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# Pneumonia promotes pulmonary metastasis of HCC after transplantation via VEGF regulated PI3K/AKT/Cas-9 signaling and angiogenesis

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## Abstract

**Aim:** Pneumonia is the most frequent early postoperative complication in liver transplantation (LT) recipients. Inflammation may provide a favorable environment for tumor implantation, so we aimed to evaluate the impact of pneumonia on pulmonary metastasis of hepatocellular carcinoma (HCC) and reveal its underlying mechanism.

**Methods:** A training cohort with 234 LT recipients were recorded and analyzed. Using the propensity-score method, we matched covariates between patients with and without pneumonia. A model for predicting pulmonary metastasis was built and validated in an independent validating cohort containing 179 subjects. A mouse model was built to mimic HCC pulmonary metastasis. The potential pathway was revealed by cytokine array analysis and validated *in vitro*.

**Results:** Pneumonia was an independent risk factor for pulmonary metastasis in liver transplant recipients. It



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promoted pulmonary metastasis in both the clinical setting and the mouse model. *In vitro*, LPS-stimulated VEGF secretion from macrophages in the lung significantly reduced cell apoptosis and activated PI3K/AKT/cas-9 signaling. Administration of VEGF receptor2 inhibitor Vatalanib could reduce metastasis and improve prognosis in pneumonia mice.

**Conclusion:** Pneumonia promotes HCC pulmonary metastasis by activating PI3K/AKT/Cas-9 signaling in HCC cells via macrophage-originated VEGF. Vatalanib might be efficient in reducing HCC pulmonary metastasis in liver transplant recipients with pneumonia.

**Keywords:** Hepatocellular carcinoma, liver transplantation, pneumonia, pulmonary metastasis, angiogenesis

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common lethal cancer and ranks third among cancer-related deaths worldwide<sup>[1]</sup>. A net increase of 62% in HCC-related annual death rate was seen in the past two decades<sup>[2]</sup>. Liver transplantation (LT) provides a possible cure for both HCC and underlying liver disease<sup>[3]</sup>. However, HCC metastasis is a major obstacle to long-term survival after LT. Although candidate selection criteria such as Milan Criteria<sup>[4]</sup> were introduced, and various strategies such as donor liver preservation, down-staging and targeted therapy were recently developed for reducing the recurrence risk, the 5-year recurrence rates were not dramatically improved, ranging from 10.0% to 23.6%<sup>[5-7]</sup>. Looking deeper into the recurrence/metastasis type, we know that typically, HCC recurrence occurs intrahepatically after partial hepatic resection. In contrast, extrahepatic metastasis, particularly pulmonary metastasis, is the major pattern after LT. The incidence of pulmonary metastasis was 8.0%-26.7%<sup>[8,9]</sup>, accounting for roughly half of all recurrences/metastases in liver transplant recipients<sup>[10]</sup>. Nevertheless, the underlying mechanism of pulmonary metastasis after LT is elusive.

Recently, the impact of inflammation on cancer metastasis has received increasing attention. Previous studies showed that lung inflammation promoted pulmonary metastasis of several types of tumors<sup>[11-14]</sup>. Inflammation-induced lung metastasis could be attenuated by aspirin or antibiotics<sup>[12,15]</sup>. The global incidence of early postoperative infections is estimated to be over 20%. Among them, pneumonia is the predominant form for LT recipients<sup>[16,17]</sup>. Because of transplant-specific risk factors such as immunosuppression, the incidence of pneumonia is much higher in LT recipients than patients receiving partial hepatectomy (8%-48% vs. 2%-5%)<sup>[18,20]</sup>. Therefore, in this study, we aimed to assess the association between pneumonia and HCC pulmonary metastasis after LT and to elucidate the possible mechanisms.

## METHODS

After excluding patients with incomplete information, loss to follow-up, or perioperative death, 234 adult HCC patients receiving primary LT from donation after citizens' death from January 2015 to February 2019 in our center were enrolled as a training cohort, and another independent cohort of 179 subjects from Shulan Hospital Affiliated to Zhejiang Shuren University between 2017 to 2019 was designated for further validation of the predictive model. 212 patients in the training cohort and 163 patients in the validating cohort were within Hangzhou Criteria as we previously reported<sup>[5]</sup>. All cases were in accordance with the Regulations on Human Organ Transplant and national legal requirements. No organs from executed prisoners were used. Patients received triple immunosuppressant therapy incorporating tacrolimus, mycophenolate, and a steroid, as we previously reported<sup>[21]</sup>. This study complies with the guidelines of China Ethical Committee and the Declaration of Helsinki. Informed consents were obtained from all participants. Demographic and pathological information including cirrhosis, Child-Pugh score, and ischemia time were collected retrospectively. Based on their microscopic appearance, the pathologist

