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ICMJE Form for Disclosure of Potential Conflicts of Interest

Instructions

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Section 1. Identifying Information

1. Given Name (First Name): Yuanli
2. Surname (Last Name): He
3. Date: 12-August-2020

4. Are you the corresponding author? ☑ Yes ☐ No

5. Manuscript Title: Relevance of assessing the endometrial microbiota in intrauterine adhesion using high-throughput sequencing

6. Manuscript Identifying Number (if you know it): ATM-20-2813

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Section 1. Identifying Information

1. Given Name (First Name): Tianmei
2. Surname (Last Name): Qiu
3. Date: 12-August-2020

4. Are you the corresponding author? ☐ Yes ☑ No

Corresponding Author's Name: Huihua Cai, Mubiao Liu, Yuanli He

5. Manuscript Title: Relevance of assessing the endometrial microbiota in intrauterine adhesion using high-throughput sequencing

6. Manuscript Identifying Number (if you know it): ATM-20-2813

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1. Given Name (First Name): Mubiao
2. Surname (Last Name): Liu
3. Date: 12-August-2020

4. Are you the corresponding author? ☑ Yes ☐ No

5. Manuscript Title: Relevance of assessing the endometrial microbiota in intrauterine adhesion using high-throughput sequencing

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1. Given Name (First Name): Hongwei
2. Surname (Last Name): Zhou
3. Date: 12-August-2020

4. Are you the corresponding author? ☐ Yes ☑ No

Corresponding Author's Name: Huihua Cai, Mubiao Liu, Yuanli He

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1. Given Name (First Name): HuaFang
2. Surname (Last Name): Sheng
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Corresponding Author's Name: Huihua Cai, Mubiao Liu, Yuanli He

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Manuscript ID: ATM-20-5293
doi: 10.21037/atm-20-5293
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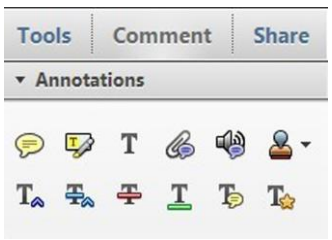

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Original Article

Irbesartan inhibits metastasis by interrupting the adherence of tumor cell to endothelial cell induced by angiotensin II in hepatocellular carcinoma

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Contributions: (I) Conception and design: LH Feng, HC Sun, XD Zhu, ZY Tang; (II) Administrative support: HC Sun, XD Zhu, ZY Tang; (III) Provision of study materials or patients: HC Sun, XD Zhu, XL Li, ZY Tang; (IV) Collection and assembly of data: LH Feng, SZ Zhang, XL Li, KS Li, XF Liu, M Lei, Y Li; (V) Data analysis and interpretation: LH Feng, SZ Zhang, XL Li, KS Li, XF Liu, M Lei, Y Li; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

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Background: The use of angiotensin II inhibitors is associated with a low risk of recurrence and metastasis in hepatocellular carcinoma (HCC) patients. Vascular cell adhesion molecule-1 (VCAM-1) is a key factor in tumor metastasis.

Methods: The effects of angiotensin II and irbesartan (an angiotensin II inhibitor) on HCC were explored with a xenograft model, microarray analysis and cell adhesion experiments. The relationship between the expression of VCAM-1 in HCC tissues and prognosis was analyzed with public and our institutional clinical databases. The effects of angiotensin II, irbesartan and VCAM-1 on adhesion and metastasis in HCC were explored with a xenograft model and cell adhesion experiments. The regulatory mechanisms were analyzed by Western blot analysis.

Results: Angiotensin II type 1 receptor and VCAM-1 were expressed in HCC tissues. Irbesartan inhibited HCC growth and metastasis in vivo and weakened the adhesion of HCC cells to endothelial cells, an effect that was enhanced by angiotensin II. VCAM-1 was found to be an independent risk factor for recurrence and survival in HCC patients with microvascular invasion. Angiotensin II upregulated VCAM-1 expression, and this upregulation was inhibited by irbesartan. Angiotensin II enhanced adhesion mainly by promoting the expression of VCAM-1 in HCC cells. Irbesartan inhibited the expression of VCAM-1 by reducing p38/MAPK phosphorylation activated by angiotensin II in HCC cells.

Conclusions: Irbesartan attenuates metastasis by inhibiting angiotensin II-activated VCAM-1 via the p38/MAPK pathway in HCC.

Keywords: Hepatocellular carcinoma (HCC); irbesartan; metastasis; vascular cell adhesion molecule-1 (VCAM-1)

Submitted Jul 18, 2020. Accepted for publication Nov 08, 2020.
doi: 10.21037/atm-20-5293
View this article at: http://dx.doi.org/10.21037/atm-20-5293

Introduction

Hepatocellular carcinoma (HCC) is the most frequently occurring primary liver cancer and the third leading cause of cancer-related death worldwide (1,2). The global estimated morbidity and related mortality rates continue to increase (3-5). Despite the tremendous progress achieved in the diagnosis and treatment of HCC, the overall efficacy remains unsatisfactory due to the high risk of recurrence and metastasis for patients undergoing curative therapy and the lack of effective drugs to target these phenomena (6,7).

Currently, accumulating evidence shows that angiotensin II (Ang II) inhibitors, which are common antihypertensive drugs, can provide survival benefits to cancer patients (8-10). These drugs can attenuate cancer progression promoted by Ang II, which promotes tumor growth or exacerbates tumor invasion and metastasis by mediating angiogenesis, inflammation and immunosuppressive microenvironments (11-13).

Ang II has been shown to promote the growth of HCC, epithelial–mesenchymal transition, and angiogenesis and mediate the inflammatory microenvironment via angiotensin II type 1 receptor (AGTR-1) (14-17). An increasing (6 clinical reports) supporting studies have confirmed that Ang II inhibitors can improve the prognosis of HCC patients by enhancing the efficacy of sorafenib, reducing the risk of recurrence and prolonging survival after curative treatments (10,18-22). We also reported that the use of Ang II inhibitors was associated with a reduced risk of disease recurrence, prolonged survival and a decreased rate of extrahepatic metastases in HCC patients after curative resection (23).

HCC is a cancer with typical hematogenous metastasis. Microvascular invasion (MVI) and circulating tumor cells (CTCs) are direct evidence of hematogenous metastasis and the main cause of metastasis (24,25). The adhesion of tumor cells to endothelial cells is a key step in tumor metastasis, and adhesion molecules play an important role in this process (26). Reports have indicated that Ang II can upregulate the expression of P-selectin, E-selectin and other adhesion molecules in endothelial cells to promote tumor cell adhesion, leading to the acceleration of tumor metastasis, and these molecules can be blocked by Ang II inhibitors (11,13). Hence, we speculated whether Ang II could also promote HCC metastasis in this way and, if so, whether this effect could be blocked by Ang II inhibitors.

In the present study, we found that irbesartan (an Ang II inhibitor and an AGTR-1 blocker) attenuated metastasis by inhibiting the adhesion of HCC cells to endothelial cells enhanced by Ang II. Additionally, we found that irbesartan mainly reduced vascular cell adhesion molecule-1 (VCAM-1), which was promoted by Ang II via the p38/MAPK pathway in HCC cells to weaken this adhesion. We present the following article in accordance with the ARRIVE Checklist (available at http://dx.doi.org/10.21037/atm -20-5293).

Methods

Cell cultures

Human HCC cell lines (HCCLM3, HMHCC97-H, HMHCC97-L, SMMC-7721, Huh-7, Hep-3B and PLC), a hepatocyte cell line (L02) and human umbilical vein endothelial cells (HUVECs) were all obtained from the Liver Cancer Institute, Fudan University, Shanghai, China, and were cultured at 37 ℃ and 5% CO_2. The HCC and hepatocyte cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, Utah, USA) containing 10% fetal bovine serum (FBS; HyClone) and 1% penicillin-streptomycin (PS; HyClone); HUVECs were cultured in endothelial cell medium (ECM; ScienCell, San Diego, California, USA) with 10% FBS, 1% PS and 1% endothelial cell growth supplement (ScienCell). It was approved by the Clinical Research Ethics Committee of Zhongshan Hospital, Fudan University, Shanghai, China (Approval No. B2012-010) and the individual consent for this retrospective analysis was waived.

Overexpression of AGTR-1 and knockdown of VCAM-1 by transfection

H-AGTR-1-OE (overexpression of AGTR-1), H-VCAM-1-sh (knockdown of VCAM-1) and a vector control lentivirus were designed and constructed by Genomeditech (Shanghai, China). The cells (2×10^5) were seeded in each well of a six-well plate the day before transfection. Subsequently, the lentiviruses were added to the well with 2 mL of DMEM containing polybrene (5 μg/mL; Genomeditech) without FBS. Forty-eight hours later, the medium containing the lentivirus was removed and replaced with medium containing 10% FBS. The expression of AGTR-1 and VCAM-1 was assessed and validated by qPCR and Western blotting.

Immunohistochemistry

The UltraVision Quanto Detection HRP DAB System

(Thermo Fisher Scientific, San Diego, California, USA) was used to perform immunohistochemical staining following the manufacturer's protocols to detect whether AGTR-1, angiotensin II type 2 receptor (AGTR-2) and VCAM-1 were expressed in HCC tissues and lung metastases. The antibodies against these three proteins were all purchased from Abcam (Cambridge, UK) and were diluted as follows: AGTR-1, 1:100; AGTR-2, 1:250; VCAM-1, 1:250.

Western blotting

Western blotting was performed following a standard procedure as described previously (27). The primary antibodies used included those against AGTR-1 (rabbit antibody; 1:1000; Abcam), VCAM-1 (rabbit antibody; 1:2000; Abcam), p38, p-p38, p65, p-p65, ERK, p-ERK, JNK and p-JNK (rabbit antibodies; 1:1000; Cell Signaling Technology, Danvers, Massachusetts, USA). The loading control antibodies, GAPDH (rabbit antibody; 1:1000) and α-tubulin (rabbit antibody; 1:1000), were purchased from BOSTER (Pleasanton, California, USA); the goat anti-rabbit IgG (1:5000) was from Yeasen (Shanghai, China).

Quantitative real-time PCR assay

RNA isolation from HCC cell lines and tissues and real-time PCR procedures were carried out according to the manufacturer's protocol (QuantStudio™3, Thermo Fisher Scientific, San Diego, California, USA). The internal reference primer, GAPDH, was purchased from Sangon Biotech (Shanghai, China). The PCR primers and sequences are shown in Table S1.

