Tex10 is upregulated and promotes cancer stem cell properties and chemoresistance in hepatocellular carcinoma

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ABSTRACT

Testis expressed 10 (Tex10), a new core component of the pluripotency circuitry, has been reported to positively regulate embryonic stem cell (ESC) super-enhancers to promote ESC self-renewal; however, the expression and function of Tex10 in hepatocellular carcinoma (HCC) and liver cancer stem cells remains unclear. The present study was designed to investigate the expression patterns of Tex10 with immunohistochemistry, western blotting and RT-qPCR in samples from HCC patients and HCC cell lines. The results obtained show that Tex10 was highly expressed in HCC tissues, and elevated Tex10 protein levels positively correlate with the poorly differentiated carcinoma. Likewise, we found that Tex10 expression in the high-metastasis HCCLM3 potential cell line was higher than that in the low-metastasis HepG2 potential cell line, and Tex10 expression in liver cancer stem cells was also higher than that in adhered HCC cells. In addition, Tex10 knockdown decreased stem cell marker expression and drug resistance. Tex10 promoted cancer stemness through activation of the STAT3 signaling pathway. Taken together, our study demonstrates that Tex10 plays a potent carcinogenic role in HCC tumorigenesis by maintaining cancer stem cell properties through activation of the STAT3 signaling pathway and promoting chemo-resistance. Thus, targeting Tex10 may provide a novel and effective therapeutic strategy to suppress the tumorigenicity of advanced HCC.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignant tumor and the third highest cause of cancer-related death in the world [1]. In China, approximately 366,000 new cases and 321,000 deaths occurred in 2012, the incidence of which ranks is in third place and the mortality of which ranks in second place for various cancers [2]. HCC is an aggressive tumor usually diagnosed at a late stage and for which effective therapies remain lacking [3]. Although the treatment for HCC has been greatly improved, the mortality rate remains high because of drug resistance and recurrence [4].

Cancer stem cells (CSCs) are a unique population of cancer cells that are responsible for tumor initiation, metastasis, self-renewal and drug resistance [5,6]. Several lines of evidence have suggested that drug resistance and recurrence of HCC are closely associated with the existence of CSCs [7,8]. Considering the potential role of CSCs in diagnosis and treatment of HCC, it is important to elucidate the molecular mechanism that regulates CSC expansion and drives carcinogenesis and tumor metastasis.

The biological functions of Testis expressed protein 10 (Tex10) are poorly understood. As a component of the five friends of methylated chotp (5FMC) and Rix complexes, Tex10 plays important roles in transcriptional regulation and regulating ribosome biogenesis and cell cycles, respectively [9,10]. The significance of Tex10 is manifested by the fact that as a new Sox2-interacting protein, Tex10 was identified as a key pluripotency factor that plays a crucial role in embryonic stem cell (ESC) self-renewal and pluripotency, early embryo development and reprogramming [11]. Interestingly, many pluripotency genes, which are highly expressed in ESCs, are also involved in tumorigenesis and tumor metastasis [12,13]. The core pluripotency transcription factor Oct4 of ESCs is frequently expressed in HCCs, and its expression correlates with those of putative CSC markers and CSC properties [14]. In addition, the key pluripotency transcription factor Nanog of ESCs contributes to facilitating formation of tumor-initiating stem-like cells in livers of mice [15,16]. Based on these findings, we propose a hypothesis that Tex10 may also be relevant to the stem cells of HCC and participate in the development of HCC. The present study therefore, investigated the expression and biological function of Tex10 in HCC and liver CSCs.

