METTL3-mediated m6A modification of lncRNA TSPAN12 promotes metastasis of hepatocellular carcinoma through SENP1-dependent deSUMOylation of EIF3I

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Abstract

In a previous study, we discovered that the lnc-TSPAN12 level was significantly elevated in hepatocellular carcinoma (HCC) and linked to a low chance of survival. In HCC, however, the function of lnc-TSPAN12 in modulating epithelial-mesenchymal transition (EMT) and metastasis is still poorly understood. This study demonstrated that lnc-TSPAN12 positively modulated migration, invasion, and EMT of HCC cells in vitro and exacerbated hepatic metastasis in patient-derived tumour xenograft metastatic models in vivo. The modification of N6-methyladenosine that is driven by METTL3 is essential because it is involved in the upregulation of lnc-TSPAN12, which contributes to the stability of lnc-TSPAN12. Mechanistically, lnc-TSPAN12 exhibits direct physical interactions with EIF3I and SENP1, and it also assumes the role of a scaffold to help enhance the SENP1-EIF3I interaction. This in turn inhibits the SUMOylation of EIF3I and the degradation of ubiquitin, eventually activating the Wnt/β-catenin signalling pathway to stimulate EMT and metastasis of HCC. Our results shed light on the lnc-TSPAN12 regulation mechanism in HCC metastasis and identify the lnc-TSPAN12-EIF3I/SENP1 axis as a novel treatment target for HCC.

Introduction

Primary liver cancer is a highly aggressive type of cancer, and it is the third major contributor to deaths associated with cancer globally [1]. The most prevalent subtype of primary liver cancer is hepatocellular carcinoma (HCC). The unfavourable prognosis recorded by HCC patients is due to the disease's tendency to reoccur and metastasise after surgery, even with the advances achieved in targeted treatments and immunotherapies [2]. To aid in the development of effective therapeutic interventions for this fatal disease, it is necessary to get a deeper understanding of the molecular pathways involved in HCC metastasis and to identify new molecular targets.

Long non-coding RNAs (IncRNAs) refer to a class of RNA molecules that typically contain > 200 nucleotides. Some IncRNAs have just recently been recognized as key mediators in a variety of biological activities, like chromatin modification, activation or suppression of transcription, post-transcriptional modulation, and modulation of protein function [3–7]. Extensive research demonstrates the frequent dysregulation of IncRNAs as well as their critical involvement in the advancement of malignancies like HCC [8–10]. As a result, more research into the discovery of IncRNAs associated with HCC and investigation into their possible activities is particularly required.

Metastasis of a tumour is a complicated process that involves a variety of different steps and factors. Tumour metastasis is thought to begin with the epithelial-mesenchymal transition (EMT), which occurs when epithelial cells exhibit a more migratory and invasive mesenchymal phenotype [11]. Accumulating evidences have indicated that EMT is closely relevant to tumour metastasis, stemness and drug resistance [12–14]. Recently, it has come to light that IncRNAs enhance tumour metastatic progression by modulating key EMT transcriptional factors, which in turn have a considerable influence on EMT [15, 16]. However, the underlying molecular mechanisms of IncRNAs in EMT regulation in HCC remain unclear.
The N6-methyladenosine (m\(^6\)A) RNA modification, as the predominant endogenous modification of eukaryotic messenger and long-noncoding RNAs, is a dynamic and reversible process catalysed by methyltransferases, eliminated by demethylases and identified via m\(^6\)A-specific binding proteins [17, 18]. In recent years, the essential function that m\(^6\)A modification plays in cancer progression, as well as the mechanisms behind it, has garnered an increasing amount of interest [19]. The significantly elevated methyltransferases 3 (METTL3) seen in gastric cancer are responsible for promoting the m\(^6\)A modification of hepatoma-derived growth factor (HDGF) mRNA, whereas insulin-like growth factor 2 mRNA-binding protein 3 (IGF2BP3), which is an m\(^6\)A reader, identifies and attaches to the m\(^6\)A domain of HDGF mRNA to promote its stabilization, which in turn stimulates tumour angiogenesis and glycolysis [20]. Nevertheless, it is still entirely unclear if modifications to m\(^6\)A are implicated in the modulation of lncRNA or what function m\(^6\)A plays in the lncRNA transcripts that are implicated in HCC metastasis.

In a previous study, we found that elevated levels of lnc-TSPAN12 expression were strongly linked to a dismal prognosis in patients with HCC [21]. In this investigation, we provided additional confirmation that the upregulation of lnc-TSPAN12 is caused by m\(^6\)A modification that is mediated by METTL3, which ultimately results in lnc-TSPAN12 stability. Additionally, in vivo and in vitro studies illustrated that lnc-TSPAN12 facilitated the invasiveness, metastases, and EMT of HCC cells. Mechanistically, lnc-TSPAN12 is capable of binding to the eukaryotic translation initiation factor 3 subunit I (EIF3I) and enhancing the activity of the SUMO-specific protease 1 (SENP1)-mediated EIF3I deSUMOylation (SUMO: small ubiquitin-like modifier). This enhanced EIF3I stability may be attributed to the stronger SENP1(EIF3I)-crosstalk, which prevented the competitive ubiquitination and degradation of EIF3I. Overall, our analyses indicate that lnc-TSPAN12 is a critical player in HCC metastasis and a promising treatment target for HCC.

