IncRNA TPT1-AS1 knockdown inhibits liver cancer cell proliferation, migration and invasion

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Abstract. Long non-coding RNA (lncRNA) tumor protein translationally controlled 1 antisense RNA 1 (TPT1-AS1) serves as an oncogene in several tumors, including ovarian and cervical cancer. However, the functional role of TPT1-AS1 in liver cancer (LC) is not completely understood. The present study aimed to explore the role of TPT1-AS1 in LC. In this study, the reverse transcription-quantitative PCR results demonstrated that TPT1-AS1 expression was significantly upregulated in LC tissues and cell lines compared with adjacent paracancerous tissues and THLE-3 cells, respectively. Elevated TPT1-AS1 expression was significantly associated with TNM stage lymph node metastasis and poor prognosis in patients with LC, as determined via χ2 and Kaplan-Meier survival analyses. By constructing TPT1-AS1 knockdown LC cell lines (HepG2 and SNU-182), loss-of-function experiments, including Cell Counting Kit-8, colony formation, flow cytometry, wound healing and Transwell assays, were performed to explore the function role of TPT1-AS1 in LC in vitro. The results demonstrated that TPT1-AS1 knockdown inhibited LC cell proliferation, G1/S transition, migration and invasion compared with the small interfering RNA (si)-negative control (NC) group. Mechanistically, TPT1-AS1 knockdown markedly decreased CDK4, N-cadherin and Vimentin expression levels, but notably increased p21 and E-cadherin expression levels compared with the si-NC group. Therefore, the results of the present study suggested that TPT1-AS1 might serve as a promising therapeutic target for LC treatment.

Introduction

Liver cancer (LC) is one of the most frequently occurring gastrointestinal malignancies and leading causes of tumor-related death worldwide (1,2). Certain primary risk factors, including alcohol abuse and hepatitis virus infection, are correlated with LC pathogenesis (3). Despite advances in early diagnosis and therapeutic strategies, including surgical resection, radiotherapy and chemotherapy (4,5), the 5-year overall survival rate for LC (34%) has not significantly improved, primarily due to the high rate of recurrence and metastasis (6). Therefore, improving the current understanding of the molecular mechanisms associated with hepatocarcinogenesis is important for improving the prognosis of LC.

Long non-coding RNAs (lncRNAs) are a class of non-protein coding RNAs that are >200 nucleotides in length. lncRNAs have become a hot research topic due to their important regulatory functions in various biological behaviors, including proliferation, differentiation and metastasis (7,8). Accumulating evidence has indicated that lncRNAs are frequently aberrantly expressed, and function as positive or negative regulators of coding genes in the progression of different types of cancer, including LC, colorectal cancer and lung cancer (9-11). For instance, Jiang et al (12) reported that upregulated double homeobox A pseudogene 8 expression predicted poor outcomes in patients with LC and promoted cell proliferation in vitro. Zheng et al (13) highlighted the oncogenic role of long intergenic non-protein coding RNA 467 in LC progression via regulating the microRNA-18a/neural precursor cell expressed, developmentally downregulated 9 axis. Moreover, IncRNA F11 antisense RNA 1 negatively regulated LC cell proliferation, migration and invasion (14). Novel IncRNA tumor protein translationally controlled 1 antisense RNA 1 (TPT1-AS1) has been reported to be associated with the prognosis of patients with glioma (15,16). Moreover, the oncogenic function of TPT1-AS1 has been further validated in other tumors. For example, Jiang et al (17) not only demonstrated that high TPT1-AS1 was correlated with adverse prognostic characteristics, but also reported that TPT1-AS1 promoted cell proliferation and metastasis in cervical cancer. Jiang et al (17) demonstrated the oncogenic effects of TPT1-AS1 on epithelial ovarian cancer cell proliferation, migration and invasion. In addition, Jia et al (18) demonstrated that TPT1-AS1 inhibited glioma cell autophagy and promoted cell proliferation. In gastric cancer, TPT1-AS1 knockdown significantly inhibited cell proliferation, G1/S transition and epithelial-mesenchymal transition (EMT) (19). However, the clinical significance and functional roles of TPT1-AS1 in LC are not completely understood.

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The present study aimed to explore the role of TPT1-AS1 expression in LC tissues and cells, as well as the association between TPT1-AS1 and LC clinicopathological features or survival prognosis were investigated. Subsequently, loss-of-function experiments, including Cell Counting Kit-8 (CCK-8), colony formation, flow cytometry, wound healing and Transwell assays, were performed to explore the potential effects of TPT1-AS1 on LC cell functions. The results of the present study may improve the current understanding of LC progression to aid with the identification of novel therapeutic targets for LC.