Results

Increased expression of Tex10 in primary HCC tissues

The expression of Tex10 was detected with immunohistochemical staining (IHC) in pairs of human normal liver tissues and HCC tissues, which were randomly chosen from six poorly-differentiated HCC patients who underwent surgical resection in the Nanchong Central Hospital in Nanchong, China. In normal liver tissue, the expression of Tex10 was negative (Figure 1(a)); however, Tex10 was detected in a nuclear location in cancerous tissue (Figure 1(b)), suggesting that Tex10 expression is up-regulated in HCC.
The expression of Tex10 is positively correlated to poorly-differentiated HCC

Nine HCC patients were enrolled in this study (Table 1). The tumor grade according to the World Health Organization (WHO) classification was Grade 1 in three patients and Grade 2 in six patients. The expression of Tex10 was negative in CD44− well-differentiated HCC cancer cells (Figure 2(a,c)). Conversely, in CD44+ poorly-differentiated HCC tissues [17], Tex10 was highly expressed at the nucleus of the cancer cells (Figure 2(b,d)). Taken together, it emerged that a higher level of Tex10 expression was observed in poorly differentiated HCC samples, compared to that in well-differentiated HCC samples.

Tex10 expression increases in HCC high-metastasis potential cell lines

To study the expression of Tex10 in HCC cells, western blotting, qRT-PCR and ICC staining were performed. The expression of Tex10 in mouse liver tissues is negative and compared with LO2 immortalized human normal liver cells, Tex10 protein and mRNA were highly expressed in HCC cell lines (Figure 3(a,b)). In addition, Tex10 expression in HCC was further analyzed by immunocytochemical staining, which suggested that HCC cell line HCCLM3 with high-metastasis potential has the highest intensities of Tex10 nucleus staining, whereas HepG2, the low-metastasis potential cell line, has the lowest intensities. Secondary antibodies alone did not

Table 1. Expression of Tex10 and CD44 in HCC tissue.

| Case                  | Tex10 | CD44 |
|-----------------------|-------|------|
|                       | n     | Positive | Negative | Positive | Negative |
| Poorly-differentiated | 6     | 5       | 1        | 6        | 0        |
| HCC                   |       |         |          |          |          |
| Well-differentiated HCC| 3    | 0       | 3        | 1        | 2        |

Figure 1. Tex10 expression is up-regulated in hepatocellular carcinoma (HCC). (A) Immunohistochemical staining showing normal liver cells was negative for Tex10. (B) Representative IHC images of Tex10 expression in HCC. Tex10 was strongly positive in the nucleus of the cancer cells in HCC tissue. Scale bar: 100 μm.

Figure 2. High expression of Tex10 related to HCC degree of differentiation. (A, C) Representative IHC images of Tex10 and CD44 expression in highly differentiated HCC. (B, D) Representative IHC images of Tex10 and CD44 expression in poorly differentiated HCC. Scale bar: 100 μm.
generate any signal (Figure 3(c)). These data suggested that Tex10 expression was significantly increased in HCC and it might be correlated with HCC metastasis.

**Tex10 promotes a stem cell-like phenotype in HCC**

The expression level of Tex10 was significantly increased in poorly differentiated HCC clinical samples and HCC cell line with high-metastasis potential. To dissect the biological functions of Tex10, we first infected HCCLM3 cells with lentivirus vectors containing Tex10 shRNA or negative control to generate stable cell lines that constitutively down-regulated Tex10 and the control cells (Figure 4(a–c)). We found that mRNA expression of the CSC markers ALDH1, ABCG2 and EpCAM was significantly decreased in HCCLM3 cells after Tex10 knockdown. Importantly, qRT-PCR analysis showed that mRNA expression of stem cell-associated genes in HCC such as Oct4 and Sox2 were also markedly inhibited in HCCLM3 cells with down-regulated Tex10 (Figure 4(d), *P < 0.05, **P < 0.01). To further investigate the functional role of Tex10 in the CSC properties of HCC, spheroid culture of cancer cells is a routine approach to enrich liver cancer stem cells (LCSCs). The results from the HCCLM3 cell line showed that expression of Tex10 in spheroids was dramatically higher than that in adherent cells (Figure 4(e)). In addition, supporting the significance of Tex10 in maintaining cancer stemness, we found that the number of spheroids formed in HCCLM3 cells with down-regulated expression of Tex10 was remarkably fewer and lower compared with control HCCLM3 cells as shown by the spheroid formation assay (Figure 5(a), *P < 0.05). The role of Tex10 in HCC migration was investigated. The wound healing assay showed that the closure of shTex10 cells was significantly slower than that of scramble cells (Figure 5(b), *P < 0.05). All these results indicated that Tex10 regulates CSC properties in HCC.

