Long non-coding RNA LINC00152 regulates cell proliferation and migration by epigenetically repressing LRIG1 expression in cholangiocarcinoma

Ni Wang
Medical Center for Digestive Diseases, Second Affiliated Hospital, Nanjing Medical University, Nanjing, Jiangsu Province

Yang Yu
Medical Center for Digestive Diseases, Second Affiliated Hospital, Nanjing Medical University, Nanjing, Jiangsu Province

Boming Xu
Department of Gastroenterology, The Quanzhou First Hospital Affiliated to Fujian Medical University, Quanzhou, Fujian Province.

Chunmei Zhang
Medical Center for Digestive Diseases, Second Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu Province

Jie Liu
The Reproduction Center of Xuzhou Maternity and Child Health Care Hospital, Xuzhou, Jiangsu Province.

Liyang Dong
Department of Nuclear Medicine, The Affiliated Hospital of Jiangsu University, Zhenjiang, Jiangsu Province.

Xuezhi He
Research Centre for Bone and Stem Cells, Nanjing Medical University, Nanjing, Jiangsu Province.

Lin Miao (linmiao@njmu.edu.cn)
Medical Center for Digestive Diseases, Second Affiliated Hospital, Nanjing Medical University, Nanjing, Jiangsu Province  https://orcid.org/0000-0003-0248-2544

Quanpeng Li
Department of Cardiology, the People's Hospital of Kizilsu Kirghiz Autonomous Prefecture, Xingjiang.

Keywords: Cholangiocarcinoma, LINC00152, LRIG1, cell proliferation and migration
Abstract

Background: Recently, long non-coding RNAs (lncRNAs) have been verified to have significant regulatory roles in multiple human cancer processes. Long non-coding RNA LINC00152, located on chromosome 2p11.2, was identified as an oncogenic IncRNA in various cancers. However, the biological function and molecular mechanism of LINC00152 in cholangiocarcinoma (CCA) are still unknown.

Methods: Bioinformatic analysis was performed to determine LINC00152 expression levels in the CCA and normal tissues by using raw microarray data downloaded from Gene Expression Omnibus (GSE76297) and The Cancer Genome Atlas (TCGA). Quantitative reverse transcription PCR (qRT-PCR) was used to validate LINC00152 expression in the CCA tissues compared with that in the paired normal tissues. CCK8, colony formation, Edu assays, transwell assays, flow cytometry, and in vivo tumor formation assays were performed to investigate the biological function of LINC00152 on CCA cell phenotypes. RNA-seq was carried out to identify the downstream target gene which was further examined by qRT-PCR, western bolt and rescue experiments. RNA immunoprecipitation (RIP) and Chromatin immunoprecipitation (ChIP) assays were performed to reveal the factors involved in the mechanism of LINC00152 functions in CCA.

Results: LINC00152 is significantly upregulated in cholangiocarcinoma. LINC00152 regulated the proliferation and migration of cholangiocarcinoma cells both in vitro and in vivo. RNA-seq revealed that LINC00152 knockdown preferentially affected genes linked with cell proliferation, cell differentiation and cell adhesion. Furthermore, mechanistic investigation validated that LINC00152 could bind EZH2 and modulate the histone methylation of promoter of leucine rich repeats and immunoglobulin like domains 1 (LRIG1), thereby affecting cholangiocarcinoma cells growth and migration.

Conclusion: Taken together, these results demonstrated the significant roles of LINC00152 in cholangiocarcinoma and suggested a new diagnostic and therapeutic direction of cholangiocarcinoma.

Background

Cholangiocarcinoma (CCA) is one of the most invasive and lethal cancers derived from ductal epithelial cells lining the intrahepatic and extrahepatic biliary ducts [1, 2]. CCA is a devastating malignancy with an abysmal 5-year overall survival rate of less than 10% [3]. In recent years, molecular targeted therapy with powerful pertinence shows wide application prospects, which may control the malignant progression [4]. Therefore, it is of paramount importance to understand the carcinogenesis and progression mechanisms underlying CCA, which make for finding novel diagnosis markers and therapeutic targets.

Over the past decade, with the development of human genome sequencing and the Encyclopedia of DNA Elements (ENCODE) project, ENCODE annotation have revealed that over 90% human genome is transcribed, while less than 2% could be subsequently translated, which reveals that most genome generates many thousands of non-coding RNAs (ncRNAs) transcriptions [5–7]. Long non-coding RNAs (IncRNAs), members of ncRNA, are classically characterized as regulatory RNA members longer than 200
nt without detectable open reading frame (ORF) to encode protein [8, 9]. Recently, more and more studies have revealed that IncRNAs participate in various biological processes, including cellular proliferation [10], migration [11] and differentiation [12], etc. Notably, the dysregulation of IncRNAs have been linked with a broad-spectrum of human cancers, including CCA [13, 14].