classified HCC into four grades: well-differentiated (Grade 1), moderately differentiated (Grade 2), poorly differentiated (Grade 3), or undifferentiated (Grade 4). Pneumonia was diagnosed based on chest imaging showing infiltrates, accompanied by fever (temperature  $> 38.3^{\circ}\text{C}$  or  $< 36^{\circ}\text{C}$ ), leukocytosis (white blood cell count  $> 10$  or  $< 4$  g/L), and clinical symptoms such as a new or worsening cough, dyspnea, and purulent secretions<sup>[22]</sup>. To exclude the possibility that pneumonia was induced by lung metastatic tumors, pneumonia in this study was defined as a lung infection that occurred before the detection of metastasis. Pulmonary metastasis was suspected by imaging and finally confirmed by pathology.

For the mouse model, six-week-old male Balb/c mice were used for the experiments. Each experimental group comprised ten randomly assigned animals. All animals were treated humanely in accordance with the guidelines detailed in the “Guide for the Care and Use of Laboratory Animals” by the National Academy of Sciences, as published by the National Institutes of Health. Institutional and national guidelines for the care and use of laboratory animals were followed. Mouse pneumonia model was induced as reported in<sup>[11]</sup>.  $1 \times 10^6$  H22 cells in 100  $\mu\text{L}$  PBS were injected into the tail vein, and immediately thereafter, 20  $\mu\text{g}$  of LPS in 50  $\mu\text{L}$  PBS were intra-nasally administered. Controls received PBS only. To deplete macrophages, clodronate liposomes (1.4 mg/20 g body weight) or an equal volume of PBS liposomes were injected intraperitoneally every other day. Vatalanib was administrated by gavage at a dose of 50 mg/kg per day as recommended<sup>[23]</sup>. Mice were sacrificed and lung tissues were fixed in 4% formaldehyde.

### Hematoxylin-eosin staining

Preparation of lung sections and histopathological techniques was performed according to standard protocols<sup>[24]</sup>. Paraffin-embedded tissues were sectioned at 4  $\mu\text{m}$  for histological and immunohistochemical analysis. The sections were stained with hematoxylin-eosin and evaluated under a microscope for histological examination. Two independent observers performed all histological assessments in a blinded fashion.

### Immunocytochemistry

Cells were seeded on slides, incubated for 24 h, and then fixed with paraformaldehyde for 30 min at  $4^{\circ}\text{C}$ . After rinsing three times in PBS, nonspecific proteins were blocked with serum for 30 min at room temperature. The cells were then incubated overnight at room temperature with the anti-VEGF receptor 2 antibody and subsequently washed twice in PBS. Following this, the cells were incubated for 1 hour with goat anti-rabbit immunoglobulin G-conjugated horseradish peroxidase diluted at 1:1,000, followed by a PBS wash. Finally, the cells were stained with diaminobenzidine.

### Immunohistochemistry, and immunofluorescence staining

Specimens were sliced into 4  $\mu\text{m}$  sections, de-waxed with xylene, and rehydrated using graded ethanol. Antigen retrieval was conducted in a microwave at  $95^{\circ}\text{C}$  for 20 min, followed by cooling to room temperature. To block nonspecific binding sites, sections were treated with 5% bovine serum albumin (BSA) for 1 h. Subsequently, the sections were incubated with anti-VEGF and anti-CD31 antibodies. Representative fields were selected from each section.

### Cell culture

The human HCC cell lines Huh-7 and SK-Hep-1, mononuclear cell line THP-1, lung fibroblasts HFL-1 and epithelium-derived lung adenocarcinoma cell line A549 were cultured in the supplier-recommended complete growth medium. All cells were maintained at  $37^{\circ}\text{C}$  in a humidified incubator with 5%  $\text{CO}_2$ . To induce monocyte-macrophage differentiation, THP-1 cells were treated with 150 nM phorbol 12-myristate 13-acetate (PMA) for 48 h. Cells were washed with cold PBS and then cultured with medium containing 50 ng/mL LPS or an equal volume of PBS for another 48 h. The supernatant was filtered and centrifuged at a

speed of 3,000 g to remove cells and cell debris. The supernatants (LPS-conditioned and Control-conditioned) were then used to culture HCC cells for 48h. The VEGFR2 inhibitor Vatalanib<sup>[25,26]</sup>, PI3K inhibitor LY294002<sup>[27,29]</sup>, and AKT inhibitor MK-2206 2HCl<sup>[30]</sup> were provided by Selleck (Selleck Chemicals, Houston, TX, USA), and used at recommended concentrations.

