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Angiopoietin-2 (Ang-2) is a useful serum tumor marker for liver cancer in the Chinese population

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ABSTRACT

Background: We estimated the diagnostic and prognostic value of serum angiopoietin-2 (Ang-2) in liver cancer patients.

Methods: Tissue Ang-2 was measured using immunohistochemistry (IHC). Cell localization of Ang-2 was tested using immunofluorescence (IF). Cell viability and apoptosis were evaluated using MTT and caspase3/7 assays, respectively. Colony-formation was measured using a soft agar assay. Serum Ang-2 was examined using enzyme-linked immunosorbent assay (ELISA) and Western blotting.

Results: Ang-2 was up-regulated in liver cancer compared to the levels in normal tissues. Serum Ang-2 concentrations were much higher in liver cancer patients than in healthy individuals and those with chronic liver disease (CLD). Inhibitions of Ang-2 using specific shRNA decreased cell proliferation. Serum Ang-2 decreased significantly after surgery. Serum Ang-2 was positively correlated with serum alpha-fetoprotein (AFP; $R = 0.375$, $P = 0.005$). Receiver operating characteristic (ROC) curves suggested that serum Ang-2 could be used with relatively high sensitivity and specificity in differentiating liver cancer patients from CLD patients or healthy controls, with corresponding AUC values of 0.742 and 0.924, respectively. Serum Ang-2 was negatively correlated with overall survival. Subgroup analysis also showed that Ang-2 retained its prognostic value in overall survival prediction in different risk subgroups.

Conclusion: Serum Ang-2 may be a useful tumor marker in predicting liver cancer prognosis.

1. Introduction

Liver cancer is the fifth-most common cancer in the world. Its high mortality rate also makes liver cancer the third-leading cause of cancer deaths worldwide [1]. There are several pathogenic risk factors, including hepatitis B and C, aflatoxins, and alcohol abuse [2]. Liver resection and liver transplantation are currently the only curative treatments. These treatments offer good prognoses but are confined to early liver cancer patients [3,4]. Most patients with advanced liver cancer receive a poor prognosis, resulting in a low survival rate. Approximately 90% liver cancer generally occurs in patients with CLD, such as viral hepatitis, alcoholic liver disease, autoimmune hepatitis and primary biliary cholangitis [5]. The formation of new blood vessels is essential for the progression, invasion, and metastasis of cancers, especially liver cancer, which requires sufficient blood supply [6]. Therefore, angiogenesis is an important process in the research and therapy of liver cancer. Angiopoietin 1 (Ang-1) and Ang-2, which belong to a family of angiogenic factors, have been specifically implicated in liver cancer. Unlike Ang-1, which stabilizes vessels, Ang-2 plays a role as a competitive antagonist of Ang-1, reducing vascular stabilization [7,8]. A study by Scholz et al. showed that Ang-2 levels were elevated in patients with liver cancer and cirrhosis [9]. Moreover, a

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high proportion of Ang-2 mRNA was found to be significantly correlated to a poor prognosis [10]. These results indicate that Ang-2 is involved in the development of liver cancer.

In the present study, we performed bioinformatics analysis, quantitative real-time PCR (qPCR) and IHC, which revealed that Ang-2 is highly upregulated in liver cancer. Cell function tests, including cell proliferation, caspase 3/7 activity, and colony-formation assays were also performed, and the results showed that Ang-2 promotes cell proliferation. Additionally, we found that Ang-2 is a better serum predictor of liver cancer according to the receiver operating characteristic (ROC) curve and overall survival curves.

2. Materials and methods

2.1. Patients and blood samples

One hundred and seventy-three patients who had been diagnosed with liver cancer (mean age ± SD, 54.13 ± 10.65 years; male: female ratio, 3.81:1) at Shanghai Cancer Center (Shanghai, China) and Shanghai Tenth People's Hospital (Shanghai, China) between March 2006 and April 2016 were considered appropriate for this study. One hundred and sixty-eight patients with CLD (mean age ± SD, 53.29 ± 9.34 years; male: female ratio, 3.2:1) at Shanghai Tenth People's Hospital (Shanghai, China) were also recruited in the study. Three hundred and three healthy individuals (mean age ± SD, 52.11 ± 9.86 years; male: female ratio, 3.28:1) were recruited at Shanghai Tenth People's Hospital (Shanghai, China) and treated as control subjects. The diagnosis of Liver cancer was based on an enhanced computed tomography (CT) or magnetic resonance imaging (MRI), and histopathological analysis of ultrasound-assisted fine-needle biopsies confirmed the final diagnosis. The following exclusion criteria were applied: insufficient extractable data, diagnosis of hepatic cholangiocarcinoma, metastastic liver cancer and loss to follow up. The diagnosis of CLD was based on the detection of hepatitis B surface antigen (HBsAg) in the serum, indicating the presence of HBV (chronic HBV infection), or based on a daily alcohol intake of > 40 g/ethanol per day for > 15 years, indicating alcoholic liver. Serum HBsAg was measured by Enzyme-linked immunosorbent assay (ELISA) using a commercially available ELISA kit (R&D Systems, Inc. MN, USA) according to the manufacturer's instructions. Healthy volunteers with no history of liver disease, alcohol consumption of < 40 g/week, and no risk factors for viral hepatitis were recruited and regarded as controls. The Liver cancer patients underwent treatments including radiofrequency ablation (RFA), transcatheter arterial chemoembolization (TACE) and surgical resection. Not all the patients received surgical resection. The first treatment for liver cancer was curative, but the information regarding liver cancer recurrence was not obtained. The study protocol was approved by the institutional review boards of Shanghai Tenth People's Hospital (SHSY-IEC-KY-4.0/17-50/01). Written informed consent was obtained from each patient and healthy volunteers. Five-milliliter blood samples were collected from all patients and healthy individuals by venipuncture. The blood samples were subsequently centrifuged at 4 °C for 10 min. The plasma samples were stored at − 80 °C and thawed until ready to be tested. The study protocol was conducted in accordance with the ethical guidelines of the 1975 Helsinki Declaration.

