Long non-coding RNAs affect the metastasis of hepatocellular carcinoma cells by regulating the epithelial-to-mesenchymal transition

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Abstract. Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer, and, in terms of its global incidence and mortality rates across all types of cancer, HCC is ranked sixth and third, respectively. Therefore, HCC is a notable global health burden. Dysregulation of long non-coding RNAs (lncRNAs) has a key role in human tumorigenesis. However, the lncRNAs involved in the epithelial-to-mesenchymal transition (EMT) that affects the migration and recurrence of HCC are yet to be fully elucidated. In the present study, the EMT was induced in Huh7 cells using transforming growth factor-β (TGF-β) and it was revealed that this process may promote HCC metastasis. RNA sequencing was then used to screen the lncRNAs and mRNAs that were differentially expressed (DE) during the TGF-β-induced EMT process. Subsequently, Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses were carried out on the identified DE lncRNAs and mRNAs. By constructing a co-expression network of DE lncRNAs and mRNAs, key lncRNAs possibly involved in the TGF-β-induced EMT that may promote the metastasis of HCC were predicted. Therefore, although preliminary, the present study suggested a list of potential candidates for further investigation of the molecular mechanism of the TGF-β-induced EMT in HCC. Further research may reveal key information on tumorigenesis and potential therapeutic targets in HCC in the future.

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Introduction

The latest data indicates that liver cancer is the sixth most prevalent malignant tumor worldwide and the fourth most prevalent in China, and primary liver cancer caused ~757,948 mortalities worldwide in 2022 (1,2). Hepatocellular carcinoma (HCC) is the main histopathological type of primary liver cancer, accounting for 75-85% of cases, with chronic hepatitis B or C viral infections being the primary risk factors (1,2). HCC has an insidious onset and rapid progression, with the majority of patients diagnosed at a late stage. In addition, HCC tumors are heterogeneous (3). At present, the HCC treatment options are diverse and include surgical resection, liver transplantation, chemotherapy, radiotherapy, targeted therapy and interventional therapy. However, recurrence and metastasis following treatment are the main contributors to the poor prognosis of patients with HCC (4-7). Therefore, investigating the mechanisms of HCC recurrence and metastasis, identifying the specific metastatic pathways and developing novel treatment strategies are key to improving patient prognosis.

The epithelial-to-mesenchymal transition (EMT) is a biological process in which epithelial cells lose their polarity and tight connections, transforming into mesenchymal cells. During this process, the expression of epithelial cell markers (such as E-cadherin) decrease, while mesenchymal markers (such as N-cadherin, vimentin and fibronectin) increase. Concurrently, the cells lose their polarity and acquire a motile phenotype (8-10). Based on this characteristic, the EMT is a key process in tumor invasion and metastasis. Long non-coding RNAs (lncRNAs) are functionally defined as transcripts of >200 nucleotides in length that lack protein-coding potential. lncRNAs exhibit aberrant expression in various types of tumors (such as endometrial and ovarian cancer) and are notable regulators of the tumor metastasis process (11-13). Increasing evidence demonstrates that lncRNAs participate in the regulation of the EMT, affecting tumor invasion and metastasis and are hypothesized to be potential targets for

tumor diagnosis and treatment (14-16). For example, a study by Yuan *et al* (17) reports that lncRNA UCID enhances Snail mRNA stability, promotes the EMT and accelerates the metastasis of HCC cells by interacting with Snail mRNA. Additionally, a study by Zhao *et al* (18) demonstrates that lncRNA PNUTS facilitates metastasis and invasion of HCC cells by targeting zinc finger E-box binding homeobox 1 and activating the EMT pathway.

The functional role of lncRNAs in metastasis is often associated with the activity of microRNA (miRNA/miR). A predominant mechanism involves the lncRNA acting as a competing endogenous RNA or molecular sponge, sequestering specific miRNAs and preventing them from binding and repressing their target mRNAs (19). For example, in HCC, lncRNAs such as H19, MALAT1 and HULC facilitate metastasis by sponging tumor-suppressive miRNAs (such as miR-200a-3p and miR-15b), which derepresses oncogenic mRNAs that promote the EMT and metastatic progression (20,21). This lncRNA-miRNA-mRNA axis represents a key layer of epigenetic regulation in cancer cell dissemination. Therefore, comprehensively profiling lncRNA and mRNA expression changes during the EMT process is a key step towards identifying novel nodes within this regulatory network.

In the present study, RNA sequencing was used to identify differentially expressed (DE) lncRNAs and mRNAs in a transforming growth factor-β (TGF-β)-induced EMT model of Huh7 cells. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were carried out on the identified DE lncRNAs and mRNAs in order to highlight potential lncRNAs and signaling pathways that may be involved in the regulation of the EMT and HCC migration.

Materials and methods

Cell culture. Huh7 cells (cat. no. SCSP-526) were obtained from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Huh7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) enriched with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin at 37˚C and under 5% CO_2. Routine medium replacement was carried out according to cell proliferation status, as assessed by microscopic confluence and medium color change, which is indicative of metabolic activity.