### ELISA

The concentration of VEGF in the supernatant was measured using the Human ELISA Kit (Raybiotech, ELH-VEGF-1), following the manufacturer's instructions. Results were read at 450 nm with an Absorbance Reader (BIO-TEK ELX800). Each sample was tested in triplicate, and the average value was used for analysis.

### Western blot

Total proteins were extracted from cells. Anti- $\beta$ -actin antibody (Abcam, ab8226, 1:2,000), anti-PI3K antibody (CST, #3358, 1:1,000), anti-PI3K p85 (19H8) (CST, #4257, 1:1,000), anti-AKT (CST, #4691, 1:1,000), anti-Phospho-AKT (Ser473) (CST, #4060, 1:1,000), anti-Caspase 9 (CST, #9508, 1:1,000), and anti-Cleaved Caspase-9 (Asp330) (CST, #7237, 1:1,000) were used. Immunodetection was carried out using an EZ-ECL chemiluminescence detection kit (BeitHaemek, Israel).

### Tumor migration and invasion assay

For migration assay, polycarbonate membrane transwell and Matrigel were used. A total of  $1 \times 10^4$  HCC cells were suspended in 200  $\mu$ L of serum-free medium and seeded into the upper chamber. The lower chamber was added with 700  $\mu$ L of MEM containing 10% FBS. For invasion assay, 40  $\mu$ L 1:8 diluted Matrigel was placed into the inserts and medium was removed without disturbing the layer of Matrigel on the membrane after rehydration. After incubation at 37 °C for the specified duration, cells remaining on the upper surface were removed using cotton swabs. The membranes were then fixed in paraformaldehyde and stained with 0.5% crystal violet. Cells on the lower surface were counted in randomly selected fields under a microscope (100X). The experiments were repeated independently three times.

### Cell cycle and apoptosis assay

For cell cycle analysis, cells were harvested and fixed in ice-cold 75% ethyl alcohol at 4 °C overnight. After incubation with DNA PREP kit solution in the dark for 30 min, the cell cycle was detected by FACS and analyzed using ModFit LT 3.1 software. Apoptosis was assessed using an FITC Annexin V Apoptosis Detection Kit II and analyzed with a flow cytometer. All experiments were performed in triplicate.

### Statistical analysis

Continuous variables were analyzed using Student's *t*-test, while categorical variables were evaluated using Pearson's chi-square test. Univariate analysis was performed for the potential prognostic factors, and the parameters that exhibited statistical significance were then included in multivariate logistic regression to identify the most critical characteristics of pulmonary metastasis. SPSS for Windows version 22.0 was used for all statistical analyses, and *p* value less than 0.05 was considered statistically significant. R software (version 4.0.2) was applied to integrate independent predictors of pulmonary metastasis into a nomogram in the training cohort. Propensity scores were estimated using a logistic model with the SAS software package 9.4.

## RESULTS

### Pneumonia independently increased the pulmonary metastasis risk in liver transplant recipients

For the training cohort, the median follow-up time was 31.6 months (ranging from 8 to 55 months). Among 75 patients with recurrence/metastasis, 48 had pulmonary metastasis (16 had sole pulmonary metastasis, 32

had both pulmonary and other site recurrence/metastasis, including liver graft, bone and other sites). As seen in Table 1 and [Supplementary Table 1], Patients with pneumonia had a higher rate of pulmonary metastasis when compared with recipients without pneumonia (38.6% vs. 10.6%,  $P < 0.001$ ).

As shown in Table 2, tumor number, AFP level, microvascular invasion (MVI), and pneumonia were significantly associated with pulmonary metastasis and overall survival ( $P < 0.05$ ). History of ICU stay was correlated only with recipients' overall survival ( $P < 0.05$ ). Multivariate analysis showed that history of ICU stays, AFP level, and pneumonia impacted overall survival. AFP level, MVI, and pneumonia were independent risk factors for pulmonary metastasis [Table 3]. Recipients with post-LT pneumonia exhibited an increase in pulmonary metastasis ( $P < 0.001$ , Figure 1A) and a significant reduction in overall survival ( $P < 0.001$ , Figure 1B). To reduce the impact of bias, a propensity analysis was achieved using SAS, incorporating preoperative variables. Matching based on propensity scores yielded 72 patients in each group for analysis of postoperative outcomes. Consequently, pneumonia was found to exacerbate pulmonary metastasis ( $P < 0.001$ , Figure 1C) and adversely impact overall survival ( $P = 0.015$ , Figure 1D) in this selected cohort.