As modulators in epigenetic process, IncRNAs could regulated gene expression in chromatin modification, transcriptional, as well as post-transcriptional processing [15, 16]. For example, the H3K4me3 and H3K27Ac-activated IncRNA HOXCAS3 could lead transcriptional activation of the downstream target genes through binding to transcriptional activator YBX1 in gastric cancer [17]. Moreover, IncRNA-ATB have the capacity to upregulate zinc-finger E-box–binding homeobox 1 (ZEB1) and ZEB2 by competitively binding the miR-200 family in hepatocellular carcinoma [18].

Long intergenic non-coding RNA 152 (LINC00152), a 828-bp IncRNA that maps to chromosome 2p11.2, was initially found to be differentially hypomethylated in hepatocarcinogenesis [19]. Continuously, LINC00152 have exhibited carcinogenic properties in a variety of cancers, including gastric cancer, hepatocellular carcinoma, colon cancer, gallbladder cancer, lung adenocarcinoma, breast cancer, non-small-cell lung cancer, papillary thyroid carcinoma, tongue squamous cell carcinoma, glioblastoma and ovarian cancer [20–32]. However, the expression pattern, biological function, and underlying mechanism of LINC00152 in human CCA remain largely unknown.

In this study, we found that LINC00152 was significantly upregulated in CCA tissues compared with normal tissues in CCA. In addition, the contributions of LINC00152 to CCA development were investigated in vitro and in vivo. RNA-seq analysis showed that downregulated LINC00152 preferentially affected the expression of proliferation and migration-related genes. More importantly, LINC00152 could directly bind to EZH2 to suppress the leucine rich repeats and immunoglobulin like domains 1 (LRIG1) expression, thereby promoting CCA tumorigenesis.

Materials And Methods

Tissue gathering and Ethics statement

A total of 17 cholangiocarcinoma patients analyzed in this study had undergone surgeries at the Second Affiliated Hospital of Nanjing Medical University (China). All tissue samples were instantly snap-frozen in liquid nitrogen until extraction of RNA. This study was approved by the Research Ethics Committee of Nanjing Medical University (Nanjing, Jiangsu, People's Republic of China). Written informed consent was obtained from all participants.

RNA extraction and qRT-PCR analysis

All RNA was isolated from specimens or cultured cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with manufacturer's instructions. Then, RNA (1 µg) was reverse transcribed to cDNA through a Reverse Transcription Kit (Takara, Dalian, China). For Real-time PCR analyses, we used SYBR
Green (Takara, Dalian China). The expression data were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers sequences were listed in Supplementary Table S1.

**Cell culture**

CCA cell lines HuCCT1 and RBE were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cell lines were cultured in DMEM (Life Technologies Corporation Attn, Grand Island, USA) medium supplemented with 10% fetal bovine serum (FBS) (Scicell, Carlsbad, CA), 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen, Shanghai, China) in humidified air at 37 °C/5% CO2.

**Cell transfection**

LINC00152 and LRIG1 cDNA was synthesized by Generay (Shanghai, China) and Generay (Shanghai, China), respectively. And they were ligated into the expression vector pcDNA3.1 (Invitrogen). Scrambled negative control siRNA (si-NC) and LINC00152 siRNAs were purchased from Invitrogen (Invitrogen, CA, USA). EZH2 siRNAs was bought from Realgene (Nanjing, China). The shLINC00152 was cloned into pENTR™/U6 vector. Severally, lipofectamine2000 (Invitrogen) and X-tremeGENE HP DNA Transfection Reagent (Roche, Basel, Switzerland) were used to transfect the siRNAs and plasmid into cells. Cells were harvested for analyses 48 h after transfections. All the siRNAs and shRNA sequences were presented in Supplementary Table S1.

**Cell proliferation analysis**

Cell viability was measured with CCK8 kit (Houston TX, USA) according to manufacturer's instruction. In colony formation test, the transfected cells were placed in 6-well plates with suitable media containing 10% FBS. After 2 weeks, colonies were fixed with methanol and stained with 0.1% crystal violet (Sigma). The numbers of visibly stained colonies were counted to determine the colony formation. Edu assays were performed using the Edu Cell Proliferation Assay Kit (Ribobio, Guangzhou, China) following the manufacturer's instructions. Then, the percentages of Edu-positive cells were examined in the sample. Experiments were independently repeated three times in triplicate.

**Cell migration assays**

For migration assays, 3 × 10^4 transfected cells in media with 1% FBS were placed into the upper chamber of the insert (Millipore, Billerica, MA, USA), while the medium in lower chamber contained 10% FBS. After 24 h of incubation, cotton wools were used to remove the cells remaining on the upper chamber. The cells migrated through the membrane were fixed with methanol, stained with 0.1% crystal violet, and imaged and counted with an IX71 inverted microscope (Olympus, Tokyo, Japan). All wells were assessed three times.