In vitro HCC EMT cell model. To induce the *in vitro* EMT cell model, Huh7 cells were cultured at 37˚C in DMEM with 2.5% FBS and 10 ng/ml TGF-β (cat. no. HY-P7118; MedChemExpress) for 4 days. This treatment was used to construct an *in vitro* EMT model (n=3). Cells in the control group were cultured in DMEM containing 2.5% FBS without TGF-β (n=3). The successful induction of the EMT was confirmed by observing a transition from an epithelial cobblestone-like to a mesenchymal spindle-shaped morphology under a phase-contrast microscope. Successful induction of the EMT was further confirmed using reverse transcription-quantitative PCR (RT-qPCR) analysis after a downregulation of E-cadherin and an upregulation of N-cadherin, Slug and Snail were revealed.

Cell migration assay. After the 4-day EMT induction, both TGF-β-treated and control Huh7 cells were detached using trypsin and resuspended in serum-free medium with or without 10 ng/ml TGF-β, respectively. Subsequently, the cells were seeded into the upper chamber of Transwell inserts (8 μm pore size with a polycarbonate membrane; cat. no. 3422; Corning Life Sciences) without Matrigel at a density of $1x10^5$ cells/well. The lower chamber was filled with 600 μl medium containing 10% FBS. The plate was then cultured at 37˚C with 5% CO_2 for 24 h. The cells were then fixed with 4% paraformaldehyde at room temperature for 30 min and stained with 1% crystal violet for 5 min at 37˚C. The migrated cells were imaged in five randomly selected fields of view using an inverted light microscope (Olympus IX73) at a x200 magnification and then manually counted.

RT-qPCR. According to the manufacturer's protocol, total RNA was extracted from the aforementioned TGF-β-treated and control Huh7 cells using the Total RNA Miniprep kit (cat. no. UE-MN-MS-RNA-50; Suzhou UELandy Biotechnology Co., Ltd.), which included an RNA extraction buffer. The concentration of RNA was quantified using a UV spectrophotometer. Subsequently, cDNA synthesis was carried out following the protocol provided with the RevertAidTM First Strand cDNA Synthesis kit (cat. no. K1622; Thermo Fisher Scientific, Inc.). qPCR was carried out using the synthesized cDNA as a template, following the manufacturers protocol of the TB Green® Premix Ex Taq™ II (Tli RNase H Plus) kit (cat. no. RR820A; Takara Biotechnology Co., Ltd.). The thermocycling conditions used were as follows: Initial denaturation at 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and then 60˚C for 34 sec; and subsequently, a melt curve analysis was carried out at 95˚C for 15 sec, 60˚C for 1 min and finally 95˚C for 15 sec. The relative expression level of the target gene was calculated using the $2^{-\Delta\Delta Cq}$ method (22), with GAPDH as the internal reference. Primers were synthesized by Sangon Biotech Co., Ltd., (Table I).

RNA sequencing. RNA sequencing was carried out by Guangzhou Genedenovo Biotechnology Co., Ltd (http://www.genedenovo.com/). Total RNA was isolated using the TRIzol® reagent kit (cat. no.15596026, Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. The integrity and quality of the RNA were evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.) and further confirmed via RNase-free agarose gel electrophoresis. Following the extraction of total RNA, ribosomal RNAs were eliminated to enrich mRNAs and ncRNAs. Sequencing libraries were prepared using the NEBNext® UltraTM II Directional RNA Library Prep kit (cat. no. E7760S; New England Biolabs). The resultant mRNAs and ncRNAs were fragmented using the fragmentation buffer from the aforementioned kit. These fragments were reverse-transcribed into cDNA using random primers. The synthesis of the second-strand cDNA was facilitated by DNA polymerase I, RNase H and dNTPs (with dUTP replacing dTTP) in DNA polymerase reaction buffer from the same aforementioned kit. The cDNA fragments were then purified using the QIAquick PCR Purification kit (cat. no. 28104; Qiagen Benelux B.V.), followed by end repair, the addition of poly(A) tails and ligation to Illumina sequencing

Table I. Primers for reverse transcription-quantitative PCR.

Gene	Primer direction	Primer sequences (5'-3')
E-cadherin	F	GAATGACAACAAGCCCGAAT
	R	GACCTCCATCACAGAGGTTCC
N-cadherin	F	GGTGGAGGAGAAGAAGACCAG
	R	GCATCAGGCTCCACAGT
Slug	F	TGGTTGCTTCAAGGACACAT
	R	GTTGCAGTGAGGGCAAGAA
Snail	F	GCTGCAGGACTCAATCCAGA
	R	ATCTCCGGAGGTGGGATG
NNMT-205	F	CAGGAGCTGGAGAAGTGGCTG
	R	TGCTTGACCGCCTGTCTCAAC
CASC15-204	F	GCACTGACCTCCTTCATTCTGC
	R	AAGCAACTCCAGATGAATCCAGG
UBASH3B-202	F	TGTGTCGGCATGGTGAGAGGAT
	R	GGCATGTTCAGGTTGGTGCGTA
CAPN2-206	F	AGGACATGCACACCATCGGCTT
	R	CGGAGGTTGATGAAGGTGTCTG
CAV2-214	F	ATCGCAGAGCCGGTGACTAC
	R	AAGCATCGTCCTACGCTCGT
COL1A1	F	GAGGGCCAAGACGAAGACATC
	R	CAGATCACGTCATCGCACAAC
BMP6	F	TTCCTCAACGACGCGGACATG
	R	CACCCTCAGGAATCTGGGATAAG
TUBA1A	F	TGCCCCTGTCATCTCTGCTG
	R	CGCTTGGTCTTGATGGTGGC
ATP2B2	F	CATCCTCAACGAACTCACCTGC
	R	GATATTGTCGCCAGTGACCATGC
F2	F	ATGGGCTGGATGAGGACTCAGA
	R	CGGTTTTGTCCTCCAGCGACTT
GAPDH	F	GAGAAGGCTGGGGCTCAT
	R	TGCTGATGATCTTGAGGCTG