Materials and methods

Clinical sample collection. Tumor tissues and matched adjacent paracancerous tissues (2 cm away from the tumor margin) were collected from 50 patients with LC at Taizhou People's Hospital (Jiangsu, China) between March 2019 and March 2021. Tissues were pathologically confirmed. The patients had not received any antitumor treatments. Collected tissues were immediately frozen in liquid nitrogen and stored at -80˚C until further analysis. The basic clinicopathological characteristics, including gender, age and TNM stage (20), of the patients are presented in Table I. Written informed consent was obtained from all patients. The present study was performed in accordance with the Declaration of Helsinki and approved by the Ethical Research Committee of Taizhou People's Hospital.

Cell transfection. Human LC cell lines (HepG2 and SNU-182) and a transformed human liver epithelial-3 cell line (THLE-3) were purchased from American Type Culture Collection. All cell lines were authenticated via STR profiling. Cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO2. Specific small interfering (si)RNAs targeted against TPT1-AS1 were used, including si-TPT1-AS1#1 (5'-AGGTTACCGAAGACGATTA-3'), si-TPT1-AS1#2 (5'-AACCTACCTTGAAGAAACA-3') and a scrambled siRNA control (si-NC) (5'-AACCATCAGTTTACAAGAA ACC-3'), which were purchased from Shanghai GeneChem Co., Ltd. HepG2 and SNU-182 cells (2x10^4 cells/well) were transfected with 50 nM si-TPT1-AS1#1, si-TPT1-AS1#2 or si-NC using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h at 37˚C. At 48 h post-transfection, cells were used for subsequent experiments.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from tissue samples or cell lines using TRIzol® reagent (Takara Bio, Inc.). Total RNA was reverse transcribed into cDNA using the PrimeScript TM RT Master Mix kit (Takara Bio, Inc.) according to the manufacturer's protocol. Subsequently, qPCR was performed using an ABI 7500 Real-time PCR instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.) with SYBR-Green Master mix (Toyobo Life Science). The following thermocycling conditions were used for qPCR: Initial denaturation at 95˚C for 1 min; followed by 40 cycles at 95˚C for 20 sec and 60˚C for 40 sec. The following primers were used for qPCR: TPT1-AS1 forward, 5'-AGCCTTGAGGCTATGCCCATC-3' and reverse, 5'-AACAGTGTGGAGGCTCTGAA-3'; and GAPDH forward, 5'-GCCCTCCGACACCCACTACCTT-3' and reverse, 5'-TGAATTCTGTAGCCAGTTTGATCA-3'. mRNA expression levels were quantified using the 2^−ΔΔCq method (21) and normalized to the internal reference gene GAPDH.

CCK-8 assay. At 48 h post-transfection, cells were seeded (3x10^3 cells/well) into 96-well plates and incubated with 10 µl CCK-8 solution (Sigma-Aldrich; Merck KGaA) for 24, 48 or 72 h. Then, the culture medium was changed and following incubation for a further 2 h, cells were harvested and absorbance was measured at a wavelength of 450 nm using a microplate reader.

Colony formation assay. Transfected cells were seeded (5x10^3 cells/well) into 6-well plates and cultured for 2 weeks under standard culture conditions. Subsequently, colonies were washed twice with PBS, fixed with 70% methanol for 30 min and stained with 0.1% crystal violet for 1 h at room temperature. Colonies (>50 cells) were observed and counted using a light microscope (magnification, x40).

Cell cycle analysis. At 48 h post-transfection, cells were washed twice with precoolied PBS and fixed with precooled 70% ethanol overnight at 4˚C. Cells were centrifuged for 5 min at 112 x g at 4˚C, and the supernatant was discarded. Subsequently, cells were incubated with 20 µl RNase A solution and 0.2% Triton X-100 at 37˚C for 10 min, followed by incubation with 400 µl PI staining solution (Beyotime Institute of Biotechnology) at 37˚C for 20 min in the dark. Cell cycle distribution was analyzed using a flow cytometer (BD FACSLyric™ Flow Cytometer; BD Biosciences). FCSalyzer 0.9.22 alpha software (https://sourceforge.net/projects/fcsalyzer/) was used for data analysis.

