatures concerning defined phenotypes or signaling pathways was derived from the data of cell or animal model studies.^[49,51,56,58,60] For examples, Lee et al.^[49] developed a gene signature of stemness from the gene profiling data of rat fetal liver tissue and Kaposi-Novak et al.^[51] developed a gene signature of Met signaling pathway using the Met deficient mouse model. The predictive value of novel gene signatures

was usually evaluated in cohort studies. High risk patients that were identified through cluster analysis or score model based on gene signatures were prone to have unfavourable clinical outcomes, such as poor overall survival and early recurrence.

Although the tumor gene signatures were identified by different studies with various comparison strategies, they shared some genes conferring cancer stemness. For instance, a group of genes related to proliferation and epithelial cell adhesion molecule (EpCAM)-positive phenotype were included in 8 gene signatures summarized in different studies and all associated with poor prognosis.^[48] Gene signatures from adjacent non-tumor tissues were also reported to be significantly associated with HCC recurrence, indicating that the histological “normal” adjacent tissue may be at the early stage of cancer evolution. That highlights the need of biopsy-based gene signature detection for specific individuals, like HBV-infected patients. However, signatures from adjacent tissues obtained in different studies are lack of genes in common. Cross validations are needed to consolidate the criteria. Altered expression patterns of the genes in HCC are usually caused by epigenetic modifications in their regulatory elements and somatic mutations of their repressors.

Somatic mutation profiles

Somatic mutations are genetic basis of carcinogenesis. The values of somatic mutations depend on their impacts on related signaling pathways. By changing patterns of signaling transduction, somatic mutations on a small proportion of genes can promote cancer evolution, which are categorized as “driver mutations”.^[19] As a matter of fact, some outstanding somatic mutations in HBV-HCC occur in the genes responsible for epigenetic modifications-chromatin remodeling including *ARID1A* and *ARID2* and methylation such *MLL4*.^[67,68] Due to survival competition and the positive selection of inflammatory microenvironment, driver mutations accumulate sufficiently to promote malignant transformation of hepatocytes.

The distribution, combination, and dynamic patterns of driver mutations reflex the pressure of microenvironmental selection and growth advantage of hepatocyte subsets. The high frequent mutations can have clinical values as biomarkers for targeted therapy, classification, and prognostic prediction.^[67-71] For instance, homozygous deletions were detected in 40% of HCC patients and were significantly associated with poor survival ($P < 0.0001$).^[68]