### **Nomogram constructed was effective in predicting pulmonary metastasis**

Based on risk factors including pneumonia, a nomogram predicting pulmonary metastasis of LT recipients was constructed [Figure 1E], and this nomogram worked effectively (C-index 0.776, Supplementary Figure 1). This model was further verified in an independent validating cohort containing 179 subjects from another LT center, and the C-index reached 0.794 when predicting pulmonary metastasis [Figure 1F].

### **Pneumonia promoted pulmonary metastasis of HCC cells in mice model**

To better illustrate the association between pneumonia and pulmonary metastasis, we established an LPS-induced mouse pneumonia model [Supplementary Figure 2] and injected H22 cells into the tail vein to mimic the HCC recurrence. The preliminary experiment showed a median survival of 13 days of the H22 cells-injected mice. Therefore, we performed Magnetic Resonance Imaging 10 days after cell injection to evaluate the tumor growth in the lung. We found significantly exacerbated pulmonary metastasis in the pneumonia group as compared with control [Figure 2A]. The following pathology confirmed the results that pneumonia mice had higher tumor burden in the lung [Figure 2B and C].

### **Pneumonia increased VEGF secretion in pulmonary macrophages**

Since angiogenesis is a key process in regulating tumor metastasis<sup>[31-33]</sup>, we assessed the microvessel density using CD31 staining and found significantly higher CD31 intensity in the pneumonia group compared with control [Figure 2D and E]. We further examined the expression of VEGF and TGF- $\beta$ , cytokines regulating angiogenesis. In the mice lung, we found a time-dependent elevated VEGF level after LPS stimulation [Figure 2F], while the intensity of TGF- $\beta$  remained almost constant [Supplementary Figure 3]. To reveal the origin of VEGF, we tested the concentration of VEGF in the supernatants of THP-1-derived macrophages, lung fibroblasts HFL-1, and lung epithelium-derived cell line A549 stimulated with LPS or not. Although they produced comparable levels of VEGF in normal conditions, when stimulated with LPS, only THP-1-derived macrophages produced dramatically elevated VEGF [Figure 3A]. Consistently, *in vivo*, macrophage-depleted mice [Supplementary Figure 4] showed significantly decreased VEGF intensity in the lungs when instilled with LPS [Figure 3B]. Taken together, VEGF was mainly originated from macrophages following LPS instillation.

**Table 1. Clinicopathological features of LT recipients**

		Pneumonia (-)	Pneumonia (+)	P value
Gender	male	131	78	0.087
	female	20	5	
Age		52.61 ± 9.23	52.89 ± 9.04	0.826
HBV	-	13	3	0.148
	+	138	80	
Cirrhosis	-	6	2	0.715
	+	145	81	
Tumor number	single	66	30	0.270
	multiple	85	53	
Diameter (cm)		5.22 ± 4.52	6.41 ± 5.2	0.330
WIT (min)		9.63 ± 7.49	8.77 ± 5.89	0.335
CIT (h)		9.43 ± 3.28	9.52 ± 3.06	0.824
AFP level (ng/mL)		4028.6 ± 14074.1	3344.8 ± 11683.1	0.708
ICU stay (days)		222.7 ± 104.7	242.6 ± 179.7	0.359
MELD score		15.61 ± 10.49	14.96 ± 9.27	0.640
MVI	-	91	53	0.689
	+	60	30	
HCC Differentiation	1	17	11	0.971
	2	78	41	
	3	54	30	
	4	2	1	
TMN stage	I	46	25	0.932
	II	49	24	
	III	53	32	
	IV	3	2	
Tacrolimus (ng/mL)		7.94 ± 2.51	7.71 ± 2.46	0.791
Pulmonary metastasis	-	135	51	< 0.001
	+	16	32	

### Macrophage-derived VEGF promotes carcinogenesis via inhibition of apoptosis through PI3K/AKT/Cas-9 signaling besides angiogenesis

We found HCC cells expressed abundant VEGF receptor 2 (VEGFR2) [Figure 4A]. When cocultured with LPS-conditioned medium, HCC cells presented significantly reduced cell apoptosis [Figure 4B], although there were no significant changes in proliferation, migration, or invasion after co-culturing [Supplementary Figures 5-7]. The KEGG analysis based on the cytokine array indicated that PI3K/AKT signaling was significantly affected [Figure 5A]. Moreover, we also observed increased expression of PI3K p85, phosphorylated AKT (Ser473), Caspase-9, but decreased expression of cleaved caspase-9 in HCC cells cocultured with LPS-conditioned medium [Figure 5B]. The VEGFR2 inhibitor Vatalanib, PI3K inhibitor LY294002, or AKT inhibitor MK-2206 2HCl reversed the effect caused by coculture [Figure. 5B and C]. The decrease in apoptosis was also attenuated by Vatalanib [Figure 5D].