Results

Lnc-TSPAN12 enhances the metastasis of HCC cells in vitro and in vivo

Previously, we showed that the elevated lnc-TSPAN12 expression level in HCC is linked to adverse clinical and pathological characteristics and a dismal prognosis. To get a deeper understanding of the possible involvement of lnc-TSPAN12 in HCC, we transfected Huh7 and Hep3B HCC cell lines with shRNA (pEX-2) or overexpressed a plasmid (pcDNA3.1) to either knock down or overexpress lnc-TSPAN12 and validated the efficiency of the transfection using qRT-PCR (Fig. S1A, B). In contrast with the scramble control group, wound healing and transwell experiments demonstrated that lnc-TSPAN12 knockdown hindered the migration and invasion of Hep3B and Huh7 cells in vitro (Fig. 1A, B, Fig. S1C, D). Conversely, HCC cells migration and invasion were enhanced by the ectopic expression of lnc-TSPAN12 (Fig. 1A-B, Fig. S1C, D).

EMT plays a pivotal role in HCC metastasis [22, 23]. Consequently, we investigated whether lnc-TSPAN12 plays a role in triggering EMT in HCC cells. As depicted in Fig. 1C, D, E-cadherin, a hallmark of epithelial cells, was expressed at a remarkably higher level after lnc-TSPAN12 was knocked down, whereas
mesenchymal markers Vimentin and Fibronectin were expressed at a remarkably lower level. However, opposite results were observed when Inc-TSPAN12 was overexpressed (Fig. 1C, D). We employed confocal imaging following immunofluorescence staining to further illustrate this phenomenon. Vimentin and E-cadherin expression profiles in Huh7 cells matched those shown by western blotting (Fig. 1E, F). Collectively, our findings raise the possibility that Inc-TSPAN12 regulates EMT to enhance HCC metastasis.

We initially established stable Inc-TSPAN12-knockdown Huh7 HCC cells (sh-Inc-TSPAN12#1, 2) to probe the possible enhancing influence of this gene on HCC cell metastasis. By injecting nude mice with tumour cells into their inferior hemispleen, patient-derived xenograft liver metastatic models were developed. Knocking down Inc-TSPAN12 in Huh7 cells remarkably reduced the numbers and luciferase activity of liver metastatic nodules relative to control cells (Fig. 2A-D). In hepatic sections stained with hematoxylin and eosin (H&E), the metastatic foci of sh-Inc-TSPAN12#1,2 cells were greatly shrunken (Fig. 2E). As per IHC staining, we discovered that Vimentin was downregulated and E-cadherin was upregulated in xenograft tumour tissues after Inc-TSPAN12 knockdown (Fig. 2F-H). Together, our results suggested that Inc-TSPAN12 acts as an oncogenic promoter in HCC metastasis.

**Lnc-TSPAN12 binds specifically to EIF3I**

Since the subcellular localization of IncRNA is directly linked to its function, we initially used RNA FISH to determine where Inc-TSPAN12 was located in Huh7 cells. Based on the findings, it seemed that the majority of Inc-TSPAN12 was located in the cytoplasm (Fig. S2A). To truly comprehend the significance of Inc-TSPAN12 in HCC metastasis, we conducted RNA-seq analysis to compare the gene expression patterns of Huh7 cells before and post-Inc-TSPAN12 knockdown. After comparing the Inc-TSPAN12 knockdown Huh7 cells to the control cells using hierarchical clustering, we discovered that 4,505 genes were substantially changed (FDR 0.05 and|log2FC|>1.0) (Fig. S2B, Table S4). KEGG enrichment analysis revealed that the enriched differential genes belonged to several key biological processes and signalling pathways, including cell adhesion, gap junctions, extracellular matrix receptor interactions, and TGF-β, which are closely associated with EMT and cancer metastasis (Fig. S2C). Altogether, our findings point to Inc-TSPAN12 as a possible key modulator in HCC metastasis.

We conducted an RNA pull-down experiment in conjunction with mass spectrometry (MS) to identify proteins that interact with Inc-TSPAN12 and delve further into the regulatory role of Inc-TSPAN12 in HCC metastases. In brief, the extracted proteins were subjected to silver staining and SDS-PAGE analysis, and several distinct bands were selected for mass spectrum analysis (Fig. 3A and Table S5). Band 1's top Inc-TSPAN12-interacting protein, EIF3I, was linked to important functions in the β-catenin pathway and cancer progression [24, 25]. Wnt/β-catenin signalling is well acknowledged as a potent EMT driver [23]. As a result, we decided to validate this protein further. Western blotting additionally confirmed EIF3I's status as a protein that binds Inc-TSPAN12 (Fig. 3B). Furthermore, antibodies directed against EIF3I were utilized in RIP assays to provide evidence of the association of Inc-TSPAN12 with EIF3I (Fig. 3C). Moreover, the cytoplasmic colocalization of EIF3I and Inc-TSPAN12 in Huh7 cells was validated by Inc-
TSPAN12 FISH followed by immunofluorescence of EIF3I, providing additional evidence for their association (Fig. 3D). Overall, our results suggested that Inc-TSPAN12 physically interacts with EIF3I.