Human gene expression microarray

Total RNA was extracted from SMMC-7721-AGTR-1-OE, SMMC-7721-vector (control), Ang II-treated-HMHCC97-H, control-HMHCC97-H, Ang II-treated HCCLM3 and control-HCCLM3 cells and was analyzed by performing a human gene expression microarray (Agilent, Santa Clara, California, USA) from OE Biotech (Shanghai, China) to determine differential gene expression and biological behaviors.

Cell adhesion assay

The cell adhesion kit was purchased from KeyGEN BioTECH (Nanjing, Jiangsu, China), and the assay was performed according to the manufacturer's protocol. The bottom of the 96-well plate was plated with HUVECs, and 100 μL of a single HCC cell suspension with 5×10^5 cells stained by calcein AM was added to the wells of a 96-well plate, which was then placed in the incubator for 30–120 minutes (depending on the attachment time of HCC cells). After incubation, the cell suspensions in the wells were removed and washed with 200 μL of FBS-free DMEM 5 times to remove nonadhered HCC cells. PBS (200 μL) was added to each well, after which the absorbance value was read (excitation wavelength =494 nm), from which the absorbance value of a blank control well was subtracted.

Experiments on nude mice

As described in our previous study, an orthotopic tumor xenograft model and a lung metastasis model were set up with 5-week-old male BALB/c nude mice (Weight =18–20 g) obtained from the Beijing Vital River Laboratory Animal Technologies Co., Ltd and maintained under specific pathogen-free conditions (27,28). The animals were grouped randomly, and each group contained six mice. For the orthotopic tumor xenograft model, 200 μL of the tumor cell suspension (5.0×10^7 cells/mL) was injected subcutaneously, and when the tumor grew to 1.0 cm in diameter (approximately 4 weeks), it was cut into small nodules ($2.0\times2.0\times2.0$ mm^3) and implanted into the liver. For the lung metastasis model, 150 μL of the tumor cell suspension (1.0×10^6 cells) was injected into nude mice through the tail vein. The groups and time axes of animal experiments are shown in Figures S1,S2. After the mice were euthanized, the size of the liver tumors and number of lung metastases were measured (27). The animal experiments were approved by the Shanghai Medical Experimental Animal Care Committee (Approval date, December 2017). All procedures were performed following the Guide for the Care and Use of Laboratory Animals and complied with institutional ethical guidelines.

Drug dosage and mode of administration

Ang II was administered by an ALZET osmotic pump (ALZA, Cupertino, California, USA; model: 1004; sustained release rate: 0.11 μL/hour; duration: 4 weeks), which could release Ang II continuously, homogeneously and stably; avoid stress due to repeated administration; and protect the short half-life of the drug. Intragastric administration

was used for irbesartan (trade name: Aprovel; Sanofi, Paris, France).

Referring to previous studies and the conversions of doses between humans and animals, we used a dose of Ang II (Sigma, St. Louis, Missouri, USA) of 100 ng/kg/min for 4 weeks in nude mice and a dose of irbesartan (Aprovel) of 30 mg/kg/day (13,29,30). For cytology experiments, Ang II (CSNpharm, Chicago, Illinois, USA) was used at 0.1 μM, irbesartan (CSNpharm) was used at 1 μM, PD123319 (Abcam) was used at 1 μM, and SB203580 (p38/MAPK signaling pathway inhibitor, Absin, Shanghai, China) was used at 10 μM (14,15,31-35). When Ang II was combined with irbesartan, PD123319 or SB203580, the HCC cells were treated with these drugs for 0.5–2 hours before the addition of Ang II to the medium.

Tissue preparation from patients and follow-up

The KM plotter public database and cases from our hospital were used for survival analysis (36). After excluding cases with recurrent HCC or combined hepatocellular cholangiocarcinoma or those with a medical history of hepatic or other malignant tumor resection and/or perioperative mortality, 128 continuous HCC cases with MVI after curative resection were selected from the Department of Hepatology, Zhongshan Hospital, Fudan University, between January 2009 and December 2010. RNA from the HCC tissues was extracted from the frozen samples.

The data were extracted from medical records. The times to recurrence and overall survival were used as endpoint events. Follow-up and survival time calculations were performed as outlined in our previous report (23).

Statistical analysis

MedCalc software (version 18.2.1; Ostend, West-Vlaanderen, Belgium) and R software (version 3.5.2) were used to analyze the data (37,38). All statistical tests were 2-tailed and considered to be significantly different when $P<0.05$. Continuous variables were analyzed with a T test or a nonparametric test, and categorical variables were analyzed with the chi-square test, Fisher's exact test or the Wilcoxon signed-rank test, where appropriate. Kaplan–Meier and Cox proportional hazards regression analyses were used for survival. The optimal cutoff values of RNA expression of the adhesion factors were generated using R software with the *survminer* package (39).

Results

Irbesartan weakened the adhesion of HCC cells enhanced by Ang II

Selection of the HMHCC97-H and HCCLM3 HCC cell lines as models based on their expression profiles of AGTR-1

By immunohistochemical staining, we confirmed that AGTR-1 was expressed in human HCC tissues and that AGTR-2 was expressed weakly (*Figure 1A*). Next, the RNA and protein expression levels of AGTR-1 in commonly used HCC cell lines and the immortalized liver cell line L02 were analyzed (*Figure 1B,C*). The Hep-3B line had the highest expression level of AGTR-1, and the SMMC-7721 cell line had the lowest expression level of AGTR-1. The expression levels of AGTR-1 in the HMHCC97-H and HCCLM3 cell lines were relatively high. Considering the tumorigenic capacity of each HCC line in animals, the HMHCC97-H and HCCLM3 lines, which have highly aggressive and metastatic abilities, were finally selected as the main model cells in our study (40,41).

Irbesartan inhibited the growth and lung metastasis of HCC *in vivo*

The HMHCC97-H cell line was used to perform orthotopic tumor xenograft experiments in nude mice, and the HCCLM3 line was used to perform lung metastasis experiments (*Figure 2A,B*). The experimental animals were divided into 4 groups: control group, Ang II group, irbesartan group, and Ang II + irbesartan group (n=6 per group; no adverse events). The orthotopic tumor xenograft analysis indicated that the irbesartan group had the smallest tumor volume (367.7±189.2 mm^3) and smallest number of lung metastases (1.5±1.6/cm^3) and that the tumor volume in the Ang II group was the largest (1,238.7±675.9 mm^3) with the highest number of lung metastases (3.9±1.5/cm^3). Compared with that in the Ang II group, the tumor volume and lung metastases were significantly reduced in the Ang II + irbesartan group. Irbesartan significantly inhibited tumor growth ($P<0.001$) and reduced lung metastases from HCC ($P=0.036$). Lung metastasis analysis showed that the irbesartan group had the lowest tumor formation rate (16.7%) and fewest lung metastases (average of 0.2±0.4) and that the Ang II group had the highest formation rate of lung metastasis (100.0%) and most lung metastases (average, 1.5±0.5). Compared with the that of the control group, the average numbers of lung metastases in the Ang II group and irbesartan

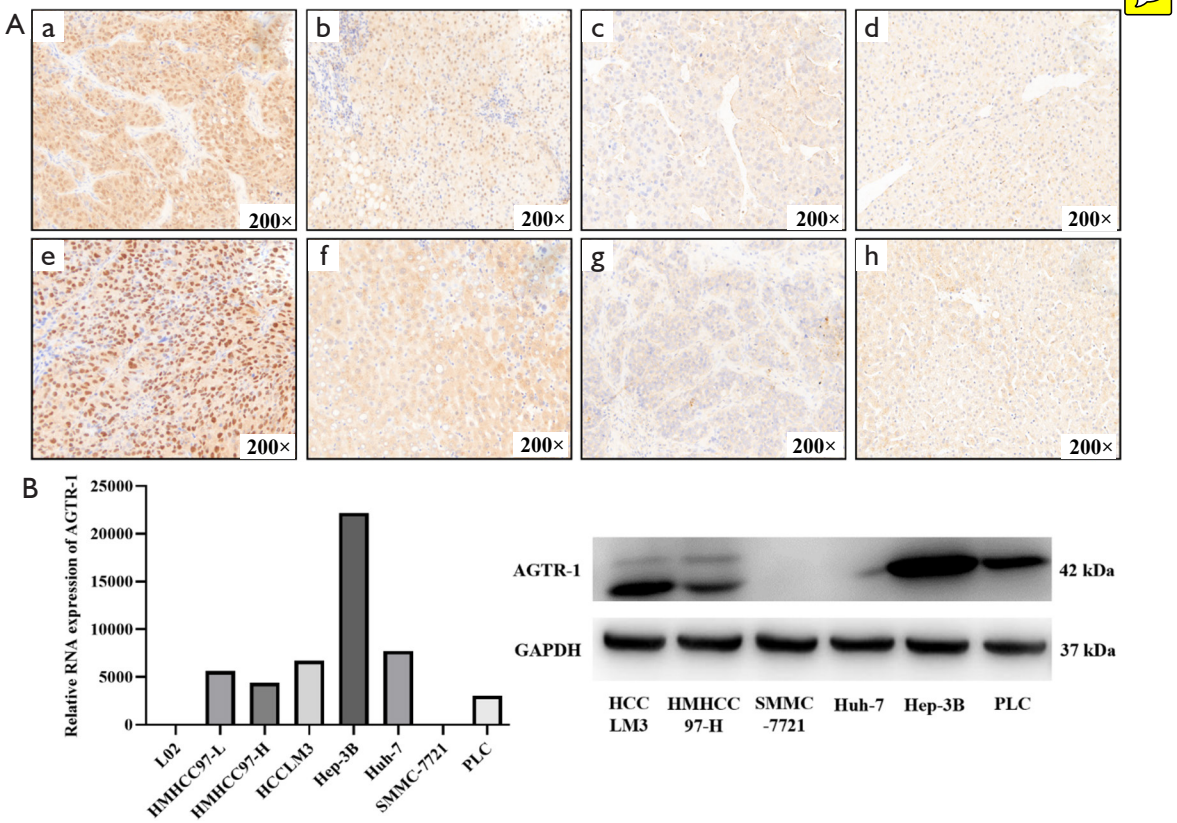

Figure 1 AGTR-1 was expressed in HCC tissues and HCC cell lines. (A) Immunohistochemistry staining of HCC and paired peritumoral tissues. AGTR-1 protein was expressed in HCC tissues on HCC cells (a and e) but weakly expressed in peritumoral tissue (b and f); AGTR-2 protein was expressed weakly on both HCC (c and g) and paired peritumoral tissues (d and h). (B) Real-time PCR and (C) Western blotting of AGTR-1 expression in HCC cell lines and a hepatocyte cell line (L02).

group were significantly different (P=0.038 and P=0.018, respectively). Compared with that in the Ang II group, the lung metastasis rate and number of lung metastases were reduced when Ang II was combined with irbesartan. These *in vivo* experiments further confirmed that Ang II could promote the growth and metastasis of HCC, which could be inhibited by irbesartan.

