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Original Articles

AZGP1 suppresses epithelial-to-mesenchymal transition and hepatic carcinogenesis by blocking TGFβ1-ERK2 pathways

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ABSTRACT

Zinc-α2-glycoprotein 1 (AZGP1) has been found to play important roles in TGF-β1 induced epithelial-to-mesenchymal transition (EMT). However, the mechanisms of AZGP1 inhibiting EMT and its therapeutic potential remain unknown in hepatocellular carcinoma (HCC). AZGP1, TGF-β1 or ERK2 expressions were examined in liver tissues of HCC patients and rat model. The effect of AZGP1 on EMT and crosstalking of TGFβ1-ERK2 signaling in human hepatic cancer cell was tested in vitro and in vivo. Hepatic expression of AZGP1 was nearly deficient in HCC patients and rats. It was proved that AZGP1 has the ability of down-regulating mesenchymal markers, up-regulating epithelial marker, inhibiting cell invasion and suppressing EMT in human HCC cells. The results clarified that AZGP1 has the effect on blocking TGF-β1 mediated ERK2 phosphorylation leading to depressing EMT and invasive potential in vitro. Local injection of AZGP1 mimic in vivo could significantly withhold lung metastasis in HCC. In conclusion, loss of AZGP1 could trigger EMT induced by TGFβ1-ERK2 signaling, confuse in energy metabolism, reduce cell proliferation and apoptosis, activate survival signals and promote invasion. Up-regulation of AZGP1 should be proposed to reverse EMT and might be a new promising therapy for HCC.

Introduction

Hepatocellular carcinoma (HCC), the fifth most popular tumor in the world, has a high mortality because of a lack of effective treatments [1]. About 230,000 cases die from HCC every year in China [2]. Emerging evidence suggests that epithelial to mesenchymal transition (EMT) contributes to tumor metastasis and recurrence, including in HCC [3,4]. Although several lines of evidence have demonstrated the significance of EMT in HCC progression, the molecular mechanisms that regulate EMT remain unclear.

Zinc-α_2-glycoprotein 1 (AZGP1), a 40-kDa protein assigned to the chromosome 7q22.1, is expressed mainly in epithelial cells [5] and involved in carcinogenesis [6,7]. The structure and sequence of AZGP1 are highly homology to major histocompatibility complex class I (MHC I) family which may function importantly in immunity [8] and so AZGP1 may afford some protective effect and benefit to prevent the cancer progression [9]. AZGP1 is actively involved in both inhibition of tumor growth and proliferation [10], inhibit the enzyme mediated tumor invasion and activate apoptosis [11]. Recently, it is identified that AZGP1 could be a potential marker for diagnosis and prognosis of tumors [12–15]. However, the role of AZPG1 in HCC has not yet been fully elucidated.

Interestingly, it has been found that the capacity of AZGP1 attenuating transforming growth factor-β1 (TGF-β1) mediated EMT makes it possible to inhibit pancreas tumor invasion [16]. TGF-β1 can induce EMT in HCC [17] and has been shown to enhance tumor dissemination in later stages of tumor progression. While the complex molecular mechanisms of AZGP1, TGF-β1 and EMT are not yet understood. We aim at clarify how AZGP1 regulating TGF-β1 induced EMT in HCC progression and discuss new insights on promising drug targets for future therapeutic approaches against HCC.

Materials and methods

Patients and liver tissue samples

A total of 164 human liver tissues were obtained through percutaneous liver biopsy of HBV related fibrosis (HBV-fibrosis) patients (n = 103) or receiving partial liver resection of HBV related HCC (HBV-HCC) patients (n = 61) in our hospital from 01/2010 to 12/2012. Patients with HBV related diseases were defined those who had HBV infection with positive hepatitis B surface antigen (HBsAg) for at least 6 months. All of the included HBV-HCC patients must met the following requirements: (1) HBV infection; (2) early HCC with single tumor nodule and T1N0-1M0 stage, in accordance with clinical and histological diagnosing criterion of HCC in China [18]; (3) Para-cancerous tissue showed cirrhosis no high atypical hyperplasia was observed. Those who were co-infected with HIV or HCV, consumed >30 g of alcohol per day, at risk of other chronic liver disease, received previous anti-tumor treatment, or recurred were excluded from the study.