Wound healing assay. Cell migration was assessed by performing a wound healing assay. Briefly, transfected cells were seeded [5x10^3 cells/well containing 5% (vol/vol) FBS] into 6-well plates. Subsequently, a 200 µl pipette tip was used to make a scratch in the (95-100%) confluent cell monolayer. The wound was observed at 0 and 24 h, which were recorded as W0 and 24, respectively, using a light inverted microscope (magnification, x40). Cell migration (%) was calculated according to the formula: (W0-24)/W0 x 100.

Cell invasion assay. Cell invasion was assessed using 24-well Matrigel-coated Transwell invasion inserts (BD Biosciences). Briefly, cells (5x10^3) were resuspended in serum-free DMEM and plated into the upper chamber, whereas DMEM supplemented with 20% FBS was added to the lower chamber. Following incubation for 24 h at 37˚C, invading cells were fixed with methanol for 15 min and stained with 0.2% crystal violet for 30 min at room temperature. Invasive cells were visualized using an inverted microscope (magnification, x40) and photographed. Cells were counted in five randomly selected fields.

Western blotting. Total protein was extracted from tissues and cultured cells using RIPA lysis buffer (Beyotime Institute of Biotechnology) and quantified using a BCA Protein Assay kit (Beyotime Institute of Biotechnology). Proteins (10 µg/well) were separated via 10% SDS-PAGE and transferred onto PVDF
membranes (EMD Millipore), which were blocked with 5% skimmed milk for 1 h at room temperature. Subsequently, the membranes were incubated overnight at 4˚C with primary antibodies (all purchased from Abcam) targeted against: CDK4 (cat. no. ab137675; 1:1,000), Cyclin D1 (cat. no. ab134175; 1:1,000), p21 (cat. no. ab188224; 1:1,000), E-cadherin (cat. no. ab416; 1:1,000), N-cadherin (cat. no. ab76057; 1:1,000), Vimentin (cat. no. ab24525; 1:1,000) and GAPDH (cat. no. ab8245; 1:1,000). Following primary incubation, the membranes were washed twice with PBS and incubated with a HRP-conjugated anti-Rabbit secondary antibody (cat. no. ab6721; 1:10,000; Abcam) for 2 h at room temperature. Protein bands were visualized using an ECL Detection kit (Thermo Fisher Scientific, Inc.). GAPDH was used as the loading control.

Statistical analysis. Statistical analyses were performed using GraphPad Prism software (version 6.0; GraphPad Software, Inc.). The χ² test was used to analyze the association between TPT1-AS1 and clinicopathological characteristics. Survival curves were plotted using the Kaplan-Meier method and statistically compared using the log-rank test. Quantitative data are presented as the mean ± SD of three independent experiments. Comparisons between two groups were analyzed using the paired or unpaired Student's t-test. Comparisons among multiple groups were analyzed using one-way ANOVA followed by Bonferroni's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Significantly upregulated TPT1-AS1 expression is associated with poor survival prognosis in patients with LC. To evaluate the clinical significance of TPT1-AS1 in LC, TPT1-AS1 expression levels were determined in 50 paired LC and adjacent paracancerous tissues via RT-qPCR. TPT1-AS1 expression levels were significantly increased in LC tissues compared with adjacent paracancerous tissues (Fig. 1A). Subsequently, the 50 patients with LC were divided into TPT1-AS1 high expression group (n=25) and low expression (n=25) groups using the median TPT1-AS1 expression level (cut-off, 0.62) in LC tissues as a cut-off value. Moreover, high TPT1-AS1 expression was more frequent in patients with late TNM stage and lymph node metastasis compared with patients with early TNM stage and no lymph node metastasis, respectively (Table I). The Kaplan-Meier survival analysis demonstrated that patients with LC with high TPT1-AS1 expression displayed significantly worse overall survival compared with patients with LC with low TPT1-AS1 expression (Fig. 1B).

TPT1-AS1 knockdown inhibits LC cell proliferation and induces G₀/G₁ phase arrest. Consistent with TPT1-AS1 expression in LC tissues, TPT1-AS1 expression levels were significantly upregulated in the LC cell lines (HepG2 and SNU-182) compared with the THLE-3 cell line (Fig. 2A). Subsequently, TPT1-AS1 expression was knocked down to investigate the function of TPT1-AS1 in HepG2 and SNU-182 cells. The RT-qPCR results indicated that si-TPT1-AS1#1 and si-TPT1-AS1#2 significantly decreased TPT1-AS1 expression levels in HepG2 and SNU-182 cells compared with the si-NC group (Fig. 2B). The CCK-8 assay results demonstrated that compared with the si-NC group, TPT1-AS1 knockdown significantly suppressed HepG2 (Fig. 2C) and SNU-182 (Fig. 2D) cell viability at 48 and 72 h. Moreover, si-TPT1-AS1#1 displayed enhanced suppressive effects on TPT1-AS1 expression and cell viability compared with si-TPT1-AS1#2, thus si-TPT1-AS1#1 was selected for

