



Long noncoding TMPO antisense RNA 1 promotes hepatocellular carcinoma proliferation and epithelial-mesenchymal transition by targeting the microRNA-126-3p/LRP6/ β -catenin axis

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Background: Long noncoding RNAs (lncRNAs) play a central role in the pathogenesis of various tumors, including hepatocellular carcinoma (HCC). TMPO antisense RNA 1 (TMPO-AS1) has been reported in many tumors. Nevertheless, the underlying mechanism whereby TMPO-AS1 influences HCC remains unclear. Our research aimed to reveal the molecular mechanism governing the function of TMPO-AS1 in HCC.

Methods: TMPO-AS1 expression levels in HCC tissues/cells were evaluated using reverse transcriptase-polymerase chain reaction. The effect of TMPO-AS1 on the progression of HCC was observed by Cell Counting Kit-8 (CCK8), clone formation, wound healing, and transwell. The direct interaction between TMPO-AS1 and microRNA (miR)-126-3p was observed using a dual-luciferase reporter.

Results: We found TMPO-AS1 expression to be remarkably higher in HCC specimens and associated with poor prognosis. Silencing of TMPO-AS1 not only inhibited HCC cell proliferation but also significantly reduced epithelial-to-mesenchymal transition-induced invasion and migration to a remarkable degree. According to the results from the online database analysis tools implemented to identify if TMPO-AS1 could target miR-126-3p, we found that miR-126-3p had a negative relationship with TMPO-AS1 in HCC specimens. Meanwhile, the luciferase reporter assay confirmed that TMPO-AS1 could directly act on miR-126-3p. Moreover, the silencing of miR-126-3p dramatically abolish the inhibitive influence of sh-TMPO-AS1 on HCC development.

Conclusions: Our research demonstrated that TMPO-AS1 acts as a sponge for the tumor suppressor miR-126-3p in HCC and promotes the expression of LRP6 indirectly. Taken together, our results show that TMPO-AS1 may be regarded as a novel therapeutic target in the treatment of liver cancer.

Keywords: TMPO antisense RNA 1 (TMPO-AS1); miR-126-3P; epithelial-mesenchymal transition (EMT); proliferation; invasion

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Introduction

Liver cancer is one of the most commonly diagnosed cancers and a leading cause of global cancer-related mortalities. Hepatocellular carcinoma (HCC) is the most

common primary liver cancer, accounting for about 75–85% (1). Although there are several treatments available for HCC, it continues to have a poor prognosis with a high recurrence rate after traditional therapies (2,3). Recent

studies report that long noncoding RNA (lncRNA) may function as a novel prognostic marker and therapeutic target for HCC (4).

Long noncoding RNAs belong to a group of noncoding RNA molecules containing more than 200 nucleotides (5) and were once thought to be transcriptional 'garbage'. However, emerging evidence has demonstrated that lncRNAs are not genomic 'noise' but rather functional RNA molecules, which participate in critical cellular processes such as growth and differentiation as well as the pathogenesis of diseases, including cancer (6). Thus, lncRNAs have become an increasingly popular focus of cancer research. A large body of emerging studies has proven that lncRNAs exhibit aberrant expression in HCC and extensively participate in cancer pathogenesis by regulating cell anti-tumor drug resistance, growth, apoptosis, and epithelial-to-mesenchymal transition (EMT) and invasion (7-10). The TMPO antisense RNA 1 (TMPO-AS1) gene has been proposed to have a carcinogenic role in various cancers. In cervical cancer, TMPO-AS1 is positively correlated with an aggressive phenotype and a poor prognosis (11). Moreover, recent studies have shown TMPO-AS1 overexpression stimulates cell division and migration in prostate cancer, thus serving as a biomarker for disease diagnosis and prognosis (12). Also, TMPO-AS1 has been shown to promote gastric cancer cells migration and invasion by activating the expression of EMT modulators (13). However, the underlying role of TMPO-AS1 in HCC development remains elusive.

MicroRNAs (miRNAs) are small noncoding, endogenously expressed RNAs comprised of 19–25 nucleotides, which regulate cellular biological processes in combination with messenger RNAs (mRNAs) (14,15). At present, a few studies have reported on a type of competitive endogenous RNA (ceRNA), also known as microRNA sponge, which indirectly regulates the expression of mRNA through competitive binding of miRNAs (16,17). Many lncRNAs can act as miRNA sponges to regulate tumor progression (18). However, the capacity of TMPO-AS1 to regulate proliferation, migration, and EMT of HCC, and if this regulation is derived from sponging a particular miRNA, remains to be elucidated.

This study aimed to investigate the biological function and molecular mechanism of TMPO-AS1 in HCC, to verify the expression profile of TMPO-AS1 in HCC tissues and cell lines and to explore the relationships between TMPO-AS1, miR-126-3p, LRP6, and β -catenin signaling pathway in HCC. Results from this study may lead to a new understanding of HCC occurrence and development and

may provide a new therapeutic option for the treatment of HCC. We present the following article in accordance with the MDAR reporting checklist (available at <https://dx.doi.org/10.21037/atm-21-5593>).

Methods

Patients and tissue specimens

Tissue specimens were obtained from 53 Chinese HCC patients undergoing hepatectomy for primary tumors at the Department of Hepatobiliary Surgery of The First Affiliated Hospital of Chongqing Medical University between March 2018 and July 2020. The basic characteristics of all patients are outlined in *Table 1*. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Chongqing Medical University (No. 2021-156) and informed consent was taken from all the patients.

Cell culture

The human HCC cell lines Hep3B, Huh7, SMMC-7721, Bel-7402, SK-Hep-1, LM9, and human normal liver cell line L-02 were procured from the Shanghai Institute of Biological Sciences, Chinese Academy of Sciences, and were subsequently cultured in Dulbecco Modified Eagle Medium (DMEM, Gibco, Amarillo, TX, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Amarillo, TX, USA; Life Technologies, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Hyclone, Logan, UT, USA) in an incubation chamber with 5% CO₂ at a constant temperature of 37 °C.