Human gene expression microarray confirmed that Ang II could affect the expression of adhesion molecules in HCC cells

RNA from SMMC-7721-AGTR-1-OE, Ang II-treated-HMHCC97-H, Ang II-treated HCCLM3 cells and the corresponding control HCC cells was analyzed with an Agilent human gene expression microarray. Compared with the respective control group, all three groups indicated that Ang II could affect the expression of adhesion molecules in HCC cells (Figure S3).

Irbesartan could inhibit the adhesion of HCC cells to endothelial cells enhanced by Ang II *in vitro* experiments

The HMHCC97-H and HCCLM3 HCC lines were used to perform cell adhesion experiments. Each HCC line was divided into six groups: the control group, Ang II group, irbesartan group, Ang II + irbesartan group, PD123319 (AGTR-2 blocker) group, and Ang II + PD123319 group. The corresponding treatments were administered for 48 hours, after which the adhesion between HCC cells and HUVECs in each group was measured. Compared with that in the control group, the adhesion of HCC cells to HUVECs was enhanced in the Ang II group ($P_{HMHCC97-H}$ =0.002; P_{HCCLM3} =0.011), and cell adhesion was decreased in the Ang II + irbesartan group but not in the Ang II + PD123319 group (*Figure 2C*). These results suggested that Ang II may enhance the adhesion of HCC cells through the AGTR-1 pathway. Compared with that in the control

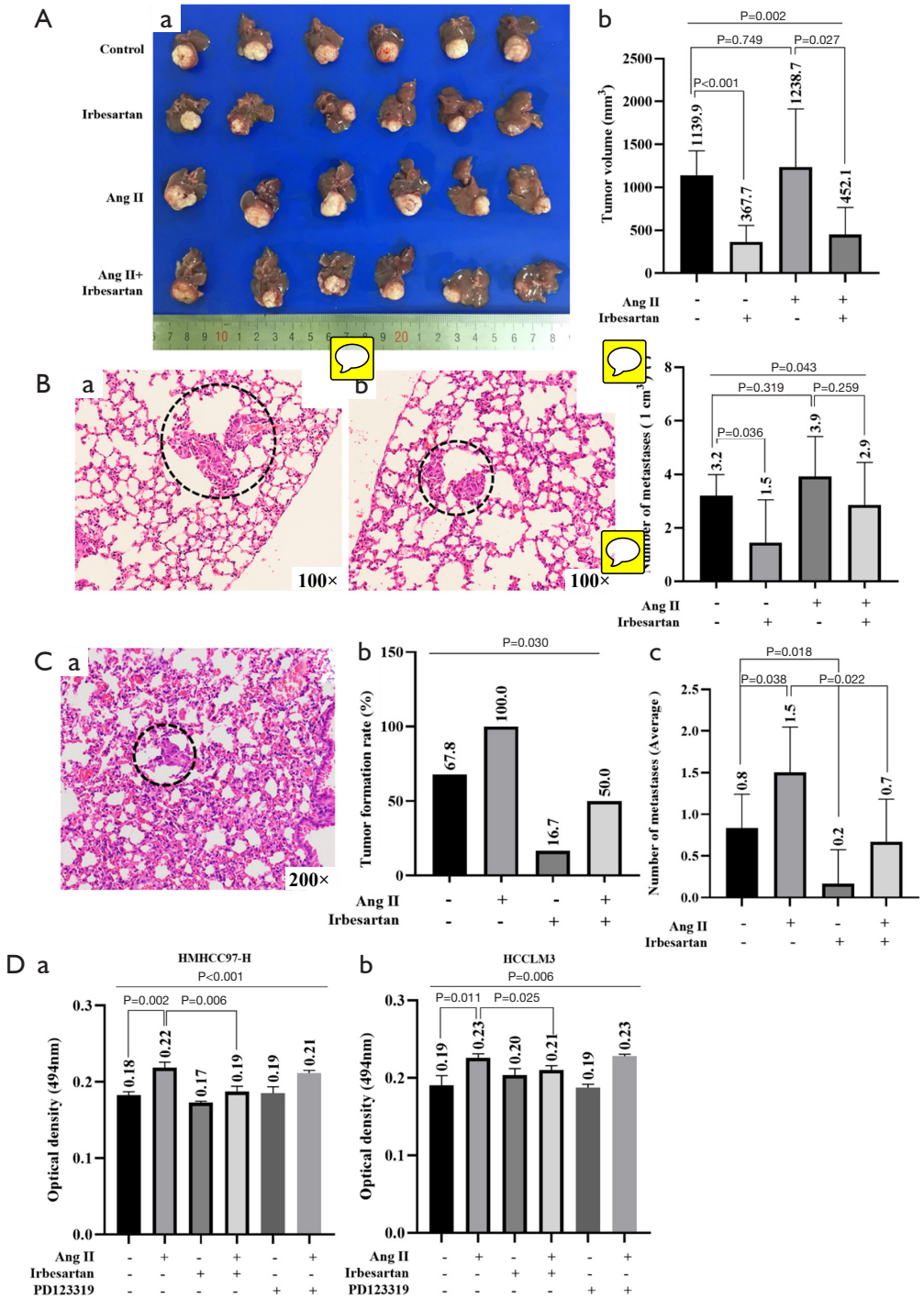

Figure 2 Irbesartan inhibited the growth and metastasis of HCC and weakened the adhesion of HCC cells to endothelial cells promoted by Ang II (~~metastasis foci, the dotted circle~~). (A) Irbesartan inhibited the growth of HCC in the liver (P<0.001), and Ang II could promote the growth of HCC but without statistical significance (P=0.749). (B) Irbesartan inhibited lung metastasis of HCC in the liver (P=0.036), and Ang II could promote the metastasis of HCC but without statistical significance (P=0.319). (C) Lung metastasis model: irbesartan and Ang II affected metastasis formation in HCC (Figure b; P=0.030). Irbesartan inhibited the lung metastasis of HCC (P=0.018), and Ang II promoted the metastasis of HCC (P=0.038), which could be inhibited by irbesartan (P=0.022). (D) Irbesartan, but not PD123319 (AGTR-2 blocker), could inhibit the adhesion of HCC cells to endothelial cells enhanced by Ang II in HMHCC97-H and HCCLM3 cells.

Table 1 Multivariate analysis of clinicopathological parameters associated with recurrence and survival for hepatocellular carcinoma with microvascular invasion

Clinicopathological parameters	HR	95% CI	P values
Time to recurrence			
HBsAg	0.49	0.28–0.86	0.013
γ-glutamyl transpeptidase	1.67	1.05–2.67	0.030
ICAM-2	0.54	0.34–0.86	0.010
VCAM-1	2.67	1.68–4.25	0.001
Overall survival			
AFP	1.76	1.07–2.88	0.025
Size	1.07	1.01–1.13	0.016
ICAM-2	0.40	0.23–0.70	0.001
NRCAM	0.59	0.39–0.90	0.014
VCAM-1	2.15	1.38–3.35	0.001

HR, hazard ratios; CI, confidence interval; ICAM-2, Intercellular cell adhesion molecule-2; NRCAM, Neuronal cell adhesion molecule; VCAM-1, Vascular cell adhesion molecule-1.

group, no significant differences in adhesion were found in the irbesartan and PD123319 alone groups, suggesting that the two inhibitors had no significant effect on the adhesion of HCC cells or enhancement of adhesion induced by Ang II (*Figure 2C*). Therefore, irbesartan could inhibit the adhesion of HCC cells enhanced by Ang II, and Ang II promoted adhesion mainly through AGTR-1 and not AGTR-2.

Irbesartan inhibited adhesion by reducing VCAM-1 in HCC cells

The adhesion molecule VCAM-1 was associated with a poor prognosis of HCC with MVI and was highly expressed in HCC tissues and lung metastases

Based on the human adhesion molecule array (RayBiotech, Atlanta, Georgia), 17 adhesion molecules were screened out, and the relationship between the RNA expression of the adhesion molecules in HCC tissues and prognosis was analyzed first with the KM plotter public database (Table S2). The adhesion molecules CEACAM-1, ICAM-1, ICAM-2, NRCAM, VCAM-1 and ICAM-3 were associated with poor outcomes in HCC patients with MVI (Figure S4). Subsequently, the relationships between these 6 adhesion molecules and the prognosis for HCC patients with MVI were reanalyzed and reverified with new cases from our hospital (Figure S5). Ultimately, we found that the high expression of VCAM-1 was an independent risk factor for both recurrence (hazard ratio =2.7; 95% confidence interval: 1.68–4.25; P<0.001) and survival (hazard ratio =2.2; 95% confidence interval: 1.38–3.35; P<0.001) in HCC patients with MVI after resection (*Table 1*; Table S3; *Figure 3A*,*B*). Additionally, immunohistochemical staining revealed that VCAM-1 was expressed in HCC tissues and lung metastases (*Figure 3C*).

Irbesartan could inhibit VCAM-1 in HCC cells activated by Ang II

To verify whether VCAM-1 was regulated by Ang II in HCC cells, the HMHCC97-H and HCCLM3 lines were used and divided into 4 groups: the control group, Ang II group, irbesartan group and Ang II + irbesartan group. The protein level of VCAM-1 in each group was assessed after the corresponding treatment measures were administered for 48 hours. VCAM-1 in the Ang II group increased, and the effect was inhibited by irbesartan in both the HMHCC97-H and HCCLM3 cell lines (*Figure 3D*,*E*). These data suggested that irbesartan could inhibit VCAM-1 in HCC cells activated by Ang II. In other words, VCAM-1 in HCC cells could be promoted by Ang II through the AGTR-1 pathway.

