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Original Article

Auxo-action of tissue transglutaminase 2 on tumors in hepatitis B virus-related HCC

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Abstract: It has been reported that tissue transglutaminase-2 (TGM2) possesses the potential to assist in early diagnosis of HCC. The aim of this study was to explore the effects of TGM2 expression on hepatitis B virus-related HCC progress. Patients clinically diagnosed with hepatitis B related-HCC were enrolled. They were divided into I-IV periods, according to TNM staging, and HCC cell lines were cultivated at the same time. This study processed gene chip analysis and immune imprinting analysis regarding correlation between TGM2 genes and expression of protein and staging of HCC. Using the competitive ability of cysteine to restrain TGM2 pathways, this study explored the effects of cysteine on cell apoptosis. Microarray analysis results showed that, compared with HCC of stage I, gene expression of TGM2 in stage IV increased significantly ($p < 0.05$). Immunohistostaining analysis showed that expression of TGM2 in stage III and IV of HCC was higher than that in stage I-II tumor tissues ($p < 0.05$). Also, 48 hours after using cystamine, the survival rate of HCC cells reduced to about 36.5% of the maximum value, reducing the activation of Caspase-3 by 1.5 times. Finally, AKT expression was reduced and apoptosis was induced. Results of this study preliminarily showed that expression level of TGM2 was related to invasiveness and staging of tumor in hepatitis B virus-related HCC. TGM2 pathways could induce apoptosis of tumor cells by means of competition.

Keywords: Hepatitis B, hepatocellular carcinoma, tissue transglutaminase-2

Introduction

At present, hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the world, accounting for 30% of primary carcinomas of the liver. In China, more than 50% of new cancer patients are diagnosed with HCC every year. Common pathogenic factors include cirrhosis caused by a various pathogenesis, chronic type B, hepatitis C virus infection, excessive drinking, metabolic disturbances, and so forth. In Asian populations, chronic hepatitis B virus infection and cirrhosis are the most common pathogenic factors [1-3]. Research on the pathogenesis of HBV has been carried out but the final survival of patients still has not significantly improved, possibly due to missed early diagnosis and lack of effective therapeutic intervention [4]. Alpha-fetoprotein (AFP) is the most common serum marker for HCC. Some studies have shown that almost 40% of HCC patients had no significant increase in AFP while some patients with obviously increased AFP were in cirrhosis or the active period of HBV infection. Therefore, it is necessary to seek a new type of marker to improve the accuracy of early detection of HCC and to improve prognosis of patients in later stages. The latest study used cDNA microarray to analyze and explore the correlation between gene expression factors and tumor invasiveness. This study possessed high accuracy, strong targeting, and other advantages. At the same time, some studies have shown that tissue transglutaminase-2 (TGM2) possesses the potential to detect HCC markers by quantitative analysis of HCC cells [5-7]. A previous study confirmed that expression levels of TGM2 increased in HCC, related to the invasion of tumors. Therefore, this present study preliminarily assumed that TGM2 inhibitors could be used as target direction for treatment, using cystamine to act on

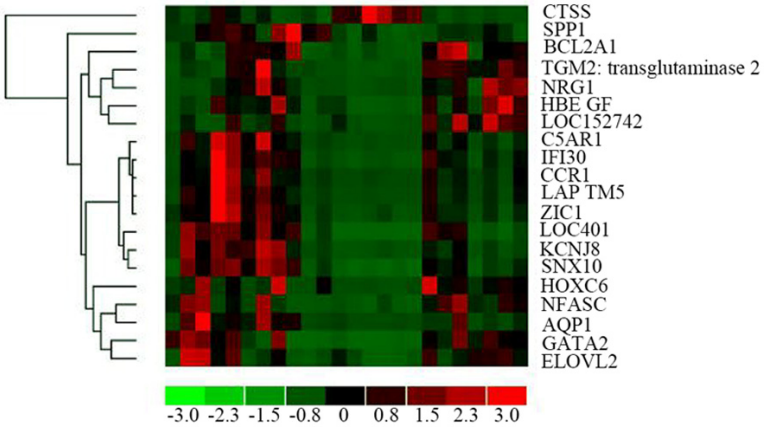

Figure 1. Microarray analysis on HCC TNM stage I stage and IV gene expression (fold change > 4; false discovery rate (FDR) of *p* value < 0.05).

Table 1. The average percentage of TGM2 positive immune cells (%)

	The average percentage of TGM2 positive immune cells (%)
Stage I	22±1.11
Stage II	43±2.13
Stage III	74±3.21
Stage IV	92±4.58

HepG2 cells. Cystamine mainly competes for glutamine enzyme active sites. This study preliminarily explored correlation between progression of hepatitis B liver cell carcinoma and expression of TGM2 by microarray analysis and immunohistochemical staining, aiming to provide reference and guidance for clinical treatment and basic research.