Transfection

Short hairpin RNAs (shRNAs) against TMPO-AS1 (sh-TMPO-AS1) were purchased from HanBio (Shanghai, China) along with the short hairpin negative control (sh-NC). The miR-126-3p inhibitor was purchased from GenePharma (Shanghai, China). Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) was employed for cell transfection following the protocols available on the manufacturer's website. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis was then used to monitor and quantify transfection efficiency after 48 h.

Table 1 Sociodemographic and medical characteristics of patients

Clinicopathological characteristics	Number of cases	
	High expression (n=33)	Low expression (n=20)
Age (years)		
<50	21 (63.6%)	9 (45%)
≥50	12 (36.4%)	11 (55%)
Gender		
Male	26 (78.8%)	17 (85%)
Female	7 (21.2%)	3 (15%)
Mass size (cm)		
≤5	13 (39.4%)	18 (90%)
>5	20 (60.6%)	2 (10%)
Liver cirrhosis		
Absence	23 (69.7%)	18 (90%)
Presence	10 (30.3%)	2 (10%)
Histological differentiation		
Poor	3 (9%)	1 (5%)
Moderate	25 (75.8%)	17 (70%)
Well	5 (15.2%)	2 (25%)
Serum alpha-fetoprotein (μg/mL)		
>400	20 (60.6%)	12 (60%)
≤400	13 (39.4%)	8 (40%)
Hepatitis B virus		
Positive	29 (87.9%)	19 (95%)
Negative	4 (12.1%)	1 (5%)
Microvascular invasion		
Absence	5 (15.2%)	5 (25%)
Presence	28 (84.8%)	15 (75%)

Quantitative real-time polymerase chain reaction (qRT-PCR) assay

Total cellular RNA was isolated from HCC cells via TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's guidelines. Reverse transcription of TMPO-AS1 into single-stranded complementary DNA (cDNA) was conducted using a reverse transcription system kit (Takara, Shiga, Japan). Hairpin-it miRNA real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) kit (GenePharm, Sunnyvale, CA, USA) was utilized

for the reverse transcription of miR-126-3p. Then, qRT-PCR assays were conducted with 2× SYBR Green qPCR Master Mix (Bimake, Houston, TX, USA) on the Bio-Rad CFX96 Real-Time PCR Detection machine (Bio-Rad Laboratories, Hercules, CA, USA). U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) functioned as internal references for miR-126-3p and TMPO-AS1, respectively. The gene-specific primers were as follows: GAPDH, 5'-CTTTGGTATCGTGGAAGGACTC-3' (F), and 5'-GTAGAGGCAGGGATGATGTTCT-3' (R); TMPO-AS1, 5'-GACCTCTACAATCGGGCACTT-3'

(F), and 5'-AGGATTCTTGCGGGTGGTG-3' (R); U6, 5'-CAGCACATATACTAAAATTGGAACG-3' (F), and 5'-ACGAATTTGCGTGTCCATCC-3' (R); miR-126-3p: 5'-CGCGTCGTACCGTGAGTAAT-3' (F) and 5'-AGTGCAGGGTCCGAGGTATT-3' (R). All primers were synthesized by Takara (Shiga, Japan). The comparative $2^{-\Delta\Delta C_t}$ method was then implemented to analyze relative gene expression.

Cell counting kit-8 (CCK-8) assay

To assess cell viability, a CCK8 assay was performed according to the instructions of the manufacturer (Dojindo, Japan). After being transfected with sh-TMPO-AS1 and sh-NC for 24 h, cells were reseeded into 96-well plates (2×10^3 cells/well) and cultured for several days. Each well was replaced with a solution of 100 μ L of complete medium containing 10 μ L CCK8. Multiskan Spectrum (Thermo Fisher, Rockford, IL, USA) was employed to measure absorbance at 450 nm.

Colony formation assay

Cells that had been transfected for 24 hours were reseeded onto 6 cm plates at a density of 500 cells/plate and were subsequently shaken well and cultured for 2 weeks, such that individual cells grew into a mass of cells visible to the naked eye. Next, the plates were fixed followed by staining with 3.7% methanol with 0.1% crystal violet.

Western blot assay

After 72 h of transfection, the total cellular protein was separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred into polyvinylidene fluoride membranes. Membranes were incubated in QuickBlockTM blocking buffer (Beyotime, Shanghai, China) for 15 min, then washed with Tris Buffered Saline with Tween (TBST, Solarbio, Beijing, China) buffer solution (20 mM Tris-HCl, 137 mM NaCl, and 0.1% Tween 20). The protein bands were then incubated overnight with the relevant primary antibodies at 4 °C. The primary antibodies utilized include anti-LRP6 (1:1,000, Abways, Shanghai, China), anti- β -catenin (1:1,000, Cell Signaling Technology, Danvers, MA, USA), anti-E-cadherin (1:2,000, Affinity, Piscataway, NJ, USA), anti-N-cadherin (1:1,000, Cell Signaling Technology, Danvers, MA, USA), anti-vimentin (1:1,000, Bimake, Houston, TX, USA),

anti-Snail (1:1,000, Cell Signaling Technology, Danvers, MA, USA), and anti-GAPDH (1:20,000, Proteintech, Wuhan, China). The next day, the blots were washed with a mixture of TBS and Tween 20. The membranes were then exposed to secondary antibodies at room temperature for 1 hour. Next, the membranes were washed with TBST three times (5 min each). The protein bands were analyzed with an enhanced chemiluminescence (ECL) kit (Merck Millipore, Ltd., Darmstadt, Germany) and a Vilber Fusion FX7 (Fusion, Paris, France). Protein expression was quantified with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Wound healing assay

The transfected HCC cells were seeded into six-well plates. Afterward, cell scratches were created with 10- μ L sterile pipette tip. Representative images were captured under a bright inverted microscope (Olympus, Tokyo, Japan). Then, the wound infliction area was measured using the Image J system (National Institutes of Health, Bethesda, USA).