Using next generation sequencing technology, some

Table 1: Representative gene signature studies of hepatocellular carcinoma

| Study | Population | Sample type | Etiology | Gene No. | Different clinical outcomes of subgroups |
|--|--|---------------------------------|----------------------|---|--|
| Lee <i>et al.</i> ^[49] | <i>n</i> = 61 (validation 1, Chinese) <i>n</i> = 78 (validation 2, European) | Tumor tissue | HBV, HCV | 907 | Overall survival (<i>P</i> < 0.001) |
| Budhu <i>et al.</i> ^[50] | <i>n</i> = 20 (training, Chinese) <i>n</i> = 95 (validation, Chinese) | Adjacent liver tissue | HBV, | 17 | Risk of survival/recurrence HR (95% CI) in validation set: 15.1 (5.0-45.8)/7.9 (2.5-25.0) |
| Kaposi-Novak <i>et al.</i> ^[51] | <i>n</i> = 249 (Caucasian) | Tumor tissue | HBV, alcohol, HCV | 24 | Overall survival (<i>P</i> < 0.001) |
| Wang <i>et al.</i> ^[52] | <i>n</i> = 23 (training, Asian) <i>n</i> = 25 (validation, Asian) | Tumor tissue | HBV, HCV | 57 | Rate of vascular invasion (accuracy: 84%; sensitivity: 86%; specificity 82%) |
| Boyault <i>et al.</i> ^[53] | <i>n</i> = 57 (training, French) <i>n</i> = 63 (validation, French) | Tumor tissue | HBV, alcohol, HCV | 16 | Overall survival (<i>P</i> < 0.001) |
| Woo <i>et al.</i> ^[54] | <i>n</i> = 65 (Chinese) | Tumor tissue | HBV | 628 | Risk of early recurrence (within 2 years after surgery) HR (95% CI): 12.539 (3.59-43.76) |
| Hoshida <i>et al.</i> ^[55] | <i>n</i> = 82 (training, Japanese) <i>n</i> = 225 (validation, European) | Adjacent liver tissue | HBV, HCV | 132 | Risk of late recurrence (more than 2 years after surgery) HR (95% CI) in the validation set: 2.08 (1.03-4.18) |
| Coulouarn <i>et al.</i> ^[56] | <i>n</i> = 139 (Caucasian) | Tumor tissue | HBV, alcohol, HCV | 249 | Overall survival (<i>P</i> < 0.001) |
| Yoshioka <i>et al.</i> ^[57] | <i>n</i> = 42 (training, Japanese) <i>n</i> = 97 (validation, Japanese) | Tumor tissue | HBV, HCV | 172 | Risk of early recurrence (within 2 years after surgery) HR (95% CI) in the validation set: 3.29 (1.83-5.91) |
| Woo <i>et al.</i> ^[58] | <i>n</i> = 61 (validation 1, Chinese) <i>n</i> = 78 (validation 2, Caucasian) | Tumor tissue | HBV, HCV | 625 | Risk of recurrence HR (95% CI) in the Chinese set: 2.84 (1.51-5.34) |
| Roessler <i>et al.</i> ^[59] | <i>n</i> = 247 (validation 1, Chinese) <i>n</i> = 139 (validation 2, GEO data) | Tumor tissue | HBV, HCV | 161 | Risk of early recurrence (within 2 years after surgery) HR (95% CI) in the Chinese set: 2.72 (1.48-4.5) |
| Villanueva <i>et al.</i> ^[60] | <i>n</i> = 287 (Japanese) | Tumor and adjacent liver tissue | HBV, HCV | 16 for tumor; 17 for adjacent liver tissue | Risk of recurrence HR (95% CI): 1.75 (1.20-2.53) for tumor signature; 1.92 (1.20-3.06) for adjacent signature |
| Minguez <i>et al.</i> ^[61] | <i>n</i> = 79 (training, Caucasian) <i>n</i> = 135 (validation, Caucasian) | Tumor tissues | HCV, HBV, alcohol | 35 | Risk of vascular invasion HR (95 % CI) in the validation set 3.12 (1.29-7.51) |
| Weng <i>et al.</i> ^[62] | <i>n</i> = 80 (Chinese) | Tumor tissue | HBV | 3 | Risk of early recurrence (within 1 year after surgery) HR (95% CI): 4.762 (1.764-12.856) |
| Kim <i>et al.</i> ^[63] | <i>n</i> = 139 (training, South Korea) <i>n</i> = 292 (validation, South Korea) | Tumor tissue | HBV | 65 | Risk of poor survival HR (95% CI) in validation the set: 1.36 (1.13-1.64) |
| Kim <i>et al.</i> ^[64] | <i>n</i> = 56 (training, South Korea) <i>n</i> = 40 (validation, South Korea) | Tumor and adjacent liver tissue | HBV | 127 | Overall survival (<i>P</i> < 0.001) |
| Lim <i>et al.</i> ^[65] | <i>n</i> = 286 (training, South Korea) <i>n</i> = 83 (validation, China) | Tumor tissue | HBV | 30 | Risk of poor prognosis HR (95% CI) in validation set: 2.048 (1.130-3.712) |
| Kim <i>et al.</i> ^[66] | <i>n</i> = 396 (Chinese) | Tumor tissues | HBV | 233 for late recurrence, 65 for early recurrence | Risk of late recurrence HR (95% CI): 2.2 (1.3-3.7) Risk of early recurrence HR (95% CI): 1.7 (1.1-2.6) |