*Figure 3.* Tex10 is highly expressed in the HCC high-metastasis potential cell line. (A) Tex10 expression in mouse normal liver tissues and distinct HCC cell lines was analyzed by western blotting. (B) qRT-PCR analysis of Tex10 expression in different HCC cell lines. (C) Immunocytochemical staining of Tex10 in different HCC cell lines. Scale bar: 100 μm.
Figure 4. Suppression of stemness expression via knockdown of Tex10 in HCC. Tex10 increases the stemness of HCC cells. (A) The stable cell lines were established by transfection with scramble and shTex10 with high infection efficiency. (B, C) qRT-PCR and western blot analysis showing knockdown of Tex10 in HCCLM3. (D) The mRNA expression of stem cell markers (EpCAM, CD90, ALDH1, Bmi-1, ABCG2) in HCCLM3 cells was analyzed by qRT-PCR. The error bars represent SD. (E) The protein expression levels of Tex10 were measured by western blotting in spheroids (LM3-CSCs) and adhered cells of HCCLM3. GAPDH expression was used as the loading control. Scale bar: 100 μm. (*P < 0.05, **P < 0.01).

Figure 5. Knockdown of Tex10 suppresses CSC behaviors. (A) Self-renewal potency was evaluated by formation of tumor spheres. The knockdown of Tex10 decreased the tumor sphere-forming abilities. (B) Wound healing assay showed that Tex10 knockdown suppressed the migration capacity of HCC cells at 0h, 24h, and 48h post wounding. Scale bar: 100 μm. (*P < 0.05).
Tex10 affects the cell cycle and drug chemoresistance of HCC to sorafenib and cisplatin

To further investigate the effect of Tex10 on the cell cycle of HCC cells, the distributions of three cell subpopulations (G0/G1, S and G2/M) were analyzed by flow cytometry. In the HCCLM3 and scramble groups, more cells were found in the S phase and G2/M phase of the cell cycle compared with the shTex10 groups (Figure 6(a)). There were no differences in the three cell subpopulations between HCCLM3 and scramble. The data suggests that there were fewer Tex10-silenced cells in the proliferative phase. Resistance to chemotherapy is an important characteristic of CSCs. The role of Tex10 in the drug resistance of HCC to sorafenib and cisplatin was investigated, and it was found that when exposed to the same dosages of sorafenib or cisplatin, sorafenib or cisplatin treatment alone significantly reduced cell proliferation compared with DMSO treatment; the sensitivity of Tex10-silenced cells to sorafenib or cisplatin was increased compared with scramble cells (Figure 6(b,c), *P < 0.05, **P < 0.01). Moreover, western blotting also showed that ABCG2, in the ABC family of multidrug resistant genes, was significantly decreased in the HCC cell line with knocked down Tex10 (Figure 6(d)). Therefore, these results showed that Tex10 knockdown significantly increased drug sensitivity of HCC to sorafenib and cisplatin, suggesting a possible role of Tex10 in the treatment of HCC drug resistance.

Tex10 promotes cancer stem cell properties in HCC through STAT3 signaling

Signal transducer and activator of transcription 3 (STAT3) remains constitutively active in many types of cancer including HCC and plays a critical role in proliferation and self-renewal of CSCs [18,19]. Therefore, we investigated the relationship between Tex10 and STAT3 signaling in the regulation of CSC properties of HCC. As shown in Figure 7(a), the protein expression of tyrosine phosphorylated STAT3 (pSTAT3 Y705) was dramatically decreased in the HCC cell line with Tex10 knockdown, whereas knockdown Tex10 had no effect on the total STAT3 protein.