### Table I. Association between TPT1-AS1 expression and clinicopathological characteristics of patients with liver cancer.

| Characteristic          | n (n=50) | High (n=25) | Low (n=25) | P-value |
|-------------------------|----------|-------------|------------|---------|
| Age, years              |          |             |            |         |
| <60                     | 20       | 12          | 8          | 0.248   |
| ≥60                     | 30       | 13          | 17         |         |
| Sex                     |          |             |            | 0.777   |
| Male                    | 27       | 13          | 14         |         |
| Female                  | 23       | 12          | 11         |         |
| Differentiation         |          |             |            | 0.371   |
| Well/moderate           | 33       | 18          | 15         |         |
| Poor                    | 17       | 7           | 10         |         |
| TNM stage               |          |             |            | 0.024‡  |
| I-II                    | 26       | 9           | 17         |         |
| III-IV                  | 24       | 16          | 8          |         |
| Lymph node metastasis   |          |             |            | 0.048‡  |
| Negative                | 25       | 9           | 16         |         |
| Positive                | 25       | 16          | 9          |         |

‡P<0.05. TPT1-AS1, tumor protein translationally controlled 1 antisense RNA 1.
subsequent experiments. Similarly, HepG2 and SNU-182 cell proliferation was significantly inhibited by TPT1-AS1 knockdown, as evidenced by a significantly decreased number of colonies in the si-TPT1-AS1#1 group compared with the si-NC group (Fig. 2E). Furthermore, the effects of TPT1-AS1 knockdown on LC cell cycle distribution were assessed. The flow cytometry results demonstrated that compared with the si-NC group, si-TPT1-AS1#1 transfection significantly increased the percentage of cells in the G₀/G₁ phase and significantly decreased the percentage of cells in the S phase in HepG2 (Fig. 3A) and SNU-182 (Fig. 3B) cells, which indicated that TPT1-AS1 knockdown induced G₀/G₁ phase arrest in LC cells.

**TPT1-AS1 knockdown suppresses LC cell migration and invasion.** In addition, the effects of TPT1-AS1 knockdown on LC cell motility were assessed. The wound healing assay results demonstrated that the relative migration rate was significantly decreased in the si-TPT1-AS1#1 group compared with the si-NC group (19.6±1.0 vs. 56.4±0.6) in HepG2 (Fig. 4A) and SNU-182 (25.2±0.8 vs. 46.5±0.8) cells (Fig. 4B). Similarly, the Transwell invasion assay demonstrated that TPT1-AS1 knockdown significantly reduced the number of invasive cells compared with the si-NC group in HepG2 (73.3±4.5 vs. 134.7±5.5) and SNU-182 (47.3±4.7 vs. 105.3±9.1) cells (Fig. 4C and D).

**TPT1-AS1 knockdown alters the expression of G₀/S transition- and EMT-associated markers.** To further assess the suppressive role of TPT1-AS1 knockdown on LC cell cycle G₀/S transition, migration and invasion, the expression
levels of G1/S transition- and EMT-associated markers were measured via western blotting. Compared with the si-NC group, si-TPT1-AS1#1 transfection obviously downregulated the expression levels of CDK4, N-cadherin and Vimentin, and markedly upregulated the expression levels of p21 and E-cadherin, but did not obviously alter the expression levels of Cyclin D1 in HepG2 cells (Fig. 5A). Similar effects of TPT1-AS1 knockdown on the expression levels of the aforementioned protein markers were also observed in SNU-182 cells (Fig. 5B). Collectively, the results demonstrated that TPT1-AS1 knockdown negatively regulated LC cell proliferation, migration and invasion.

Discussion

The identification of specific and reliable biomarkers for early-stage diagnostic modalities to improve prognostic outcomes in LC is important, of which lncRNAs have been highlighted as potential markers in cancer diagnosis and prognosis (22). In the present study, TPT1-AS1 expression was significantly upregulated in LC tissues compared with adjacent paracancerous tissues. Moreover, high TPT1-AS1 expression was significantly associated with adverse clinical features, including TNM stage, lymph node metastasis and poorer prognosis in patients with LC. Consistent with the results of the present study, ectopic TPT1-AS1 expression was strongly associated with unfavorable clinicopathological features and poor survival in epithelial ovarian (23), cervical (18) and gastric (19) cancer.

