

# Roles of p53 in extrinsic factor-induced liver carcinogenesis

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## ABSTRACT

Liver cancer remains one of the most common human cancers with a high mortality rate. Therapies for hepatocellular carcinoma (HCC) remain ineffective, due to the heterogeneity of HCC with regard to both the etiology and mutation spectrum, as well as its chemotherapy resistant nature; thus surgical resection and liver transplantation remain the gold standard of patient care. The most common etiologies of HCC are extrinsic factors. Humans have multiple defense mechanisms against extrinsic factor-induced carcinogenesis, of which tumor suppressors play crucial roles in preventing normal cells from becoming cancerous. The tumor suppressor *p53* is one of the most frequently mutated genes in liver cancer. *p53* regulates expression of genes involved in cell cycle progression, cell death, and cellular metabolism to avert tumor development due to carcinogens. This review article mainly summarizes extrinsic factors that induce liver cancer and potentially have etiological association with *p53*, including aflatoxin B1, vinyl chloride, non-alcoholic fatty liver disease, iron overload, and infection of hepatitis viruses.



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## INTRODUCTION

Liver cancer is the 6th most common cancer in men and the 9th most common cancer in women with the 3rd highest mortality rate of all cancers globally.<sup>[1,2]</sup> The majority of these cases (about 80%) occur in Eastern Asia, South-Eastern Asia, Mid-Africa, and West Africa, within the context of viral hepatitis.<sup>[2-4]</sup> Although there are genetic etiologies for hepatocellular carcinoma (HCC) including hereditary hemochromatosis and  $\alpha$ 1-antitrypsin deficiency,<sup>[5-7]</sup> viral hepatitis, as well as exposure to other extrinsic factors, such as aflatoxin B1 (AFB1), polyvinyl chloride (PVC), a poor diet inducing non-alcoholic fatty liver disease (NAFLD), and excess iron exposure, remain among the most common causes of liver cancer.<sup>[8,9]</sup> Despite vaccinations for hepatitis B virus (HBV), new treatments for hepatitis C virus (HCV), regulations governing PVC production, and restrictions preventing AFB1 contamination of food products, countries still struggle to prevent liver cancer.<sup>[9,10]</sup>

Surgical resection is currently the preferred treatment, and liver transplantation is ultimately the most effective therapeutic modality of HCC; however, it is limited by the availability of suitable organs.<sup>[11,12]</sup> Due to a high