Materials and methods

Study subjects

This study included 120 patients with hepatitis B related-HCC. They were divided into I-IV stages, according to TNM staging. There were 30 cases for each stage and brief information of each stage included: Stage I, single tumor nodule, no vascular invasion; Stage II, single nodule with minor vascular invasion or the diameter of many nodules < 5 cm; Stage III: multiple tumor nodules with blood vessel and peripheral tissue invasion or local lymph node metastasis; and Stage IV: had distant metastasis. Tissue samples were obtained by standard liver puncture method. All operations in this study were informed and agreed upon by patients, family members, and the Ethics Committee.

Cell lines

In this study, human HCC cell line HepG2 was used because it carried the whole HBV gene group and could produce all virus associated proteins, HBV DNA, and Dane particles. Cell lines were obtained from Cell Research Institute of Chinese Academy of Sciences. All cells were cultured in Modified Eagle's culture medium with high glucose (HyClone) and 10% fetal bovine serum (Gibco). Temperature was 37°C and the environment contained 5% CO_2.

Microarray analysis

RNA from primary cell culture was extracted by RNA Extraction Kit. Quality and completeness were detected. Analysis was made by the expression of U133 plus 2.0 spectrum array from Affymetrix Company (classification was made according to the number of samples and grade of liver cancer). Synthesis of cDNA, probe labeling, hybridization, and array scanning were processed according to standard methods. Visualization and data filtering for basic microarray data were processed by methods previously reported in the literature [8]. dCHIP program was used to analyze and compare array data among groups. Sample groups were established and a medium intensity probe was used to standardize variables. It was regarded as baseline level of array parameters. The model was calculated and analyzed by the perfect match/mismatch model.

Immunohistochemical staining analysis

For IHC analysis, 4% paraformaldehyde phosphate buffer solution (PBS) was used to fix tissue samples or cells. Paraffin embedding was processed and stained according to standard steps. Briefly, dimethylbenzene was used to remove paraffin wax, then dehydration of ethanol was processed at different concentrations for samples, with 2% Lowlenthal serum adopt-

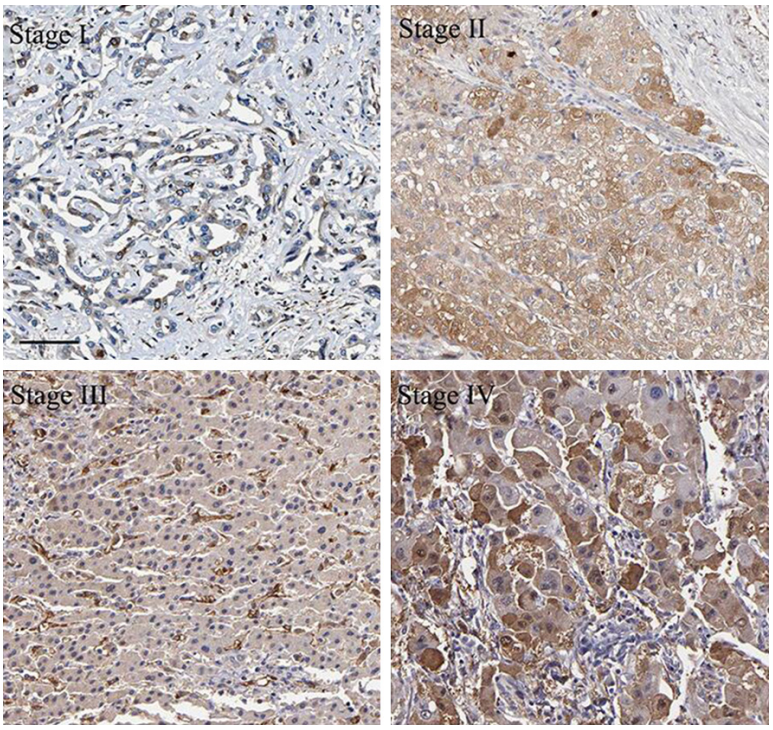

Figure 2. TGM2 antibodies against HCC in different stages (right). Bar = 50 μm. Expression of TGM2 in stage III and stage IV of HCC was obviously higher than the level of tumor tissue in stage I-II.

ed for sealing. Samples were incubated with primary antibody overnight (4°C), combining the secondary antibody with horse radish peroxidase (HRP) for one hour, which could visualize signal strength through HRP substrate. Primary antibody included: polyclonal rabbit antibody GFAP (Chemicon International), monoclonal mouse antibody EMA (Thermo Scientific), rabbit polyclonal antibody against S100, and monoclonal mouse antibody vimentin (Dako). Glutamine enzyme 2 (TGM2) antibody was bought from Abcam. At least 4 high intensity regions in gross tumor volume were randomly chosen for analysis. Researchers counted cells of HPF, independently, and recorded them as %/HPF.