2.2. Cell culture and vectors

The liver cancer cell lines Bel-7402 and SMMC-7721 were cultured in Dulbecco's Modified Eagle's Medium (DMEM). shRNAs against human Ang-2 were obtained from Open Biosystems(Lafayette, USA).

2.3. Immunofluorescence (IF) and immunohistochemistry (IHC)

For IF, SMMC-7721 or Bel-7402 cells were cultured on glass slides and fixed with 4% paraformaldehyde for 15 min. Then, the cells were washed three times with phosphate-buffered saline (PBS) for 5 min and incubated with blocking buffer (PBS solution containing 3% fetal bovine serum (FBS), 1% goat serum, and 0.1% Triton X-100) for 2 h at room temperature. Subsequently, cells were incubated with the primary antibody diluted in PBS at 4 °C overnight. Anti-Ang-2 (Abcam #ab155106) and anti-CD63 (Abcam #ab59479) antibodies were used as the primary antibodies. The slides were then washed with PBS three times prior to being incubated with Alexa Fluor®-488/555 fluorescent conjugated secondary antibody (CST, 4408 or 4413 s) for 1 h in the dark. Afterward, the slides were washed three times in PBS prior to being mounted with Pro-Long® Gold Antifade Reagent with 4′,6-diamidino-2-phenylindole (DAPI: Molecular Probes, Eugene, OR, USA). The slides were observed using an LSM 800 Confocal Microscope (Carl Zeiss AG, Oberkochen, Germany).

For IHC, the details of these procedures were described previously [11]. Anti-Ang-2 (Abcam #ab155106) antibodies were used for detection.

2.4. Western blotting (WB) and quantitative RT-PCR (qPCR)

Serum samples were resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels followed by standard WB. The following primary antibodies were used: albumin (Epitomics), Ang-2, ACAN, B4GALNT1, C11ORF82 and CNTNAP3 (all from Abcam). Total RNA was extracted from cells using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. qPCR assays were performed to evaluate the expression of Ang-2 and other proteins, and primers were as follows: Ang-2-Fwd: 5′-TCCAAGAGCTCGGTTGC TAT-3′; Ang-2-Rev:5′-AGTTGGGGAAGGTCAGTGTG-3′;GAPDH-Fwd:5′--GAGTCAACGGATTTGGTCGT-3′, GAPDH-Rev: 5′-GACAAGCTTCCCGT TCTCAG-3′. ACAN-Fwd: 5′-GTGTGGGACTGAAGTTCTTGGAG-3′,ACAN-Rev: 5′-GATGCTGACACTCAGCGAGTTG-3′; B4GALNT1-Fwd:5′-TTTCTGTCGAGGAGCCAGTCCCCAG-3′; B4GALNT1-Rev: 5′-ATACCTCC TGACCAGAAGCTGCCTG-3′; C11ORF82-Fwd:5′-AGGGTCTTGCTCTGT-CACCCAG-3′; C11ORF82-Rev: 5′-GAGGCTAGAAGAAATTTTCGTC-3′; CNTNAP3-Fwd: 5′-GGTCATCCTTCAGCAGCTCCTCAG-3′; CNTNAP3-Rev: 5′-ACCTCCATTCTCTCTCCAAGGTC-3′. All experiments were performed at least three times.

2.5. ELISA

The levels of Ang-2 in serum samples from 173 patients with liver cancer, 168 patients with CLD and 303 healthy people were measured using a commercially available ELISA kit (R&D Systems, Inc. MN, USA) according to the manufacturer's instructions. Signals were determined by measuring the absorbance at 450 nm using a plate reader (Σ960, BioTek).

2.6. Cell proliferation, caspase3/7 activity, and soft agar colony formation assay

Cell proliferation was measured by a CellTiter proliferation assay kit (Promega), and caspase3/7activity was determined using a caspase-Glo assay system (Promega), according to the manufacturers' instructions. For anchorage-independent soft agar colony formation assays, cells were seeded onto six-well plates at a density of 5 × 10^3 cells per well and maintained. Colonies were then counted until foci were evident.

2.7. Mice experiments

For xenograft mouse experiments, SMMC-7721 or Bel-7402 cells (5×10^6 cells) under different treatments were subcutaneously injected into 8-week-old athymic nude mice (Bikai). Tumor size was measured every 6 days, and the tumor volume was calculated as $0.5 \times L \times W^2$, with L representing length and w representing width. All mouse experiments were performed according to the institution guidelines of Shanghai Tenth People's Hospital.

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