As per Inc-TSPAN12’s anticipated secondary structure in the catRAPID database (http://service.tartaglialab.com/page/catrapid_group, Fig. 3E), we generated a series of truncated fragments to determine the binding region of Inc-TSPAN12 that interacts with EIF3I [26]. Thereafter, RNA pull-down tests were carried out using these deletion mutant constructs. The 430–493 nt Inc-TSPAN12 segment was identified as the critical region for its interaction with EIF3I (Fig. 3F). Notably, EIF3I is a protein containing the WD40 motif and has seven WD40 repeats encompassing practically the whole sequence of EIF3I [27]. We then conducted RIP tests with FLAG-tagged full-length and five truncated EIF3I to identify the domains of EIF3I responsible for its interactions with Inc-TSPAN12. EIF3I's interaction with Inc-TSPAN12 was drastically attenuated when its RNA-binding sites (128–325 and 218–325 aa) were deleted (Fig. 3G), implying that the interaction with Inc-TSPAN12 could depend on these sites.

**Lnc-TSPAN12 enhances protein EIF3I stability**

We initially evaluated EIF3I levels in Inc-TSPAN12-knockdown HCC cells to ascertain the possible interplay between Inc-TSPAN12 and EIF3I. After Inc-TSPAN12 was knocked down, there was no change in EIF3I mRNA levels (Fig. S3A). Conversely, knocking down Inc-TSPAN12 resulted in a significant drop in EIF3I protein level, illustrating that Inc-TSPAN12 may elevate EIF3I protein levels post-transcriptionally (Fig. 4A). Likewise, immunohistochemical staining analysis against previous xenograft tumour tissues suggested that Inc-TSPAN12 knockdown was correlated with the lowered level of EIF3I (Fig. S3B). In Huh7 and Hep3B cells, we discovered that knocking down Inc-TSPAN12 increased the rate of EIF3I degradation and decreased its half-life after treatment with the protein synthesis inhibitor cycloheximide (CHX) (Fig. 4B, C). In addition, knockdown of Inc-TSPAN12 downregulated endogenous EIF3I in HCC cells, but after treatment with the proteasome inhibitor MG132, this effect was prevented, and this finding was accompanied by increased EIF3I ubiquitination levels, implying that Inc-TSPAN12 could prevent EIF3I from being degraded via the ubiquitin-proteasome pathway (Fig. 4D, Fig. S3C). Using the Ubibrowser database (Fig. S3D) (http://ubibrowser.ncpsb.org/), we uncovered more evidence that many E3 ligases could be implicated in the ubiquitination and degradation of EIF3I [28].

The lysine at position 298 was identified as EIF3I's primary ubiquitination domain in previous research [29, 30]. We then mutated this lysine to alanine (K298A mutant) and tested if this mutant might prevent the degradation of EIF3I by ubiquitination. Co-transfection with a lentiviral vector expressing either a FLAG-tagged EIF3I K298A mutant (FLAG-EIF3I-K298A) or a FLAG-tagged wild-type EIF3I (FLAG-EIF3I-WT) was implemented to assess the expression levels and ubiquitination of EIF3I in Inc-TSPAN12 knockdown HCC cells. Additionally, knocking down Inc-TSPAN12 may reduce EIF3I's stability and enhance its degradation by increasing its ubiquitin modification, whereas the K298A mutant greatly attenuated the Inc-TSPAN12 knockdown's promoting impact on EIF3I ubiquitination degradation, leading to EIF3I upregulation (Fig. 4E, F). As per these findings, it appears that the Inc-TSPAN12 gene has a detrimental effect on the ubiquitination of the EIF3I protein. Consequently, we investigated how Inc-TSPAN12 affected EIF3I's ability to bind to its predicted ubiquitin E3 ligases, such as STUB1 and SMURF1. Co-IP assays
demonstrated that the knockdown of Inc-TSPAN12 strengthened the EIF3I-SMURF1 interaction, but had no prominent effect on the adherence of EIF3I to STUB1 (Fig. 4G). Thus, to prevent EIF3I from being ubiquitinated and degraded, Inc-TSPAN12 reduces the protein's ability to bind to the ubiquitin E3 ligase SMURF1.