The included 32 liver tissues (Fibrotic stage S0: n = 7; S4: n = 10; HCC: n = 15) were performed for microarray and other 132 liver tissues for qPCR (Fibrosis: n = 86; HCC: n = 46). Immunohistochemistry was performed in 45 human liver tissues (these patients involved in qPCR cohort, Fibrosis: n = 28; HCC: n = 17). All patients were

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given written informed consent and the study was approved by the Ethics Committee of our hospital. Fibrotic stage was determined using Scheuer's classification. Other clinical data were also collected, including routine blood test, HBV DNA, liver function test (Supplementary Table S1).

Experimental animal models

Diethylinitrosamine (DENA) induced cirrhosis or HCC rat model

Sixty male cirrhosis or HCC Wistar rats model was induced by watering of 0.05 g/l DENA daily. Rats were sacrificed in 8, 12 and 16 weeks after DENA watering and then separated into 4 groups (Control, Cirrhosis, Advanced Cirrhosis and HCC, each group: n = 15).

Tumor xenograft experiment

Using Lipofectamine 2000 transfection reagent, recombinant plasmid vector with plenti-AZGP1 transfected into 293T cells prepared transfected AZGP1 lentiviruses. For xenograft implantation, a total of 5×10^6/0.2 ml HepG2 cells over-expression of AZGP1 or vector transfection as controls were injected subcutaneously into the bilateral lower extremity hip joint of 6-week-old male BALB/c nude mice (HepG2-AZGP1 and HepG2-GFP groups, each group: n = 8). Tumor volume was calculated as (length × width^2)/2 and measured twice a week. After 4 weeks of operation, the mice were sacrificed.

Orthotopic liver transplantation tumor and metastasis experiment

In another group of 6-week-old nude mice (HepG2-AZGP1 and HepG2-GFP groups, each group: n = 7), we take 1 cm subcutaneous tumor and transplanted to liver. After 6 weeks of operation, the mice were sacrificed and the liver, lung and the incidence rate of lung metastasis were observed.

These studies were approved by the Ethical Committee of our Hospital.

Cell culture

Human hepatic cancer cell lines (Bel7402, HepG2, HUH7 and HCCLM3) and normal hepatic cell line (LO2) were used in this study. Cells were cultured in RPMI-1640 medium (Hyclone) supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin, and 10% fetal bovine serum (Invitrogen, Gaithersburg, MD, USA). Cells were treated with 1 μg/ml rAZGP1, 10 ng/ml TGF-β1 (Sigma), SB525334 (inhibitor of TGF-βI/ALK5 receptor), LY364947 (inhibitor of TGF-βI/II receptor), PD98059 [inhibitor of extracellular regulated protein kinase (ERK)] (SELLECK), respectively for 24–48 hours. Lentivirus encoding siRNA gene of AZGP1 (siAZGP1) or ERK2 (siERK2) for transduction (Origene) and the control virus showed green fluorescent protein (GFP) expression in the cells (Microbix, Ontario, Canada).

Immunohistochemistry (IH)

Formaldehyde-fixed, paraffin-embedded sections of human, rat and nude mice tissue were subjected to hematoxylin and eosin (H&E) staining and IH following routine protocols as described [14,19,20]. Antibodies were as follows: TGF-β, AZGP1 and ERK2 (Abcam, USA). IH was performed in 45 human liver tissues (S0–S1 group: n = 15; S3–S4 group: n = 13; HCC group: n = 17). Scoring was used the manual microscopic counting method. Representative images were captured with an Olympus IX70.

Quantitative polymerase chain reaction (qPCR)

The qPCR was performed using SYBR Green PCR Kit (Applied Biosystems, Foster City, CA) and ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). The messenger RNA (mRNA) level of specific genes was normalized against β-actin. Primers used are listed (Supplementary Table S2).

Western blotting (WB)

Western blotting was performed using antibodies as follows: anti-AZGP1 (1:1000); anti-TGFβ1 (1:1000); anti-CDH1 (E-cadherin, 1:1000); anti-VIM (Vimentin, 1:1000); anti-pSmad2/3/4 (1:500); anti-pERK2 (1:1000); anti-p21 (1:1000) and secondary antibody was HRP-IgG (1:10,000) (Promega, USA).

Cell proliferation assay

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was employed to examine the proliferation of cells. The optical density (OD) value was measured by a spectrophotometric plate reader (Thermo, Waltham, MA, USA) at 570 nm. The assay was performed three times independently.