F, forward; R, reverse.

adapters. Subsequently, uracil-N-glycosylase was used to digest the second-strand cDNA. The resulting digested products underwent size selection via agarose gel electrophoresis, followed by PCR amplification. Sequencing was then carried out by Guangzhou Genedenovo Biotechnology Co. Ltd. using the Illumina HiSeq™ 4000 platform with paired-end 150 bp strand-specific reads. For cluster generation and sequencing, the HiSeq 3000/4000 PE Cluster kit (cat. no. PE-410-1001; Illumina, Inc.) and SBS kit (cat. no. FC-410-1003; Illumina, Inc.) were used according to the manufacturer's protocols. The final library was loaded at a concentration of 20 nM as measured using a Qubit fluorometer. For bioinformatic analysis, raw sequencing reads were subjected to quality control using FastQC software (version 0.12.1; https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), followed by adapter trimming and quality filtering. The cleaned reads were then aligned to the reference genome using HISAT2 software (version 2.2.1; https://daehwankimlab.github.io/hisat2/download/). Gene expression levels were then quantified using featureCounts software (version 2.0.6; http://subread.sourceforge.net). Subsequently, the sequencing data were analyzed using edgeR software (version 4.0.16;https://bioconductor.org/packages/edgeR). The screening threshold used to identify the significance of DE lncRNAs and mRNAs was false-discovery rate (FDR)<0.05 and [log2 fold-change (FC)]>1.

GO and KEGG analyses. GO and KEGG analyses were carried out using the 'clusterProfiler' R package (version 4.0.5; R Development Core Team) to elucidate the potential molecular mechanisms through which DE lncRNAs and mRNAs influence HCC migration by regulating the EMT process. GO analysis was used to classify and annotate the functions of the DE genes, as well as to perform enrichment analysis. KEGG analysis was used to identify significantly enriched signaling pathways or metabolic pathways associated with the DE genes. Q<0.05 was set as the screening criterion.