**Lnc-TSPAN12 enhancement of SENP1-mediated EIF3I deSUMOylation suppresses its ubiquitination**

The mechanism via which Inc-TSPAN12 causes EIF3I to be ubiquitinated and degraded was further explored. SUMOylation is now recognized as a crucial ubiquitin-like, post-translational modification (PTM) of proteins by SUMO proteins, which can regulate the stability, subcellular localization, and interaction of the targeted substrate proteins [31–33]. Intriguingly, previous studies have shown that SUMOylation and ubiquitination are often present at the same lysine residues of a substrate protein, and SUMOylation can sometimes promote the ubiquitination and degradation of modified proteins [34, 35]. Notably, an SUMO1-specific protease SENP1 was identified in band 4 in addition to EIF3I. SENP1 was subsequently shown to directly bind to Inc-TSPAN12 by RNA pull-down followed by western blot and RIP assay (Fig. 5A, B). FISH followed by immunofluorescence of SENP1 further verified the colocalization of Inc-TSPAN12 and SENP1 in the cytoplasm of Huh7 cells (Fig. S4A). The interplay of Inc-TSPAN12 with SENP1 was found to occur through the 430–493 nt region of the gene (Fig. S4B). Since SENP1 is in charge of removing the SUMO family [36], we speculated that SENP1 may also function as a lncRNA-binding protein, endowing Inc-TSPAN12 with the ability to perform deSUMOylation- and/or deubiquitination-associated PTM on EIF3I. The knockdown of Inc-TSPAN12 seemed to have a negligible influence on the SENP1 protein level, as expected (Fig. S4C). The knockdown of Inc-TSPAN12, nonetheless, attenuated the combined effect of SENP1 and EIF3I as shown by co-IP assays, suggesting that Inc-TSPAN12 could act as a scaffold to facilitate the EIF3I-SENP1 binding (Fig. 5C). Furthermore, the binding of EIF3I with SUMO1, SUMO2, and SUMO3 was improved to different levels following Inc-TSPAN12 knockdown, implying that Inc-TSPAN12 facilitated EIF3I deSUMOylation (Fig. 5D).

Furthermore, we investigated whether EIF3I's SUMOylation exerts influence on how EIF3I interacts with ubiquitination enzymes and becomes ubiquitinated. We discovered that SENP1 knockdown strengthened the combination of EIF3I with SUMO1 and SMURF1, as well as EIF3I ubiquitination (Fig. 5E, F). However, after SMURF1 was silenced, EIF3I's binding to SENP1, SUMO1, SUMO2, and SUMO3 did not change (Fig. 5G), demonstrating that EIF3I's SUMOylation could act as a mediator for its ubiquitination, whereas EIF3I's ubiquitination showed no impact on its SUMOylation level. Coincidentally, K298 is also the underlying SUMOylation site according to the bioinformatics website prediction (http://ubibrowser.ncpsb.org/ubibrowser). To confirm this prediction, we mutated K298 and observed alterations in EIF3I SUMOylation levels once Inc-TSPAN12 was knocked down in Huh-7 cells. Accordingly, this mutant remarkably decreased the EIF3I SUMOylation (Fig. 5H). Likewise, the sh-Inc-TSPAN12 + FlagEIF3I-K298A group exhibited a reduced intracellular level of EIF3I in contrast with the FLAG(EIF3I-WT group (Fig. 5H). These results demonstrate that Inc-TSPAN12 promotes SENP1-induced deSUMOylation of EIF3I K298, thereby inhibiting its ubiquitination.
**Lnc-TSPAN12 promotes EMT in HCC via regulation of EIF3I/SENP1**

We postulated that lnc-TSPAN12 could exhibit its bioactivities by interfacing with EIF3I/SENP1 in HCC due to its influence on EIF3I stability. We found that knocking down EIF3I/SENP1 with shRNA led to a significant decrease in Huh7 cell migration and invasion (Fig. 6A, B). EIF3I/SENP1 knockdown also reversed the enhanced cell migration and invasion observed in lnc-TSPAN12-overexpressing HCC cells (Fig. 6A, B). According to our results, lnc-TSPAN12-induced metastasis in HCC may be reversed by knocking down EIF3I/SENP1.

To comprehend the function of lnc-TSPAN12 in Wnt/β-catenin signalling, we initially explored how lnc-TSPAN12 affects the expression of downstream target genes of this pathway. In Huh-7 cells with lnc-TSPAN12 knockdown, the levels of cyclin D1, Axin2, and c-myc mRNA and proteins were considerably downregulated, as depicted in Fig. 6C, D. Subsequently, we explored whether lnc-TSPAN12 may trigger EMT in HCC by modulating EIF3I/SENP1 activity and activating Wnt/β-catenin signalling. Western blot outcomes further demonstrated that ectopic lnc-TSPAN12 expression contributed to an upregulation of β-catenin as well as the associated downstream target genes and promoted EMT in HCC. However, the stimulation of the Wnt/β-catenin signalling pathway caused by lnc-TSPAN12 was attenuated by knocking down EIF3I (Fig. 6E) or SENP1 (Fig. 6F). Our findings indicate that EIF3I and SENP1 are necessary for lnc-TSPAN12 to trigger the Wnt/β-catenin signalling pathway, which may then lead to the induction of EMT and the promotion of metastasis in HCC.

**Lnc-TSPAN12 upregulation in HCC is promoted by m^6^A modification**

Initial research indicates that m^6^A modifications are prevalent in mRNA and IncRNA and serve as a functional modulator of the transcriptome, influencing RNA translation, exportation, localisation, and stability [19, 37]. We subsequently checked to see whether the increase of lnc-TSPAN12 in HCC is linked to m^6^A modification. HCC cells were subjected to methylated RIP qPCR evaluation. The outcomes demonstrated that Huh7 and Hep3B cells have greatly enriched levels of m^6^A methylation of lnc-TSPAN12 (Fig. 7A). According to our bioinformatics research, the key m^6^A methyltransferase METTL3 was evidently upregulated in HCC (Fig. 7B). Additionally, the overall survival (Fig. 7C) and disease-free survival (Fig. 7D) of HCC patients with elevated METTL3 levels were both considerably reduced relative to those with lowered METTL3 levels, pointing to its significance in the prognosis of HCC.