Transwell assay

To assess cell migratory or invasive ability, transwell inserts (Costar, Cambridge, MA, USA) were used either uncoated or coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), respectively. After 24 h of transfection, cells were incubated in the upper chamber with serum-free medium. At the same time, DMEM (Gibco, Amarillo, TX, USA) containing 10% FBS was placed in the lower chamber. The cells were incubated for 24 h at 37 °C, and noninvaded cells were carefully eliminated via cotton swabs. Meanwhile, the invaded cells located on the membrane's underside were fixed with 95% methanol and stained with crystal violet for 30 min at room temperature. Five randomized images were captured using the Carl ZEISS microscope system (ZEISS, Jena, Germany). Finally, the stained cells were counted and averaged across five random fields.

Dual-luciferase reporter assay

Dual-luciferase reporter assay was implemented to confirm the interaction between TMPO-AS1 and miR-126-3p. Wild-type (Wt) and mutant (Mut) miR-126-3p 3'-UTR were inserted into the pSI-Check2 vector (HanBio, New Taipei City 221, Taiwan). Then, sh-NC or sh-TMPO-AS1 was cotransfected with Wt miR-126-3p 3'-UTR or

Mut miR-126-3p 3'-UTR into Huh7 cells. After 48 hours, luciferase activity was measured with the dual-luciferase reporter system (Promega, Madison, WI, USA).

Bioinformatics analysis

A Kaplan-Meier plotter (<https://www.kmplot.com/analysis/>) was employed to detect the relationship between TMPO-AS1 level and overall patient survival quality. The possible target gene of TMPO-AS1 was predicted using starBase Version 3.0 (<http://starbase.sysu.edu.cn/>). The difference in the expression of TMPO-AS1 between normal and tumor liver tissues was analyzed using the online Atlas of Noncoding RNAs in Cancer (TANRIC) database (https://ibl.mdanderson.org/tanric/_design/basic/main.html).

Statistical analyses

GraphPad Prism (version 8.0, GraphPad, San Diego, CA, USA) was used for statistical analysis. Data are presented as mean \pm standard deviation (SD). The normality of quantitative data was also examined. The student's *t*-test was used to analyze differences between two groups of the normally distributed data. One-way analysis of variance was employed to compare statistical differences among three or more groups using the Tukey-Kramer test. The differences between paracarcinoma and HCC tissues were examined using Wilcoxon matched-pairs signed rank test. $P < 0.05$ was considered as indicating statistical significance.

Results

TMPO-AS1 is overexpressed and associated with poor prognosis in HCC

A qRT-PCR analysis conducted to measure TMPO-AS1 levels revealed that TMPO-AS1 was remarkably overexpressed in HCC tissues compared to pericarcinomatous tissues (*Figure 1A*). It exhibited elevated levels in HCC lines when compared to healthy liver cells (L-O2). Of all HCC cell lines, TMPO-AS1 expression was greatest in Hep3B and Huh7 (*Figure 1B*). Additionally, TANRIC database results consistently indicated higher TMPO-AS1 expression in HCC tissues than in normal liver tissues (*Figure 1C*). As shown in *Figure 1D*, patients with lower levels of TMPO-AS1 expression had a distinctly better median overall survival time than individuals that exhibited higher TMPO-

AS1 expression levels (59.7 vs. 46.57 months, respectively).

Decreased TMPO-AS1 expression blocks proliferation in HCC cells

To investigate the effect of TMPO-AS1 on the biological function of HCC cells, we transfected Hep3B and Huh7 cells with sh-TMPO-AS1#1/2/3 and found inhibition efficiency was caused by sh-RNA#2 (*Figure 2A*). We therefore selected sh-TMPO-AS1#2 to silence TMPO-AS1 in subsequent experiments. Firstly, CCK8 assay results indicated that knockdown of TMPO-AS1 remarkably reduced the proliferative capacity of Hep3B and Huh7 cells (*Figure 2B*), and a colony formation assay showed that TMPO-AS1 silencing prominently suppresses HCC cell growth compared to sh-NC populations (*Figure 2C*). These findings suggest that TMPO-AS1 regulates the progression of HCC through the regulation of HCC cell proliferation.

Knockdown of TMPO-AS1 represses HCC cell migration and invasion in vitro

As shown earlier, elevated TMPO-AS1 levels were observed in HCC patients with advanced-stage disease (i.e., higher tumor-node-metastasis stages) and portal vein tumor thrombosis. Therefore, we speculated TMPO-AS1 might be crucial in the metastasis of liver cancer cells. To verify this conjecture, two experimental schemes were adopted to explore differences in cell migration and invasion following TMPO-AS1 downregulation. First, we performed a wound healing assay to analyze HCC cells' motility in 24 h and found silencing of TMPO-AS1 repressed cell migration in Hep3B (*Figure 3A*) and Huh7 (*Figure 3B*) cells. Consistent with these results, transwell assays indicated that the downregulation of TMPO-AS1 minimized the quantity of migrated and invaded cells compared with sh-NC (*Figure 3C, 3D*). Since EMT is critical for the enabling the metastasis of cancer cells, we further explored whether TMPO-AS1 regulates the induction of EMT-related proteins in HCC cells. Western blot analysis illustrated that TMPO-AS1 silencing intensified the epithelial protein expression but repressed the expression levels of mesenchymal proteins, such as N-cadherin, Vimentin, and Snail (*Figure 3E*). Taken together, these findings suggest TMPO-AS1 contributes to HCC metastasis by promoting cell migration and invasion.