The expression of VCAM-1 in HCC cells was shown to be related to adhesion in *in vitro* and *in vivo* experiments

The expression of VCAM-1 at the RNA level in commonly used HCC cell lines was tested, and the HMHCC97-H and HCCLM3 cell lines, which have high VCAM-1 expression, were selected for the knockdown of VCAM-1 by lentiviral transfection (*Figure 4A*). Cell adhesion experiments were performed to test the adhesion of these two cell lines with VCAM-1 knockdown. The adhesion of HMHCC97-H and HCCLM3 cells to HUVECs decreased after VCAM-1 was knocked down ($P_{HMHCC97-H}$ =0.003; P_{HCCLM3} =0.006; *Figure 4B*-a and b). In vivo, lung metastasis model experiments showed that the number of metastases was significantly reduced after VCAM-1 knockdown ($P_{HMHCC97-H}$ =0.013; P_{HCCLM3} =0.018; *Figure 4B*-c and d; n=6 per group; no adverse events). The *in vitro* and *in vivo* results suggested that VCAM-1 expression in HCC cells was related to the adhesion of HCC cells to endothelial cells. Cells with a high expression of VCAM-1

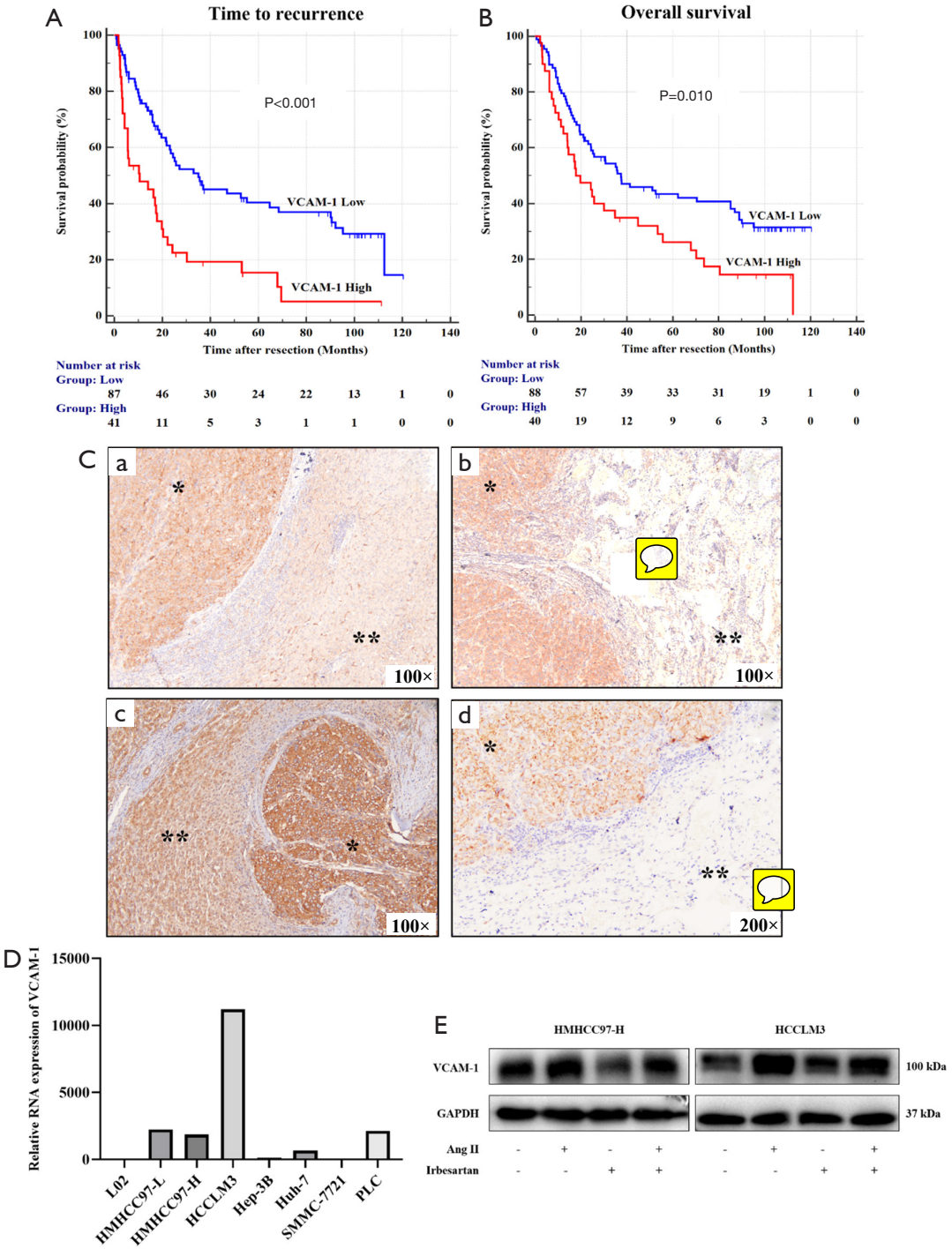

Figure 3 A high level of VCAM-1 (RNA) expression in HCC tissues was associated with a poor prognosis; this expression could be promoted by Ang II and blocked by irbesartan. Survival analysis: a high level of VCAM-1 expression was found to be an independent risk factor for recurrence (A) and survival (B) in HCC patients with microvascular invasion after resection. (C) Immunohistochemical staining of HCC tissues and lung metastases: Case 1, primary HCC in the liver (a) and lung metastases (b); Case 2, primary HCC in the liver (c) and lung metastases (d). VCAM-1 was expressed on HCC tissues and HCC cells located in metastases and at a higher level than that in peritumoral tissues; *, tumor; **, metastases. (D) Real-time PCR: VCAM-1 was expressed on HCC cell lines. (E) The expression of VCAM-1 could be promoted by Ang II in HMHCC97-H and HCCLM3 cells and was inhibited by irbesartan (Ang II=0.1 μM, irbesartan 1 μM).

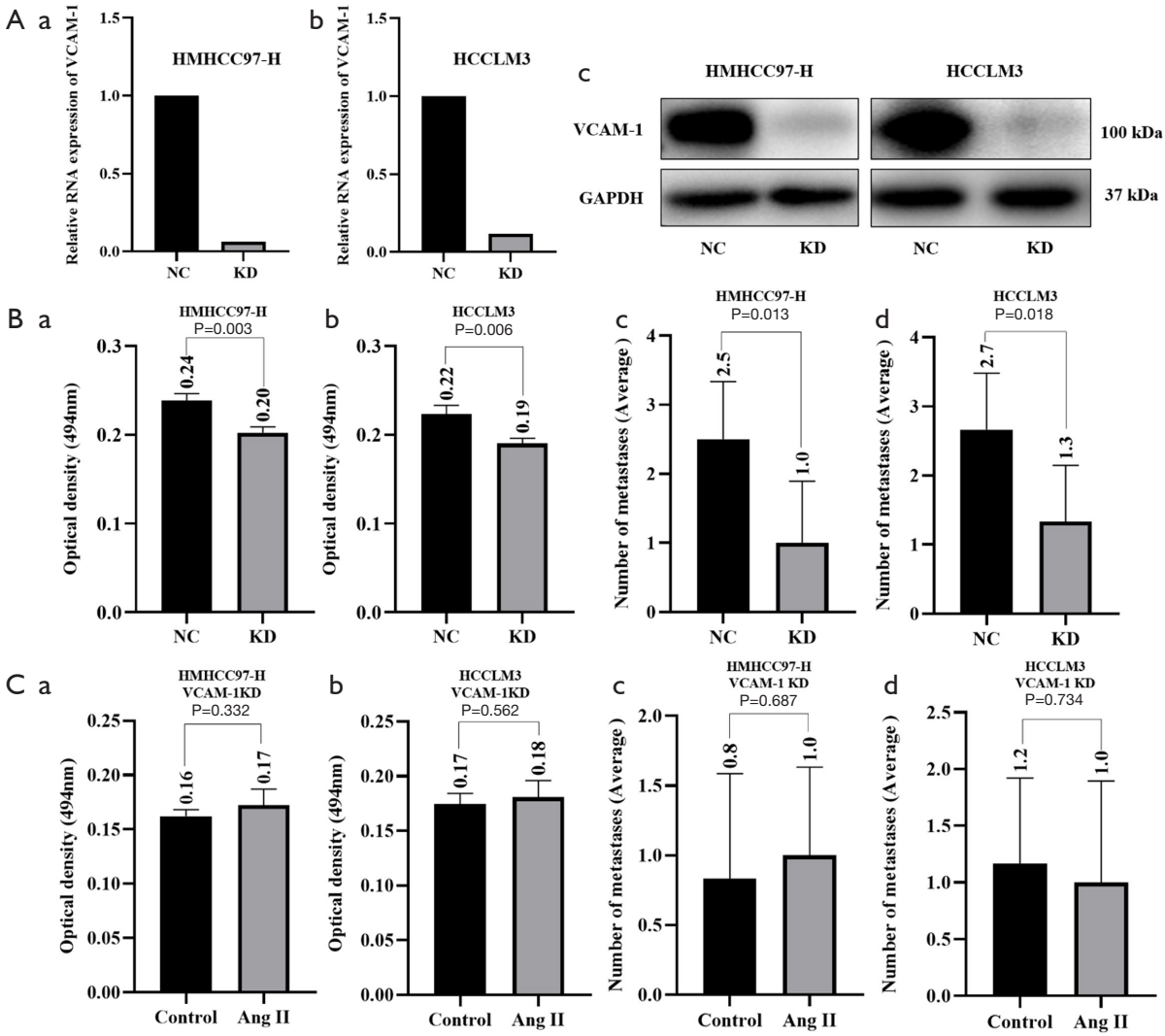

Figure 4 VCAM-1 played a role in cell adhesion. Ang II enhanced adhesion mainly by promoting the expression of VCAM-1 in HCC cells. (A) Real-time PCR and Western blotting: verification of VCAM-1 knockdown in HMHCC97-H and HCCLM3 cells. (B) Cell adhesion experiment and lung metastasis model: after VCAM-1 was knocked down in HMHCC97-H and HCCLM3 cells, the adhesion of HCC cells to HUVECs decreased (a, b), and the number of lung metastases was significantly reduced (c, d). (C) Cell adhesion experiment and lung metastasis model: no significant difference was observed in absorbance (a, b) or in the number of lung metastases (Figure c, d) between the control and Ang II groups in HMHCC97-H and HCCLM3 cells with VCAM-1 knockdown. NC, control group; KD, VCAM-1 knockdown group.

had a strong adhesion ability.