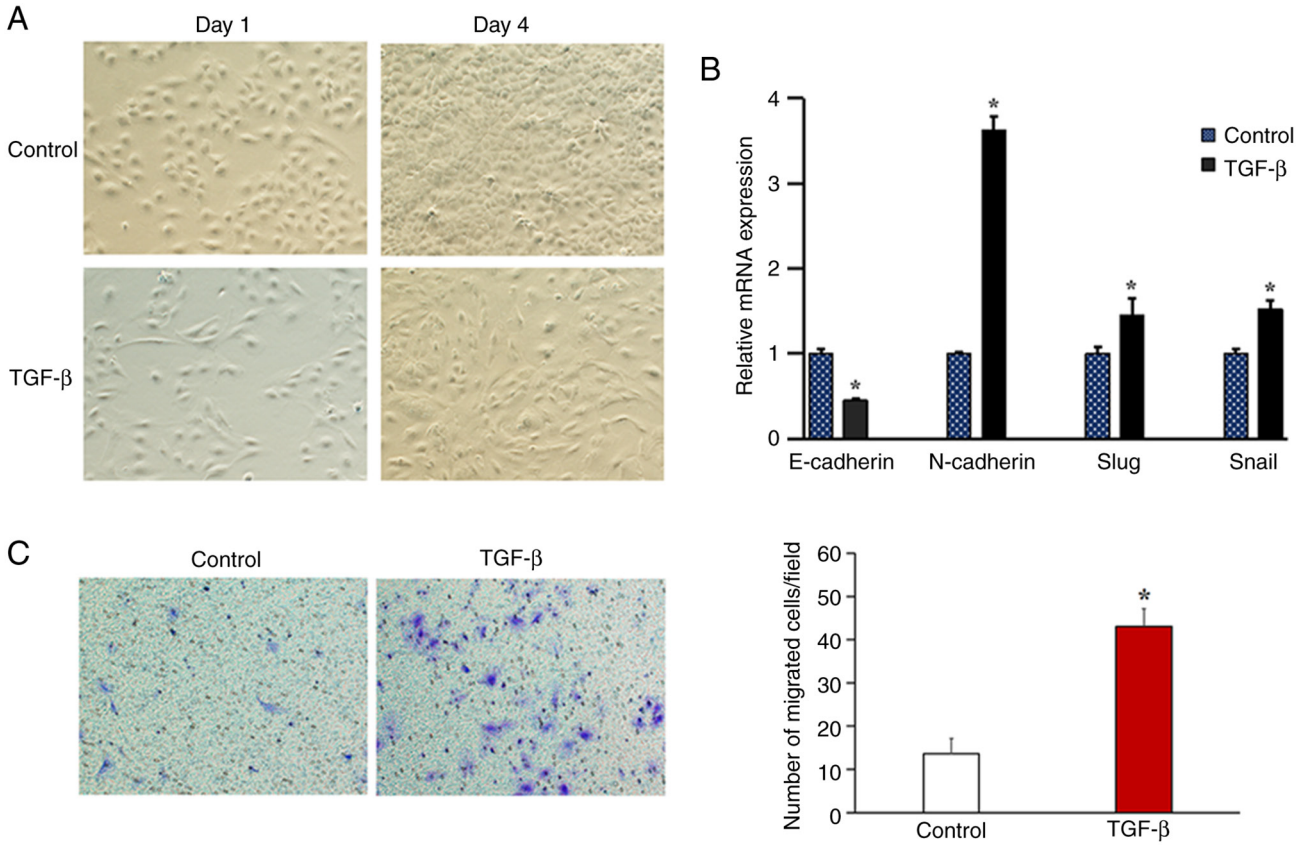

Figure 1. Validation of the epithelial-to-mesenchymal transition model. (A) Micrographs of Huh7 cells treated with or without 10 ng/ml TGF-β for 4 days. (B) Relative mRNA levels of E-cadherin, N-cadherin, Snail and Slug in Huh7 cells treated with or without 10 ng/ml TGF-β for 4 days. (C) *In vitro* migration of Huh7 cells treated with or without 10 ng/ml TGF-β for 4 days. *P<0.05 vs. the control group. TGF-β, transforming growth factor-β.

Construction of the lncRNA-mRNA interaction network. To investigate the potential roles of DE lncRNAs in HCC migration and the EMT, a regulatory network was constructed based on the lncRNA-mRNA pairs predicted by trans-action analysis. The interaction pairs were formatted as a two-column source-target list and imported into Cytoscape software (version 3.10.3; https://cytoscape.org/download.html) using default settings to generate the network visualization.

Statistical analysis. All data were statistically analyzed using SPSS (version 16.0; SPSS, Inc.). Quantitative data are expressed as the mean ± SD. Differences between two groups were compared using an unpaired t-test. All of the experiments were carried out in three independent biological replicates. P<0.05 was considered to indicate a statistically significant difference.

Results

Validation of the HCC cell EMT model. To construct an *in vitro* EMT model, Huh7 cells were stimulated with 10 ng/ml TGF-β for 4 days, with untreated cells serving as the control group. Compared with this control, the TGF-β-treated Huh7 cells lost their original polarity and transformed into a spindle or typical fibroblast-like phenotype (Fig. 1A). At the molecular level, TGF-β treatment significantly downregulated the mRNA expression of the epithelial marker E-cadherin and upregulated the expression of mesenchymal markers (N-cadherin, Snail and Slug) compared with the control group (Fig. 1B). Functionally, the induction of the EMT led to a significant enhancement in the cell migration ability in the TGF-β treatment group compared with the control (Fig. 1C).

Analysis of the DE lncRNAs and mRNAs in TGF-β-treated Huh7 cells. RNA sequencing was carried out to detect the DE mRNAs and lncRNAs in Huh7 cells treated with or without TGF-β. The volcano plots revealed 231 upregulated and 81 downregulated DE lncRNAs (Tables SI and SII) as well as 1,580 upregulated and 810 downregulated DE mRNAs (Tables SIII and SIV) (Fig. 2A). In addition, hierarchical clustering of the DE lncRNAs and mRNAs was carried out and notable differences between the Huh7 cells with or without TGF-β treatment were identified (Fig. 2B). These findings potentially identify key DE lncRNAs (for example, CAV2-214 and TACC1-226) and DE mRNAs (for example, TUBA1A and H4C11) that regulate the EMT process and promote HCC migration.

GO and KEGG enrichment analyses. The function of lncRNAs is associated with their co-expressed protein-coding genes. Trans-action analysis was conducted to identify associations between DE lncRNAs and mRNAs. The prediction results indicated that 312 lncRNAs may act on 2,367 target genes, forming a total of 101,780 lncRNA-gene connections (Table SV). To further investigate the potential function of these identified DE lncRNAs, both GO and KEGG functional