Apoptosis and cell cycle analysis

Cells were washed with PBS and re-suspended in 500 μl binding buffer containing 2 μl Annexin V-FITC. After incubation, the samples were analyzed using flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA). Cells were harvested after 48 hours and fixed in 70% ice-cold ethanol overnight. Cells were then washed with PBS, and stained with propidium iodide (50 mg/ml) in PBS supplemented with RNase (50 mg/ml) in the dark at room temperature for 30 minutes. Tests were performed in triplicate for each sample, and analyses of cell cycle distribution were performed by flow cytometer in accordance with the manufacturer's guidelines (FACS, BD Bioscience, USA).

Invasion assays

To assess cell invasion in vitro, we used 24-well Matrigel invasion chambers and reconstituted them with 600 ml serum-free Dulbecco's modified Eagle's medium in both the top and the bottom chambers for 2~4 hours. Cells were trypsinized and seeded into the top chamber at a density of 5×10^4 cells per well in 500 ml Dulbecco's modified Eagle's medium containing 0.5% fetal calf serum. The outer chambers contained 0.4 ml of medium (30% fetal calf serum). After incubation at 37 °C for 24 hours, cells remaining attached to the upper surface of the membrane were carefully removed with cotton swabs, whereas cells that reached the underside of the chamber were stained with H&E and were counted. All experiments were repeated three times.

Wound scratch assay

Cell migration was monitored in a wound scratch assay. Briefly, the HCC cancer cells were seeded on a 6 well plate at a density of 2×10^5 cells/well. A scratch was made with a sterile 10 μl pipette tip in a confluent cell monolayer. Images of the wells were captured at the beginning of the experiment and after 24 hours on an inverted microscope (CK40 F200; Olympus, Japan). Digital images were obtained with an Optronics MicroFire digital camera (Optronics, Goleta, CA, USA) driven by the Picture Frame imaging software (Optronics). All experiments were repeated three times.

Statistical analysis

Results are expressed as mean ± SE and the experiment was repeated three times. Data were analyzed using ANOVA test. All statistical analysis was done using SPSS 19.0 (SPSS Inc, Chicago). A p value <0.05 was considered statistically significant.

Results

Gradually down-regulated AZGP1 was closely related to cirrhosis and tumor in chronic hepatitis B (CHB) patients

Because there were few reports on the expression of mRNAs and their relationship of HBV related cirrhosis (HBV-cirrhosis) progression to HCC, we conducted mRNA profiling and prediction analysis of microarray (PAM) in 32 liver tissues of HBV-fibrosis and HBV-HCC patients (S0: n = 7; S4: n = 10; HCC: n = 15; Supplementary Fig. S1A). AZGP1 mRNA as one of the "Predictor Genes" was found statistically down-regulated in liver tissues of HBV-HCC and HBV-cirrhosis patients compared to no-fibrosis patients; and statistically down-regulated in HBV-HCC compared to HBV-cirrhosis patients ($p < 0.05$, Supplementary Fig. S1B–C).

Then expressions of AZGP1 were verified in liver tissues of other 86 HBV-fibrosis and 46 HBV-HCC patients. The levels of AZGP1 mRNA gradually decreased from fibrotic stages S1–3, cirrhosis to HCC comparing to no-fibrosis patients ($p < 0.05$, Fig. 1A); and it was also dramatically decreased in HCC comparing to cirrhosis patients ($p < 0.001$, Fig. 1A). AUROC of AZGP1 mRNA distinguishing HCC was higher than cirrhosis (HCC = 0.797, Cirrhosis = 0.660, Fig. 1B–C). To examine the protein level of AZGP1 in liver tissues, WB was performed in pairs of 6 HBV-cirrhosis and HBV-HCC patients. AZGP1 proteins in tumor tissues were on average 4.36-fold lower than that in cirrhotic tissues (Fig. 1D). Comparing to no- or mild fibrosis (S0–1 group), AZGP1 proteins presented at lowest extent and 82.4% with negative or weak positive expression in HCC tissues (AZGP1 semi quantitative score = 2.53 ± 2.21, HCC versus S0-1 group: $p = 0.002$); then presented at second lower extent and 69.1% with weak positive expression in severe fibrotic or cirrhotic (S3–4 group) tissues (score = 3.46 ± 2.50, S3–4 versus S0–1 group: $p = 0.036$) in IH examination (Fig. 1E). It revealed that AZGP1 expression was significantly decreased 2.9-fold in HCC tissues and 2.1-fold in S3–4 tissues compared to that in S0–1 tissues (Supplementary Table S3). **Results indicated that AZGP1 is closely related to cirrhosis progressing to HCC in CHB patients.**