Significance of TRPS1 in the development and clinicopathologic of hepatocellular carcinoma

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Abstract. – OBJECTIVE: The purpose of this paper was to explore roles and significance of transcriptional repressor GATA binding 1 (TRPS1) in the process of hepatocellular carcinoma (HCC) and clinicopathological parameters of HCC patients, respectively.

PATIENTS AND METHODS: The expression of TRPS1 was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot. Chi-square test was used to investigate the association between TRPS1 and clinicopathological parameters of HCC patients. SiRNA was designed to suppress the expression of TRPS1. Moreover, transwell assay and cell cycle analysis were adopted to determine the invasion ability and proliferation ability of HCC cells.

RESULTS: It was demonstrated that TRPS1 was abnormally over-expressed in HCC tissues and cells, and was closely associated with the vascular invasion, tumor size, and TNM stage of HCC patients. Besides, the results indicated that the inhibition of TRPS1 in HCC cells could impede the cell invasion ability and proliferation ability.

CONCLUSIONS: TRPS1 plays a key role in the development of HCC and is closely associated with the clinicopathological parameters of HCC patients. Hence, it is proposed that TRPS1 may serve as a novel prognostic marker and a potential therapeutic target for HCC treatment.

Key Words: Hepatocellular carcinoma, Transcriptional repressor GATA binding 1, Invasion, Proliferation.

Introduction

Primary liver cancer is one of the most common tumors worldwide, with the incidence rate ranking sixth among all malignant tumors and mortality rate ranking fourth among cancer-related deaths. Belonging to primary liver cancer, hepatocellular carcinoma (HCC) is the most common one. At the same time, HCC patients account for about 95% of patients with primary liver cancer, and only 10-23% of HCC patients have the opportunity for surgical treatment due to the strong proliferation, invasion, and metastasis abilities of HCC. Currently, radiotherapy and chemotherapy have been applied in the treatment of HCC, but the clinical efficacy is still poor due to the insensitivity to these therapies. In recent years, mounting molecules (mRNA, miRNA, circRNA, and IncRNA) have been reported to participate in and regulate a wide range of biological activities of HCC. Therefore, in-depth investigation of the molecular mechanism of HCC occurrence and development will contribute to the exploration of new vital molecular targets, thus providing a theoretical basis for the molecular diagnosis and targeted therapy of HCC.

Members of the GATA transcription factor family are widely considered as key transcription factors in human cancers. Transcriptional repressor GATA binding 1 (TRPS1), located in human chromosome 8q23-24, is the 7th atypical transcription factor found in the GATA family. Mainly present in the nucleus of the organism, TRPS1 is widely expressed in various tissues and organs, and involved in the development and differentiation of a variety of cells. The role of TRPS1 in tumors has been gradually researched. It is reported that TRPS1, as a transcription factor, is related to the occurrence and development of various tumors, whose expression is upregulated in breast cancer, prostate cancer, non-small cell lung cancer, and colon cancer. Besides, TRPS1 is closely related to such biological processes as EMT and angiogenesis and plays vital...
roles in them\textsuperscript{18}. However, the expression pattern of TRPS1 in HCC and its impacts on HCC patients remain unknown.

This study mainly aims to investigate the molecular function and role of TRPS1 in the invasion and proliferation of HCC cells, as well as the association between TRPS1 and clinicopathological parameters of HCC patients.

**Patients and Methods**

**Tissue Obtaining**

HCC tissues that were surgically resected and confirmed by pathology were collected, and the corresponding adjacent tissues were at least 2 cm away from the cancer tissues. All patients did not receive radiofrequency ablation, interventional therapy, radiotherapy, and chemotherapy or drug targeted therapy before surgery. The patients were informed of the use of all specimens and the acquisition of data and signed the informed consent. The investigation was approved by the Ethics Committee of the Yantai Yuhuangding Hospital.

