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The association of renin-angiotensin system genes with the progression of hepatocellular carcinoma

Guanxiong Ye, Yong Qin*, Xianghong Lu, Xiangdong Xu, Shengqian Xu, Chengjun Wu, Xinmei Wang, Shi Wang, Debiao Pan

Department of Hepatobiliary Surgery, The People's Hospital of Lishui, The Sixth Affiliated Hospital of Wenzhou Medical University, Lishui 323000, PR China

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ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide. Angiogenesis is reported to play a pivotal role in the occurrence, development and metastasis of HCC. The renin-angiotensin system (RAS) is involved in the regulation of angiogenesis. Here, based on the analysis of HCC datasets from Gene Expression Omnibus (GEO) database and The Cancer Genome Atlas (TCGA), we found that there was a negative correlation between the mRNA levels of angiotensin converting enzyme 2 (ACE2) and CD34. To explore the association of RAS with the progression from fibrosis to cirrhosis to HCC, liver specimens and serum samples were collected from patients with hepatic fibrosis, cirrhosis and HCC. Relative hepatic mRNA levels of CD34 and ACE2 were determined by real-time PCR, and the serum concentrations of Angiotensin II (Ang II), Ang (1–7) and vascular endothelial growth factor (VEGF) were detected by ELISA. We found that ACE2 mRNA was gradually decreased, while CD34 mRNA was progressively increased with the increasing grade of disease severity. Concentrations of Ang II, Ang (1–7) and VEGF were higher in the sera of patients than in that of healthy volunteers. These proteins' concentrations were also progressively increased with the increasing grade of disease severity. Moreover, a positive correlation was found between VEGF and Ang II or Ang (1–7), while negative correlation was observed between mRNA levels of CD34 and ACE2. More importantly, patients with higher level of ACE2 expression had longer survival time than those with lower level of ACE2 expression. Taken together, our data suggests that the low expression of ACE2 may be a useful indicator of poor prognosis in HCC. The RAS may have a role in the progression of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumor worldwide. The five-year survival of HCC patients is extremely low because of the high rate of recurrence and metastasis [1]. Angiogenesis is now widely recognized as playing a pivotal role in the occurrence, development and metastasis of HCC [2]. The renin-angiotensin system (RAS) may be involved in both beneficial angiogenesis and pathological vessel growth. Angiotensin converting enzyme (ACE) is a key enzyme in the RAS and converts angiotensin (Ang) I to the vasoconstrictor Ang II [3,4]. ACE2 is a homologue of ACE and cleaves a single residue from Ang I to generate Ang (1–9) and degrades Ang II to the vasodilator Ang (1–7) [5,6]. Overexpression of ACE2 inhibits cell invasion, angiogenesis and vascular endothelial growth factor (VEGF) production in a non-small cell lung cancer (NSCLC) cell line [7]. It is reported that Ang II can accelerate VEGF-induced cell growth and tube formation in bovine retinal microcapillary endothelial cells [8]. Ang II has also been shown to activate in vivo angiogenesis, which involved activation of the VEGF/eNOS-related pathway and of the inflammatory reaction [9]. Although previous studies suggest the association of RAS with tumor growth and development, few clinical study of the RAS has been done on HCC.

CD34 is widely used as a marker for evaluating angiogenesis in NSCLC [10], cervical cancer [11], gastric adenocarcinoma [12] and HCC [13]. In the present study, bioinformatics analysis was carried out based on HCC datasets from Gene Expression Omnibus (GEO) database and The Cancer Genome Atlas (TCGA). A negative correlation between the mRNA levels of ACE2 and CD34 was observed in

* Corresponding author. Department of Hepatobiliary Surgery, The People's Hospital of Lishui, The Sixth Affiliated Hospital of Wenzhou Medical University, 15 Dazhong Street, Liandu District, Lishui 323000, PR China. Fax: +86 0759 2780009.
E-mail address: bingmayong811@163.com (Y. Qin).

