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

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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.

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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

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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.

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