*In vivo*, Vatalanib administration dramatically improved the prognosis of the pneumonia mice [Figure 5E]. No obvious adverse effects such as diarrhea were observed in mouse model. Moreover, microvessel density in the metastatic nodules was also decreased [Figure 5F and G].

**Table 2. Univariate analysis for overall survival and pulmonary metastasis**

	Overall survival		Pulmonary metastasis	
	Hazard Ratio	P value	Hazard Ratio	P value
Age	1.018	0.170	0.991	0.587
MELD score	1.017	0.078	1.013	0.329
ICU stay	1.002	0.009	0.998	0.295
Tumor number	1.897	0.012	2.308	0.012
Tumor diameter	0.998	0.543	1.001	0.127
Tumor Differentiation	0.900	0.114	0.853	0.087
AFP level	2.204	< 0.001	2.644	< 0.001
CHILD score	1.089	0.074	1.023	0.724
WIT	1.012	0.452	1.020	0.333
CIT	1.064	0.066	1.032	0.481
SEX	0.686	0.375	0.729	0.544
Cirrhosis	1.158	0.804	1.654	0.503
HBV infection	1.862	0.291	NA	NA
MVI	2.539	< 0.001	2.215	0.007
Pneumonia	1.857	0.006	5.435	< 0.001

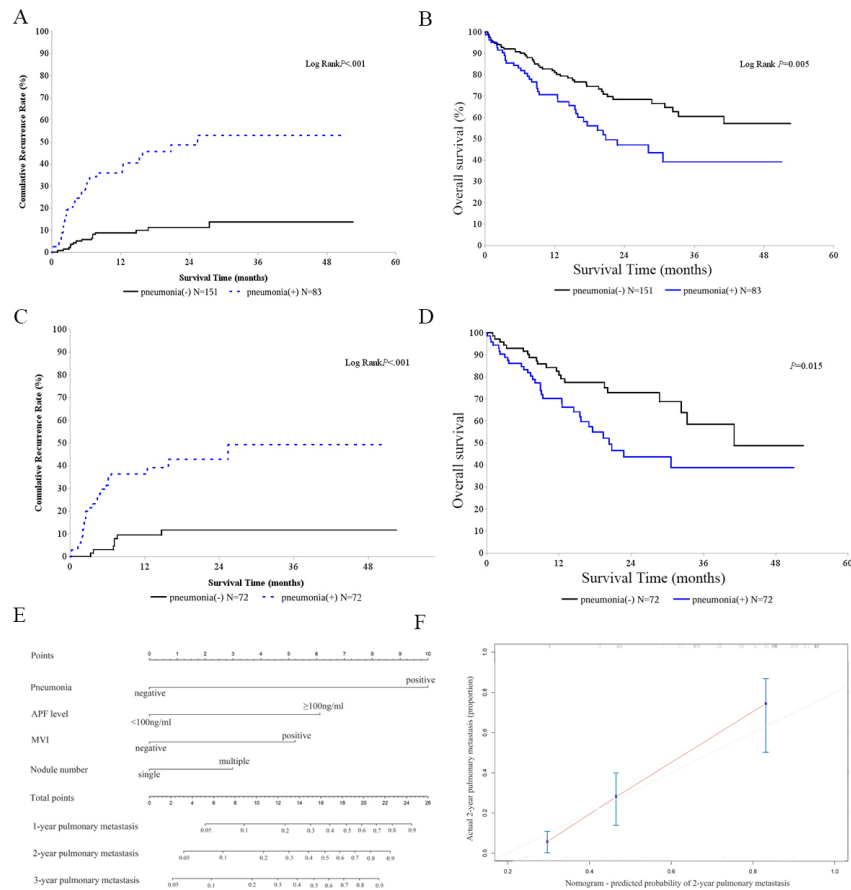
**Table 3. Multivariable hazard ratios for overall survival and pulmonary metastasis**