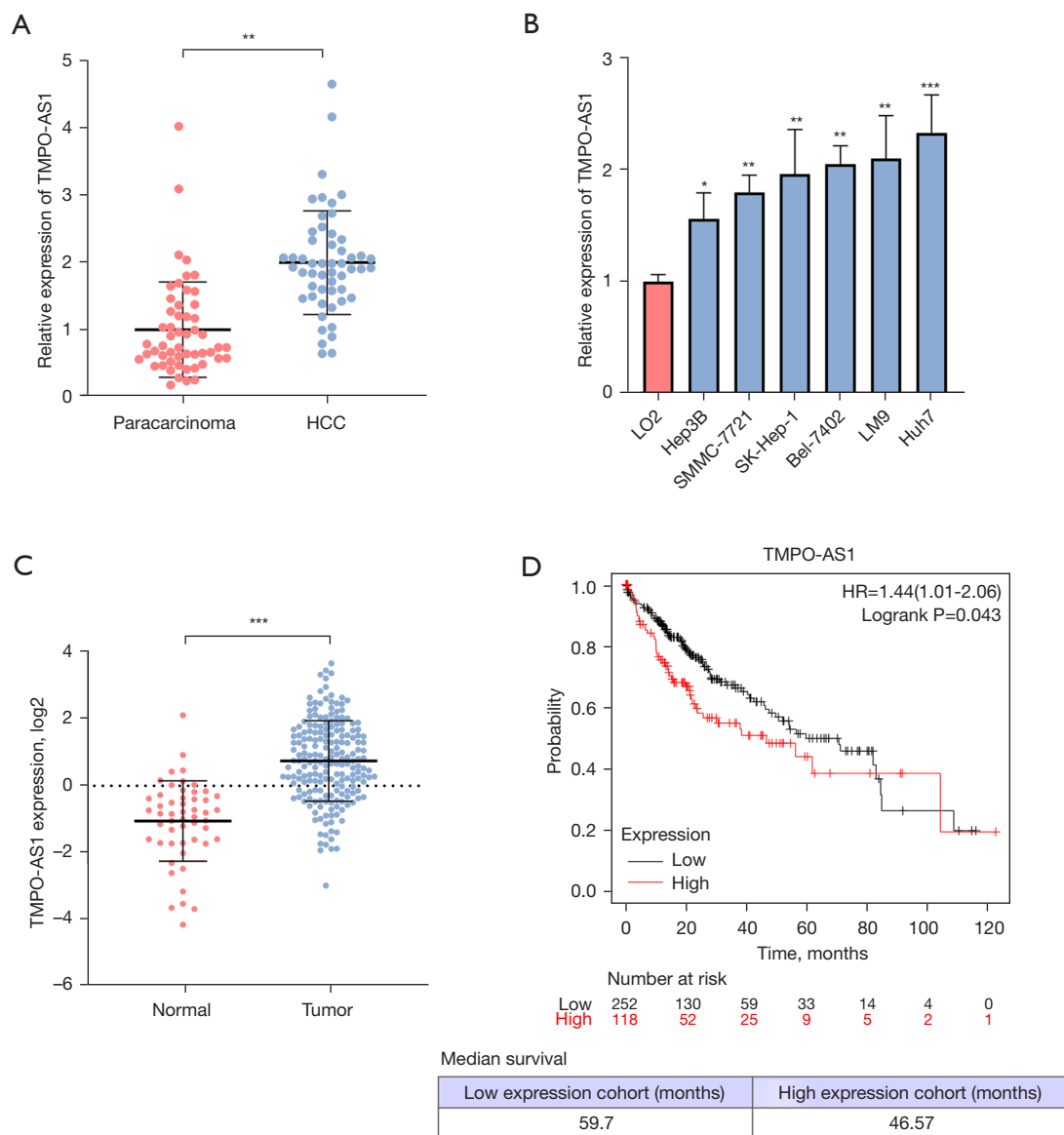


Figure 1 *TMPO* antisense RNA 1 (*TMPO-AS1*) expression in HCC tissues and cell lines. (A) Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed to evaluate the expression of *TMPO-AS1* in 53 paired hepatocellular carcinoma (HCC) tissues. (B) *TMPO-AS1* levels in control L-O2 cells and HCC cell lines were measuring using qRT-PCR. (C) Patient information was obtained from The Cancer Genome Atlas database to analyze *TMPO-AS1* expression in the HCC tumor group (n=200) compared with the normal control group (n=50). (D) The survival percentage was lower in HCC patients with higher *TMPO-AS1* expression levels than in those with lower *TMPO-AS1* expression levels. *, P<0.05; **, P<0.01; ***, P<0.001.

TMPO-AS1 promotes HCC progression by competitively binding to miR-126-3p and activating the LRP6/ β -catenin axis

To investigate the underlying molecular mechanisms by which *TMPO-AS1* promotes HCC cell progression, we

used the starBase (<https://starbase.sysu.edu.cn/>) 3.0 online database to predict the potential target genes of *TMPO-AS1*. MiR-126-3p was identified as prospective loci *TMPO-AS1* may bind with. As illustrated in *Figure 4A*, *TMPO-AS1* formed complementary base pairs with miR-126-3p. To