To examine the impact of METTL3 on the upregulation of lnc-TSPAN12 in HCC, we used shRNA to knock down the METTL3 levels in Hep3B and Huh7 cells. The effectiveness of the knockdown was examined using a western blot, as depicted in Figure S5A. The expression level and m^6^A levels of lnc-TSPAN12 were discovered to be substantially reduced in the METTL3-knockdown group as opposed to the control group (Fig. 7E, F). We subsequently used the methylation inhibitor 3-DAA to treat two HCC cell lines and
discovered that Inc-TSPAN12 expression was greatly diminished (Fig. 7G). Lastly, the RNA FISH experiment demonstrated that Inc-TSPAN12 and METTL3 were colocalized in the cytosol (Fig. 7H).

The invasion and migratory capacities of Hep3B cells were also inhibited by the knockdown of METTL3 (Fig. S5B, C). Additionally, the upregulation of E-cadherin and downregulation of Vimentin and Fibronectin levels were seen following METTL3 knockdown in Hep3B and Huh7 cells (Fig. S5D). The above findings raised the possibility that METTL3-elicited m^6^A modification have contributed to the transcriptional stability of Inc-TSPAN12, which may partially be responsible for its upregulation in HCC.

**Discussion**

Mounting research proof suggests that IncRNAs perform important functions in the advancement of many different human malignancies. Our previous study illustrated that the Inc-TSPAN12 level was significantly elevated in HCC, particularly in HCC with microvascular invasion, and was linked to a dismal prognosis [21]. Herein, we established that Inc-TSPAN12 had potent pro-metastatic activity by enhancing the migration, invasion, and EMT of HCC cells in vitro and in vivo. Notably, Inc-TSPAN12 strengthened SENP1-mediated EIF3I deSUMOylation by facilitating SENP1’s interaction with EIF3I after binding to EIF3I, thus restraining the ubiquitination and proteasomal degradation of EIF3I. These results point to Inc-TSPAN12 as a potential treatment target for HCC, as it exerts a significant effect on HCC metastasis.

Over the past two decades, the involvement of EMT in cancer advancement and metastasis has come into focus. Nonetheless, additional research is needed to uncover the molecular mechanism via which IncRNAs regulate EMT. By means of gain- and loss-of-function investigations, we determined that Inc-TSPAN12 can trigger HCC cells migration, invasion, and EMT in vitro and in vivo. Similar to our discoveries, the IncRNA TTN-AS1 may act as a competitive endogenous RNA to control the level of CDK5 by sponging miR-142-5p, hence promoting EMT in lung adenocarcinoma progression [38]. Furthermore, IncRNA RP11 promotes colorectal cancer cell metastasis and EMT by increasing levels of the critical EMT transcriptional activator Zeb1 [39]. Our results strongly support a regulatory involvement of Inc-TSPAN12 in EMT and HCC metastasis, mediated by the interaction of Inc-TSPAN12 with EIF3I to stimulate Wnt/β-catenin signalling.

Cytoplasmic IncRNAs generally exert their biological functions by acting as miRNA sponges or physically interacting with regulatory proteins. Using an RNA pull-down experiment and MS, we determined that Inc-TSPAN12 binds directly to EIF3I and then serves as a scaffold to enhance the binding of SENP1 to EIF3I, which in turn increases EIF3I stability. The commencement of mRNA translation, control of the cell cycle, proliferative potential, and tumorigenesis are all impacted by EIF3I, a subunit of the eIF3 complex [27]. Research shows that EIF3I is expressed at high levels in many human malignancies, like colon adenocarcinoma [24], HCC [25], and melanoma [40], suggesting that it plays a role in tumour onset and development. Although EIF3I overexpression leads to carcinogenesis, no studies have yet to be investigated the IncRNA-mediated PTM of EIF3I thus far. Herein, we provided proof that EIF3I deSUMOylation driven by SENP1 blocks its ubiquitination and degradation, hence enhancing its stability.
These findings offer additional evidence that Inc-TSPAN12 modulates the PTM of EIF3I and shed light on the functional link between IncRNA and protein SUMOylation modifications.

With the ability to modulate the whole RNA life cycle, m$^6$A has quickly become one of the most important internal RNA modifications [41]. Over the past few years, evidence has accumulated demonstrating that m$^6$A is implicated in the occurrence of multiple different types of cancer by influencing the targeted IncRNA or mRNA[19, 42]. There are currently not many research publications on m$^6$A modification in IncRNAs, particularly in HCC. For example, the modification of m$^6$A that is caused by METTL3 leads to an increase in LINC00958 because it stabilizes the RNA transcript of that gene [43]. LNCAROD upregulation in HCC was revealed to be maintained by increased m$^6$A methylation-induced improvement of its RNA stability, according to a recent research report [44]. In this investigation, we discovered that m$^6$A was enriched within Inc-TSPAN12 in HCC cells. Additionally, Inc-TSPAN12's stability was increased as a result of m$^6$A modification driven by METTL3, implying that m$^6$A modification could contribute to the upregulation of Inc-TSPAN12 in HCC.

In conclusion, the novel m$^6$A-regulated Inc-TSPAN12 exerts a pro-metastatic role in HCC advancement via the SENP1-EIF3I interaction (Fig. 8). Our results shed light on the mechanisms of HCC metastasis and identified a novel treatment target for expanding anti-metastatic strategies against HCC.