**Culture of Cells**

Normal cell line L-02 and the hepatoma cell lines HuH-6, Hep-3B, HepG2, SMMC7721 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) complete medium containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) and 1% penicillin-streptomycin mixture. At the passaging process, an appropriate amount of trypsin was added into cell culture for digestion, and then 2 mL of complete medium was quickly added to terminate trypsin digestion. Next, cell suspension was collected and centrifuged at 800 rpm × 5 min. The supernatant was then discarded and fresh RPMI-1640 complete medium (HyClone, South Logan, UT, USA) was added. Thereafter, the cells were re-suspended and passaged at 1:2 or 1:3.

**Transient Transfection**

An appropriate amount of HCC cell line was seeded in a 6-well plate and transfected with plasmid containing TRPS1 siRNA (si-TRPS1 group) or siRNA NC (NC group) when the density reached 70-90%, respectively. First, Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) and plasmid were separately diluted with serum-free RPMI-1640 cell culture medium and allowed to stand at room temperature for 5 min. Then, the above two systems were mixed and allowed to stand at room temperature for 15 min. Finally, the incubated plasmid-lipid complex was mixed and added to a 6-well plate. Subsequent experiments were performed after cell transfection for 24-48 h.

**Transwell Assay**

The cell invasion ability was detected at 48 h after transfection. Briefly, after washed twice with phosphate-buffered saline (PBS), cells were trypsinized and re-suspended in serum-free RPMI-1640 medium. Then, a total of 2×10\textsuperscript{4} cells were added to the upper chamber after the transwell chamber was placed in a 24-well plate. After culturing in a 5% CO\textsubscript{2}, 37°C cell culture incubator for 24 h, cells were washed twice with PBS again, and then a cotton swab was used to scratch the cells in the upper chamber. Later, the cells in the chamber were fixed with formaldehyde for about 30 min, followed by staining in 0.1% crystal violet for 20 min. Finally, the chamber membrane was air-dried, sealed with a neutral resin, and observed under a microscope (Olympus, Tokyo, Japan).

**Cell Cycle Analysis**

At 48 h after transfection, the cells were trypsinized and then washed twice with PBS, followed by fixing with 1% paraformaldehyde at 4°C overnight. Then, 500 μL of propidium iodide (PI) staining regents were added into the cells and co-cultured at 4°C in the dark for 30 min. The cell samples were then loaded onto flow cytometer (FACSCalibur; BD Biosciences, Detroit, MI, USA). Results were further analyzed by cycle fitting software.

**Quantitative Real-Time Polymerase Chain Reaction (q-PCR) Analysis**

RNA in tissues and cells was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Briefly, TRIzol reagent was added into the tissues or cells and allowed to stand on ice for 5 min, and then the mixture was collected and added with appropriate chloroform and isopropanol. The RNA was later dissolved in diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China) after centrifugation at 1000 g for 10 min, and the concentration and purity of the RNA were determined using Nanodrop. After reverse transcription, 1 μg of comple-
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Molecular deoxyribonucleic acid (cDNA) was taken from each group for qPCR to detect gene expression. The applied primers are listed below: GAPDH forward primer, 5’-GTCAGCGCCATCTTTTTT-3’, and reverse primer, 5’-CGCCCAAATACGACCAAAT-3’, and TRPS1 forward primer, 5’-CAAATCTCAGGCTGAGTGA-3’ and reverse primer, 5’-GTGAAGAGCTGATCCTGCAG-3’. The expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference, and the results calculated by the 2^{-ΔΔCT} method based on CT values were used as relative gene expression levels in each group.

Western Blot Analysis

The cells were collected first, washed 3 times with cold PBS, and then added with radioimmunoprecipitation assay (RIPA) lysate (containing phenylmethylsulfonyl fluoride (PMSF) at the ratio of 100:1) (Beyotime, Shanghai, China). Subsequently, the concentration of protein was measured by a bicinchoninic acid (BCA) protein quantification kit (Pierce, Rockford, IL, USA) according to the manufacturer’s instructions. Later, the protein was mixed with the 5× loading buffer, and boiled at 100°C for 10 min. Then, a total of protein sample (40 mg/well) was separated by a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland). After blocking with Tris-Buffered Saline and Tween-20 (TBST) + 5% skim milk powder for 2 h, the PVDF membrane was incubated with the primary antibody at 4°C overnight and the secondary antibody at 37°C for 30 min, respectively. After washing 3 times with TBST, the PVDF membrane was placed on a chemiluminescence imager for imaging.