Subsequently, two LC cell lines (HepG2 and SNU-182) with significantly higher TPT1-AS1 expression levels compared with the THLE-3 cell line were selected to perform loss-of-function experiments to assess the biological function of TPT1-AS1 in LC in vitro. The results demonstrated that TPT1-AS1 knockdown significantly inhibited LC cell proliferation, G1/S transition, migration and invasion compared with the si-NC group. Moreover, antisense lncRNAs have been reported to serve pivotal regulatory roles in sense mRNA stability, sense-encoded protein translation and cis-antisense RNA, and are highly expressed in various tumors, such as breast cancer and colorectal adenocarcinoma (24-26). In line with the results of the present study, a variety of antisense RNAs, including MCM3AP antisense RNA 1 (27), LIM and SH3 protein 1 antisense RNA 1 (28) and DSCAM antisense RNA 1 (29), have been reported to display oncogenic roles in LC. Moreover, TPT1-AS1 promoted cell proliferation, migration and invasion in cervical cancer in vitro and in vivo (17). Wu et al (23) reported that TPT1-A1 overexpression remarkably induced cell proliferation, migration and invasion, whereas TPT1-AS1 knockdown results in the opposite effects in epithelial ovarian cancer. Additionally, TPT1-AS1 knockdown significantly inhibited gastric cancer cell proliferation, cell cycle G1/S transition, migration and invasion (19). The results indicated that TPT1-AS1 displayed oncogenic effects, thus served a critical role in LC progression.

In the present study, the possible molecular mechanisms underlying TPT1-AS1 knockdown-mediated effects on LC cell malignant behaviors were explored by performing western blotting. Compared with the si-NC group, TPT1-AS1 knockdown decreased CDK4 expression, but increased p21 expression in LC cells. CDK4 and CDK inhibitor p21 serve an important role in cell cycle G1/S transition, and have been investigated in tumor cell proliferation (30,31). Moreover, Wang et al (32) demonstrated that cell adhesion molecule 1 antisense RNA 1 induced G0/G1 phase arrest by decreasing CDK4 and enhancing p21 expression. Consistently, Tang et al (19) indicated that TPT1-AS1 knockdown downregulated the expression of CDK4 and
upregulated the expression of p21, negatively regulating G_{1}/S transition and proliferation in gastric cancer cells. According to the close association between uncontrolled proliferation and cell cycle dysfunction, it was hypothesized that TPT1-AS1 knockdown suppressed LC cell proliferation by inducing G_{0}/G_{1} phase arrest via regulating CDK4/p21 expression. EMT is a pivotal mechanism contributing to cancer invasion and metastasis, whereby epithelial cells lose their polarity and acquire the migratory properties of mesenchymal cells (33). In the present study, the suppressive effects of TPT1-AS1 knockdown on EMT were observed in LC cells, as evidenced by increased E-cadherin expression.
and decreased N-cadherin and Vimentin expression in the si-TPT1-AS1#1 group compared with the si-NC group. In recent years, it has been reported that knockdown of antisense lncRNAs, including HOXA cluster antisense RNA 2 (34) and SBF2 antisense RNA 1 (35), suppressed LC cell migration and invasion by modulating EMT ability. Interestingly, the effects of TPT1-AS1 knockdown on E-cadherin and Vimentin have also been reported in gastric cancer cells, resulting in decreased cell migration and invasion in vitro (19). The aforementioned results strongly supported the suppressive role of TPT1-AS1 on LC cell proliferation and metastasis. Additionally, the present study had a number of limitations, including the lack of overexpression experiments, and recurrence rate and disease-free survival analyses, which require further investigation in future studies.

In summary, the present study demonstrated that TPT1-AS1 may serve as an important predictor in the clinical outcomes of patients with LC. Functionally, TPT1-AS1 facilitated LC cell proliferation, migration and invasion by affecting G1/S transition and EMT-associated markers. Collectively, the results of the present study suggested that TPT1-AS1 may serve as a promising therapeutic target for LC.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
HL drafted the work and performed the experiments. JJ was responsible for the conception and design of the present study, and gave final approval of the work. JX researched the literature and performed the experiments. WW performed data analysis and interpretation. All authors have read and approved the final manuscript. HL and WW confirm the authenticity of all the raw data.
Ethics approval and consent to participate

The present study was performed in accordance with the Declaration of Helsinki and approved by the Ethical Research Committee of Taizhou People’s Hospital (approval no. XCV/20190842; Taizhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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