**Western blot assay and antibodies**

Cells protein lysates were divided using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) transferred to 0.22 µm NC membranes (Sigma) and incubated with specific antibodies. The ECL chromogenic substrates were quantified by densitometry (Quantity One software; Bio-Rad). An anti-
GAPDH antibody was employed as a control. Anti-EZH2 was from Millipore (Billerica, MA, USA) and Anti-LRIG1 was purchased from GeneTex (Irvine, USA).

**Flow cytometric analysis**

The HuCCT1 and RBE cells were harvested by trypsinization 48 h after transfection with si-NC or si-LINC00152. After double staining with fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide (PI), the cells were analyzed by flow cytometry (FACScan; BD Biosciences) equipped with CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA). Cells were stained with PI using the CycleTEST™ Plus DNA Reagent Kit (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed by FACScan for cell cycle. The proportions of cells in G0/G1, S, and G2/ M phase were calculated.

**In vivo tumor formation assays**

Four-week-old Athymic male mice bought from the Animal Center of the Nanjing University (Nanjing, China) were kept under specific pathogen-free conditions. The HuCCT1 cells stably transfected with sh-LINC00152 or empty vector were harvested and washed with phosphate-buffered saline (PBS). The cells re-suspended at 2 × 10^7 cells/mL were subcutaneously xenografted into the ventral side of each BALB/c male nude mice. Then, the tumor volumes were calculated as V = 0.5 × D × d^2 (V, volume; D, longitudinal diameter; and d, latitudinal diameter) every 3 days. 16 days after injection, the mice were asphyxiated by CO₂ and the tumor weights were measured and analyzed. This study was strictly consistent with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was licensed by the Committee on the Ethics of Animal Experiments of Nanjing Medical University.

**Chromatin immunoprecipitation assays**

Chromatin immunoprecipitation (ChIP) assays were conducted using EZ-CHIP KIT following the manufacturer’s instructions (Millipore, USA). EZH2 and H3 trimethyl Lys 27 (H3K27me3) antibodies were from Millipore and Abcam, respectively. The ChIP primer sequences were shown in Supplementary Table S1. Quantification of precipitated chromatin DNA was analyzed by qPCR. ChIP data were calculated as a proportion relative to the input DNA using the following equation: 2^{[\text{Input Ct} - \text{Target Ct}] × 0.1 × 100}.

**RNA immunoprecipitation assays**

RNA immunoprecipitation (RIP) assays were conducted using a Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) in accordance with the manufacturer’s protocols. The EZH2 antibody used in RIP was obtained from Millipore (Billerica, MA, USA).

**Whole transcriptome deep sequencing**

Total RNA from LINC00152 knockdown and control HuCCT1 cells were separated and quantified. The concentration of each sample was measured with NanoDrop 2000 (Thermo Scientific, USA). The quality was evaluated with the Agilent2200 (Agilent, USA). The sequencing library of each RNA specimen was prepared using Ion Proton Total RNA-Seq Kit v2 (Life technologies, USA). Data of six samples are available in Supplementary Table S2 and Table S3.
Statistical analysis
Statistical analyses were performed with GraphPad Prism5 (GraphPad Software, La Jolla, USA). Student’s t-test, #2 test or Wilcoxon test were used to estimate the significance of the distinctions between groups. The dys-regulated genes from GEO and TCGA datasets were obtained by limma R package and edger R package, respectively. All resulting data were recounted as mean ± SD. Two-sided P-values were calculated, and less than 0.05 was considered for statistical significance.

Result

LINC00152 is upregulated in the human CCA tissues

To identify the dysregulated lncRNAs involved in CCA, we investigated the CCA and normal tissue RNA-Seq data of the public gene profiling datasets from Gene Expression Omnibus (GEO) database (GSE76297; 91 pairs of cancer tissue specimens and normal tissue specimens, as well as a single normal tissue specimen without pairing; we discarded the single normal tissue specimen data and analyzed the 91 paired data synthetically) [33] and The Cancer Genome Atlas (TCGA) (9 normal and 36 cancer specimens) (Fig. 1A, B and C). LncRNA LINC00152 which is consistently highly upregulated in these datasets caught our attention. Accordant with these results, remarkable increasing expression levels of LINC00152 from normal tissues to carcinoma tissues were confirmed in a cohort of the 17 paired CCA and non-tumor tissues (Fig. 1D).

Knockdown of LINC00152 inhibits CCA cell proliferation and migration

The siRNA-mediated knockdown and plasmid-mediated overexpression were used to manipulate the expression of LINC00152 exogenously, both in HuCCT1 and RBE cell lines, to evaluate the biological functions of LINC00152 in CCA (Supplementary Figure S1A and S1B).