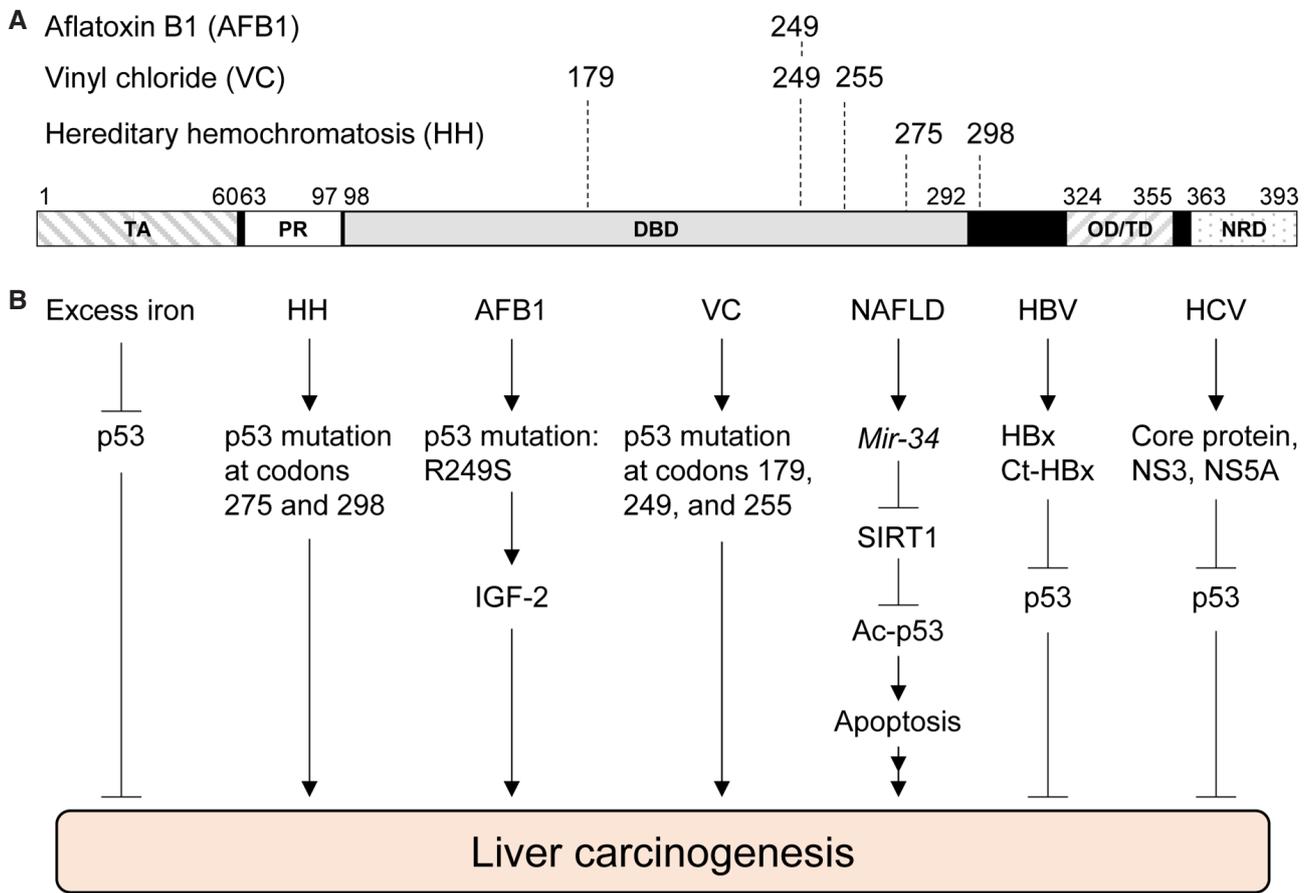
probability of being diagnosed at advanced stages, as well as poor responses to systematic chemotherapy and radiation therapy, prognosis of HCC is particularly bleak with an incidence to mortality ratio of 0.95 and a 5-year survival rate around 17.5%.<sup>[2,13]</sup>

Molecular mechanisms involved in liver carcinogenesis remain unclear. The tumor suppressor p53, a transcription factor that regulates many downstream target genes regulating cell cycle progression, apoptosis, DNA repair, senescence, and metabolism,<sup>[14,15]</sup> is one of the most commonly mutated genes in HCC.<sup>[16,17]</sup> Indeed, p53 is the most commonly mutated human gene, occurring in > 50% of all human cancers.<sup>[18]</sup> Additionally, in some HCC cases, proteins such as a 26S proteasome regulatory protein, gankyrin, and a p53-specific ubiquitin ligase, murine double minute 2 (MDM2), are elevated, hence decreasing p53 protein levels.<sup>[19,20]</sup> MicroRNAs (miRNAs) can also inhibit p53 activity; specifically, *miRNA-24*, when dysregulated in HCC, is shown to promote invasion and metastasis by decreasing p53 levels.<sup>[21]</sup> Thus, p53 activity is impaired by multiple mechanisms in HCC, hence contributing to HCC genesis. In this review article, we focus on HCC-inducing extrinsic factors that are etiologically associated with p53 [Table 1].