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HCC. Because HCC development follows liver fibrosis and cirrhosis, we evaluated the mRNA levels of CD34 and ACE2 in liver specimen from patients with hepatic fibrosis, cirrhosis and HCC. The concentrations of Ang II, Ang (1–7) and VEGF were detected in the sera of healthy volumteers and patients with hepatic fibrosis, cirrhosis or HCC. We found that ACE2 mRNA was gradually decreased, while CD34 mRNA and serum concentrations of Ang II, Ang (1–7) and VEGF were progressively increased with the increasing grade of disease severity. VEGF showed a positive correlation with Ang II and Ang (1–7), while negative correlation was observed between mRNA levels of CD34 and ACE2. These data suggests the association of RAS with angiogenesis, which may play an important role in the development of HCC.

2. Materials and methods

2.1. Bioinformatics analysis

HCC datasets were downloaded from the NCBI Gene Expression Omnibus database (Access ID: GSE54236 and GSE51401) and The Cancer Genome Atlas (TCGA). The relationship between mRNA levels of CD34 and ACE2, and between mRNA levels of VEGF and Ang II was evaluated by Pearson correlation analysis.

To further investigate the biological pathways involved in HCC pathogenesis through ACE2 pathway, we performed a gene set enrichment analysis (GSEA) by using GSEA version 2.0 from the Broad Institute at MIT. The data in question were analyzed in terms of their differential enrichment in a predefined biological set of genes (representing pathways). The KEGG gene sets biological process database (c2.KEGG.v4.0) from the Molecular Signatures Database–Msig DB (http://www.broad.mit.edu/gsea/msigdb/index.jsp) were used for enrichment analysis.

2.2. Serum samples and liver specimens

From 2006 to 2010, 20 patients with no evidence of liver disease, 117 patients with hepatic fibrosis, 117 patients with cirrhosis and 117 patients with HCC admitted to the People's Hospital of Lishui, the Sixth Affiliated Hospital of Wenzhou Medical University were enrolled in this study. Sera samples were obtained from these patients before treatment. Sera from age matched 117 healthy volunteers were used as control samples. The control samples was obtained from screening clinics that were open to the general public during March 2010. All of the samples were obtained in the morning before food intake and were stored at −80 °C until use. Liver specimens were obtained from 20 patients with no evidence of liver disease, 26 patients with fibrosis, 36 patients with cirrhosis and 75 patients with HCC with informed consent. All tissues were snap frozen in liquid nitrogen immediately after resection.

Ethical approval for the study was provided by the independent ethics committee, the People's Hospital of Lishui, the Sixth Affiliated Hospital of Wenzhou Medical University. Informed and written consent was obtained from each individual according to the ethics committee guidelines.

2.3. Enzyme-linked immunosorbent assay (ELISA) analysis

Serum concentrations of VEGF, Ang II and Ang (1–7) were assessed by using ELISA assay (Bio-Swamp life science, Shanghai, China). Assays were performed following the instructions of the manufacturer. Plates were read at 450 nm on a using a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA).

2.4. RNA extraction and real-time PCR

Total RNA was extracted from liver **s**pecimens using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. One μg of total RNA was reverse transcribed using with cDNA synthesis kit (Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Real-time PCR was performed to detect mRNA levels of indicated genes. GAPDH was served as an internal control. The primers used were list as follows: ACE2, 5′-GAATAGCGCCCAACCCAAG -3′ and 5′-CTGAGAAGGAGCCAGGAAGAG-3’; CD34, 5′- ACTGGCTATTTCCTGATG -3′ and 5′-GTGTTGTCTTGCTGAATG -3’; GAPDH, 5′- AATCCCATCACCATCTTC -3′ and 5′-AGGCTGTTGTCATACTTC-3’; All reactions were conducted on an ABI 7300 real-time PCR machine (Applied Biosystems, Foster City, CA, USA) using the following cycling parameters, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 45 s. The gene expression was calculated using the ΔΔ Ct method. All data represent the average of three replicates.

2.5. Immunoblotting

Tissue samples were homogenized on ice in radioimmunoprecipitation assay buffer (JRDUN Biotehnology, Shanghai, China). Protein concentration was measured by BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Equal amounts of protein were separated via sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electro-blotted onto nitrocellulose transfer membrane. Immunodetection of proteins was performed using specific antibodies. Densitometric analysis was performed with Image-J (NIH Imag). Anti-ACE2 was from Abcam (Cambridge, MA, USA). Antibody against GAPDH was purchased from Cell Signaling Technology (Danvers, MA, USA).