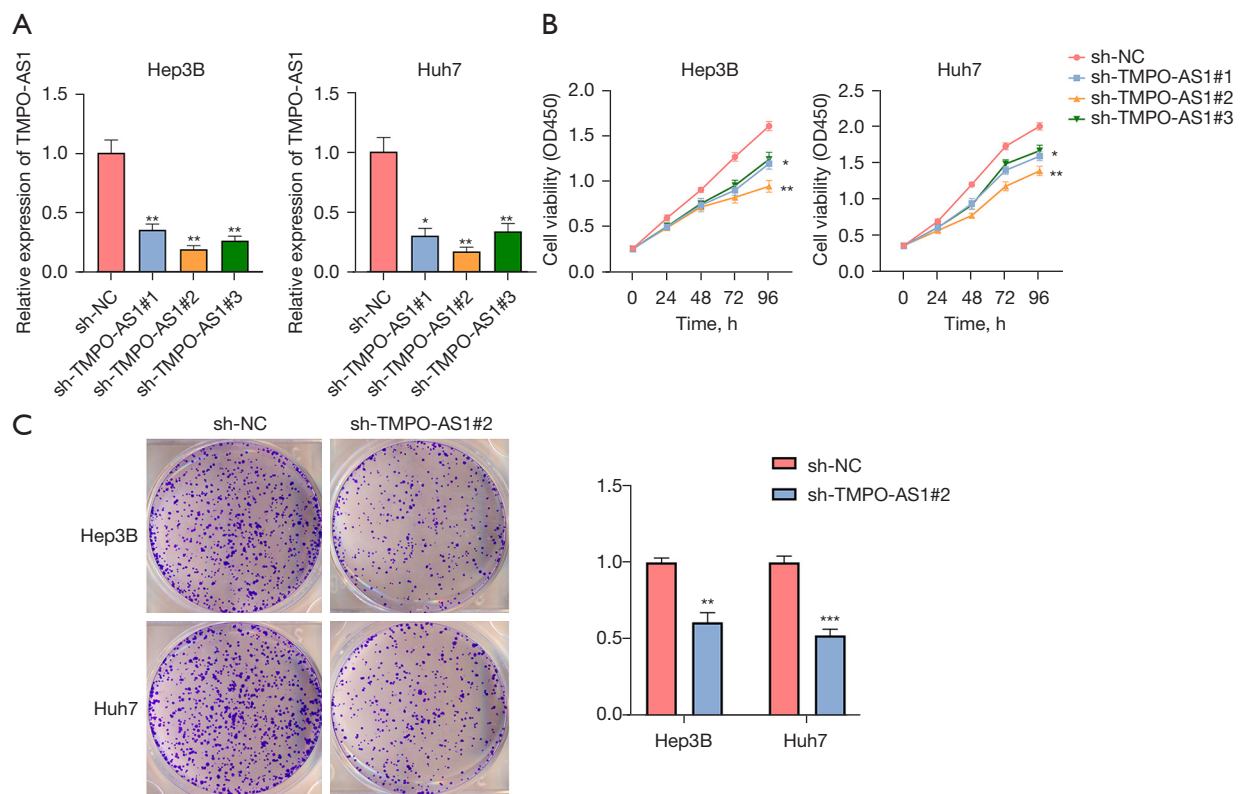


Figure 2 *TMPO* antisense RNA 1 (*TMPO-AS1*) regulates hepatocellular carcinoma (HCC) cell proliferation. (A) Real-time quantitative reverse transcription polymerase chain reaction analysis of *TMPO-AS1* induction in Hep3B and Huh7 cells following transfected with three different types of sh-*TMPO-AS1*. (B) Cell Counting Kit-8 assays revealed *TMPO-AS1* knockdown inhibits Hep3B and Huh7 cell proliferation. (C) Downregulation of *TMPO-AS1* weakens the colony formation ability of HCC cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

evaluate whether *TMPO-AS1* directly interacts with miR-126-3p, transfected Huh7 cells were analyzed using a dual-luciferase gene reporter assay. As highlighted by *Figure 4B*, the luciferase activity was dramatically reduced following co-transfection with sh-*TMPO-AS1* and Wt-miR-126-3p. However, the luciferase activity remained unchanged in the cells co-transfected with sh-*TMPO-AS1* and Mut-miR-126-3p.

To further assess the correlation between *TMPO-AS1* and miR-126-3p, the expression level of miR-126-3p in 53 paired HCC tissues was evaluated through qRT-PCR analysis (*Figure 4C*). The induction of miR-126-3p was considerably lower in HCC tissues than in their paired normal tissues. Interestingly, Pearson's correlation analysis revealed an inverse relationship between *TMPO-AS1* and miR-126-3p (*Figure 4D*). Since previous research has reported that LRP6 is the downstream target of miR-126-3p (19), we speculated that LRP6 and subsequent

signaling pathways might be modulated by *TMPO-AS1*. As hypothesized, downregulation of *TMPO-AS1* decreased the expression levels of LRP6 and beta-catenin in Hep3B and Huh7 cells (*Figure 4E*). Moreover, a miR-126-3p inhibitor was transfected into Hep3B and Huh7 cells (*Figure 4F*) to ascertain if miR-126-3p plays an intermediary role between *TMPO-AS1* and LRP6 in liver cancer cells. Because Huh7 cells were observed to have a higher degree of transfection efficiency than Hep3B cells, Huh7 cells were used for successive experiments. Western blot analysis revealed that co-transfection with sh-*TMPO-AS1* and the miR-126-3p inhibitor restored the expression of LRP6 and beta-catenin, which were downregulated by sh-*TMPO-AS1* (*Figure 4G*). These findings prove *TMPO-AS1* can interact with miR-126-3p to control LRP6 induction. In summary, we provided evidence that *TMPO-AS1* could act as an endogenous 'sponge' by combining with miR-126-3p to counteract the miRNA-induced repression of LRP6.