**Methods**

**Cell culture**

From the American Type Culture Collection (ATCC, Rockville, MD, USA), we obtained human liver cancer cell lines (Hep3B and Huh7). Dulbecco's Modified Eagle Medium was used to culture the Hep3B and Huh7 cells (DMEM; Gibco). In addition, 10% foetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Sigma) were added to all media. Each cell line was grown in a 37°C incubator in a humidified atmosphere containing 5% CO$_2$.

**RNA immunoprecipitation (RIP)**

A Magna RIP Kit was used as directed by the manufacturer (Millipore, Schwalbach, Germany) to facilitate the RIP assay. In brief, immunoprecipitation (IP) lysates were produced in RIP lysis solution containing a protease inhibitor cocktail. The protein A/G Magnetic Beads were treated with 5µg of the relevant antibody for half an hour at ambient temperature. The RIP lysate was added to each bead–antibody complex in the RIP immunoprecipitation buffer and incubated overnight at 4°C. Each immunoprecipitant was subjected to resuspension for half an hour at 55°C. After obtaining purified RNA, a qRT-PCR procedure was performed on the sample.

**Methylated RIP (MeRIP)-qPCR**
MeRIP was implemented as per the procedures specified in the Magna MeRIP m⁶A Kit (Millipore, Schwalbach, Germany). In brief, 18µg of mRNA was subjected to a chemical process that resulted in the creation of fragments that were around 100 nucleotides in length. The m⁶A-specific antibody was incubated in IP buffer and treated with the Magna ChiP Protein A/G Magnetic Beads at ambient temperature for 1 hour. The MeRIP reaction solution was added to the mixture and subjected to incubation at 4°C for a whole night. Analysis of the m⁶A enrichment was accomplished using RT-qPCR.

**RNA binding protein pull-down assay**

Thermo Fisher Scientific™ RNA pulldown Kit (Thermo Fisher Scientific, Pittsburgh, PA, USA) was utilized as recommended by the manufacturer to conduct the RNA binding protein pull-down test. Ribo Biotech (RiboBio, Guangzhou, China) performed the production of both the IncRNA cDNA sense and antisense strands. In vitro transcription utilizing T7 RNA polymerase and Biotin RNA Labeling Mix (Roche) resulted in the production of Biotin-labelled IncRNAs. In this experiment, cell lysates isolated from HCC cells were treated for one hour at 4 °C with 50 pmol of pure biotinylated transcripts with rotation. Afterwards, streptavidin magnetic beads were introduced into the lysate of cell proteins to precipitate the RNA–protein complex. To isolate proteins for use in western blotting, the beads were boiled in SDS buffer after three washes.

**Fluorescence in situ hybridization (FISH)**

GenePharma Co., Ltd. (Shanghai, China) developed the Inc-TSPAN12 FISH probe. Utilizing a confocal microscope and a FISH kit allowed for the detection of the subcellular localisation of Inc-TSPAN12 (Zeiss, Germany) following the guidelines provided by the manufacturer (RiboBio). Immunofluorescence (IF) with anti-SENP1 and anti-EF3I antibodies was utilised to detect the levels of these proteins in HCC cells (1:100; Abcam, Cambridge, UK), as per the previous descriptions.

**Immunoprecipitation (IP)**

On the ice, a lysis buffer was used to lyse HCC cells for one hour. After centrifuging the samples at 14000 g for half an hour, the samples were clarified, and supernatants were incubated overnight with the designated antibodies at 4°C. Protein A/G agarose beads (Life Technologies, 20421) were utilised to collect protein-antibody complexes at 4°C for 2 h mixing by rotation. After that, immunoblotting analysis was conducted on the beads after they had been rinsed and boiled.

**Co-immunoprecipitation (Co-IP) assay**

The Co-IP was implemented in the following manner. In brief, the HCC cells that had been pretreated were harvested and then lysed in an ice-cold IP lysis buffer. The cell lysates were utilised for the Co-IP experiment, during which they were first treated with anti-Flag antibodies at 4 °C throughout the night, and then with human anti-IgG as the negative IP control. Protein A/G agarose beads were used to incubate the immune complexes for two to three hours. Following that, the beads were rinsed a minimum of 5 times, followed by treating and boiling them for 5 minutes with a sample buffer containing 1x SDS. The Western blot tests were conducted as was previously reported.
**In vivo experiment**

Research with animals was conducted in conformity with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals (NIH publication 80–23, revised 1996), with the approval of the Institutional Committee of the West China Hospital, Sichuan University, China. For the development of the liver metastasis model, five male nude mice were inoculated per group. Each mouse received an injection of $2 \times 10^6$ Huh7NC, Huh7sh-TSPAN12#1, and Huh7sh-TSPAN12#2 cells into their spleens. After that, the cells were transferred to the liver for the spontaneous formation of metastatic lesions. To track metastases with an IVIS@ Lumina III system (PerkinElmer, Waltham, MA, USA), 1.5 mg luciferin (Gold Biotech, St. Louis, MO, USA) was given weekly for 4 weeks.