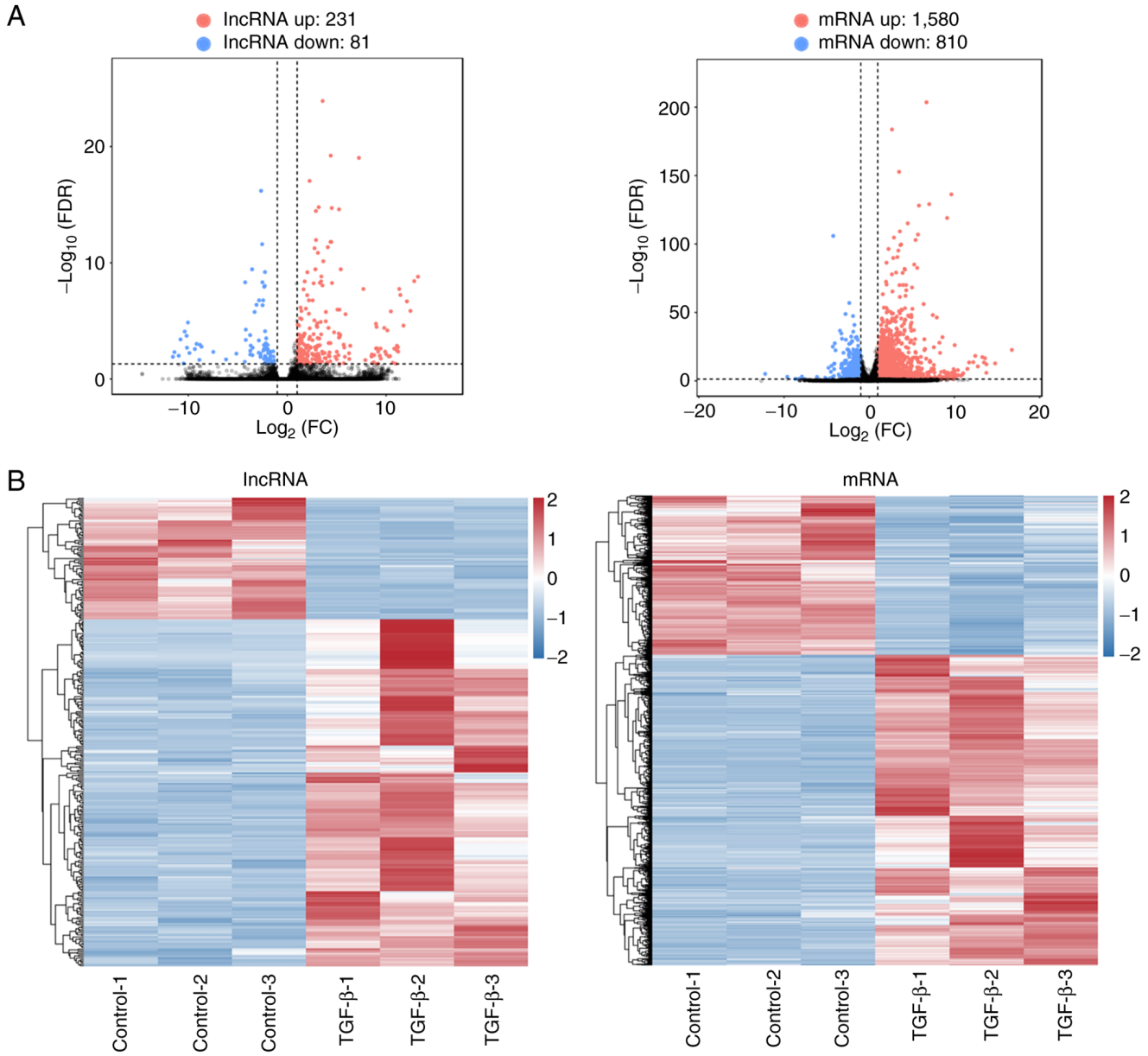

Figure 2. DE lncRNAs and mRNAs in TGF-β treated Huh7 cells. (A) Volcano plot demonstrating DE lncRNAs and mRNAs. Red points represent upregulated RNAs, blue points represent downregulated RNAs and black points represent RNAs with no significant differences. (B) Hierarchical clustering analysis based on the significantly DE lncRNAs and mRNAs. Red indicates high relative expression levels, blue indicates low relative expression levels and white indicates no change in the gene expression levels. The color brightness indicates the extent of the upregulation or downregulation of the gene expression. TGF-β, transforming growth factor-β; DE, differentially expressed; lncRNA, long non-coding RNA; FC, fold-change; FDR, false-discovery rate.

enrichment analyses were carried out. Within the GO molecular function classification, several key GO terms were enriched, including 'extracellular matrix structural constituent', 'extracellular matrix binding' and 'signaling receptor binding' (Fig. 3A). Within the GO cellular component classification, several key GO terms were enriched, including 'collagen-containing extracellular matrix', 'extracellular matrix' and 'external encapsulating structure' (Fig. 3B). Within the GO biological process classification, several key GO terms were enriched, including 'anatomical structure morphogenesis', 'system development' and 'tissue development' (Fig. 3C). A number of these GO terms have been reported to be associated with EMT processes, such as 'actin binding', the 'extracellular region' and 'cell migration' (23-25). The results of the KEGG enrichment analysis revealed that the associated signaling pathways included 'ECM-receptor interaction', 'focal adhesion' and 'axon guidance' (Fig. 3D). These pathways have also been reported to be associated with the EMT process (26-28). These results were used to select the lncRNAs that are potentially involved in the regulation of the EMT in the progression of HCC.

Establishment of the lncRNA-mRNA interaction network. To further investigate how DE lncRNAs potentially affect the migration and development of HCC by regulating the EMT, a network map was constructed based on 15 DE lncRNAs and 18 DE mRNAs. The lncRNA-mRNA interactions were predicted by the previous trans-action analysis (Fig. 4).