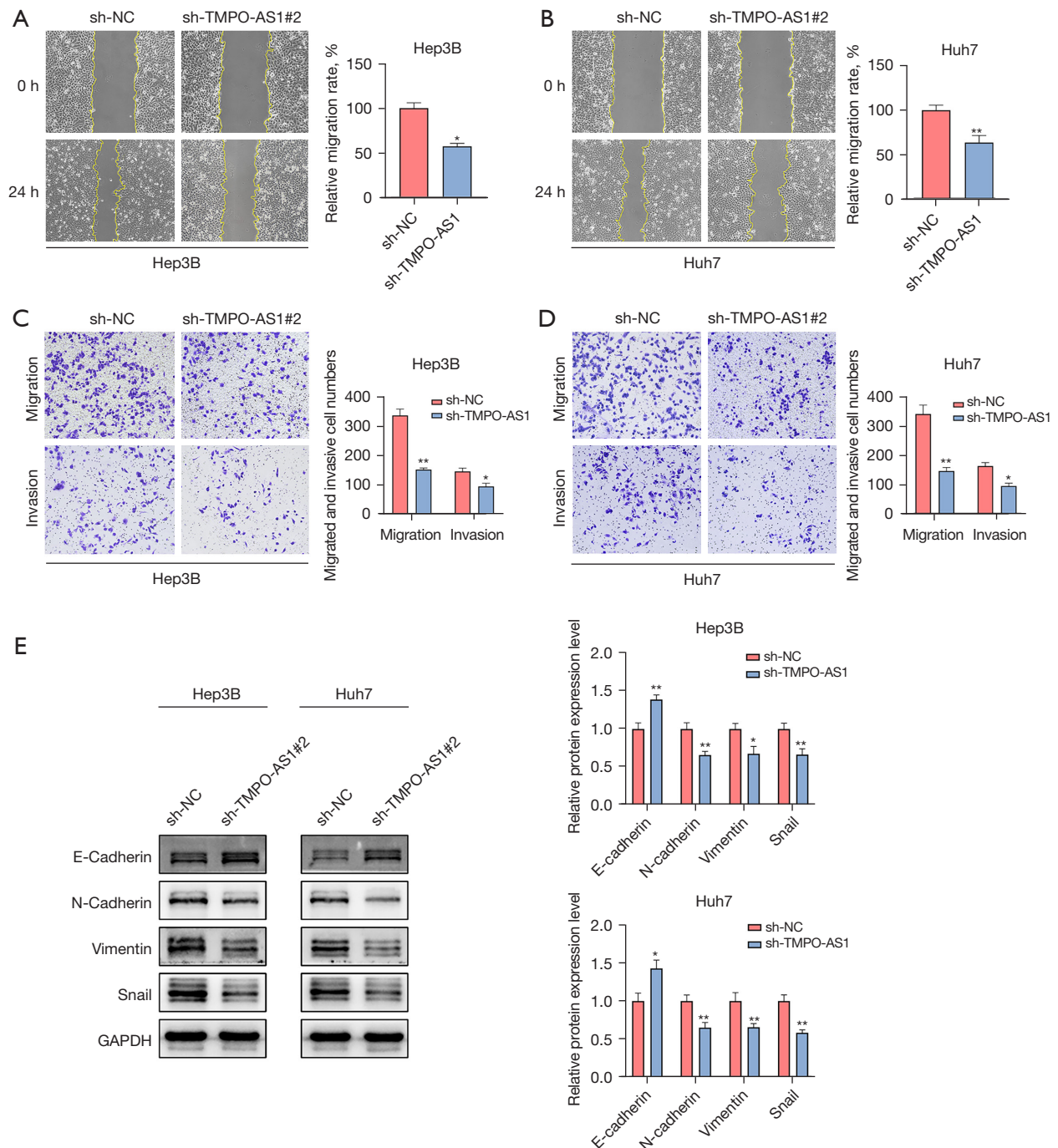


Figure 3 *TMPO* antisense RNA 1 (*TMPO-AS1*) promotes hepatocellular carcinoma (HCC) cell migration and invasion. (A,B) Wound healing analysis found that *TMPO-AS1* is positively correlated with Hep3B and Huh7 cell metastasis. (C,D) Transwell assays revealed that *TMPO-AS1* knockdown reduced the metastasis and invasion ability of Hep3B and Huh7 cells. Cells were stained with crystal violet. Magnification power 200 \times . (E) Western blot analysis was conducted to assess the expression level of epithelial-to-mesenchymal transition (EMT)-associated proteins, including E-cadherin, N-cadherin, vimentin, and Snail, in HCC cells transfected with sh-*TMPO-AS1*#2. *, $P < 0.05$; **, $P < 0.01$.