HBV: hepatitis B virus; HCV: hepatitis C virus; HR: hazard ratio; CI: confidence interval

basic patterns of HCC somatic mutations have been extensively investigated. The somatic mutations provide a novel genomic insight of molecular classification and prognostic prediction. Some genes

including *TP53*, *TERT*, *CTNNB1*, *ARID1A*, and *AXIN1* are proved to be hotspots of genetic alteration [Table 2]. However, specific mutation in a single hot gene is not frequent, ranging from 5% to 20%. Such a low rate

Table 2: Important somatic mutations and related signaling pathways of hepatocellular carcinoma

| Study | Population and sequencing method | Etiology | Mutation frequency of important genes | Global gene mutation frequency of signaling pathways |
|--|---|-------------------------|--|--|
| Guichard <i>et al.</i> ^[67] | <i>n</i> = 24 (training), whole exome sequencing; <i>n</i> = 125 (validation), Sanger sequencing | Alcohol, HBV, HCV, NASH | <i>CTNNB1</i> (32.8%), <i>TP53</i> (20.8%), <i>ARID1A</i> (16.8%), <i>PIK3CA</i> (1.6%) | Wnt/β-catenin pathway (49.6%), p53/cell cycle pathway (32.8%), chromatin remodeling (22.4%), PI3K/Ras pathway (12.8%) |
| Kan <i>et al.</i> ^[68] | <i>n</i> = 88, whole genome sequencing | HBV | <i>CTNNB1</i> (16.0%), <i>IL6R</i> (26.0%), <i>TP53</i> (35.2%), <i>AXIN1</i> (5.0%) | Wnt/β-catenin pathway (62.5%), JAK/STAT pathway (45.5%), p53 pathway (43.2%), Apoptosis (45.5%) |
| Ahn <i>et al.</i> ^[69] | <i>n</i> = 231, whole exome sequencing | HBV, HCV | <i>CTNNB1</i> (16%), <i>TP53</i> (32%), <i>CCND1</i> (5%), <i>RPS6KA3</i> (5%), <i>ARID1A</i> (7%) | Wnt/β-catenin pathway (31%), p53 pathway (37%), cell cycle pathway (23%), PI3K/Ras pathway (12%), chromatin remodeling (34%) |
| Totoki <i>et al.</i> ^[70] | <i>n</i> = 608, whole exome sequencing | HBV, HCV | <i>CTNNB1</i> (31%), <i>TP53</i> (31%), <i>ARID2</i> (10%), <i>NF1</i> (4%), <i>TERT</i> (54%), <i>NFE2L2</i> (5%) | Wnt/β-catenin pathway (66%), p53 signaling (72%), chromatin remodeling (67%), PI3k/mTOR signaling (45%), telomere maintenance (68%), Nrf2/Keap1 pathway (19%) |
| Schulz <i>et al.</i> ^[71] | <i>n</i> = 235, whole exome sequencing | Alcohol, HBV, HCV, NASH | <i>CTNNB1</i> (37%), <i>TP53</i> (24%), <i>TERT</i> (60%), <i>ARID1A</i> (13%), <i>ALB</i> (13%), <i>AXIN1</i> (11%), <i>CDKN2A</i> (9%) | Wnt/β-catenin pathway (54%), p53 pathway (49%), telomere maintenance (60%), PI3k/mTOR pathway (51%), MAP kinase pathway (43%), hepatic differentiation (34%), epigenetic regulation (32%), chromatin remodeling (28%) |