	Overall survival		Pulmonary metastasis	
	Hazard Ratio	P value	Hazard Ratio	P value
ICU	1.002	0.014	NA	NA
AFP	2.204	< 0.001	2.308	0.007
Tumor number	1.274	0.376	1.035	0.079
MVI	1.886	0.008	2.806	< 0.0001
Pneumonia	1.606	0.042	6.360	< 0.0001

## DISCUSSION

The ambiguity and complexity of pulmonary metastasis make it difficult to predict or intervene. To the best of our knowledge, this is the first study to evaluate the impact of post-LT pneumonia on HCC metastasis. We found that pneumonia was a very common phenomenon after LT and an independent risk factor for pulmonary metastasis based on two independent cohorts from different LT centers. On the one hand, pneumonia itself is one of the leading causes of sepsis and mortality after liver transplantation[18]. On the other hand, it increases the risk of pulmonary metastasis. Therefore, we should take intensive care of the post-LT recipients to prevent pneumonia. For the recipients already with pneumonia, close follow-up will be needed.

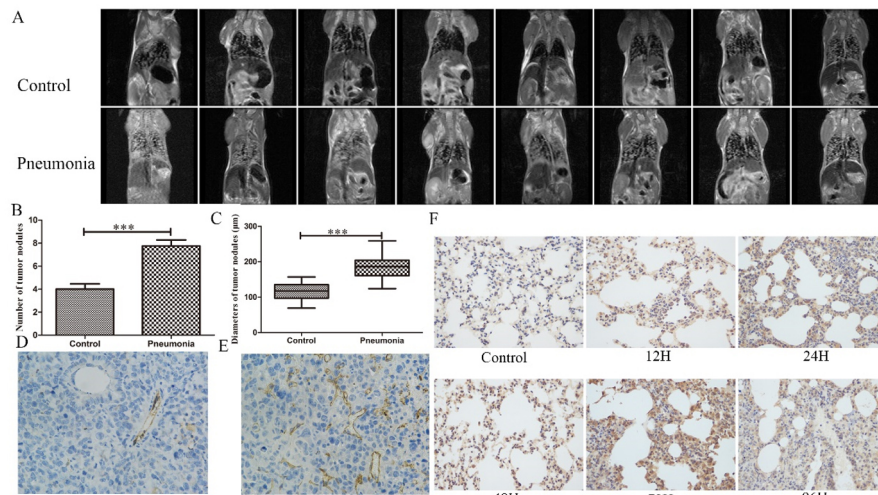
Hospital-acquired pneumonia early following LT is predominantly caused by Gram-negative bacteria. This group of pathogens induce pneumonia through the secretion of LPS. Therefore, the LPS-induced mouse pneumonia model could fully imitate the hospital-acquired pneumonia of the LT recipients during their hospital stay. In this study, not only clinical investigation but also *in vivo* models proved that pneumonia promotes HCC pulmonary metastasis. Local inflammation facilitates tumor metastasis through various mechanisms, such as triggering the formation of neutrophil extracellular traps and enhancing adhesion capacity at the metastatic site<sup>[14,34,35]</sup>. As is well known, angiogenesis plays a pivotal role in tumor metastasis<sup>[36-38]</sup>. We found that microvessel density in the metastatic nodules of pneumonia mice was significantly elevated. Furthermore, VEGF, the key angiogenesis modulator, was remarkably upregulated



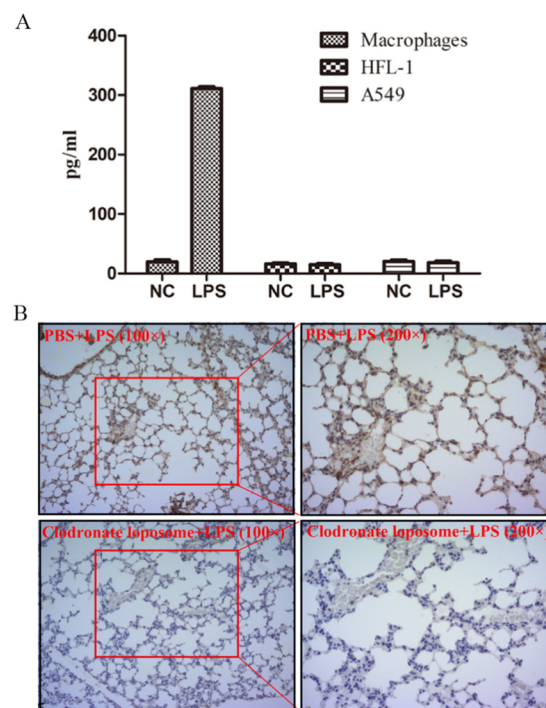
**Figure 1.** Pneumonia increases pulmonary metastasis and nomogram incorporating risk factors is effective. Liver transplantation recipients for HCC with pneumonia had increased pulmonary metastasis (A) and decreased overall survival (B). After PSM, two paired groups of patients were formed, with 72 subjects in each. Recipients with pneumonia showed increased pulmonary metastasis (C) and shortened overall survival (D); Nomogram incorporating risk factors predicted pulmonary metastasis for LT recipients (E) and was externally validated in an independent cohort (C-index 0.794, F)