Figure 6. The effect of Tex10 on cell cycle and drug resistance of HCC to sorafenib and cisplatin. (A) Flow cytometric analysis of the cell cycle in 3 groups (HCCLM3, scramble, shTex10). (B, C) Cell proliferation of HCC cell lines with knocked down Tex10 compared with control cells when exposed to the same dosages of sorafenib or cisplatin. (D) The protein expression of the multidrug resistant gene ABCG2 in HCCLM3 with and without knocked down Tex10. (*P < 0.05, **P < 0.01).
Moreover, the mRNA expression of STAT3 downstream genes, such as Bcl-xL, Survivin, CCND1, c-Myc, and Twist1 was significantly decreased (Figure 7(b), **P < 0.01). To determine whether Tex10-promoted a stem cell-like phenotype mediated by STAT3 phosphorylation, we treated shTex10 cells and scramble cells for 48 h with 10 μM of S31-201, a specific STAT3 inhibitor, which can effectively inhibit STAT3 phosphorylation, dimerization, and translocation to the nucleus. As shown in Figure 7(c), S3I-201 narrows the difference in the number of spheroids formed between Tex10 knocked down cells and scramble cells (Figure 7(c), *P < 0.05), indicating that Tex10 induced cancer stem cell properties that were pSTAT3 dependent. Taken together, these results indicated that Tex10 regulates CSC properties in HCC through STAT3 signaling.

**Discussion**

Despite advances in surgery and chemotherapy, the mortality rate of HCC remains very high due to late diagnosis and lack of effective treatment strategies. Accumulating evidence has demonstrated that liver CSCs contribute to the chemo-resistance and recurrence of HCC. Therefore, more effective therapeutic strategies could be developed if the molecular mechanism underlying liver CSC regulation were clarified. In this study, our results first demonstrated that Tex10 was up-regulated in HCC and associated with HCC metastasis. Moreover, we also first demonstrated that Tex10 promoted cancer stem cell-like traits through STAT3 signaling. Thus, Tex10 has a great clinical value for targeted therapy.

The functions of Tex10 are not well understood. It was previously reported as participating in transcriptional regulation by linking arginine methylation to desumoylation [9], and contributing to control ribosome biogenesis and cell cycle regulation through p53 [10]. Ribosome biogenesis has been intrinsically linked with cancer development [20,21] and cell cycle control is being recognized for its potential role in malignant progression of cancers [22,23]. Importantly, as a core component of the pluripotency network, Tex10 plays a central role in regulating enhancer activity in ESCs to promote ESC self-renewal and sustain pluripotency. However, Tex10 expression and its function in HCC remain unclear. In the present study, we found that Tex10 was dramatically up-regulated in human HCC tissues, compared to normal liver tissues. Moreover, Tex10 expression is significantly up-regulated in poorly-differentiated HCC tissues and the highly metastatic HCC cell line, suggesting its potential role in HCC metastasis. Together, these results confirm that targeting Tex10 could be a novel promising strategy for therapy of poorly differentiated HCC.

CSCs, a small population within the tumor mass, participate in tumor growth, invasion, and metastasis as well as in drug resistance and recurrence, including in liver cancers, and thus, CSCs are therefore important targets for cancer treatment [24,25]. Consequently, it is necessary to investigate the mechanism of CSC behavior in depth. Considering that self-renewal is a hallmark of CSCs, to test whether Tex10 initiates self-renewal features in HCC, we performed a sphere-forming ability assay, and our study demonstrated that Tex10 expression was highly expressed in hepatoma spheroids. Moreover,
Tex10-silencing significantly decreased the self-renewal capacity of HCCLM3 cell lines. Specifically, Tex10-silencing reduced the expression of CSC markers CD90, ALDH1 and BMI-1. Our results indicated that Tex10 plays a strong tumorigenic role in HCC by maintaining stem cell-like traits. As one of the main characteristics of CSCs, chemoresistance is largely ascribed to the self-renewing subpopulations of CSCs present in the bulk tumor. As chemotherapeutic agents, sorafenib and cisplatin are widely used for the treatment of HCC but with low response rates. Thus, there is an urgent need to confirm the cellular and molecular mechanisms of drug resistance in HCC. Our results indicate that acquired sorafenib and cisplatin resistance in HCC is associated with up-regulation of Tex10 expression and can partially be reversed by knockdown of Tex10. In addition, Tex10-silencing substantially decreased the expression of ABCG2, a main ABC transporter, which is attributed to drug resistance. Therefore, Tex10 could be a novel target for overcoming chemoresistance in HCC.