Immunoblotting low-temperature PBS was used to wash 2 times after collecting tumor cells, then RIPA buffer solution was used for cell disruption. The obtained cell lysate was put on ice and rested for 30 minutes, then centrifuged for 10 minutes under 13000 × g. Next, the sediment in the buffer containing SDS and 33% glycerol buffer was resuspended. Obtained proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad). Immune imprinting analysis was processed by caspase-3 phosphorylation and total AKT, α Tubulin antibodies (Santa Cruz), HRP combined secondary antibody, and enhanced chemiluminescence detection system (GE, Fairfield). Afterward, the effects of TGM2 inhibitors, before and after the use of cysteine, were observed. Finally, relative expression levels were quantified by GS-800 calibration density meter (BioRad).

Data analysis

Statistics and analysis of all data were processed by SPSS 20.0 software (SPSS Inc, Chicago, IL). Data is recorded as average value ± standard deviation and paired t-test was used to confirm *p* values. If $p < 0.05$, differences were statistically significant.

Results

Gene expression profile

A total of 20 tissue samples were used for microarray analysis. Multivariate analysis of image clustering showed 2,552 differentially expressed genes. Then, "mafdr" order was used to calculate false discovery rate (FDR) through MATLAB version (R2013a) in multiple comparison test hypothesis for I phase and IV HCC. Results showed that, compared with HCC in stage I, gene expression of TGM2 in stage IV increased significantly, with $p < 0.05$ (**Figure 1**).

Expression of TGM2 in HCC

To show the significance of differential expression of TGM2 in each stage of HCC, this study used TGM2 antibody to immunostain for tissue samples. Three cases from each stage were selected for immunohistochemical analysis. Quantitative analysis was carried out by a microscope with the view of 400 × high magnification. If TGM2 immunoreactive cells were more than 50%, then it would be defined as