2.6. Statistical analysis

All statistical analyses were carried out using MedCalc software (Mariakerke, Belgium). The results were presented as the mean value ±SEM. The two-tailed Student's t-test was used to calculate the statistical significance of difference between groups. The relationships between two factors were assessed by Pearson correlation analysis. Kaplan–Meier survival curve was conducted to evaluate the association between ACE2 mRNA level and survival rate of 75 patients with HCC. Differences were considered significant with a value of $P < 0.05$.

3. Results

3.1. Correlation analysis based on human HCC dataset

We re-analyzed data from NCBI Gene Expression Omnibus database (Access ID: GSE54236) and The Cancer Genome Atlas (TCGA). As shown in Fig. 1, the expression of CD34, a widely used marker for evaluating angiogenesis, significantly increased, while ACE2 expression notably decreased in HCC tissues compared with the adjacent tissues of patients (Fig. 1).

The correlation analysis between CD34 and ACE2 was then carried out by Pearson correlation analysis. A negative correlation was observed between the mRNA levels of ACE2 and CD34 (Fig. 1C and F), which suggested a role of the RAS in the angiogenesis of HCC.

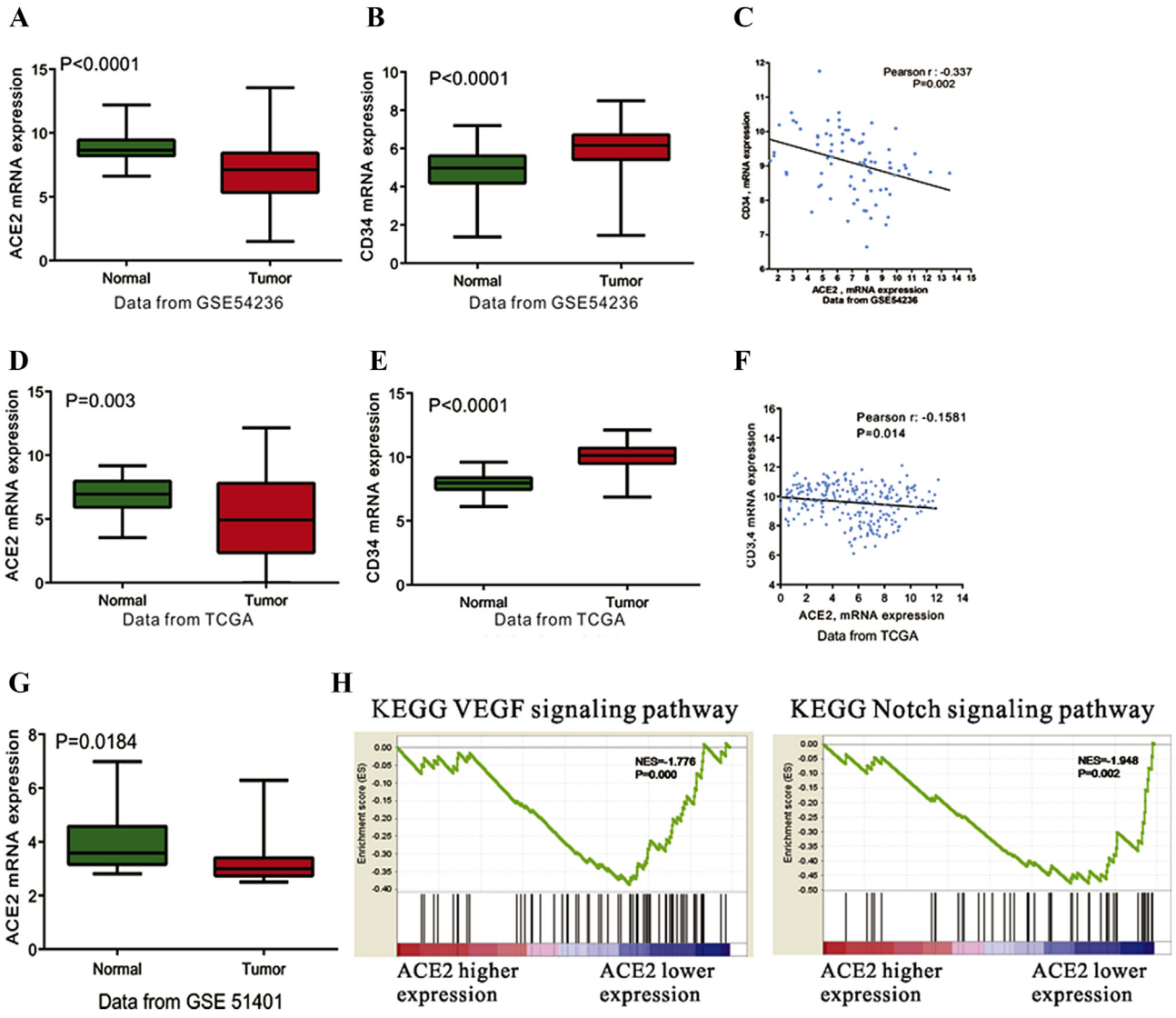

Fig. 1. Bioinformatics analysis of ACE2 and CD34 mRNA expression in HCC. ACE2 expression was significantly decreased in HCC tissues when compared with the adjacent tissues of patients from GSE54236 dataset (A) and TCGA data (D). CD34 expression was remarkably decreased in HCC tissues when compared with the adjacent tissues of patients from GSE54236 dataset (B) and TCGA data (E). A significant negative correlation was shown between the mRNA levels of CD34 and ACE2 as determined by using GSE54236 dataset (C) or TCGA data (F). (G) ACE2 expression was significantly decreased in HCC tissues when compared with the adjacent tissues of patients from GSE51401 dataset. (H) Gene Set Enrichment Analysis (GSEA) identified significant association between ACE2 and VEGF or Notch signaling pathway in GSE51401 dataset.