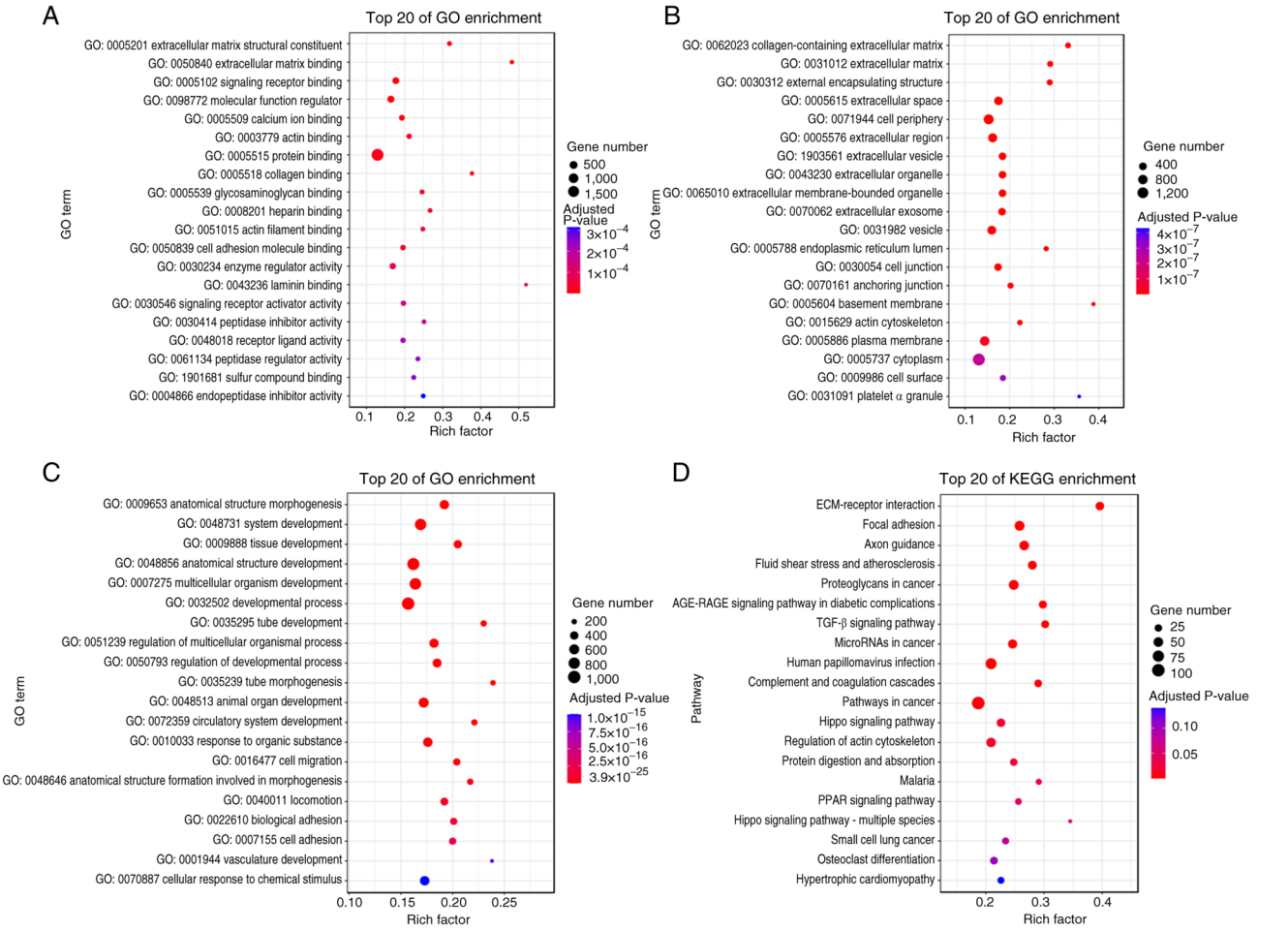

Figure 3. GO and KEGG enrichment analysis. GO term enrichment categories, including (A) molecular function, (B) cellular component and (C) biological process. (D) KEGG pathway enrichment analysis. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; ECM, extracellular matrix; TGF-β, transforming growth factor-β; PPAR, peroxisome proliferator-activated receptor; AGE-RAGE, advanced glycation end-products-receptor for advanced glycation end-products.

Validation of sequencing data using RT-qPCR. To validate the RNA sequencing results, 5 DE mRNAs (COL1A1, BMP6, TUBA1A, ATP2B2 and F2) and 6 DE lncRNAs (NNMT-205, CASC15-204, UBASH3B-202, CAPN2-206, CAV2-214 and ZSWIM8-210) were selected for RT-qPCR analysis based on their statistical significance and putative association with the EMT. The RT-qPCR results were consistent with the sequencing data. Compared with the control, TGF-β treatment significantly upregulated the expression of COL1A1, BMP6, TUBA1A, NNMT-205, CASC15-204, UBASH3B-202, CAPN2-206, CAV2-214 and ZSWIM8-210. Furthermore, TGF-β treatment significantly downregulated the expression of ATP2B2 and F2 compared with the control (Fig. 5A and B).

Discussion

The EMT is involved in the metastasis of various types of tumors (such as colorectal and breast cancer) and is a key mechanism for the recurrence and metastasis of malignant tumors, including HCC (29-31). Despite the role of the EMT in HCC progression, therapeutic strategies targeting regulators of the EMT, such as TGF-β/Smad signaling or Snail family transcription factors, have not yet been clinically successful. This