**Table 1: Extrinsic factors causing liver cancer and their association with p53**

Extrinsic factors	Mechanisms of action	Roles of p53	References
AFB1	AFB1 is metabolized to AFB1-8,9-epoxide to form AFB1-N <sup>7</sup> -guanine adducts, leading to specific mutation at p53 codon 249 ( <i>p53</i> <sup>R249S</sup> )	AFB1 frequently causes <i>p53</i> <sup>R249S</sup> mutation which enhances IGF-2 expression	[25,29,34]
VC	VC activated by CYP2E1 is converted into chloroethylene oxide, which forms bulky DNA adducts, leading to A>T transversions in the genome	It is unclear whether p53 plays protective roles in VC-induced liver cancer	[41,43,44]
NAFLD	NAFLD-induced hepatitis leads to cirrhosis and HCC, and dysregulation of NF- $\kappa$ B signaling, the PI3K-ATK-PTEN pathway, insulin resistance, and expression of certain miRNAs (e.g. <i>miR-34</i> ) is suggested; however, the molecular mechanisms behind NAFLD-mediated HCC remain unclear	The <i>miR-34</i> -SIRT1-p53 pathway plays a role in the progression of NAFLD. However, the direct role of p53 in the NAFLD-mediated HCC is unknown	[49,51-57]
Iron	Excess iron generates ROS and decreases p53 activity, leading to HCC genesis	Chronic iron overload reduces p53 protein levels by heme-mediated degradation or increased MDM2 levels, which can increase intracellular iron levels via a decrease in ISCUC2, thus further promoting HCC development	[64,68-70]
HBV	HBV-induced HCC occurs following repeated inflammation-liver regeneration-cirrhosis process, as well as through oncogenic function of HBx and Ct-HBx in both p53-dependent and -independent manners	Although direct involvement of p53 in HBV-induced HCC is unclear, functional inactivation of p53 by HBx and Ct-HBx may contribute to HCC progression	[76,81,82,85,99,100]
HCV	The majority of HCV-mediated HCC is via cirrhosis. But HCV core protein, NS3, and NS5 are implicated in HCC development in both p53-dependent and -independent manners	There is no direct evidence showing dependency of HCV-induced HCC on p53. However, HCV core protein, NS3, and NS5A inhibit p53 activity by binding to p53, altering subcellular localization, or modulating post-translational modifications	[112,118,119,123-125,131,132]

AFB1: aflatoxin B1; VC: vinyl chloride; NAFLD: non-alcoholic fatty liver disease; HBV: hepatitis B virus; HCV: hepatitis C virus; HCC: hepatocellular carcinoma; ROS: reactive oxygen species; MDM2: murine double minute 2; ISCUC2: iron-sulfur cluster enzyme 2; Ct-HBx: HBx variants with C-terminal truncations



**Figure 1:** Functional roles of p53 in liver cancer-associated diseases. (A) Functional domains in human p53 and amino acid locations mutated in liver cancer associated with aflatoxin B1 (AFB1), vinyl chloride (VC), and hereditary hemochromatosis (HH). (B) Involvement of p53 in liver carcinogenesis. Multiple hereditary and extrinsic factors cause liver cancer possibly through the p53 pathway. TA: transactivation domain, PR: proline-rich domain, DBD: DNA-binding domain, OD/TD: oligomerization/tetramerization domain, NRD: negative regulatory domain; NAFLD: non-alcoholic fatty liver disease; HBV: hepatitis B virus; HCV: hepatitis C virus; SIRT1: sirtuin 1; IGF-2: insulin-like growth factor 2; Ct-HBx: HBx variants with C-terminal truncations

## AFB1

AFB1 is a well-characterized liver mutagen produced by the fungus *Aspergillus*, and can be ingested by humans from contaminated food products.<sup>[22,23]</sup> One study estimates the population attributable risk of AFB1-mediated HCC as 17% in some parts of the world.<sup>[24]</sup> Mechanistically, AFB1 is activated by CYP40s into AFB1-8,9-epoxide, which reacts with DNA, forming 8,9-dihydro-8-(N<sup>7</sup>-guanyl)-9-hydroxyafatoxin B1 (AFB1-N<sup>7</sup>-guanine) adducts; these adducts, if left unrepaired, induce G>T transversions during DNA replication.<sup>[25,26]</sup>