following LPS instillation.

For VEGF, there are abundant existing studies concerning the pro-angiogenesis effect of VEGF<sup>[39,40]</sup>. It is capable of leading to leaky vascular networks that facilitate tumor cell invasion and induce EMT<sup>[41,42]</sup>. In this study, we found that macrophage-derived VEGF may inhibit apoptosis by activating PI3K/AKT/Cas-9 signaling. Elevation of VEGF induced by pneumonia in the lung may thus foster a pro-metastatic niche. The high incidence of pneumonia in patients receiving LT may partially explain why lung metastasis is more prevalent compared with liver resection or locoregional therapies. Anti-angiogenesis agents targeting the VEGF/VEGF receptor pathway have become a crucial component of standard therapy for various cancer types<sup>[43,45]</sup>, so we explored the potential of preventing pulmonary metastasis through the administration of VEGFR2-specific inhibitor Vatalanib<sup>[25,46,47]</sup>. We found that prophylactic administration of VEGFR2 inhibitor Vatalanib could ameliorate HCC pulmonary metastasis for the pneumonia mice. As this therapeutic effect was only observed in a mouse model with a small sample size, it is only a hypothesis and further validation is needed.

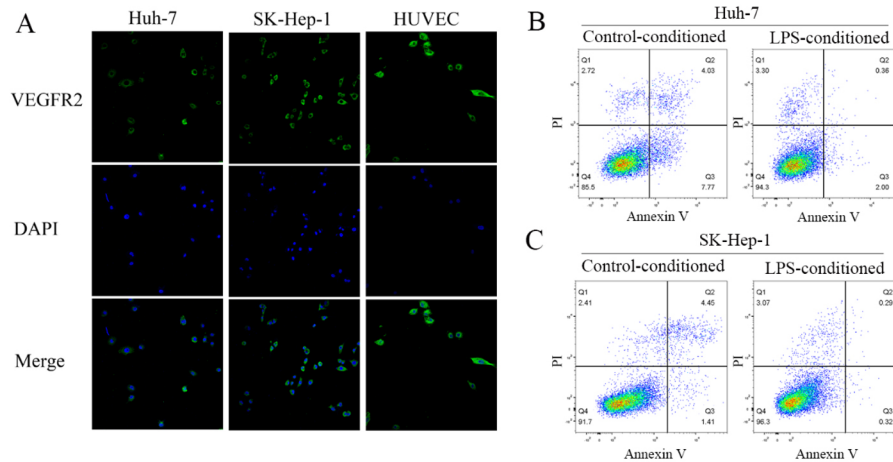


**Figure 2.** Pneumonia facilitated HCC pulmonary metastasis and angiogenesis in mouse models. (A) MRI images of the control and pneumonia mice; Mean metastatic nodule number (B) and diameter (C); Representative images of CD31 staining of the metastatic nodules of control (D) and pneumonia group (E); (F) VEGF was upregulated consecutively in the pneumonia mice lungs for at least 96 hours following LPS instillation. \*\*\*:  $P < 0.001$