3.2. Identification of genes and signaling associated biological pathways and processes by gene Set Enrichment Analysis (GSEA)

To probe the ACE2-associated pathways on an unbiased basis, we performed GSEA using data from the GEO database (Access ID: GSE51401). GSEA is designed to detect coordinated differences in expression of predefined sets of functionally related genes. Among all the 188 predefined 'KEGG pathways' gene sets, the VEGF and Notch signaling pathway was significantly associated with ACE2 expression in the GSE51401 dataset (Fig. 1H).

3.3. mRNA levels of CD34 and ACE2 detected by real-time PCR

To further explore the changes of CD34 and ACE2 during HCC progression, we detected their relative mRNA levels in normal liver tissues and liver specimens from patients with chronic liver disease and HCC (Fig. 2A and B). There were no significant difference in CD34 and ACE2 mRNA levels between normal tissues and fibrosis tissue. Comparing with hepatic fibrosis patients, the CD34 mRNA of hepatic cirrhosis patients and HCC patients increased to 1.50 fold and 3.40 fold, respectively, while the ACE2 mRNA decreased to 65% and 40%, respectively. A negative correlation between CD34 and ACE2 was observed by Pearson correlation analysis (Fig. 2C, $r = -0.6216$, $P < 0.0001$), which was consistent with the analysis results with GEO and TCGA datasets (Fig. 1).

We then detected the protein level of ACE2 in 10 pairs of HCC tissues and normal liver tissues (Fig. 2D). ACE2 protein level was also notably lower in HCC tissues than in normal liver tissues ($P < 0.0001$).

3.4. Serum concentrations of Ang II, Ang (1–7) and VEGF assessed by ELISA

The protein concentrations of Ang II, Ang (1–7) and VEGF were quantified in all specimens using ELISA (Fig. 3). Ang II concentration in the serum samples from healthy volunteers, hepatic fibrosis