Statistical Analysis

The statistical data were statistical analyzed by using Statistical Product and Service Solutions (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA) with data expressed as mean ± standard deviation (x ± s). Differences between two groups were analyzed by using the Student’s t-test. Comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). Comparison between groups was performed using GraphPadPrism7.0 software (La Jolla, CA, USA). The association between TRPS1 and clinicopathological parameters of HCC patients was analyzed using Chi-square test. p<0.05 suggested that the difference was statistically significant.

Results

TRPS1 was Highly Expressed in HCC Tissues and Cells

To explore roles of TRPS1 in HCC, the mRNA expression of TRPS1 in acquired HCC tissues and corresponding normal liver tissues were detected. As shown in Figure 1A, the expression level of TRPS1 in HCC tissues was about 1.7-fold higher than that in normal tissues. Further, the mRNA expression of TRPS1 was

Figure 1. TRPS1 is highly expressed in HCC tissues and cells. A, TRPS1 is upregulated in HCC tissues compared with that in adjacent normal tissues. B, TRPS1 is markedly over-expressed in HuH-6, Hep-3B, HepG2, SMMC7721 cell lines than that in L-012 cell line. (***p<0.001).
analyzed in five cell lines, including one normal cell line (L-02) and four hepatoma cell lines (HuH-6, Hep-3B, HepG2, SMMC7721) by qRT-PCR assay. The results (Figure 1B) revealed that TRPS1 was significantly up-regulated both in HuH-6, Hep-3B, HepG2, SMMC7721 cell lines compared to that in L-02 cell line, indicating that TRPS1 is abnormally involved in the progression of HCC.

**Clinical Significance of TRPS1 in HCC**

To explore the clinical significance of TRPS1 in HCC, 156 HCC tissues were divided into TRPS1 low expression group (78/156) and TRPS1 high expression group (78/156) according to the mRNA expression level of TRPS1. Subsequently, the association between TRPS1 and clinicopathological parameters of HCC patients was analyzed. As shown in Table I, no relationships were detected between the expression level of TRPS1 and the HCC patient’s gender ($p=0.373$), age ($p=0.518$), AFP level ($p=0.109$) and Cirrhosis ($p=0.873$). However, the expression level of TRPS1 was found related to the Vascular invasion ($p=0.001$), Tumor size ($p<0.001$), and TNM stage ($p=0.002$) of included HCC samples.

### Table I. TRPS1 expression and clinic pathological parameters of patients with HCC.

<table>
<thead>
<tr>
<th>Variable</th>
<th>TRPS1 mRNA expression level</th>
<th>$p$-value</th>
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<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
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<td>Sex</td>
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<td>Age, years</td>
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</tr>
<tr>
<td>&gt; 50</td>
<td>42</td>
<td>47</td>
</tr>
<tr>
<td>Vascular invasion</td>
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<td></td>
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<tr>
<td>Yes</td>
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<td>AFP level, µg/L</td>
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<td>Stage III-IV</td>
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AFP, alpha fetoprotein; TNM, tumor-node-metastasis. $p<0.05$, statistical difference.

**Inhibition of TRPS1 Blocked the Invasion Ability of HCC Cells**

To further illustrate the molecular function of TRPS1 in HCC, Hep-3B and HepG2 cell lines were selected as the research objects based on the expression level of TRPS1. Firstly, stable Hep-3B and HepG2 cell lines with TRPS1 downregulation were established (Figure 2), and then transwell assay was performed. The results (Figure 3) showed that the invasion ability of HCC cells was notably decreased in the si-TRPS1 compared that in NC group, implying that inhibition of TRPS1 expression manifestly damages the invasion ability of HCC cells.