**Statistical analysis**

SPSS 24.0 (IBM Corporation, Armonk, NY, USA) and GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA) was utilised to conduct all analyses of statistical data. Data are reported as mean ± standard error of the mean (SEM). The variations between two or more groups were analysed utilising either the Student's t-test or a one-way analysis of variance, correspondingly. The Kaplan–Meier (KM) approach was used to analyse the cumulative survival curves, and the log–rank test was used for comparison. A significant difference was determined to exist at $P > 0.05$ (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

**Declarations**

**Ethics approval and consent to participate**

This study was reviewed and approved by the Ethics Committees of West China Hospital of Sichuan University (Chengdu, China). The study was conducted in accordance with the International Ethical Guidelines for Biomedical Research Involving Human Subjects.

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**Author contributions**

J. L and X. X considered and designed the study. B. L, J. X, D. P, and G. N performed experiments. B. L, X. X, N. W and Y. W did data analyses. J. L and B. L wrote the manuscript. J. L, B. L and X. X contributed to the revision of manuscript. All authors read and approved the final manuscript.

**Consent for publication**
All authors agree to the content of the manuscript and to publish the manuscript as co-authors.

**Competing interests**

The authors declare no competing interests.

**Data Availability**

More detailed methods are available in the Supporting Information and Experimental Section. All relevant data supporting the findings of this study are available within the article and its Supporting Information or from the authors upon reasonable request.

Additional materials and methods are available in the Supplementary material.

**References**


Figures
Lnc-TSPAN12 promotes migration, invasion and EMT of HCC cells in vitro. A, B. Wound healing and transwell assays were performed to assess migration and invasion abilities in Huh7 and Hep3B cells transfected with lnc-TSPAN12 shRNA or lnc-TSPAN12 overexpression vector. Quantification (bottom panel) of the cell migration and invasion assay results were shown. C, D. The expression of EMT-related markers in Huh7 and Hep3B cells were verified by western blot analysis. E, F. The expression of E-
cadherin (E) and Vimentin (F) in Huh7 cell was detected by immunofluorescence assay using fluorescence microscope (200X, scale bar =50 um). Results were mean ± SD for three individual experiments, for each condition, were performed in triplicate.

Figure 2
Lnc-TSPAN12 promotes metastasis in HCC. A. Decreased tumor metastasis formed in the livers of nude mice through the inferior hemispleen implantation of Inc-TSPAN12-knockdown Hep3B PDX cells. Representative bioluminescent images of livers for each experimental group at 8 weeks. B. Statistical analysis of bioluminescent tracking plots. C. Representative images of metastatic nodules formed in the liver. D. The number of metastatic nodules formed in the livers of mice for each group. Data represent mean ± S.D.; dot plot shows data points from independent experiment. The P values were determined by a two-tailed unpaired Student’s t test. E. Representative HE staining of PDX liver metastatic lesions, original magnification, ×100, scale bar, 100 μm. F-H. The expression levels of E-cadherin and Vimentin were detected in PDX liver metastatic tissues using immunohistochemistry. *P<0.05, **P<0.01.
Figure 3

Lnc-TSPAN12 physically interacts with EIF3I and SENP1. A. Proteins interacting with Lnc-TSPAN12 were screened by RNA pull-down assay followed by mass spectrometry. B. Western blot analysis of EIF3I after pull-down assay confirming its specific combination with Lnc-TSPAN12. GAPDH served as a negative control. C. RIP assay using anti-EIF3I antibody showed that it interacts with Lnc-TSPAN12 in Huh7 cell. The relative fold enrichment of Lnc-TSPAN12 between EIF3I and IgG RIP fractions was determined by qRT-PCR. D. Lnc-TSPAN12 FISH (green), EIF3I immunofluorescence (red), nuclei staining (blue), and merged (yellow) images in Huh7 cell. FISH assay shows the colocalization of Lnc-TSPAN12 and EIF3I in cytoplasm. Scale bar, 20 μm. E. Deletion mapping of the EIF3I-binding region(s) in Lnc-TSPAN12. F. Western blot analysis of EIF3I pulled down by full-length or truncated Lnc-TSPAN12. G. Immunoblot analysis with anti-Flag in Huh7 cell transfected with plasmids encoding FLAG-tagged full-length or truncated EIF3I constructs.
Lnc-TSPAN12 enhances the stability of EIF3I protein by blocking its ubiquitination degradation. A. Western blot analysis shows the protein levels of EIF3I with or without knockdown of lnc-TSPAN12 in Huh7 and Hep3B cells. B. The protein levels of EIF3I in the indicated cells transfected with sh-lnc-TSPAN12 or sh-NC and treated with cycloheximide (CHX) (100 μg/mL). C. Induced knockdown of lnc-TSPAN12 shortened the half-life time of EIF3I protein with the treatment of CHX. D. Lnc-TSPAN12
knockdown enhanced EIF3I ubiquitination. IB of EIF3I ubiquitination in the indicated cells expressing sh-lnc-TSPAN12 or sh-NC with the treatment of 20 μM MG132. E. The Huh-7 cell was co-transfected with shRNA-Inc-TSPAN12 and Flag(EIF3I-WT or Flag(EIF3I-K298A by lentiviral vector. The expression of Flag from the indicated group was detected by western blot assay. F. The cell extracts from the indicated groups were subjected to immunoprecipitation with anti-Flag antibody, followed by immunoblot with anti-ubiquitin antibody. G. The impact of lnc-TSPAN12 on the binding of EIF3I to its predicted ubiquitin E3 ligases. The cell extracts from the indicated groups were subjected to immunoprecipitation with anti(EIF3I antibody, followed by immunoblot with anti-STUB1 or anti-SMURF1 antibody. Results were mean ± SD for three individual experiments which, for each condition, were performed in triplicate. *P<0.05, **P<0.01.
Figure 5