Ang II-enhanced adhesion mainly depended on VCAM-1 in HCC cells in *in vitro* and *in vivo* experiments

The HMHCC97-H and HCCLM3 cell lines with knocked down VCAM-1 expression were divided into the control and Ang II groups. The cell adhesion experiments showed no significant difference in the absorbance between the control and Ang II groups in these HCC cell lines (*Figure 4C*-a and b). Furthermore, the lung metastasis experiments demonstrated that the number of lung metastases was not significantly different between the control and Ang II groups (*Figure 4C*-c and d; n=6 per group; no adverse events). When VCAM-1 was knocked down in HCC lines, the adhesion and metastases enhanced by Ang II were suppressed, indicating that Ang II-enhanced adhesion was mainly dependent on promoting the expression of VCAM-1 in HCC cells.

Irbesartan inhibited VCAM-1 in HCC cells by reducing p38/MAPK phosphorylation activated by Ang II

Ang II could activate the p38/MAPK pathway in HCC cells, an effect that was blocked by its pathway inhibitor SB203580

Reports in the literature have indicated that the p38/MAPK and NF-κB/p65 pathways play important roles in regulating adhesion molecules activated by Ang II in endothelial cells (42-44). Therefore, we focused on the phosphorylated protein levels of p38, p65, JNK and ERK in HMHCC97-H and HCCLM3 cells after Ang II treatment. The phosphorylation of p38 significantly increased after Ang II treatment and was blocked by the p38/MAPK pathway inhibitor SB203580 (*Figure 5A*,*B*,*C*).

The p38/MAPK pathway was involved in the Ang II promotion of VCAM-1 in HCC cells

The HMHCC97-H and HCCLM3 lines were divided into 4 groups: the control group, Ang II group, SB203580 group and Ang II + SB203580 group. The protein levels of VCAM-1 in each group were assessed. The Ang II group had the highest expression level of VCAM-1. When Ang II was combined with SB203580, VCAM-1 expression was significantly reduced, indicating that SB203580 could inhibit the promotion of VCAM-1 by Ang II (*Figure 5D*). This finding suggested that the p38/MAPK phosphorylation pathway was involved in the Ang II promotion of VCAM-1 in HCC cells.

The Ang II-activated p38/MAPK pathway could be inhibited by irbesartan

The HMHCC97-H and HCCLM3 lines were divided into 6 groups: the control group, Ang II group, irbesartan group, Ang II + irbesartan group, PD123319 (AGTR-2 blocker) group and Ang II + PD123319 group. The phosphorylation of p38 was assessed, and irbesartan was found to inhibit the phosphorylation of p38 activated by Ang II, while PD123319 had no significant effect on this phosphorylation (*Figure 5E*). This finding not only suggested that Ang II activated the p38/MAPK pathway mainly through the AGTR-1 receptor but also indicated that irbesartan inhibited VCAM-1 by reducing p38/MAPK phosphorylation activated by Ang II in HCC cells.

Discussion

A high risk of recurrence and metastasis and a lack of effective anti-recurrence treatments are the bottlenecks restricting surgical efficacy in HCC. Based on the previous discovery that Ang II inhibitors improve prognosis and reduce metastasis, we found that irbesartan attenuated metastasis by inhibiting Ang II-activated VCAM-1 via the p38/MAPK pathway in HCC.

Ang II inhibitors include angiotensin-converting enzyme inhibitors (ACEIs, such as captopril and enalapril) and angiotensin receptor blockers (ARBs, such as irbesartan and valsartan) (45). An ARB, irbesartan, rather than an ACEI, was used in our study because it is the most commonly used antihypertensive drug in HCC patients with primary hypertension at our hospital and serves as a selective AGTR-1 receptor blocker, facilitating analysis of the subsequent mechanism. More importantly, ACEIs, but not ARBs, have been shown to cause the accumulation of bradykinin, which has a cancer-promoting effect (45,46).

In this study, the first step was to identify that AGTR-1 was expressed in HCC tissues (*Figure 1A*). Subsequently, the HMHCC97-H and HCCLM3 cell lines, which have a relatively high expression of AGTR-1, better tumorigenicity, high invasiveness and metastasis potential, were selected for *in vivo* experiments (*Figure 1B*,*C*). The orthotopic liver transplantation experiment in nude mice found that irbesartan inhibited tumor growth in the liver, which is similar to observations in previous studies (14). We also found that irbesartan inhibited lung metastases promoted by Ang II (*Figure 2B*). The tumor size in the liver may affect the formation of lung metastases. Hence, lung metastasis experiments were performed, further verifying that Ang II promoted the lung metastasis of HCC, which could be inhibited by irbesartan (*Figure 2C*).

Ang II, which has a certain organ specificity in terms of its physiological synthesis, comes from angiotensin I, which is catalyzed by angiotensin-converting enzymes on lung endothelial cells and is not only a part of the lung microenvironment but also a shaping factor. Ang II has been shown to stimulate endothelial cells to express adhesion molecules, promoting CTCs to adhere to endothelial cells and form metastases; this stimulatory effect can be blocked by Ang II inhibitors (11,13). Thus, we asked whether Ang II also stimulated the expression of adhesion molecules of HCC cells and promoted metastases in the same way and, if so, whether this stimulation was blocked by Ang II inhibitors. A human gene expression microarray was used to analyze Ang II-treated HMHCC97-H and HCCLM3 cells, as well as SMMC-7721 cells overexpressing the AGTR-1 receptor, and we confirmed that Ang II affected the

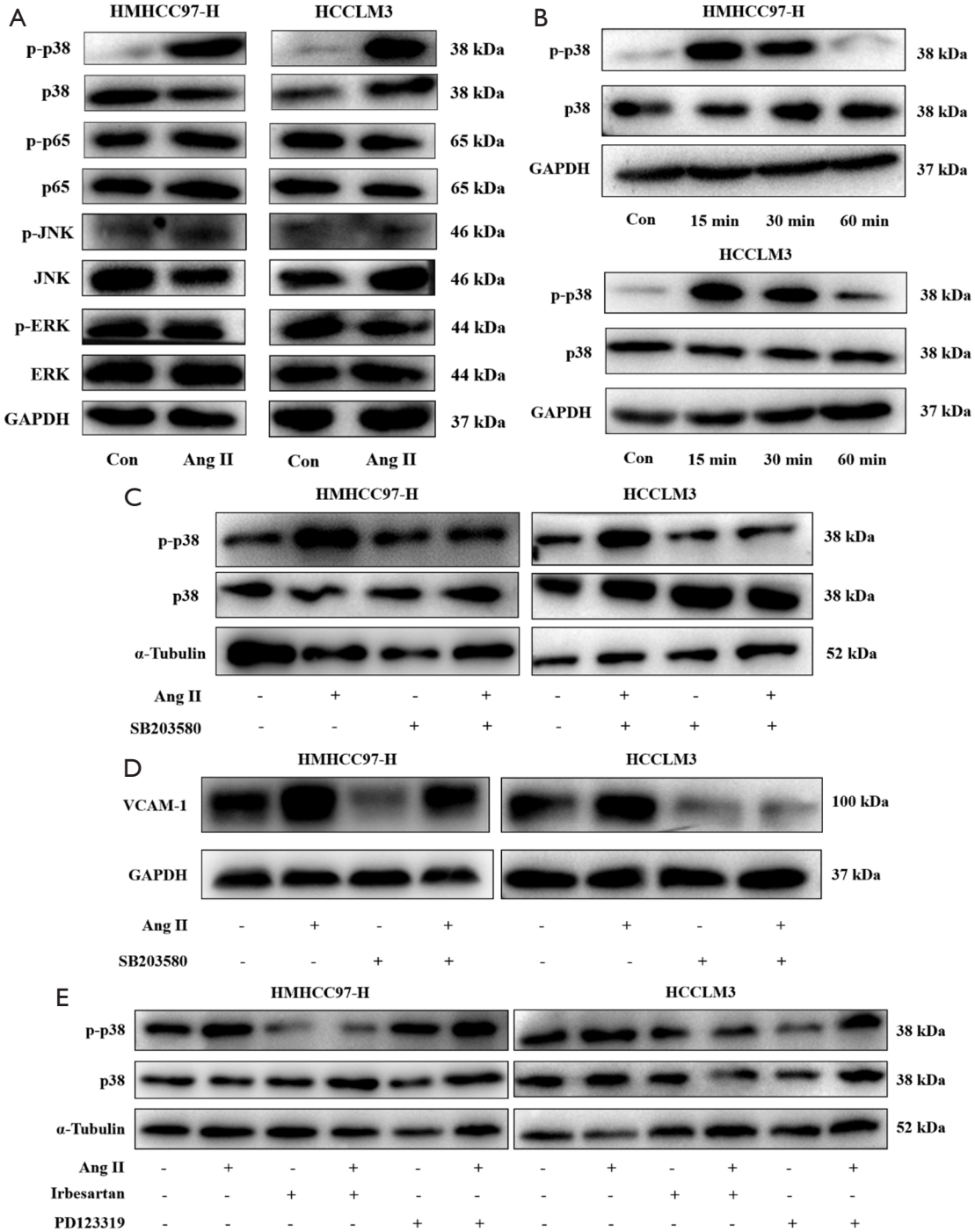

Figure 5 Irbesartan reduced p38/MAPK phosphorylation and inhibited VCAM-1 expression in HCC cells. (A) The phosphorylation of p38 was significantly increased after Ang II treatment in HMHCC97-H and HCCLM3 cells. (B) The phosphorylation of p38 activated by Ang II gradually decreased with time in HMHCC97-H and HCCLM3 cells. (C) The phosphorylation of p38 activated by Ang II could be inhibited by SB203580 (p38/MAPK pathway inhibitor) in HMHCC97-H and HCCLM3 cells. (D) The expression of VCAM-1 promoted by Ang II in HMHCC97-H and HCCLM3 cells could be inhibited by SB203580, suggesting that the p38/MAPK pathway was involved in the Ang II promotion of VCAM-1 expression in HCC cells. (E) Irbesartan, but not PD123319 (AGTR-2 inhibitor), could inhibit the phosphorylation of p38 activated by Ang II. Con, control group.

expression of adhesion molecules in HCCs. Cell adhesion experiments indicated that Ang II could promote the adhesion of HCC cells to endothelial cells, an effect that was inhibited by irbesartan (*Figure 2D*).

Numerous tumor-related adhesion molecules have been identified. Therefore, we wanted to determine the key targets regulated by Ang II and irbesartan. The prerequisite for adhesion is that tumor cells have been in the circulatory system, and MVI is a direct evidence of hematogenous metastasis for HCC (24). Hence, the prognostic values of 17 adhesion molecules based on a human adhesion molecule array for HCC patients with MVI were analyzed with the KM plotter public database and clinical case data from our hospital. Ultimately, VCAM-1 was found to be an independent risk factor for recurrence and survival in HCC patients with MVI (*Table 1*; *Figure 3A*,*B*).