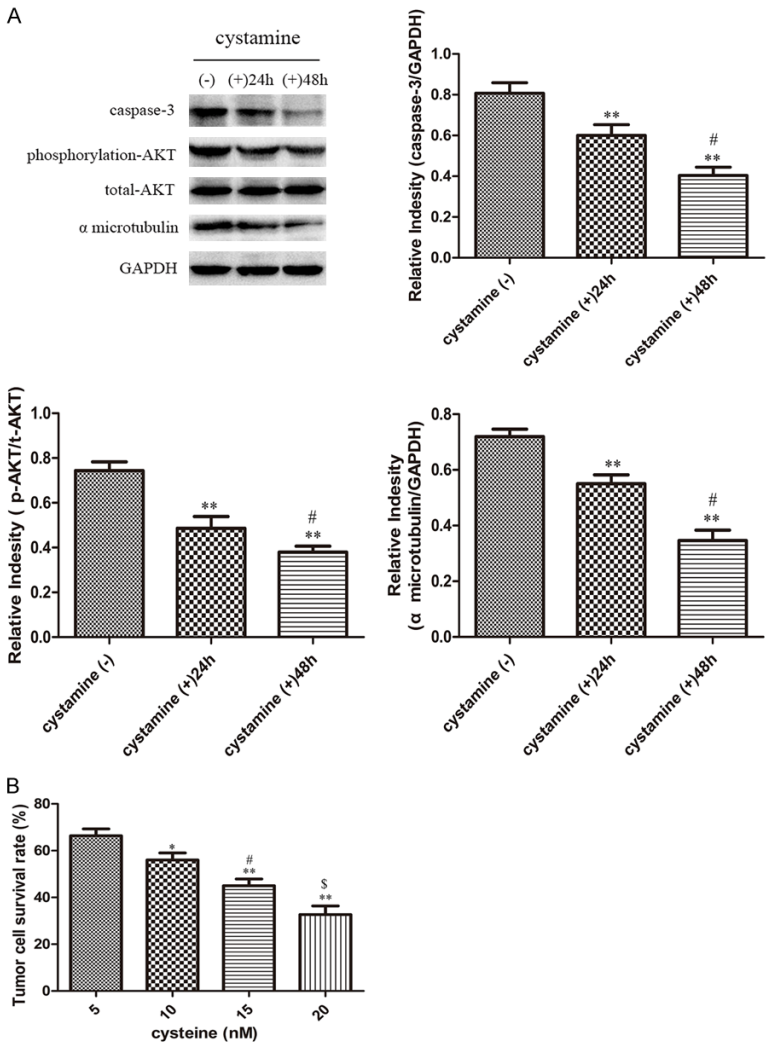

Figure 3. Western blot analysis was showed in the (A) for the expression of caspase-3, phosphorylated AKT expression and α microtubulin. *, $P < 0.05$, **, $P < 0.01$ vs. cystamine (-); #, $P < 0.05$ vs. cystamine (+) 24 h. The change of tumor cell survival rate after using competitive inhibition of cysteine TGM2 was showed in (B). *, $P < 0.05$, **, $P < 0.01$ vs. cysteine (5 nM); #, $P < 0.05$ vs. cysteine (10 nM); $, $P < 0.05$ vs. cysteine (15 nM).

large number of TGM2 expression. This study showed that large expression of TGM2 was mainly found in stage III and stage IV of HCC, obviously higher than levels of tumor tissue in stage I-II ($p < 0.05$). This indicates that expression of TGM2 might be related to invasiveness and disease progression of tumors, n = 3 (**Table 1**, **Figure 2**).

Restraining TGM2 and promoting cell death

Study results showed that with an increase of cysteine dose, the number of HCC decreased significantly. Forty-eight hours after using cystamine, the survival rate of tumor cells decreased to about 36.5% of the maximum value. In addition, cystamine obviously decreased activation of caspase-3. Finally, it decreased phosphorylated AKT expression and induced apoptosis (**Figure 3**).

Discussion

HBV-related chronic liver disease is one of the main causes of HCC. It has been reported that the odds of developing cirrhosis of the liver could be up to about 40%, without any intervention, greatly increasing the risk of HCC. Prognosis of such patients has been closely related to early diagnosis and effective treatment measures [9]. At present, known molecular markers mainly include AFP, AFP-L3, DCP, α-fucosidase, and glypican-3. Previous studies, however, have shown that accuracy and sensitivity for early diagnosis have been lacking for these markers. Therefore, more accurate early diagnosis of biomarkers and effective detection of HCC progression are extremely important.

TGM2 is regarded as multifunctional enzyme which can decorate post transcriptional proteins and form isopeptide bonds between glutamine and lysine side chains. It participates in different biological processes. Some studies have shown that it plays an important role in celiac disease, neurodegenerative disorders, and some cancer neurodegenerative disorders [10, 11]. The latest study also confirmed that upregulation of TGM2 expression levels was related to poor prognosis in colorectal cancer, non-small cell lung cancer, laryngocarcinoma, and other cancers. Some studies have thought it could be used as an effective marker for mammary cancer and lung cancer chemotherapy resistance, which could obviously decrease

the survival time of patients by up regulating TGM2 expression for patients with brain glioma [12]. If using cystamine, aminoglucose, or KCC009 to restrain TGM2, it could obviously improve glioma, mammary cancer, or cell death of pancreatic cancer. At the same time, this study demonstrated that upregulation of TGM2 expression related to strong tumor invasion. Therefore, it could be used as target point to treat some types of cancer [13-17].

Transglutaminase-2 could express greatly in many kinds of tissues and could consist in multiple regions of a cell, including extracellular matrix, cell membrane, cytoplasm, mitochondria, and nucleus. It exerts a negative effect on cell growth, differentiation, and apoptosis through multiple mechanisms of action, including transamidase, GTP enzyme, cell adhesion, protein disulfide isomerase, kinase, and protein folding [18]. At present, specific action mechanisms of TGM2 in tumor formation remain unclear, but it has corresponding existence of several mechanisms. Some studies have argued TGM2 could activate NF-κB and focal adhesion kinase (FAK) tyrosine kinase, so that anti-apoptotic pathways are activated and immortality of tumor cells is achieved [19]. Inhibitor KCC009 of TGM2 could decrease Akt phosphorylation and promote upregulation of apoptotic protein Bim expression, thus enhancing apoptosis of tumor cells [20, 21]. This present study used cysteine competitiveness to restrain the action pathways of TGM2. Results showed that cystamine decreased activation of caspase-3 and reduced AKT expression of phosphorylation and apoptosis, consistent with the literature. Therefore, it could become one of the main target points of HCC treatment in the future.

In conclusion, this study preliminarily confirms that expression level of TGM2 is related to invasiveness and tumor staging in hepatitis B virus-related HCC, promoting tumor cell apoptosis by competitive inhibition of TGM2 pathways.

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Disclosure of conflict of interest

None.

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