HBV: hepatitis B virus; HCV: hepatitis C virus; NASH: nonalcoholic steatohepatitis; *CTNNB1*: catenin beta 1; *TP53*: tumor suppressor p53; *ARID1A*: AT rich interactive domain 1A; *PIK3CA*: phosphoinositide-3-kinase catalytic alpha polypeptide; *IL6R*: interleukin 6 receptor; *CCND1*: cyclin D1; *RPS6KA3*: ribosomal protein S6 kinase polypeptide 3; *ARID2*: AT rich interactive domain 2; *NF1*: neurofibromin 1; *TERT*: telomerase reverse transcriptase; *NFE2L2*: nuclear factor (erythroid-derived 2)-like 2; *CDKN2A*: cyclin-dependent kinase inhibitor 2A; JAK: Janus kinase; STAT: signal transducer and activator of transcription; MAP: methionine aminopeptidase

limits the application of a single mutation. For example, *RB1* somatic mutation can serve as an independent predictor for poor cancer-specific survival (HR 2.5, 95% CI: 1.05-5.93, *P* = 0.038) and early recurrence (OR 3.93, 95% CI: 1.29-11.90, *P* = 0.015). But the frequencies of *RB1* somatic mutation were only 3.4% and 7% among different studies.^[68,69] Similarly, somatic mutations of *CDKN2A* and *FGF-CCND1* were proved to be significantly associated with overall survival (*P* = 3.0×10^{-4} and *P* = 7.4×10^{-6} respectively) and their frequencies were both less than 5%.^[70]

Although the spectrums and frequencies of altered genes vary greatly among individuals, they are clustered to pathways or function groups that are closely related with stemness and embryonic characteristics. In this regard, global mutation rates of functionally related genes are added together to define the mutation rate of a given signaling pathway. Mutation rates of Wnt/β-catenin, p53/cell cycle control, JAK/STAT, PI3k/mTOR, and MAP kinase signaling pathways range from 12% to 72%. Similar outstanding outcomes are also observed in function gene groups of chromatin remodeling and telomere maintenance. Ahn *et al.*^[69] developed a somatic mutation signature

of cell cycle pathway which comprised 4 genes including *RB1*, *MYC*, *CCND1*, and *RBL2*. The total mutation rate of those 4 genes were 23% and the signature was significantly associated with poor cancer-specific and recurrence-free survival (*P* = 0.002 and *P* = 0.007, respectively). Therefore, it is promising to use combo somatic mutations as predictive and prognostic biomarkers.

DETECTING CELLS WITH MALIGNANCY POTENTIAL AND THEIR HALLMARKS IN PERIPHERAL BLOOD

Circulating tumor cells

Release of cancer cells into the circulation is common in HCC patients. The appearance of circulating tumor cells (CTC) in the blood stream characterizes the intermediate stage of tumor metastasis process.^[72] CTC test can be applied to monitor early metastasis, assess the effectiveness of therapeutic options, and predict the prognosis.^[73] A study examining blood samples of 123 HCC patients one month before and after tumor resection indicated that EpCAM⁺ CTCs were presented in 66.67% of patients and that CTCs count in 7.5 mL blood (CTC7.5) is an independent prognostic factor

of tumor recurrence.^[74] Therefore, EpCAM⁺ CTCs may be used as a real-time parameter for monitoring treatment response. In addition, EpCAM⁺ CTCs are positive in HCC patients with different BCLC stages and the positive rates of EpCAM⁺ CTCs in patients of BCLC stage A, B, and C are 11.1%, 19.4%, and 57.9%, respectively.^[75] Thus, EpCAM⁺ CTC is prognostic and predictive in HCC.

Cell-free DNA

Biopsy of HCC may be restricted by the special position of tumors or the poor condition of patients, resulting in the limitation of HCC gene analysis for prognostic and predictive purposes.^[76] The necrosis and apoptosis of tumor cells usually release cell-free DNA (cfDNA) into circulation. Based on sequencing technology, genetic and epigenetic information can be obtained from these cfDNA. Detecting cfDNA is a minimally invasive method to find early HCC, termed as “liquid biopsy”.^[77] The abnormalities including methylation changes and point mutations in cfDNA can be detected in peripheral blood even before the solid tumor nodule can be detected.