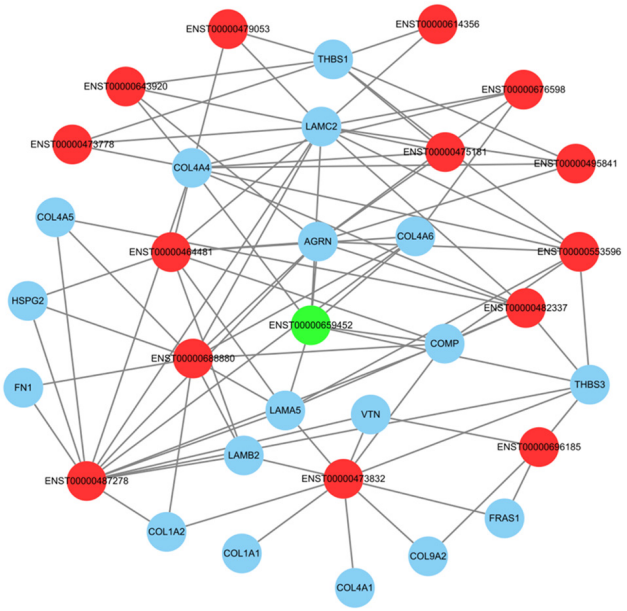

Figure 4. lncRNA-mRNA network. Red represents the upregulated lncRNAs, green represents the downregulated lncRNAs and blue represents mRNAs. lncRNAs, long non-coding RNAs.

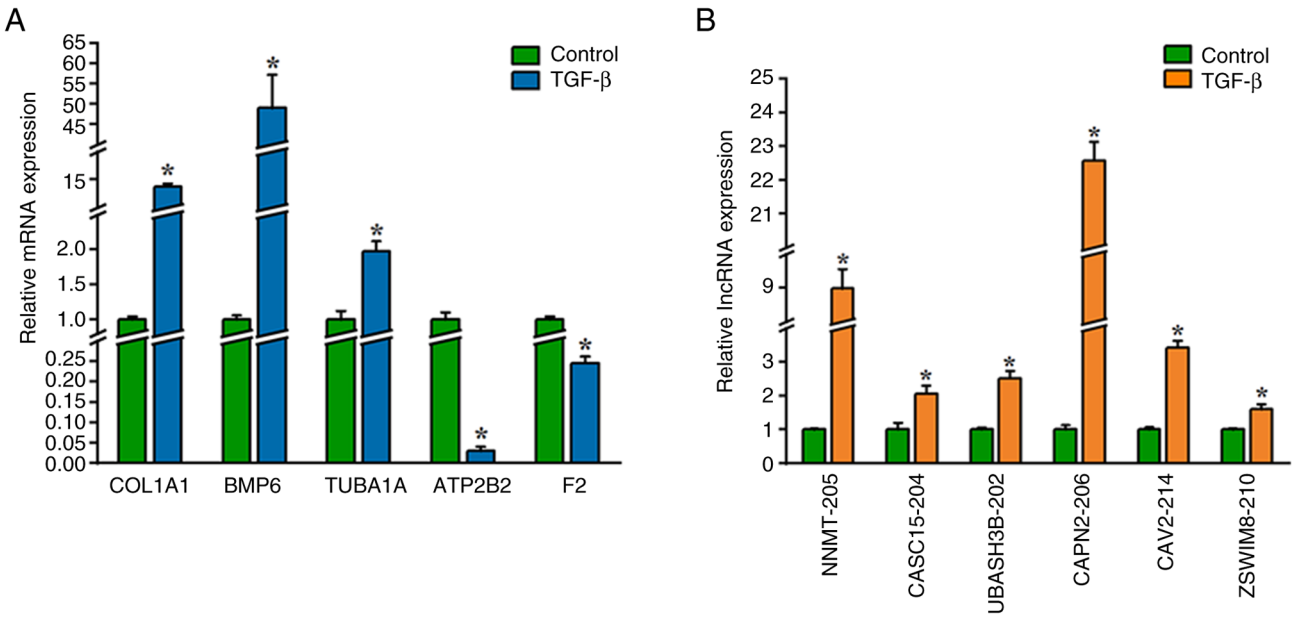

Figure 5. Validation of the sequencing data using reverse transcription-quantitative PCR. (A) Relative mRNA levels of COL1A1, BMP6, TUBA1A, ATP2B2 and F2 in Huh7 cells treated with or without 10 ng/ml TGF-β for 4 days. (B) Relative lncRNA levels of NNMT-205, CASC15-204, UBASH3B-202, CAPN2-206, CAV2-214 and ZSWIM8-210 in Huh7 cells treated with or without 10 ng/ml TGF-β for 4 days. *P<0.05 vs. the control group. lncRNA, long non-coding RNA; TGF-β, transforming growth factor-β.

highlights that the complexity and compensatory mechanisms in the EMT regulatory network are intricate.

Increasing evidence suggests that lncRNAs can affect the migration and progression of HCC by regulating the EMT process (32,33). For example, a study by Chen *et al* (34) reports that lncRNA MALAT1 is highly expressed in HCC cells and promotes the EMT through the miRNA22-Snail family transcriptional repressor 1 axis, which accelerates the progression of HCC. Furthermore, a study by Zhang *et al* (35) identifies that lncRNA SNHG 3 is aberrantly activated in highly metastatic HCC and can accelerate the EMT process and invasion of HCC cells. However, despite a small number of well-characterized molecules (such as MALAT1 and SNHG 3), the majority of lncRNAs that are dynamically expressed during the EMT are still functionally unannotated, suggesting that there may be a number of potential therapeutic targets that still require investigation. Therefore, the present study aimed to generate a catalog of potential candidate lncRNAs to suggest novel, uncharacterized regulators beyond these established molecules, in order to potentially provide insights into the mechanisms of HCC tumorigenesis and potential therapeutic targets.