VCAM-1 is a 110-kDa transmembrane sialic acid glycoprotein belonging to the immunoglobulin superfamily of proteins. VCAM-1 can be expressed on tumor cells, endothelial cells and immune cells and plays an important role in tumor metastasis (47,48). Particularly in breast cancer, VCAM-1 participates in lymphatic metastasis, lung metastasis, bone metastasis and brain metastasis through different mechanisms (49). Therefore, VCAM-1 is a target not only for tumor therapy but also for metastasis detection by imaging (50).

However, we also wanted to determine whether VCAM-1 was expressed in HCC tissues, regulated by Ang II and irbesartan, and involved in the adhesion of HCC cells to endothelial cells. Our immunohistochemical results showed that VCAM-1 was expressed in HCC tissues and lung metastases (*Figure 3C*). Furthermore, Western blot experiments confirmed that Ang II could promote the expression of VCAM-1 in HCC cells, which could be inhibited by irbesartan (*Figure 3E*). Finally, cell adhesion experiments and lung metastasis experiments confirmed that the expression of VCAM-1 in HCC cells was related to adhesion (*Figure 4B*). The effect of Ang II mainly depended on promoting the expression of VCAM-1 in HCC cells to enhance the adhesion of HCC cells to endothelial cells (*Figure 4C*).

We also wanted to know how Ang II and irbesartan affected the expression of VCAM-1 in HCC cells. In cardiovascular diseases, VCAM-1 is an important inflammatory factor in endothelial cell damage induced by Ang II through the p38/MAPK and/or NF-κB/p65 pathways (42-44). In Ang II-treated HCC cells, the phosphorylation of p38 was enhanced most significantly, decreased gradually over time and was inhibited by its inhibitor, SB203580 (*Figure 5A*,*B*,*C*). These results supported the hypothesis that Ang II activates the p38/MAPK pathway in HCC cells. Furthermore, we confirmed that the p38/MAPK pathway was involved in the promotion of VCAM-1 by Ang II in HCC cells (*Figure 5D*). It can be easily speculated that irbesartan inhibits VCAM-1, probably because it inhibits the phosphorylation of p38 activated by Ang II. We confirmed that the phosphorylation of p38 activated by Ang II could be inhibited by irbesartan but not by PD123319 (AGTR-2 blocker; *Figure 5E*). Therefore, we finally speculated that Ang II could activate the p38/MAPK pathway through the AGTR-1 receptor pathway to promote VCAM-1 in HCC cells, which was blocked by irbesartan.

Our study possessed several limitations. First, the drug doses used in the cytology and animal experiments mainly relied on previous studies and were not directly determined. Second, cytology and animal experiments on VCAM-1 were primarily performed to establish knockdown HCC lines, which should be verified by the overexpression of VCAM-1 in HCC lines. Third, the results of the study should be verified with more HCC cell lines.

In conclusion, we found that the Ang II inhibitor irbesartan blocked the binding of Ang II and AGTR-1, reduced the phosphorylation of the p38/MAPK pathway activated by Ang II, inhibited VCAM-1 expression in HCC cells, weakened the adhesion of HCC cells to endothelial cells and attenuated metastasis. The high expression level of VCAM-1 in HCC tissues is an independent risk factor for the poor prognosis of HCC patients with MVI.

Acknowledgments

Funding: This work was supported by the Leading Investigator Program of Shanghai municipal government (17XD1401100), the National Key Basic Research Program (973 Program; 2015CB554005) from the Ministry of Science and Technology of China, and the National Natural Science Foundation of China (81672326).

Footnote

Reporting Checklist: The authors have completed the ARRIVE Checklist. Available at http://dx.doi.org/10.21037/atm-20-5293

Data Sharing Statement: Available at http://dx.doi.org/10.21037/atm-20-5293

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/atm-20-5293). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. It was approved by the Clinical Research Ethics Committee of Zhongshan Hospital, Fudan University, Shanghai, China (Approval No.: B2012-010) and the individual consent for this retrospective analysis was waived. The animal experiments were approved by the Shanghai Medical Experimental Animal Care Committee (Approval date, December 2017). All procedures were performed following the Guide for the Care and Use of Laboratory Animals and complied with institutional ethical guidelines.

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Cite this article as: Feng LH, Sun HC, Zhu XD, Zhang SZ, Li XL, Li KS, Liu XF, Lei M, Li Y, Tang ZY. Irbesartan inhibits metastasis by interrupting the adherence of tumor cell to endothelial cell induced by angiotensin II in hepatocellular carcinoma. Ann Transl Med 2021. doi: 10.21037/atm-20-5293

Supplementary

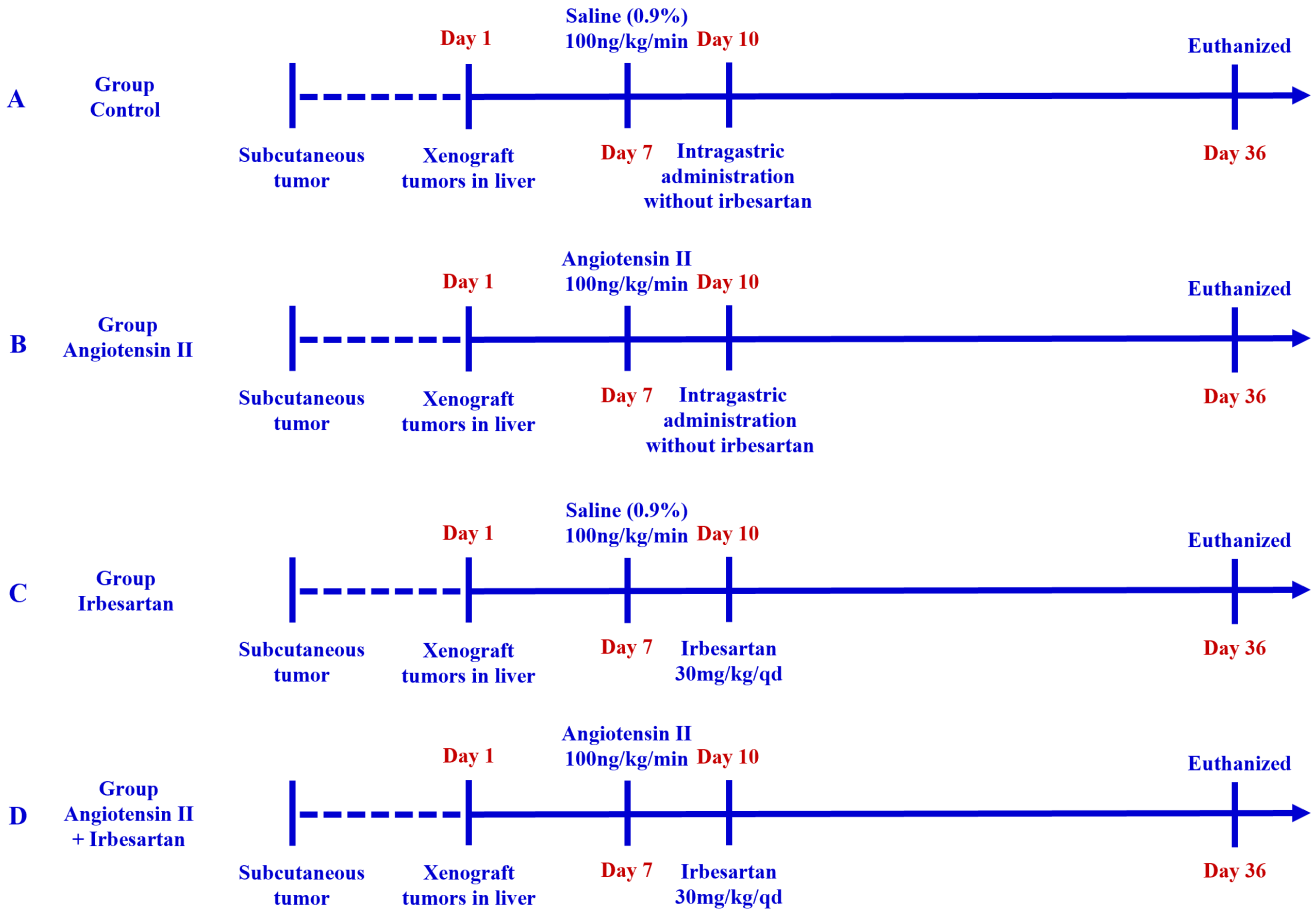

Figure S1 The groups and time axes of animal experiments (Orthotopic tumor xenograft model).