AFB1 is well-known to generate a specific p53 mutation in the DNA binding domain from an arginine to serine missense mutation at codon 249 (R249S), which is caused by a G>T transversion at the third base of codon 249 [Figure 1A].<sup>[27,28]</sup> In geographic areas exposed to high levels of AFB1, such as the Qidong City in China, about 50% of HCC cases have the p53<sup>R249S</sup> mutation,<sup>[29]</sup>

suggesting the involvement of p53 in AFB1-induced HCC. AFB1-8,9-epoxide also reacts with guanines of the p53 gene other than those at codon 249, but these guanine adducts do not form cancer-causing mutations as frequently as p53<sup>R249S</sup>.<sup>[26,28,30]</sup> Although AFB1-mediated DNA damages initially activate p53 to induce cell cycle arrest at S to G2/M phases,<sup>[31-33]</sup> liver cells that gain p53<sup>R249S</sup> would escape this cellular defense mechanism with a selective advantage for proliferation, which could further proceed toward liver cancer. Indeed, p53<sup>R249S</sup> is shown to increase transcription of insulin-like growth factor 2 (IGF-2) in Hep3B (p53<sup>null</sup>) cells, suggesting a possible gain-of-function activity of p53<sup>R249S</sup>.<sup>[34]</sup> IGF-2 is over-expressed in 16-40% of human HCC and is implicated in promoting HCC progression.<sup>[35]</sup> Also, a positive correlation is observed between IGF-2 +3580 AA genotype and the risk of HCC.<sup>[36]</sup> Intriguingly, silencing of IGF-2 in HepG2 cells leads to decrease in cell survival and proliferation.<sup>[37]</sup> Thus, AFB1-mediated mutation in p53 plays a crucial role in HCC genesis, possibly through enhanced IGF-2 signaling [Figure 1B].

## VINYL CHLORIDE

Vinyl chloride (VC) is a carcinogenic gas used in the manufacture of PVC which induces mainly angiosarcomas of the liver (ASL) and rarely HCC, although it remains controversial whether VC can induce HCC in humans.<sup>[38-40]</sup> VC is absorbed in the lungs and then metabolized to chloroethylene oxide by CYP2E1 in the liver, which forms bulky DNA adducts, leading to liver cancer.<sup>[41,42]</sup> There are four VC-associated DNA adducts detected *in vivo*, including 7-(2-oxoethyl)-deoxyguanosine, 3,N<sup>4</sup>-etheno-deoxycytidine, 1,N<sup>6</sup>-etheno-deoxyadenosine, and N<sup>2</sup>,3-etheno-deoxyguanosine.<sup>[41]</sup>

VC-induced human ASLs are reported to have an increase in A>T transversions at codons 179, 249, and 255 of the *p53* gene [Figure 1A].<sup>[43,44]</sup> A study using Sprague Dawley rats also indicates that the majority of *p53* mutations in ASL and HCC following VC exposure are A>T transversions; the A>T transversions in ASLs are detected at codon 253 of rat *p53*, which is equivalent with codon 255 in humans.<sup>[45]</sup> Moreover, serum samples from workers exposed to VC have an increase in the levels of p53 protein with mutant conformation, detected by a conformation-specific p53 antibody PAb240, as well as other antibodies for p53.<sup>[44,46]</sup> However, it is still unclear whether p53 plays protective roles in VC-induced DNA damages and liver cancer development, and how mutations in *p53* contribute to the VC-induced liver cancer [Figure 1B].

## NAFLD

NAFLD represents a range of disorders including non-alcoholic fatty liver (NAFL), non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and HCC. NAFLD is associated with metabolic syndrome, type 2 diabetes mellitus, and obesity.<sup>[47]</sup> It is estimated that 20-30% of individuals in the Western world suffer from NAFLD.<sup>[48]</sup> However, only 11.5% of patients with NAFLD-induced cirrhosis eventually develop HCC, and about 50% of NASH-induced HCCs occur without cirrhosis.<sup>[49,50]</sup> These observations indicate the requirement of additional oncogenic events toward NAFLD-associated HCC. However, the molecular mechanisms behind NAFLD-mediated HCC are not fully understood. Several mediators have been implicated in its genesis, including dysregulation of NF- $\kappa$ B signaling, the PI3K-ATK-PTEN pathway, insulin resistance, and expression of certain miRNAs (e.g. *miR-34*).<sup>[51,52]</sup>