The present study investigated the molecular mechanisms by which lncRNAs affect HCC migration and development by regulating the EMT. An *in vitro* EMT model was successfully constructed by treating Huh7 cells with 10 ng/ml TGF-β for 4 days. RNA sequencing analysis of the lncRNAs and mRNAs in the model and control groups was then carried out. The results revealed 312 significantly DE lncRNAs and 2,390 significantly DE mRNAs (FDR<0.05 and $|\log_2 FC|>1$). The expression profiles of the lncRNAs and mRNAs in the model and control groups were then systematically investigated through trans-action analysis, which predicted 101,780 potential lncRNA-mRNA target gene pairs by analyzing 312 significantly DE lncRNAs and 2,367 significantly DE mRNAs. To further reveal the key lncRNAs, mRNAs and their regulated signaling pathways, GO and KEGG enrichment analyses of the significantly DE lncRNAs were carried out. The GO analysis results revealed that DE lncRNAs were significantly enriched in cellular components such as 'collagen-containing extracellular matrix', 'extracellular matrix' and 'external encapsulation structure'. KEGG pathway analysis revealed several key signaling pathways associated with disease or biological processes, such as 'ECM-receptor interaction', 'focal adhesion' and 'axon guidance'. These findings are consistent with previous reports and confirm the relevance of the ECM-receptor interaction, focal adhesion and collagen-containing extracellular matrix components in the regulation of the EMT (36,26).

Furthermore, a lncRNA-mRNA network was constructed in the present study, which further elucidated that lncRNAs affect cellular molecular functions or signaling pathways by regulating mRNAs, thereby influencing the EMT process. The lncRNA-mRNA network results demonstrated that lncRNAs such as ZSWIM8-210 (ENST00000487278), BMS1P23-222 (ENST00000688880) and DGCR2-204 (ENST00000473832) were core nodes that interacted with multiple mRNAs; suggesting that these lncRNAs may serve a key role in the regulatory network. Upon reviewing the literature, it was identified that lncRNAs ZSWIM8-210, BMS1P23-222 and DGCR2-204 are yet to be elucidated in the field of HCC research. However, the expression level of ZSWIM8 is associated with drug resistance in breast cancer cells and epithelial ovarian cancer cells (37,38). In the present study, the role of ZSWIM8-210 as a core node in the lncRNA-mRNA network highlights its potential importance in the regulatory landscape of cancer biology, thus, further investigation into its functional roles and therapeutic relevance is warranted.

The present study has several limitations that should be considered. Firstly, the primary findings are based on sequencing and bioinformatic analyses of *in vitro* experiments. A key limitation of this approach is that it fails to recapitulate the key influence of the *in vivo* microenvironment. Secondly, although the co-expression network analysis provided valuable predictive insights and has been partially validated by the results of RT-qPCR, the functional roles of the identified lncRNAs (including the validated candidates such as ZSWIM8-210 and CAV2-214) in EMT regulation remain to be experimentally verified. Future studies should include a functional characterization through *in vitro* gain-of-function and loss-of-function experiments to establish causative relationships between the identified lncRNAs and the EMT phenotypes. Thirdly, the precise molecular mechanisms through which these lncRNAs operate, such as whether through chromatin remodeling, transcriptional regulation or post-transcriptional modulation, requires further elucidation. Investigating these mechanisms through subcellular localization studies, RNA-protein interaction assays and validation of downstream pathways are suggested experiments for future studies.

Despite these limitations, the results of the present study provided an understanding of the lncRNA regulatory networks in TGF-β-induced EMT. The aforementioned experiments that were suggested for future studies may help to translate these bioinformatic predictions into mechanistic insights with potential therapeutic implications for HCC metastasis.

In conclusion, the present study identified significantly DE lncRNAs in Huh7 cells treated with TGF-β. GO enrichment analysis revealed that these significantly DE lncRNAs were involved in 'cell migration', 'cell adhesion', 'tube morphogenesis' and 'regulation of multicellular organismal process'. KEGG pathway analysis suggested that these significantly DE lncRNAs were involved in pathways associated with 'ECM-receptor interaction', 'focal adhesion' and 'axon guidance'. Lastly, by constructing a lncRNA-mRNA network, potential DE lncRNAs and mRNAs that may affect the EMT process were highlighted. These results indicated that these DE lncRNAs may contribute to tumor development and progression by participating in the EMT process. The present study established a framework for the lncRNA regulatory network, with the lncRNAs highlighted in the present study serving as potential candidates for future research. To translate these suggested bioinformatic predictions into mechanistic insights, future work should address current knowledge gaps through *in vivo* validation, functional assays, mechanistic studies and clinical correlation. Such efforts are required in order to advance therapeutic and prognostic strategies for HCC.

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Availability of data and materials

The data generated in the present study may be found in the Sequence Read Archive database under accession number (PRJNA1017537) or at the following URL: https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA1017537.

Authors' contributions

QD and LM searched the literature, designed the present study, interpreted the findings and revised the manuscript. QD, JZ, YZ, XL, TG and QM carried out data management, statistical analysis and performed the experiments. QD, JZ, YZ, XL and TG confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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