**Suppression of TRPS1 Impaired the Proliferation Ability of HCC Cells**

To figure out whether TRPS1 can regulate the proliferation of HCC cells, cell cycle distribution was detected by flow cytometry. As shown in Figure 4, when the expression of TRPS1 in HCC cells was inhibited by siRNA transfection, a higher percent of cell number in G1 state and a lower percent in S and G2 state were observed in si-TRPS1 group than those in NC group, suggesting that suppression of TRPS1 clearly impedes the proliferation of HCC cells.
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Discussion

HCC is a common malignant tumor that seriously endangers human health. Although treatment strategies, such as hepatectomy, liver transplantation, radiofrequency ablation, interventional therapy, and targeted drug combination therapy for HCC treatment have been developed remarkably\(^{19,20}\), the prognosis of HCC patients is still unsatisfactory due to its complex pathogen-

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**Figure 2.** siRNA transfection suppresses the expression of TRPS1. A-F, Hep-3B cells and HepG2 cells transfected with TRPS1-siRNA present decreased mRNA and protein expression levels of TRPS1 compared with cells treated with scramble siRNA, respectively (**\(p<0.01\), ***\(p<0.001\)).

**Figure 3.** Inhibition of TRPS1 blocks the invasion ability of HCC cells. A-D, Inhibition of TRPS1 expression in Hep-3B cells and HepG2 cells manifestly damages their invasion ability compared to that in the cells transfected with scramble siRNA (magnification: 200×). (**\(p<0.01\)).
According to recent reports, up to 90% of HCC patients who received treatment for the first time would suffer from tumor recurrence and metastasis. Therefore, searching for biomarkers related to the early diagnosis, therapeutic intervention, and prognosis of HCC and investigating the molecular mechanism of HCC occurrence and metastasis are of great significance for HCC patients.

As a non-specific transcription inhibitor of the GATA family, TRPS1 exerts a crucial effect in regulating various tumors. Lin et al. found that the high expression of TRPS1 can improve the prognosis of breast cancer patients who received chemotherapy, so as to inhibit cancer progression. In addition, Wu et al. have shown that down-regulation of TRPS1 can promote the occurrence of EMT and enhance the migration and invasion abilities of highly metastatic breast cells, colon cancer cells, and nasopharyngeal carcinoma cells. However, researchers also discovered that high expression of TRPS1 is associated with lymph node metastasis and pathological stage of colon cancer patients, and it is an independent indicator for the poor prognosis of them. Collectively, the above reports indicated that the functions of TRPS1 varied in different types of tumors and was closely related to the prognosis of tumor patients. However, very little is known about the roles of TRPS1 on the development of HCC.

In the present study, for the first time, the expression level of TRPS1 was compared between HCC tissues and corresponding normal liver tissues, and it was found to be highly expressed in the former. Next, for further validation, normal liver cell line L-02 and four hepatoma cell lines, including HuH-6, Hep-3B, HepG2, SMMC7721, were selected to detect the TRPS1 mRNA expression. It was discovered that the expression level of TRPS1 in HCC cell lines was also significantly upregulated compared with that in normal cell lines. In view of this, the relationship between TRPS1 and clinicopathological features of patients with HCC was analyzed, and the results showed that TRPS1 was not only related to vascular invasion, but also closely related to tumor size and TNM stage. These results indicate that TRPS1 is of great significance in the progression of HCC and the prognosis of HCC patients. Considering that the invasive ability and proliferative capacity of cancer cells are closely related to the development and prognosis of cancer, the effects of TRPS1 on the invasion and proliferation of HCC cells were then investigated by transwell assay and cell cycle detection, respectively. Not surprisingly, significant impairment was found in the invasion ability and proliferation ability of HCC cells when the expression of TRPS1 was endogenously inhibited by siRNA transfection.

**Conclusions**

In summary, the data of this study suggest that acting as an oncogene, TRPS1 is closely involved in HCC development by positively regulating the invasion and proliferation of HCC cells, thus leading to worsening vascular invasion, poor tumor size, and TNM stage of HCC patients. These
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findings in this study not only improve our understanding of the molecular mechanisms of TRPS1 in HCC, but also reveal a novel therapeutic target for HCC treatment.

Conflict of Interest
The Authors declare that they have no conflict of interests.

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