Hypermethylated *RASSF1A* within cfDNA sequence is present in the sera of 93% HCC patients. When combining *RASSF1A* methylation and AFP to diagnose HCC, the sensitivity and specificity increase from 65% and 87% using AFP alone to 77% and 89%, respectively. Serum methylated *RASSF1A* is also prognostic and also reflects the tumor load in HCC patients.^[78] A study with a cohort of 151 HCC patients indicated that 4 hypermethylation genes (*RGS10*, *ST8SIA6*, *RUNX2*, and *VIM*) in sera have weak correlation with each other but the combination of the 4 genes as a classifier successfully identified HCC patients from HBV-induced cirrhosis population, with the sensitivity of 85% and the specificity of 96%.^[79]

TP53 R249S mutation in cfDNA was proved to have a remarkable ecological correlation with HCC exposure in China and Africa.^[80] In a retrospective study using short oligonucleotide mass analysis to examine *R249S* in the plasma ahead of cancer diagnosis, 9 (64%) of 14 patients who developed HCC during the follow-up were positive for *R249S*.^[81] Genetic mutation in serum is related to the mutation in tumor tissue. Another study examining the mutations of *CTNNB1*, a gene encoding β -catenin, in HCC patients' sera indicated that *CTNNB1* mutation was not present both in serum and corresponding tumor tissues, although the average mutation rate of *CTNNB1* was about 25% in previous researches.^[82] This suggests that clinical application of cfDNA mutations should be mutation signatures rather than single gene mutation.

CONCLUSION

HBV-induced HCC is a common malignancy characterized by high mortality, high recurrence rate, and significant heterogeneity. *Cancer Evo-Dev*, a novel scientific theory of HBV-induced carcinogenesis, provides an evolutionary insight of HCC occurrence/recurrence prediction. From this point of view, recent development of HCC predictive and prognostic strategies can be categorized as three main directions: evaluating the inflammatory microenvironment of cancer evolution via investigating HBV variables and characteristics of immune imbalance, identifying alteration patterns of signaling transformation through signatures of gene expression and somatic mutation, and detecting cells with malignancy potential and their hallmarks in peripheral blood. To validate predictive or prognostic biomarkers, 4 steps should be taken: (1) exploratory research, to discover promising biomarkers; (2) case-control study, to evaluate statistical association between the occurrence/recurrence and biomarkers; (3) cohort study, to validate the sensitivity and specificity of biomarkers; (4) randomized clinical control trial, to determine if the screening and related prophylaxis/treatment can reduce the occurrence/recurrence. Currently, most novel biomarkers were just validated in phase 2 or 3. Further validation and reasonable combination of novel biomarkers should be conducted under the direction of *Cancer Evo-Dev* theory.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

There is no patient involved.

Ethics approval

This review is waived for ethical approval.

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| Manuscript Type | Definition | Abstract | Keywords | Main Text Structure |
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2.3.1 Front Matter

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The title of the manuscript should be concise, specific and relevant, with no more than 16 words if possible. When gene or protein names are included, the abbreviated name rather than full name should be used.

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This section should discuss the implications of the findings in context of existing research and highlight limitations of the study. Future research directions may also be mentioned.

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It should state clearly the main conclusions and include the explanation of their relevance or importance to the field.

2.3.3 Back Matter

2.3.3.1 Acknowledgments

Anyone who contributed towards the article but does not meet the criteria for authorship, including those who provided professional writing services or materials, should be acknowledged. Authors should obtain permission to acknowledge from all those mentioned in the Acknowledgments section. This section is not added if the author does not have anyone to acknowledge.

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Each author is expected to have made substantial contributions to the conception or design of the work, or the acquisition, analysis, or interpretation of data, or the creation of new software used in the work, or have drafted the work or substantively revised it.

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