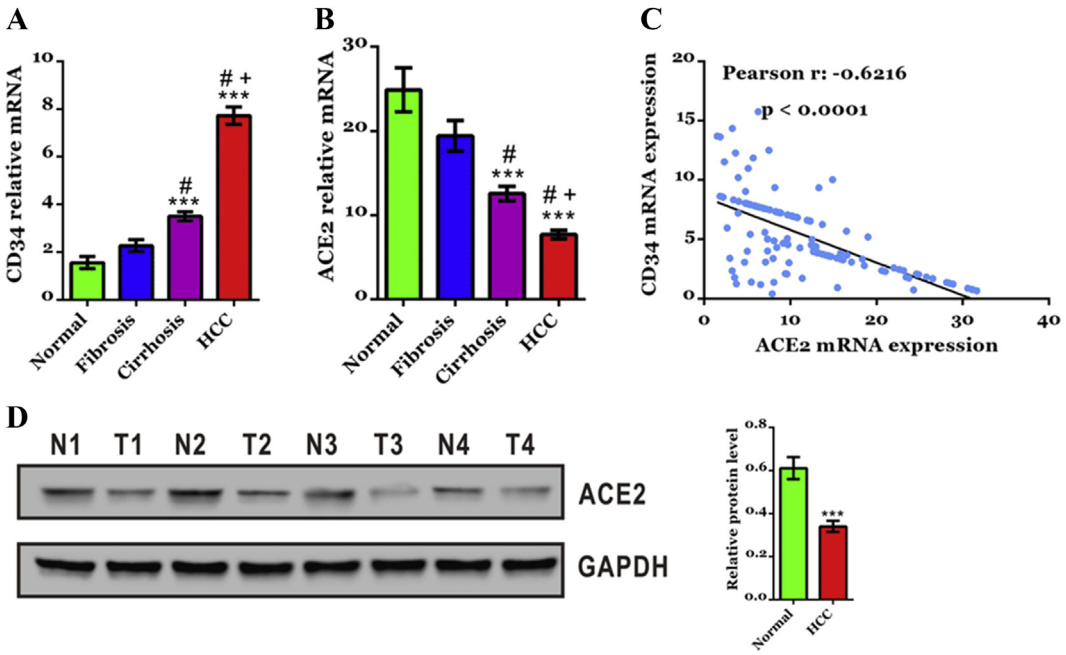

Fig. 2. he mRNA levels of CD34 and ACE2 in liver specimens were detected by real-time PCR. CD34 (A) was gradually increased, while ACE2 (B) was progressively decrease with the increasing grade of disease severity. ***P<0.0001 VS normal; # P<0.01 VS fibrosis; + P<0.01 VS cirrhosis. (C) Negative correlation between the mRNA levels of CD34 and ACE2 as determined by real-time PCR analysis (P<0.0001). (D) The protein levels of ACE2 were analyzed by western blot. GAPDH was also detected as the control of sample loading. Representative western blots were shown in the left. Quantitative results were shown in the right. N: normal tissue; T: HCC tissue (***P<0.0001 VS normal).

patients, hepatic cirrhosis patients and HCC patients were 77.77 ± 2.72, 147.9 ± 6.04, 200.80 ± 6.24 and 290.50 ± 11.70, respectively. A significant difference ($p < 0.01$) was observed between patients and healthy volunteers. Moreover, Ang II protein concentrations in the serum samples from HCC patients were significantly higher than that in hepatic fibrosis patients or hepatic cirrhosis patients ($p < 0.01$). A significant difference of Ang II protein levels was also shown between hepatic fibrosis patients and