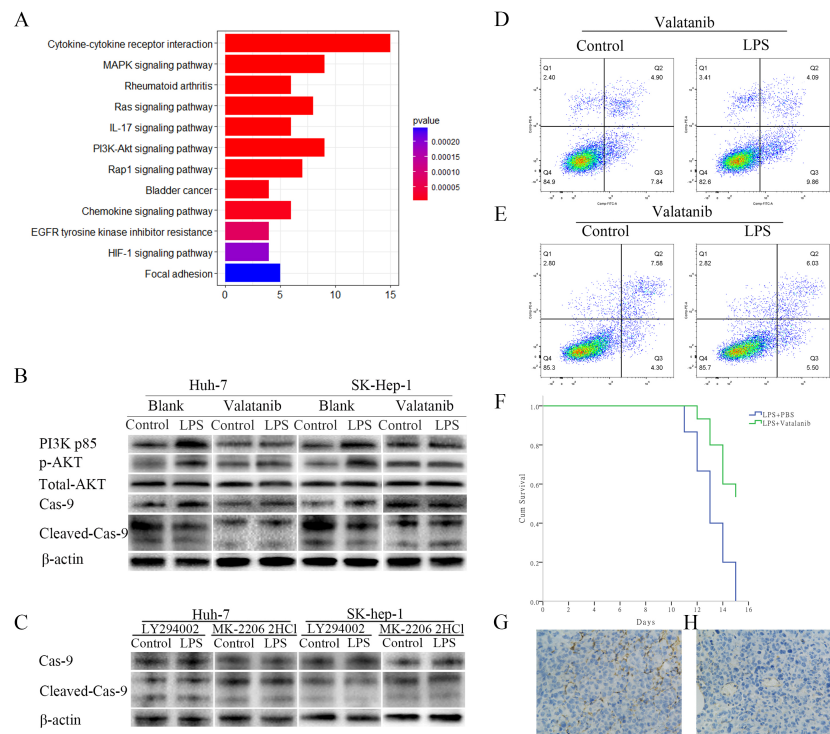


**Figure 3.** VEGF was originated from macrophage. (A) Concentration of VEGF in supernatants of THP-1-derived macrophages, lung fibroblast cell line HFL-1, and lung epithelium-derived adenocarcinoma cell line A549 stimulated with LPS or not; (B) Immunohistochemistry staining of VEGF of lungs in the control or macrophage-depleted mice.

Immunosuppressants could also influence HCC recurrence after live transplantation. Limiting the use of CNI and using m-TOR inhibitors could help reduce HCC recurrence rates<sup>[48]</sup>. However, owing to differences in metabolic rates, equal administration of immunosuppressants could result in different plasma concentrations in mice. Therefore, immunosuppressants were not used in the mouse model.



**Figure 4.** LPS-conditioned medium ameliorated HCC cell apoptosis. (A) Immunofluorescence staining of VEGFR2 for HCC cell lines Huh-7 and SK-Hep-1, with human umbilical vein endothelial cells as positive control; LPS-conditioned medium inhibited apoptosis of the Huh-7 (B) and SK-Hep-1 (C) cells.



**Figure 5.** LPS-conditioned medium activated PI3K/AKT/Cas-9 signaling and prophylactic administration of Vatalanib improved the prognosis of the pneumonia mice. (A) KEGG analysis based on cytokine array; (B) Western blot of PI3K/AKT signaling of HCC cells cultured in the Control-conditioned and LPS-conditioned medium; (C) Activation of the PI3K/AKT signaling was abrogated with PI3K inhibitor LY294002 or AKT inhibitor MK-2206 2HCl; Changes in apoptosis of Huh-7 (D) and SK-Hep-1 (E) was abrogated by Vatalanib; (F) Cumulative survival of the pneumonia mice administered with PBS or Vatalanib ( $P < 0.05$ ); CD31 staining of the metastatic nodules of the pneumonia mice administered with PBS (G) or Vatalanib (H).

The primary limitation of this study is that the cohort enrolled is small, especially the number of recipients with pulmonary metastasis. We hope to validate this as the number of transplantations increases. It is also

limited as the mouse pneumonia model, which was not immunosuppressed, could not adequately reflect the clinical practice.

In summary, this study provided a novel explanation for the extremely high incidence of pulmonary metastasis after LT. We strongly recommend an early screening and prompt treatment of pneumonia in the perioperative period in HCC patients. Anti-angiogenesis agents that target the VEGF/VEGF receptor pathway might be a promising strategy in liver recipients with pneumonia for reducing HCC pulmonary metastasis risk.

## DECLARATIONS

### Authors' contributions

Made substantial contributions to the conception and design of the study: Zhuang R, Wei Q, Xu X

Performed data analysis and interpretation: Dong S, Lu D

Performed data acquisition, *in vivo* and *in vitro* experiments: Zhuang R, Zhuo J

### Availability of data and materials

Not applicable.

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Research involving human subjects, human material, or human data was performed in accordance with the Declaration of Helsinki and approved by the Ethics Committee of The First Affiliated Hospital of Zhejiang University School of Medicine. Ethical approval and consent to participate Approval was provided by the Clinical Research Ethics Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University (2020-852). Informed consent was obtained from each subject prior to their participation in this study.

### Consent for publication

Not applicable.

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