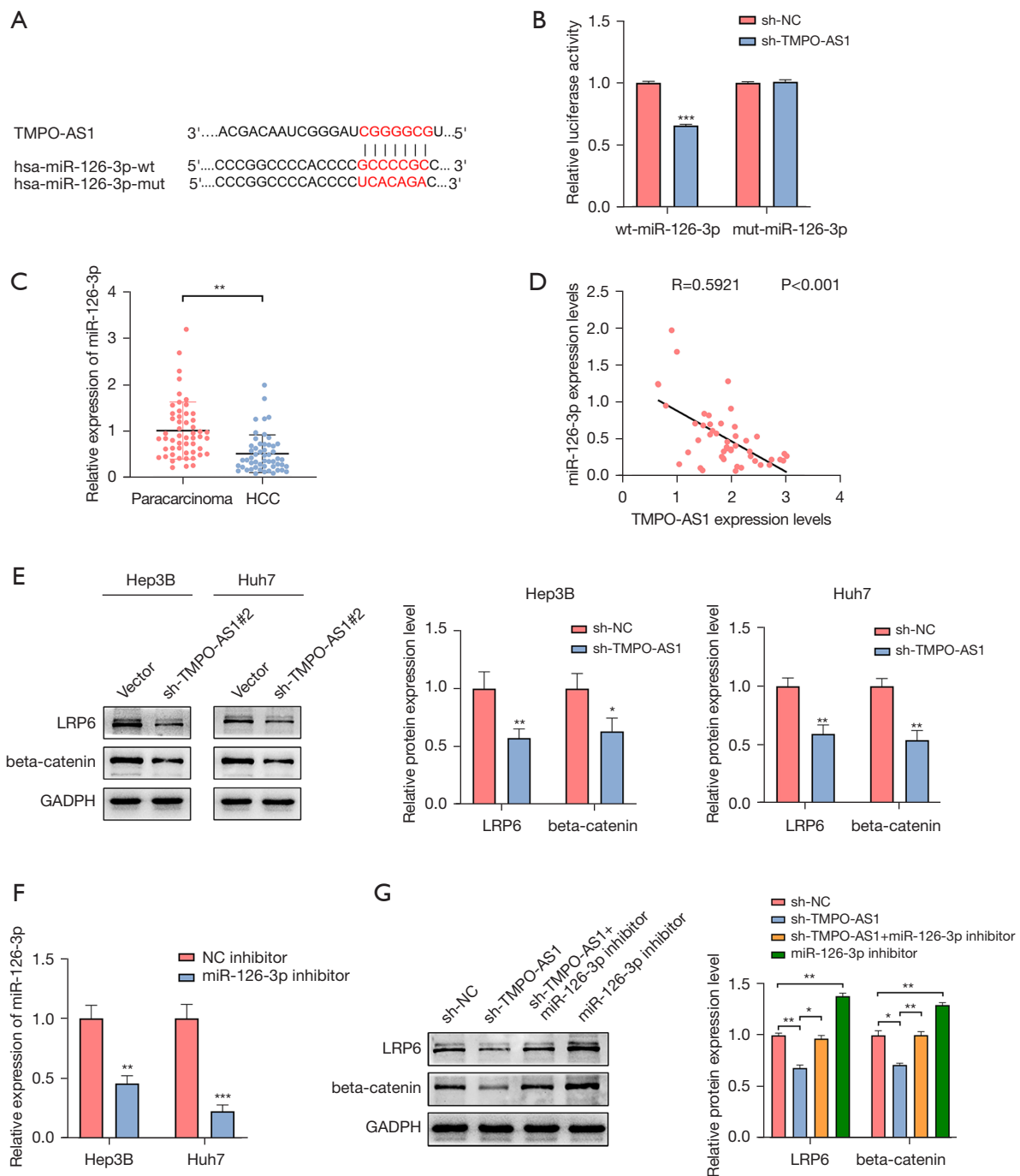


Figure 4 *TMPO* antisense RNA 1 (*TMPO-AS1*) activates the LRP6/ β -catenin signaling pathway by sponging miR-126-3p. (A) Wild or mutant type of miR-126-3p complementary binding sites for *TMPO-AS1* were identified. (B) The luciferase reporter assay was employed to verify direct binding between *TMPO-AS1* and miR-126-3p. (C) Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) was utilized to analyze miR-126-3p expression in 53 paired HCC tissues. (D) Pearson's correlation analysis found a negative correlation between the expression profiles of *TMPO-AS1* and miR-126-3p. (E) Western blotting was used to assess LRP6 and β -catenin expressions. (F) MiR-126-3p expression after transfecting Hep3B and Huh7 cells with a miR-126-3p inhibitor was analyzed using qRT-PCR. (G) Western blot analyses showed that miR-126-3p inhibition reversed the changes in LRP6 and β -catenin expression levels. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

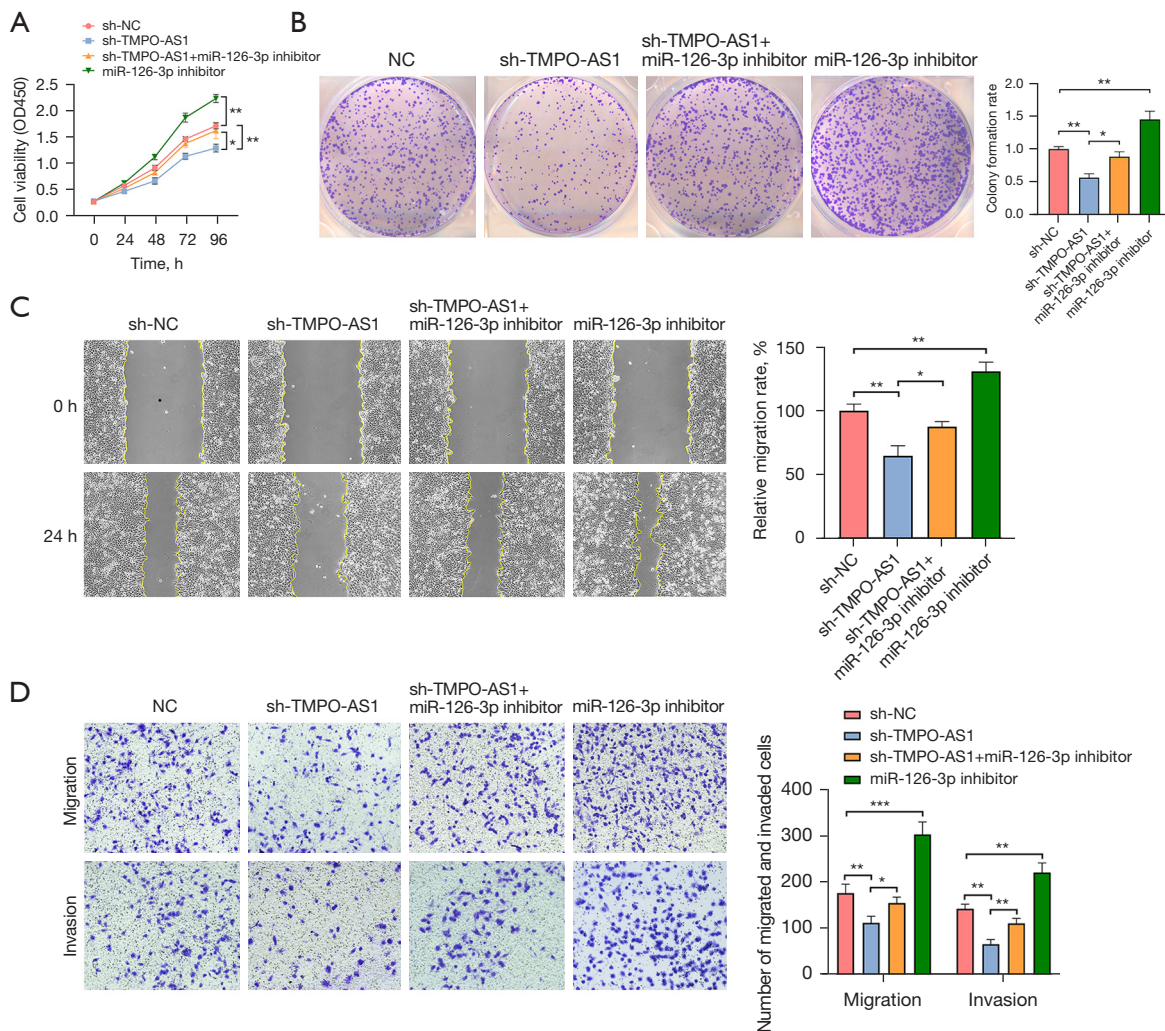


Figure 5 *TMPO* antisense RNA 1 (*TMPO-AS1*) promotes hepatocellular carcinoma progression by inhibiting the miR-126-3p/LRP6 axis. (A,B) Cell Counting Kit-8 and colony formation assays showed that decreased miR-126-3p expression recovered the growth-inhibitory role of short-hairpin (sh)-*TMPO-AS1* in Huh7 cells. (C,D) Wound healing and transwell assays were conducted to evaluate the ability of Hih7 cells to migrate and invade following cotransfection with sh-*TMPO-AS1* and a miR-126-3p inhibitor. Cells were stained with crystal violet. Magnification power 200 \times . *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