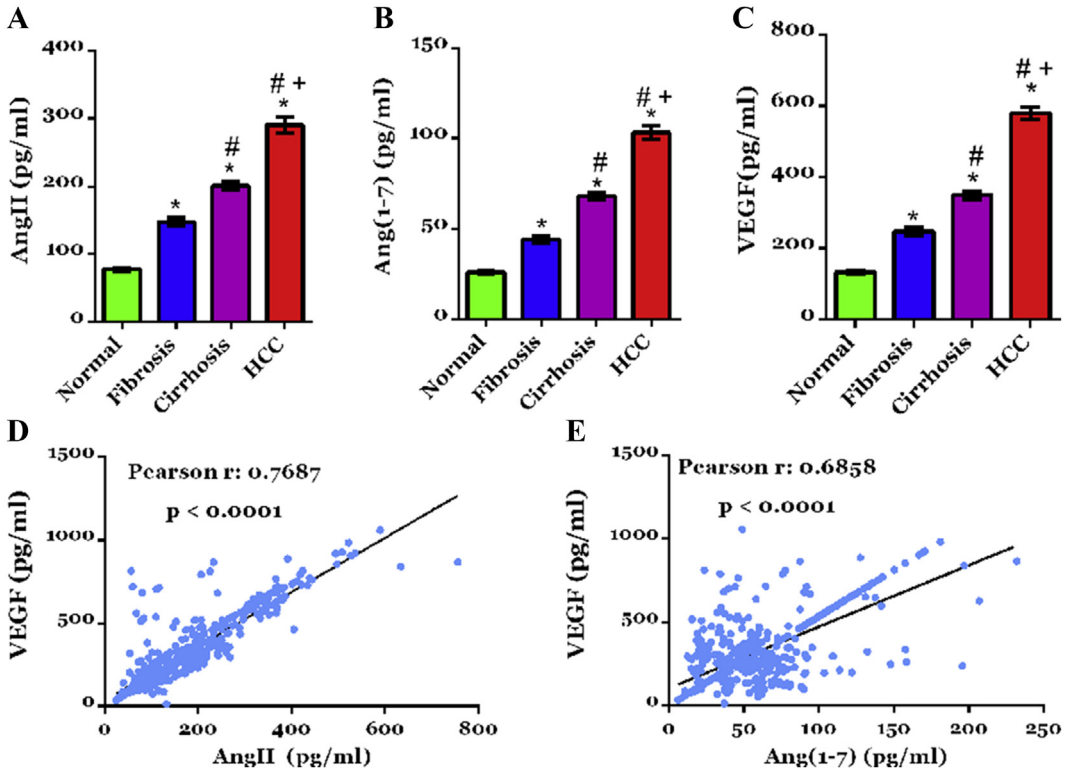

Fig. 3. The protein levels of Ang II, Ang (1–7) and VEGF were higher in the sera of patients with fibrosis, cirrhosis and HCC than that in normal sera. (A, B, C) The protein concentrations of Ang II, Ang (1–7) and VEGF in the sera from healthy volunteers and patients were evaluated by ELISA assay. ELISA data are expressed as average protein concentration. *P < 0.01 VS normal; #P < 0.01 VS fibrosis; + P < 0.01 VS cirrhosis. (D) The protein levels of VEGF and Ang II were subjected to Pearson correlation analysis, which suggested a positive correlation between these two proteins (P < 0.0001). (E) Correlation between the protein levels of VEGF and Ang (1–7) as determined by ELISA of sera samples.

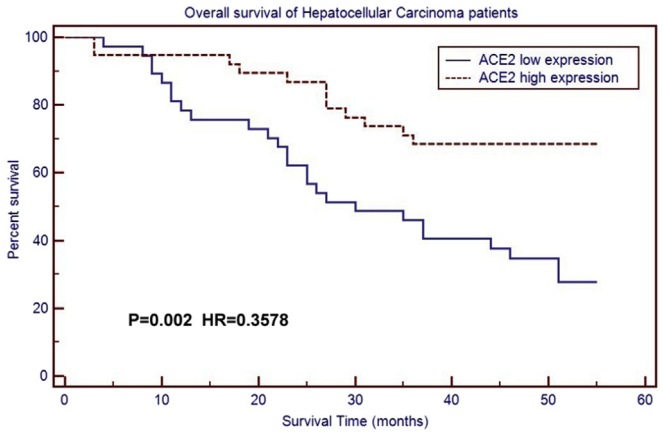

Fig. 4. The survival time of high ACE2 expression level patients was notably longer than that of low ACE2 expression patients. The overall survival time of 75 patients with HCC.

hepatic cirrhosis patients. Serum concentration of Ang (1–7) was also significantly increased (70% for hepatic fibrosis patients, 162% for hepatic cirrhosis patients and 298% for HCC patients VS healthy volunteers). These data indicated that the changes in the expression of RAS components may be associated with chronic liver diseases and HCC. The increase trend of VEGF in patients with chronic liver disease and HCC was similar to that of Ang II and Ang (1–7), which suggested a role of angiogenesis in the development of chronic liver disease and HCC.