p53 has also been implicated in the progression of NAFLD due to multiple mechanisms. In a mouse

model for NAFLD where *p53*<sup>+/+</sup> and *p53*<sup>-/-</sup> mice are fed a methionine- and choline-deficient diet, *p53*<sup>+/+</sup> mice show increases in histologically observable steatohepatitis, reactive oxygen species (ROS) formation, and fibrosis with increased protein levels of p66Shc, a protein associated with oxidative stress, as compared to *p53*<sup>-/-</sup> mice.<sup>[53]</sup> Human NASH hepatocytes display upregulated p53 activity with increased mRNA levels of *p21* and *p66Shc*, which is positively correlated with fibrosis severity.<sup>[53]</sup> The *miR-34*-Sirtuin 1 (SIRT1)-p53 pathway is also implicated in NAFLD pathogenesis; increased *miR-34* expression and subsequent decrease in SIRT1 protein levels are detected in human NAFLD liver tissues with increased acetylation of p53, which is correlated with disease severity.<sup>[54]</sup> Activation of the *miR-34a*-SIRT1-p53 axis is also shown to contribute to liver fibrosis or NASH by inducing hepatocyte apoptosis.<sup>[55,56]</sup> Moreover, p53 can upregulate *miR-34*, which inhibits *SIRT1* mRNA expression, leading to increased acetylation of p53, thus forming a positive feedback loop [Figure 1B].<sup>[57]</sup> These observations indicate that high expression of *miR-34* and p53 is associated with NAFLD. However, it should be noted that *miR-34a*-mediated apoptosis can occur in p53-dependent and p53-independent manners.<sup>[58]</sup> Nonetheless, surrounding evidence suggests involvement of p53 in the progression of NAFLD and NASH; however, further studies are required to demonstrate whether p53 directly plays a crucial role in the NAFLD-mediated HCC.

## IRON OVERLOAD

Iron is an essential mineral that takes part in numerous metabolic processes, such as heme synthesis, Fe-S cluster biogenesis, and oxygen transport via hemoglobin.<sup>[59]</sup> However, when iron homeostasis is perturbed, whether due to genetic or environmental causes, there can be severe consequences including cardiomyopathy, hepatic fibrosis, endocrine disorders, and arthropathy.<sup>[60,61]</sup> Importantly, excess iron is a risk factor for many types of neoplasia, including breast cancer, colorectal cancer, and HCC.<sup>[62]</sup> In parts of sub-Saharan Africa, dietary iron overload, mainly from beer prepared in iron pots, is strongly associated with an increased risk of HCC.<sup>[63]</sup> Experimentally, Wistar rats fed a high-iron diet are shown to develop HCC.<sup>[64]</sup> One mechanism implicated in iron overload-mediated HCC genesis is due to ROS-inducing DNA mutations, as multiple rat models and surveys of human HCCs have linked increased iron levels with increases in 8-oxo-2-deoxyguanosine adducts and oxidizing products such as malondialdehyde.<sup>[65-67]</sup>