miR-126-3p inhibition abrogates the repressive effect of *TMPO-AS1* knockdown

We next explored how the biological functions of HCC are regulated by *TMPO-AS1* in combination with miR-126-3p. The results of the CCK8 and clone formation assays indicated Huh7 cell proliferation is decreased following *TMPO-AS1* knockdown, as compared with NC. However, cell proliferation can be enhanced by miR-126-3p suppression. Nevertheless, the inhibitory effect of sh-*TMPO-AS1* on HCC growth could be recovered by the

repression of miR-126-3p (Figure 5A,5B). Correspondingly, wound healing assays (Figure 5C) and transwell (Figure 5D) revealed that the reduced ability of sh-*TMPO-AS1* to prevent HCC cell migration and invasion could be partially recuperated through miR-126-3p suppression. In general, these results imply that *TMPO-AS1* regulates HCC cell metastasis by restraining the miR-126-3p/LRP6 axis.

Discussion

The recent focus on non-coding RNAs has led to the discovery

of the biological roles lncRNAs play in distinct human tumors (20,21), providing novel insights into the development of cancer. lncRNA regulates HCC cells in a variety of ways, including DNA binding and chromatin remodeling, RNA interaction, protein interaction and regulation, and small peptide generation (22). Substantial TMPO-AS1 expression has been demonstrated in human cancers, and downregulation of TMPO-AS1 has been suggested to repress proliferation while promoting apoptosis in cancer cells by sequestering cancer-associated miRNAs (23). However, the role of TMPO-AS1's in the initiation and progression of liver cancer is not well characterized, with the underlying molecular mechanisms remaining elusive. Our research shows TMPO-AS1 is upregulated in HCC tissues and cell lines. In addition, high expression of TMPO-AS1 can lead to decreased quality of life of patients. Therefore, we suspected that TMPO-AS1 can be used as a potential biomarker for patient risk stratification and local regional metastasis hepatocellular carcinoma.

The induction function of EMT is well-known in all stages of tumor development, such as tumor initiation, tumor distant metastasis, tumor cell migration, intravascular metastasis, and malignant progression (24,25). Some studies have confirmed that EMT is closely related to immunosuppressive cytokines and T lymphocyte resistance in the development of tumors (26). In addition, there is evidence that EMT is closely associated with broader inflammatory changes in the tumor microenvironment in NSCLC (27). In the last few years, EMT has been shown to play an indispensable role in inducing the invasion and migration of HCC cells (28). It is evident from our results that TMPO-AS1 can trigger the transformation of epithelial traits of HCC cells into mesenchymal characteristics, leading to increased cell invasion and metastasis. In the clinical specimens we studied, patients with high expression of TMPO-AS1 showed more microvascular invasion. Relatedly, TMPO-AS1 also plays an essential part in promoting the proliferation of HCC cells and may therefore spur the growth of tumors in newly invaded tissues.

While our study confirmed that TMPO-AS1 promotes HCC progression, the underlying mechanism(s) by which TMPO-AS1 regulates HCC pathogenesis remain(s) to be elucidated. Recent research has put forward a novel regulatory mechanism whereby lncRNAs can serve as endogenous competitive 'sponges', which interact with miRNAs to regulate their functions (29,30). We therefore studied the potential downstream signaling pathway employed by TMPO-AS1 to act as competing endogenous

RNA (ceRNA) in the development of HCC. Bioinformatics analysis (starBase 3.0) illuminated a presumptive binding site between TMPO-AS1 and miR-126-3p. Interestingly, existing literature suggests miR-126-3p plays a vital role as a tumor suppressor in liver cancer (19,31). Thus, we postulated that TMPO-AS1 might participate in HCC development by interacting with miR-126-3p. Luciferase reporter and qRT-PCR assays indicated that TMPO-AS1 binds to miR-126-3p and decreases its expression. Meanwhile, LRP6, which has been deemed to be downstream miR-126-3p (19), is a well-established activator of beta-catenin, which can exert metastatic, proliferative, and anti-apoptotic effects in several types of cancers, including HCC. Here, we proved that miR-126-3p inhibitor largely rescued the effects of TMPO-AS1 on the biological functions of HCC cells. Meanwhile, TMPO-AS1 competitive binding to miR-126-3p prevented the miRNA-induced inhibition of LRP6 and its downstream beta-catenin expression *in vitro*. Hence, the new TMPO-AS1/miR-126-3p/LRP6 pathway could be a promising prognostic marker and therapeutic target in HCC recurrence and metastasis.

In conclusion, our study found TMPO-AS1 is a novel oncogene that promotes HCC development by competitively binding to miR-126-3p. Furthermore, TMPO-AS1 induces tumor cell proliferation, metastasis, and EMT by activating the miR-126-3p/LRP6/ β -catenin pathway. This research elucidated the specific role and mechanism of TMPO-AS1 in promoting HCC development, which will contribute to the design of new therapeutic strategies for the prevention and treatment of HCC. Moreover, TMPO-AS1 may be a prognostic marker for HCC due to its overexpression.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at <https://dx.doi.org/10.21037/atm-21-5593>

Data Sharing Statement: Available at <https://dx.doi.org/10.21037/atm-21-5593>

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://dx.doi.org/10.21037/atm-21-5593>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Chongqing Medical University (No. 2021-156) and informed consent was taken from all the patients.

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