The relationships between the serum concentration of VEGF and Ang II or Ang (1–7) were assessed by Pearson correlation analysis. VEGF concentration strongly correlated with both Ang II ($r = 0.7608$; $P < 0.0001$) and Ang (1–7) concentration ($r = 0.6858$; $P < 0.0001$).

3.5. Down-regulated ACE2 expression correlates with poor HCC patient survival

To further evaluate the clinical relevance of ACE2 mRNA in HCC in terms of prognosis, Kaplan–Meier survival analysis was performed using patient overall survival. Our results indicated that ACE2 mRNA was significantly associated with patient survival (Fig. 4). Patients with high expression of ACE2 tended to have longer survival than patients with low levels of ACE2 expression ($p = 0.002$).

4. Discussion

The role of the RAS in tumor progression or metastasis has been extensively described [7,14–17]. Changes in the expression of RAS components, particularly in local tumor tissue, appear to correlate with tumor grade and clinical outcome [18]. Restoration of ACE2 protein suppressed cell proliferation, motility and increased the sensitivity to hypoxia induced injury in pancreatic cancer cells [14]. Overexpression of ACE2 may potentially suppress the invasion and angiogenesis of NSCLC [7]. In the liver, it has been shown that the RAS is frequently activated in the patients with liver cirrhosis [19,20]. In human patients with cirrhosis, both plasma concentrations of Ang II and Ang (1–7) were markedly increased. Ang (1–7) has antifibrotic actions in a rat model of cirrhosis [20]. Ang II has also been reported to induce contraction and proliferation of hepatic stellate cells (HSC) [21], and induce VEGF in a dose- and time-dependent manner [22]. Ang II type 1 receptor blocker (ARB) significantly suppressed liver fibrosis development along with suppression of the VEGF expression [21]. These data suggests that Ang II and VEGF interaction played an important role in liver fibrosis development and HSC activation. CD34 is widely used as a marker for evaluating angiogenesis in several cancers, including HCC [23]. Here, the HCC patients' data from GEO and TCGA shows that ACE2 was low-expressed in HCC patients, while CD34 was high-expressed in HCC patients. Furthermore, a negative correlation between the mRNA levels of ACE2 and CD34 was also observed (Fig. 1). These data suggests that low expression of ACE2 and high expression of CD34 may serve as a useful diagnosis marker for HCC.

Liver fibrosis results from chronic damage to the liver, which leads to excessive matrix or scar deposition. Over time this process can result in progressive liver damage and cirrhosis, complicated by liver failure, portal hypertension and/or hepatocellular carcinoma [24]. In the present study, serum samples and liver specimens were collected from patients with liver fibrosis, cirrhosis or HCC to explore the molecular changes during the progression from liver fibrosis to HCC. We found that hepatic CD34 mRNA and serum concentrations of Ang II, Ang (1–7) and VEGF were gradually elevated, and ACE2 mRNA was gradually decreased with the increasing grade of disease severity (Figs. 2 and 3), which indicates the role of RAS in the progression of HCC. Moreover, it is noted that serum Ang (1–7) levels in patients with liver diseases were not gradually increased in proportion to Ang II, which suggested that Ang (1–7) concentration may not reflect the breakdown of serum Ang II. Ang (1–7) can be converted from Ang II by ACE2, or from Ang (1–9) by ACE [25]. Ang (1–7) may undergo subsequent metabolism by ACE to another peptide Ang (1–5). Therefore, the relative angiotensin peptide levels are dependent on ACE and ACE2 relative activity [26]. More importantly, we found that low expression of ACE2 was associated with poor survival time in patients with HCC (Fig. 4), which indicates ACE2 may serve as a prognosis marker for HCC.

Taken together, overexpression of Ang II, Ang (1–7) and VEGF with gradual progression was found from healthy volunteers to fibrosis to HCC. A gradual down-regulation of ACE2 mRNA and a progressive increase of CD34 was observed with the increasing grade of disease severity. Understanding of this process of angiogenesis might help in the design of efficient and safe antiangiogenic therapy for these liver disorders. Moreover, the low expression of ACE2 may be a useful indicator of poor prognosis in HCC.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

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