Several signaling pathways, such as Wnt/β-catenin, TGF-β, Notch, and the STAT3 pathway, have been defined as involved in the regulation of LCSCs. It is well-known that the STAT3 proteins are steadily phosphorylated on the tyrosine residue in many cancers [26], and the STAT3 signaling pathway has been identified as accelerating the progression and metastasis by inducing cancer stemness and chemotherapeutic resistance in various cancers [27]. In the present study, we confirmed that Tex10 regulates CSC properties in HCC through STAT3 signaling. However, accurate molecular target(s) need to be further confirmed in future studies.

In conclusion, our study demonstrated for the first time that Tex10 contributes to the development of malignant phenotypes of HCC and may serve as a stem cell marker for HCC. It represents a novel therapeutic target for advanced HCC treatment.

Materials and methods

Tissue samples

All patients signed an informed-consent document for diagnosis and research on tissue specimens before being enrolled in the project. All subjects gave written informed consent in accordance with the Declaration of Helsinki principles. The protocol was approved by the Ethics Committee of the North Sichuan Medical College (NSMC) and Ethical Committee of the Nanchong Central Hospital. All methods in this study were performed in accordance with the human experimentation guidelines of the People’s Republic of China.

Cell culture

Human HCC cell lines HCCLM3, were purchased from the China Center for Type Culture Collection in Wuhan. HepG2 and immortalized human normal liver cells, LO2, were obtained from the Cell Bank of Typical Culture Preservation Committee of the Chinese Academy of Science, Shanghai, China. The cells’ metastatic potential and the characterization of these cells were based on previous studies [28]. All cell lines were cultured in Dulbecco’s Modified Eagle Medium (DMEM) at 37°C in a 5% CO2 incubator. The medium was supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Lentivirus construction and cell transduction

Human Tex10 small hairpin (shRNA) plasmids (target sequence: AGCTACTGCCCTCGGAATT) and shRNA control (target sequence: TTCTCGAACGTGTCAGT) were obtained from GENECHEM (Shanghai, China), and used to transfect 293T cells for virus production. Lentiviral vector encoding shTex10 was designated as shTex10. Scramble is a non-target shRNA control. The HCCLM3 cells were infected with recombinant lentivirus-transducing units following the manufacturer's instructions. The stable suppression of target genes was achieved by selection of cells with 2.5 μg/ml puromycin for 2 weeks. Selected pools of knockdown cells were used for the following experiments.

Spheroid formation assay

The spheroid formation assay was based on the method described previously [16]. Hepatoma cells were seeded in Ultra Low Attachment 6-well plates and cultured in serum-free Dulbecco’s modified Eagle’s medium/F12, supplemented with N2, B27 (Thermo Scientific, Waltham, MA), 20 ng/ml epidermal growth factor and 20 ng/ml basic fibroblast growth factor (Millipore, Billerica, MA, USA). Seven days later, the formed spheroids were counted under the microscope.

Protein extraction and western blot analysis

Whole cell protein was extracted with RIPA lysis buffer supplemented with protease inhibitors and separated by 10% SDS-PAGE, and then transferred to the NC membrane. The membrane was blocked with 5% non-fat milk and detected with appropriate primary antibodies followed by horseradish peroxidase-conjugated secondary antibody (Sigma, Saint Louis, MO, USA; A0545, M6898). Proteins were visualized using enhanced chemiluminescence regents (Thermo Scientific, Waltham, MA). The antibody against Tex10 (17,372–1-AP) was purchased from Proteintech Biotechnology. The antibody against ABCG2 (#42,078) and antibody against total STAT3 (#9139) were obtained from Cell Signaling Technology (Danvers, MA, USA). The antibody against pSTAT3 (Y705) (ab76315) was purchased from Abcam (Abcam, Cambridge, UK). Western blotting of GAPDH (ab8245, Abcam) on the same membrane was used as a loading control.