However, there is evidence that iron overload has a

direct effect on p53 activity. C57BL/6 mice fed a high-iron diet show a decrease in p53 protein levels in the liver.<sup>[68]</sup> Also, male Sprague-Dawley rats fed a high-iron diet for prolonged periods of time present with an increase of MDM2, and a subsequent decrease of p53 in the liver.<sup>[69]</sup> Another molecular mechanism behind decreased levels of p53 due to iron excess includes that p53 is bound by heme, exported to the cytoplasm, and degraded in HepG2 cells via the proteasomal pathway.<sup>[68]</sup> Thus, both iron excess and dysregulated heme decrease p53 levels, contributing to HCC development [Figure 1B]. Intriguingly, p53 is also involved in reducing intracellular iron levels by transactivating iron-sulfur cluster enzyme 2 which contributes to reduced iron uptake.<sup>[70,71]</sup> Thus, following chronic iron overload, reduced p53 activity leads to increased intracellular iron levels, further promoting HCC genesis. It should be noted that patients with hereditary hemochromatosis show higher rates of p53 mutations (64-71%), as compared with those in sporadic HCC, supporting a role of p53 in iron overload-induced HCC genesis.<sup>[72,73]</sup> In HCC tissues from hereditary hemochromatosis, 45% A>C transitions and 33% G>C transversions, including two hotspots at codon 275 and 298, are identified in the p53 gene [Figure 1A].<sup>[73]</sup> However, in the study using British families with hereditary hemochromatosis, the p53 mutation spectrum consists of 60% A>G transitions and 40% A>T transversions.<sup>[72]</sup> Nonetheless, it remains to be elucidated whether iron overload indeed induces HCC in a p53-dependent manner in animal models.

## HBV

Globally, it is estimated that 248 million individuals have chronic HBV infection and are positive for the hepatitis surface antigen.<sup>[74]</sup> HBV is the leading cause of HCC, with the majority being attributed to chronic HBV infection.<sup>[75]</sup> HBV-mediated HCC tumorigenesis can be caused by repeated bouts of immune-mediated hepatocyte death and subsequent tissue repair, with eventual cirrhosis of the liver.<sup>[76]</sup> Importantly, 10-30% of HBV-related HCCs do not occur in the background of cirrhosis, indicating additional oncogenic mechanisms behind HBV-induced HCC genesis.<sup>[77]</sup>

HBV, a circular, partially double-stranded DNA virus, consists of four overlapping open reading frames in its genome: a core region, surface region, polymerase region, and X region which produce seven viral proteins named precore, core, polymerase, L, M, HBx, and S.<sup>[78-80]</sup> Of these, the HBx protein, which plays a pivotal role in viral replication, is most implicated in HCC genesis.<sup>[80]</sup> Indeed, HBx induces HCC by

sequestering p53 to the cytoplasm in transgenic mouse models [Figure 1B].<sup>[81,82]</sup>

HBx is also implicated in hepatocyte apoptosis.<sup>[78]</sup> In many contexts, HBx inhibits apoptosis not only by increasing levels of anti-apoptotic protein, survivin, but also by binding to and sequestering p53 to the cytoplasm.<sup>[83-86]</sup> HBx is also reported to inhibit TGF- $\beta$ -mediated apoptosis.<sup>[87]</sup> Conversely, in some contexts, HBx is shown to induce apoptosis in a p53-independent manner.<sup>[88-90]</sup> Hence, the dual roles of HBx in hepatocyte apoptosis and its association with HCC genesis warrant further investigation.

HBx variants with C-terminal truncations (Ct-HBx) are frequently detected in HCC and might also contribute to HCC development, though there is no direct evidence for it.<sup>[91-93]</sup> Ct-HBx promotes hepatocyte proliferation and inhibits apoptosis in multiple cell lines.<sup>[94-96]</sup> Transcriptional downregulation of ubiquitin specific peptidase 16 (USP16) by Ct-HBx is also shown to enhance tumorigenicity and stem-like properties of HCC cells.<sup>[97]</sup> Moreover, Ct-HBx binds to p53 and inhibits p53-mediated apoptosis similar to HBx [Figure 1B].<sup>[85,98,99]</sup> Additionally, some Ct-HBx variants have the ability to silence mRNA expression of GAS2, a modulator of p53-mediated apoptosis.<sup>[100]</sup> Thus, Ct-HBx may contribute to the pathogenesis of HBV-related HCC by downregulating USP16 and inhibiting p53-mediated apoptosis.