Lnc-TSPAN12 knockdown promotes EIF3I ubiquitination through suppressing SENP1-mediated deSUMOylation. A. Western blot analysis of SENP1 after pull-down assay confirming its specific combination with Lnc-TSPAN12. GAPDH served as a negative control. B. RIP assay using anti-SENP1 antibody showed that it interacts with lnc-TSPAN12 in Huh7 cell. The relative fold enrichment of lnc-TSPAN12 between SENP1 and IgG RIP fractions was determined by qRT-PCR. C. The effect of lnc-TSPAN12 on EIF3I and SENP1 expression.
TSPAN12 knockdown on the interaction between SENP1 and EIF3I. The cell extracts from the indicated groups were subjected to immunoprecipitation with anti-SENP1 antibody, followed by immunoblot with anti(EIF3I antibody. D. The association between Inc-TSPAN12 and EIF3I SUMOylation. The cell extracts from the indicated groups were subjected to immunoprecipitation with anti(EIF3I antibody, followed by immunoblot with anti-sumo1, anti-sumo2 or anti-sumo3 antibody. E, F. The effect of SENP1 knockdown on the combination of EIF3I with ubiquitination enzyme and EIF3I ubiquitination. The cell extracts from the indicated groups were subjected to immunoprecipitation with anti(EIF3I antibody, followed by immunoblot with anti-sumo1 and anti-SMURF1 antibody (E), or anti-ubiquitin antibody (F). G. The effect of SMURF1 knockdown on EIF3I SUMOylation. The cell extracts from the indicated groups were subjected to immunoprecipitation with anti(EIF3I antibody, followed by immunoblot with anti-sumo1, anti-sumo2, anti-sumo3 or anti-SENP1 antibody. H. The Huh-7 cell co-transfected with shRNA-Inc-TSPAN12 and FlagEIF3I-WT or Flag(EIF3I-K298A by lentiviral vector. The cell extracts from the indicated groups were subjected to immunoprecipitation with anti(EIF3I antibody, followed by immunoblot with anti-sumo1, anti-sumo2 or anti-sumo3 antibody. Results were mean ± SD for three individual experiments which, for each condition, were performed in triplicate. *P<0.05, **P<0.01.
Figure 6

Lnc-TSPAN12 exerts EMT-promoting functions in HCC by regulating EIF3I/SENP1. A, B. Knockdown EIF3I (A) or SENP1 (B) can reverse the increased migration and invasion abilities of Huh-7 cell mediated by Inc-TSPAN12 overexpression. C. qRT-PCR detecting the mRNA expression levels of cyclin D1, c-myc and Axin2 in Huh-7 cell with lnc-TSPAN12 knockdown. D. Western blot analysis detecting the protein expression levels of cyclin D1, c-myc and Axin2 in Huh-7 cell with Inc-TSPAN12 knockdown. E, F. The Huh-
7 cell was co-transfected with oe-Inc-TSPAN12 and shRNA-EIF3I (E) or shRNA-SENP1 (F) by lentiviral vector. The expression of β-catenin, E-cadherin, Vimentin, cyclin D1 and c-myc from the indicated group were detected by western blot assay. Results were mean ± SD for three individual experiments which, for each condition, were performed in triplicate. *P<0.05, **P<0.01.

Figure 7
Lnc-TSPAN12 is upregulated in HCC through METTL3-mediated m^6^A modification. A. The m^6^A methylation level of lnc-TSPAN12 in Huh7 and Hep3B cells were determined by MeRIP-qPCR assays. B. METTL3 up-regulation was found in TCGA HCC cohort. C, D. Kaplan-Meier analysis of the correlation between METTL3 and overall survival (C), disease-free survival (D) in TCGA database. E. The expression levels of lnc-TSPAN12 in Huh7 and Hep3B cells transfected with sh-METTL3 or sh-NC were detected by qRT-PCR. F. The m^6^A methylation level of lnc-TSPAN12 in the indicated cells transfected with sh-METTL3 or sh-NC were determined by MeRIP-qPCR assays. G. The Huh7 and Hep3B cells was exposed to 3-DAA (0, 100, 200 μM) for 24 h. The expressions of lnc-TSPAN12 in the indicated cells were detected by qRT-PCR. H. The location of lnc-TSPAN12 and METTL3 in Hep3B cell were detected by FISH assay and immunofluorescence assay. Representative images were captured and the cells were counted from three independent experiments. Results were mean ± SD for three individual experiments which, for each condition, were performed in triplicate. *P<0.05, **P<0.01.
Figure 8

A schematic model for the mechanism of Inc-TSPAN12 in the progression of HCC.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.
- TableS1SequencesofPCRprimersusedinRTqPCRassays.docx
- TableS2SequencesofshRNAusedinthestudy.docx
- TableS3Antibodydetailsusedinthisstudy.docx
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