http://dx.doi.org/10.21037/atm-20-5293

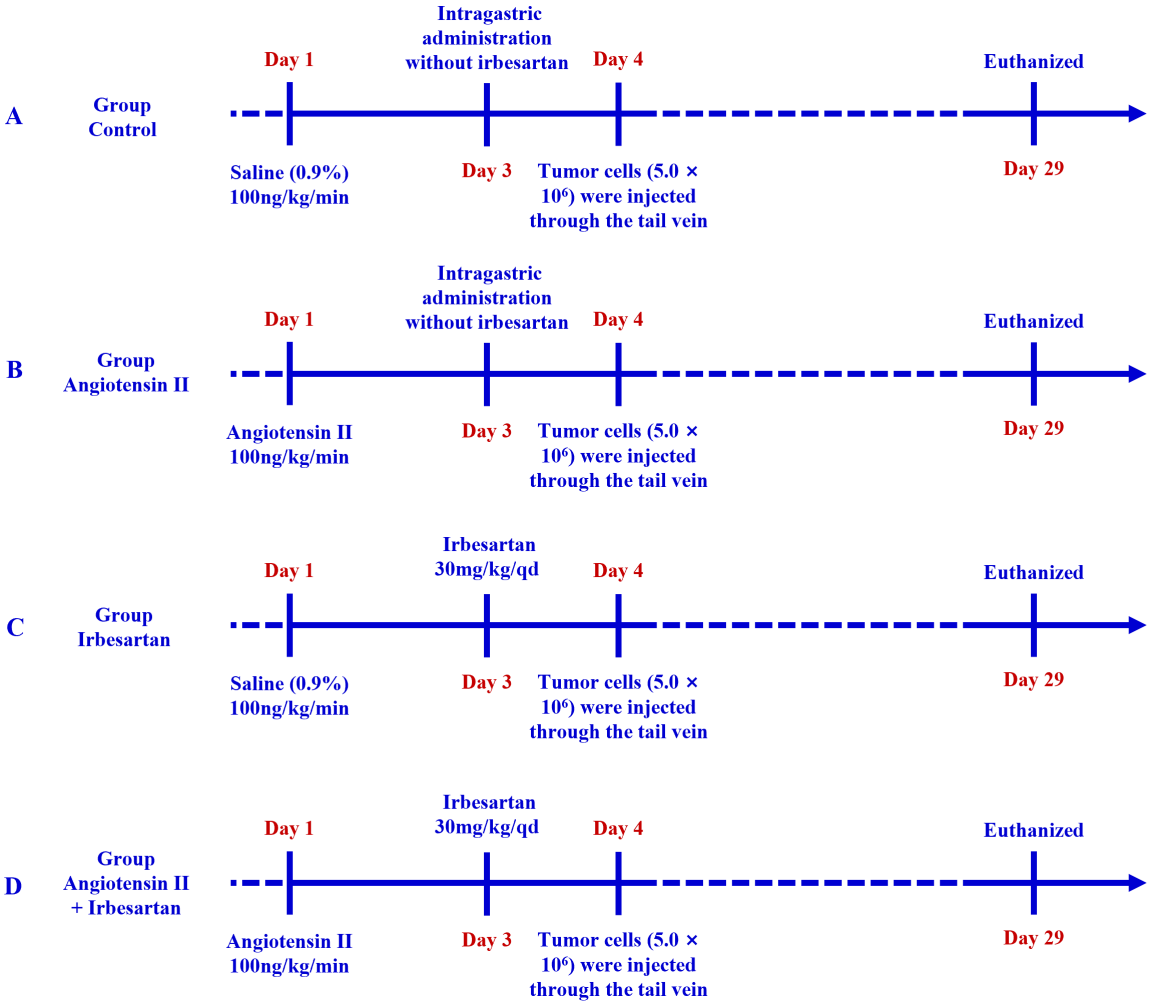

Figure S2 The groups and time axes of animal experiments (Lung metastasis model).

http://dx.doi.org/10.21037/atm-20-5293

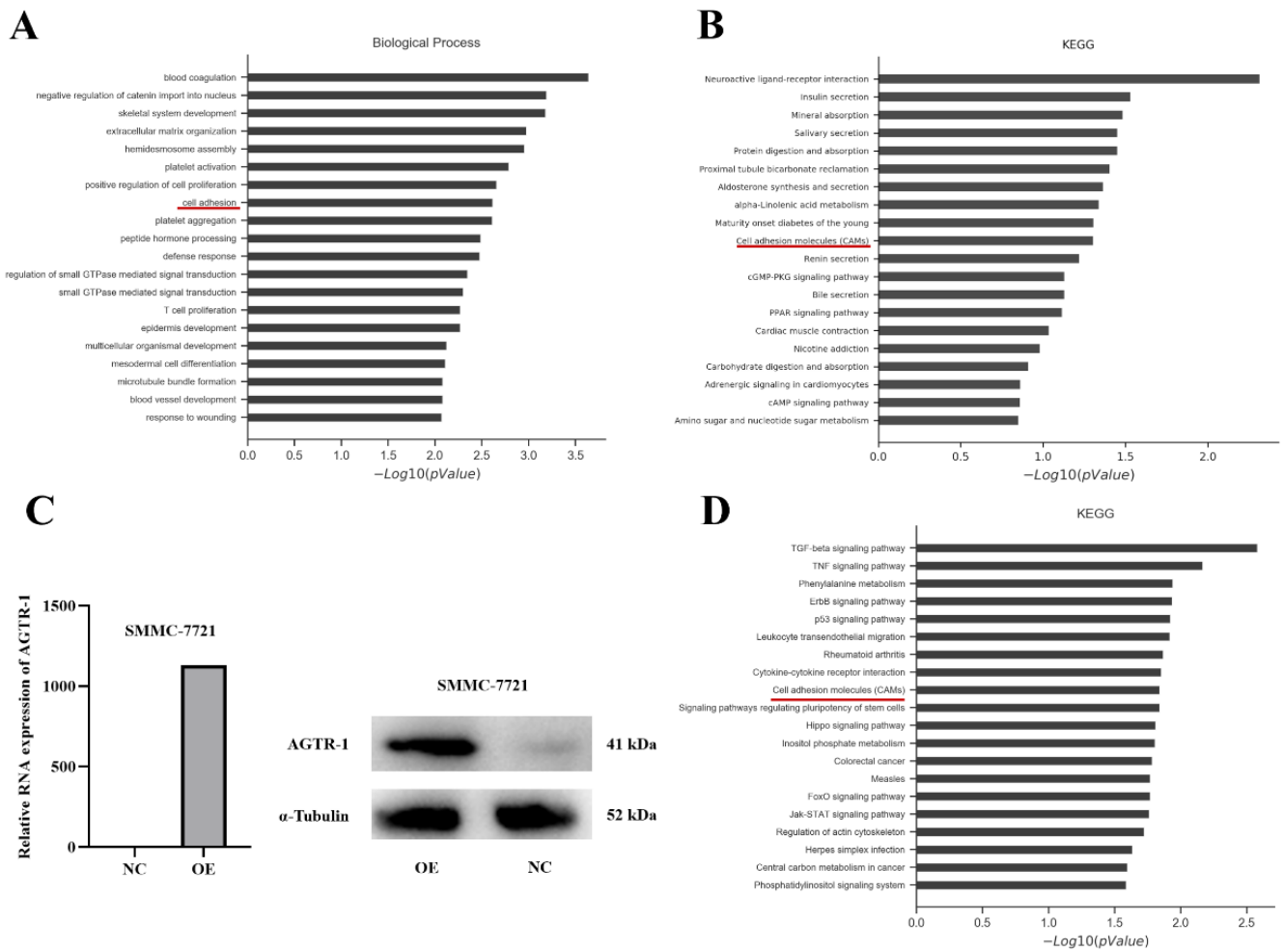

Figure S3 Human gene expression microarray: Ang II could affect the expression of adhesion molecules in HCC cells. (A) Biological behavior analysis on Ang II-treated HMHCC97-H cells and control HMHCC97-H cells. (B) Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis on Ang II-treated HCCLM3 cells and control HCCLM3 cells. (C) Real-time PCR and Western blot: verification of AGTR-1 overexpression in SMMC-7721 cells. (D) KEGG analysis on SMMC-7721-AGTR-1-overexpressed cells and SMMC-7721-Control cells. NC, control group; OE, AGTR-1 overexpression group.

http://dx.doi.org/10.21037/atm-20-5293

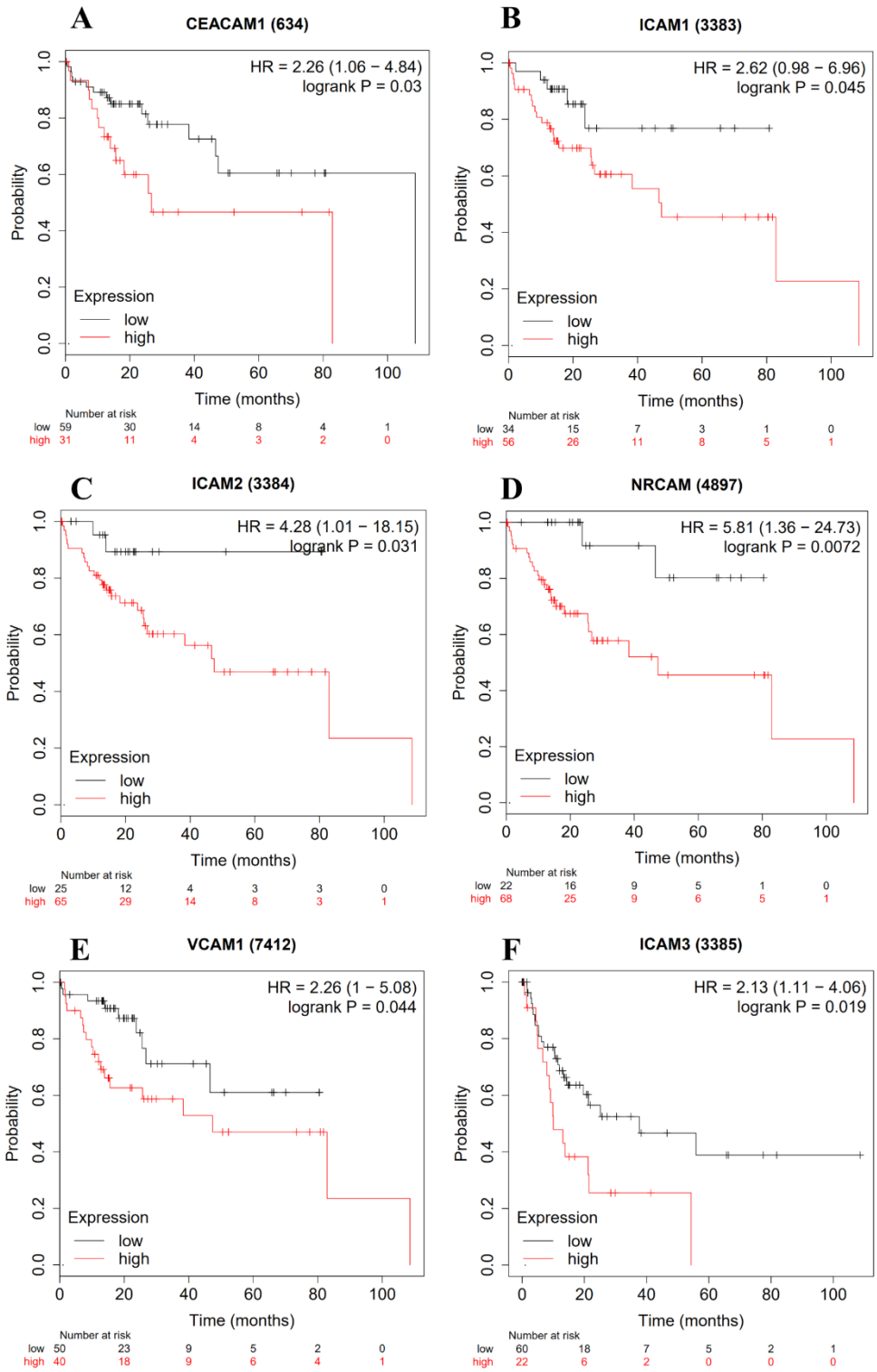

Figure S4 The relationships between the expression of six adhesion molecules in HCC tissues and prognosis in HCC patients with microvascular invasion (KM-Plotter public database, n=90). (A) CEACAM-1, overall survival; (B) ICAM-1, overall survival; (C) ICAM-2, overall survival; (D) NRCAM, overall survival; (E) VCAM-1, overall survival; (F) ICAM-3, recurrence.