Given that p53 is infrequently mutated in HBV-related HCC, p53 mutations are associated with late stage disease, and both HBx and Ct-HBx bind to and inhibit p53 function [Figure 1B],<sup>[101-103]</sup> inactivation of p53 activity may be favorable for HBV-mediated HCC tumorigenesis, rather than p53 mutation. Importantly, HCC patients with wild-type p53 have better overall survival and an increase in recurrence free survival as compared with those having p53 mutations.<sup>[104]</sup>

## HCV

Hepatitis C is estimated to have a global prevalence of 184 million individuals positive for anti-HCV, and individuals with HCV have a 15 to 20 fold increased risk for HCC.<sup>[105,106]</sup> HCV is a 9,600 nucleotide positive sense single-stranded RNA virus with a single open reading.<sup>[107,108]</sup> The HCV genome encodes for a polyprotein that is subsequently cleaved into nine viral proteins, including structural proteins (C, E1, E2), and non-structural proteins (p7, NS2, NS3, NS4A, NS5A, NS5B).<sup>[109]</sup> Although the vast majority of HCV-related HCCs occur within the context of cirrhosis, there is some evidence showing oncogenic potential

for the HCV viral proteins.<sup>[77,110,111]</sup> Specifically, HCV core, NS3, and NS5 proteins have been implicated in HCC development in both p53-dependent and -independent manners.<sup>[112]</sup>

Transgenic mice expressing the HCV core protein indeed spontaneously develop HCC, without the background of cirrhosis.<sup>[113,114]</sup> HCV core protein also increases ROS, inhibits Fas- and TNF-mediated apoptosis, and upregulates the Wnt- $\beta$ -catenin pathway.<sup>[115-117]</sup> Importantly, the core protein inhibits p53 activity by altering its subcellular localization to the perinuclear region and nuclear granular structures, as well as its post-translational modifications such as phosphorylation and acetylation of p53 in HeLa and HepG2 cell lines [Figure 1B].<sup>[118]</sup> Moreover, the core protein upregulates SIRT1, a deacetylation enzyme for p53, leading to impaired p53-dependent apoptosis in HepG2 cells [Figure 1B].<sup>[119]</sup> Thus, HCV core protein likely causes HCC in both p53-dependent and -independent manners.

A non-structural HCV protein, NS3, is another HCV protein that can transform human hepatocytes with an increase in cyclooxygenase-2 and activation of mitogen-activated protein kinase.<sup>[120-122]</sup> NS3 also complexes with p53 in HeLa and NIH3T3 cells<sup>[123,124]</sup> and inhibit p53's transcriptional activity in NIH3T3 and Huh7 cells [Figure 1B].<sup>[124,125]</sup> Moreover, NIH3T3 cells transformed by overexpression of NS3 can form tumors in mice.<sup>[126]</sup> However, it remains unclear whether transformation by NS3 is p53-dependent or not.

Another non-structural HCV protein, NS5A, can cause steatosis and HCC in transgenic mouse models.<sup>[127]</sup> NS5A is shown to inhibit TNF $\alpha$ -mediated apoptosis, transactivate c-fos, and inhibit Bax-mediated apoptosis independent of p53.<sup>[128-130]</sup> However, NS5A can also bind to and colocalize with p53 to the perinuclear membrane, leading to inhibition of p53 transcriptional activity [Figure 1B].<sup>[131,132]</sup> Moreover, NS5A binds with hTAF<sub>II</sub>32 at the nucleoplasm membrane and inhibits its ability to stabilize p53, resulting in abrogation of p53-mediated apoptosis in Hep3B cells.<sup>[132]</sup> Thus, NS5A contributes to HCC development and progression through p53-dependent and -independent mechanisms.

## CONCLUSION

In summary, there is a large body of data indicating p53's involvement in extrinsic factor-induced liver carcinogenesis. Nonetheless, demonstrating *in vivo* evidence for the protective role of p53 in HCC genesis is crucial. While many of the aforementioned

risk factors for liver cancer have become preventable or treatable, efficient therapeutic strategies are still limited. Hence, understanding the role of p53 in the molecular pathogenesis of HCC and restoring p53 activity in tumors would significantly help accelerate the development of new therapies for this therapy-resistant disease.

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## Authors' contributions

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Design, writing and revision of the paper: T. Link, T. Iwakuma

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## Conflicts of interest

There are no conflicts of interest.

## Patient consent

Not applicable.

## Ethics approval

Not applicable.

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