RNA extraction and qrt-pcr analysis

Total RNA was extracted by TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions and reverse transcribed using the ThermoScript RT-PCR System (Thermo Scientific, Waltham, MA). The cDNA was subjected to quantitative real-time PCR (qRT-PCR) using EvaGreen (Bio-Rad) on an iCycler Thermal Cycler (Bio-Rad). GAPDH was used as an internal control according to the previously described method [29]. All reactions were run in triplicate. The sequences of qRT-PCR primers are listed in the Supplementary Table 1.
**Immunohistochemistry**

Immunohistochemistry was performed according to our previous study [29]. In brief, 3-μm thickness paraffin-embedded samples were dewaxed and then antigen retrieval with 0.01 M sodium citrate buffer (pH 6.0) was carried out. This was followed with 3% H2O2 incubation for 10 min to quench endogenous peroxidase and 0.1% Triton X-100 for 10 min to permeate the membrane, and then with 3% bovine serum albumin blocking for 1h. The sections were incubated with primary antibodies against Tex10 (17372-1-AP, Proteintech, China) and CD44 (2M-0047, ZSGQ-BIO, China) overnight at 4°C, followed by horseradish peroxidase-conjugated secondary antibody (ZSGQ-BIO, China) incubation for 1h at room temperature. The sections were visualized using 3, 3’-diaminobenzidine tetrahydrochloride (DAB). The tissue sections were counterstained with hematoxylin and mounted.

**Immunocytochemical staining**

The HCC cell lines were fixed in paraformaldehyde and permeated with Triton X-100. ICC staining was conducted following routine protocols for Tex10 antibody (1:20, Proteintech). The nuclei of cells were counterstained with hematoxylin and mounted for microscopic visualization.

**Resistance analysis**

Cells were counted and seeded at 1 × 10^4 cells/well in 96-well plates, and cultured for 12 to 24h. The same dosage of 5μm/L sorafenib or 5μm/L cisplatin was added to the scramble and shTex10 HCC cells, respectively. The measurement of cell proliferation was performed with the Cell Counting Kit-8 (KeyGEN, Jiangsu, China). Three wells of each group were detected every day and incubated for 1.5h with the Cell Counting Kit-8 solution. The absorbance of the sample taken from each well was measured on a micro-plate reader (KHB, ST-360) at 450 nm. The results were plotted as the mean ± SD from three separate experiments with three replicates per experiment for each experimental condition.

**Wound healing assay**

The HCC cells were cultured for 2 days to 90% confluence, next rinsed with phosphate-buffered saline (PBS), and then serum-starved for 24h in serum-free medium. After the serum starvation, the cell monolayer was wounded with a 10μl plastic pipette tip to create three separate, parallel wounds. At the indicated times (0h, 24h, 48h), migrating cells at the wound front were photographed. A percentage of the wound area at each time point was measured using Image-Pro Plus v6.2 software. These experiments were performed in triplicate.

**FACS analyses**

Three groups of cells (HCCLM3, scramble, shTex10) were trypsinized, washed with cold PBS, and fixed in 70% ethanol at 4°C overnight. Then, 1 × 10^6 cells were stained with 50 mg/mL propidium iodide supplemented with 80 mg/mL RNase A at room temperature in the dark for 1 hr. The Cells were then subjected to FACS analysis with a FACSscan flow cytometer (Becton Dickson). The data were analyzed using the Cell Quest program.

**Statistical analysis**

All experiments were performed at least three times. The results were expressed as the mean ± standard deviation. Statistical analyses were performed using the Student’s t-test. A p-value < 0.05 was considered significantly different.

**Abbreviations**

Tex10 Testis expressed protein10  
HCC Hepatocellular carcinoma  
ESC Embryonic stem cell  
CSC Cancer stem cell  
IHC Immunohistochemical staining  
5FMC Five friends of methylated chtop  
LCSCs Liver cancer stem cells  
STAT3 Signal transducer and activator of transcription 3

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**Disclosure statement**

No potential conflict of interest was reported by the authors.

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**Author Contributions**

XX and GF designed the experiments, and XX and GF wrote the paper. XX, LD, RX, DX, KL, ZC, and YF performed the experiments.

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