http://dx.doi.org/10.21037/atm-20-5293

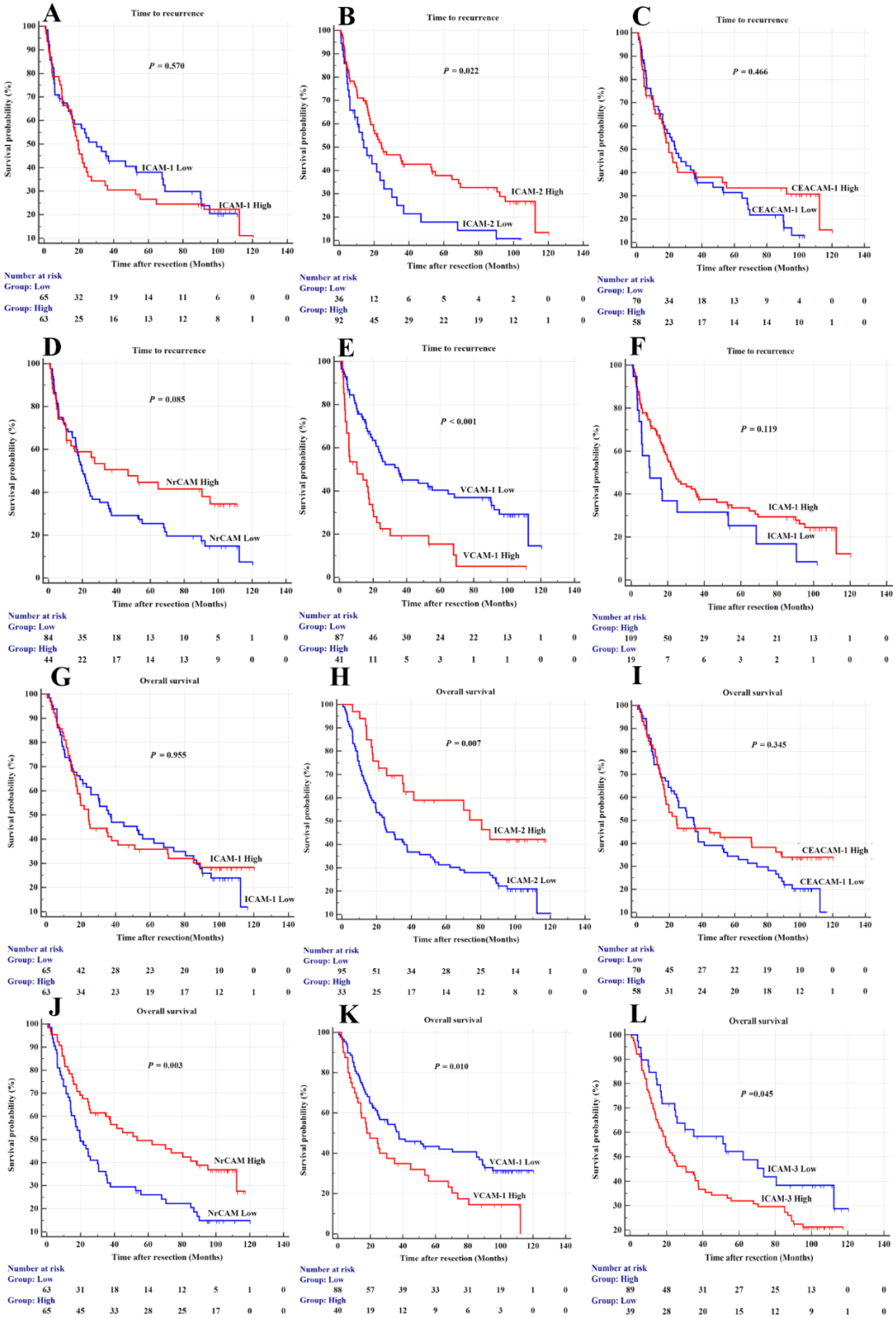

Figure S5 The relationships between the expression of five adhesion molecules in HCC tissues and overall survival in HCC patients with microvascular invasion (clinical cases from our hospital, n=128). Recurrence: (A) CEACAM-1; (B) ICAM-1; (C) ICAM-2; (D) NRCAM; (E) VCAM-1; (F) ICAM-3. Overall survival: (G) CEACAM-1; (H) ICAM-1; (I) ICAM-2; (J) NRCAM; (K) VCAM-1; (L) ICAM-3.

http://dx.doi.org/10.21037/atm-20-5293

Table S1 PCR primers and sequences

Name	Primer	Sequences (5'→3')
AGTR-1	Forward	CACTGGCTGACTTATGCTTTTT
AGTR-1	Reverse	TAGAAACACACTAGCGTACAGG
VCAM-1	Forward	CAGGCTGGAGATAGACTTACTG
VCAM-1	Reverse	CCTCAATGACAGGAGTAAAGGT
CEACAM-1	Forward	CCACAGTCAAGACGATCATAGT
CEACAM-1	Reverse	TCATCTTGTTAGGTGGGTCATT
ICAM-1	Forward	TGCAAGAAGATAGCCAACCAAT
ICAM-1	Reverse	GTACACGGTGAGGAAGGTTTTA
ICAM-2	Forward	ATGAGACTCTGCACTATGAGAC
ICAM-2	Reverse	GCTGAGTGTTTGTGAAAGATGT
NRCAM	Forward	CGGAGCTGCAGTTTCTAATAAC
NRCAM	Reverse	TGCAGGGAAGTACTAAAGACTG

AGTR-1, angiotensin II type 1 receptor; VCAM-1, vascular cell adhesion molecule-1; CEACAM-1, CEA cell adhesion molecule-1; ICAM-1, intercellular cell adhesion molecule-1; ICAM-2, intercellular cell adhesion molecule-2; NRCAM, neuronal cell adhesion molecule.

Table S2 Seventeen adhesion molecules and prognosis of hepatocellular carcinoma with microvascular invasion after resection (KM-plotter public database)

Number	Adhesion molecules	Abbreviation	OS (n=90)	RFS (n=90)
1	Activated leukocyte cell adhesion molecule	ALCAM	-	-
2	Basal Cell Adhesion Molecule	BCAM	-	-
3	CEA cell adhesion molecule-1	CEACAM-1	↑	↓
4	Epithelial Cell Adhesion Molecule	EpCAM	↓	-
5	Epithelial cadherin	E-Cadherin	-	-
6	Intercellular cell adhesion molecule-1	ICAM-1	↑	-
7	Intercellular cell adhesion molecule-2	ICAM-2	↑	-
8	Intercellular cell adhesion molecule-3	ICAM-3	-	↑
9	Neuronal cell adhesion molecule-1	NCAM-1	-	-
10	Neuronal cell adhesion molecule	NRCAM	↑	↓
11	Placental-cadherins	P-Cadherin	-	↓
12	Platelet endothelial cell adhesion molecule-1	PECAM-1	-	↓
13	Endothelial selectin	E- Selectin	-	-
14	Leukocyte selectin	L-Selectin	-	-
15	Platelet selectin	P-Selectin	-	-
16	Vascular cell adhesion molecule-1	VCAM-1	↑	-
17	Vascular endothelial cadherin	VE-Cadherin	↓	-

OS, overall survival; RFS, recurrence free survival; MVI, microvascular invasion; "↑", High expression is associated with poor prognosis; "↓", Low expression is associated with poor prognosis; "-", no difference in prognosis.

http://dx.doi.org/10.21037/atm-20-5293

Table S3 Univariate analysis of clinicopathological parameters associated with recurrence and survival in hepatocellular carcinoma patients with microvascular invasion

Clinicopathological parameters	Number	Time to recurrence	Overall survival
		P values	P values
Gender (Man/Female)	113/15	0.137	0.696
Age (Year)	128	0.662	0.637
Transfusion (Yes/No)	15/113	0.342	0.529
HBsAg (Positive/Negative)	19/109	0.004	0.044
Pringle maneuver (Yes/No)	38/90	0.867	0.651
AFP >20 μg/L (Yes/No)	93/35	0.564	0.047
CA19-9 >40 μ/mL (Yes/No)	27/101	0.732	0.799
ALT >50 U/L (Yes/No)	35/93	0.333	0.499
AST >50 U/L (Yes/No)	24/104	0.349	0.863
GGT >60 U/L (Yes/No)	77/51	0.027	0.065
ALP >135 U/L (Yes/No)	18/110	0.529	0.616
Total bilirubin (μmol/L)	128	0.365	0.470
Albumin (g/L)	128	0.693	0.426
Hemoglobin (g/L)	128	0.691	0.354
Platelet (10^9/L)	128	0.099	0.382
Prothrombin time (second)	128	0.642	0.252
Size (cm)	128	0.270	0.014
Singe nodule (Yes/No)	81/47	0.999	0.123
Intact capsule (Yes/No)	53/75	0.524	0.269
Differentiation (I-II/III-IV)	70/58	0.643	0.093
Cirrhosis (Yes/No)	74/54	0.221	0.057
Satellite nodules (Yes/No)	26/102	0.268	0.055
Tumor thrombus (Yes/No)	30/98	0.541	0.048
ICAM-1 (High/Low)	63/65 (63/65) †	0.570	0.955
ICAM-2 (High/Low)	92/36 (33/95) †	0.022	0.007
ICAM-3 (High/Low)	19/109(39/89) †	0.118	0.045
CEACAM-1 (High/Low)	58/70 (58/70) †	0.466	0.345
NRCAM (High/Low)	44/84 (65/63) †	0.085	0.003
VCAM-1 (High/Low)	41/87 (40/88) †	0.001	0.010

†, group of overall survival. ALT, alanine transaminase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, γ-glutamyl transpeptidase; ICAM-1, Intercellular cell adhesion molecule-1; ICAM-2, Intercellular cell adhesion molecule-2; ICAM-3, Intercellular cell adhesion molecule-3; CEACAM-1, CEA cell adhesion molecule-1; NRCAM, Neuronal cell adhesion molecule; VCAM-1, Vascular cell adhesion molecule-1.

http://dx.doi.org/10.21037/atm-20-5293