The CCK-8 assay revealed that LINC00152 silencing significantly decreased cell viability in HuCCT1 and RBE cell lines, while the overexpression of LINC00152 could promote the cell viability by contrast (Fig. 2A). Similarly, the colony formation capacity of the CCA cells was also greatly impaired with LINC00152 knockdown and enhanced after overexpression of LINC00152 (Fig. 2B). Furthermore, EdU staining assays demonstrated that LINC00152 had a significant impact on CCA cell proliferation as well (Fig. 2C). Then, using a transwell assay, we found that HuCCT1 and RBE cells migration were observably impaired or promoted after the knockdown or overexpression of LINC00152, respectively (Fig. 2D). Taken together, these results indicated that LINC00152 could accelerate the proliferation and migration phenotype of CCA cells.

LINC00152 knockdown induces cell cycle arrest and cell apoptosis

To determine whether LINC00152 is involved in regulating CCA cell apoptosis, flow cytometry was performed. Compared with the control group, LINC00152 silencing evidently improved the proportion of apoptotic CCA cells (Fig. 3A). We further examined if the change of cell cycle progression accounted for the influence of LINC00152 silencing on cell viability. LINC00152 downregulation made an increased cell cycle arrest at the G1/G0 phase (Figs. 3B). These results suggested that LINC00152 downregulation
could inhibit the cell proliferation and migration by influencing the apoptosis and cell cycle progression of CCA cells.

**LINC00152 downregulation suppresses CCA cell proliferation in vivo**

HuCCT1 cells stably expressing sh-LINC00152 or control vector were subcutaneously inoculated into nude mice to further confirm whether the LINC00152 influences tumorigenesis of CCA *in vivo*. After 17 days, the tumors established in the LINC00152 silencing group were substantially smaller than those in the control group (Figs. 4A-C). Correspondingly, at the final experiment, the mean tumor weight in the sh-LINC00152 group was markedly lighter than that in the control vector group (Fig. 4D). These findings indicated that LINC00152 silencing could inhibit CCA growth *in vivo*.

**Downstream Targets of LINC00152 in CCA**

To probe the LINC00152-associated potential target genes in CCA cells, we used an RNA transcriptome sequencing in control or siRNAs against LINC00152. A total of 261 genes were significantly upregulated or downregulated in HuCCT1 cells as a consequence of LINC00152 knockdown (fold change ≥ 1.5) (Fig. 5A, Supplementary Table S2 and S3). The thorough assessment of gene ontology analysis indicated that the most distinct overrepresented biological processes included pathways related to cell proliferation, cell adhesion and cell apoptosis (Fig. 5B). To prioritize most LINC00152-associated genes, more attention was paid for the overexpressed genes after silencing LINC00152. Many renowned proliferation and migration-associated genes (e.g. ACTG2, TNFAIP3, LRIG1, ADAM19, NFκB2, CMTM3, NFκBIE, et al.) were included. Their regulation was verified by qRT-PCR after LINC00152 silencing in HuCCT1 and RBE cells (Fig. 5C). Noticeably, among these transcripts, LRIG1 was significantly elevated after LINC00152 knockdown, which is locating at chromosomes 3p14 and could act as a tumor-suppressive gene in various cancers, including bladder, lung, renal, squamous cell, breast, brain, glioma, and colorectal cancers (Figs. 5C) [34–41]. Western blotting assay further demonstrated that LINC00152 knockdown significantly increased the LRIG1 protein levels in CCA, confirming that LRIG1 was a bona fide target of LINC00152 (Figs. 5D).

**LINC00152 epigenetically silences LRIG1 transcription through binding to EZH2**

A growing number of studies have reported that a large number of IncRNAs have the cooperative function with chromatin modifiers, suggesting an important role for IncRNAs in the epigenetic regulation of gene expression [42]. Remarkably, polycomb repressive complex 2 (PRC2), the histone methyltransferase constituted with EZH2, SUZ12 and embryonic ectoderm development (EED), has been reported as the catalyst in the trimethylation of lysine residue 27 of histone 3 (H3K27me3) and mediates transcriptional silencing [43, 44]. Previous study showed that approximately 20% of human IncRNAs are bound by PRC2 to exert their regulatory functions [45, 46]. Notably, LINC00152 has been reported to bind to EZH2 which is the core component of PRC2 to regulate target gene expression epigenetically in hepatocellular carcinoma, renal cell carcinoma, lung adenocarcinoma and gastric cancer [20, 26, 47–49]. But little is known whether LINC00152 could interact with EZH2 in CCA. The promoter region hypermethylation has been reported to resulted in LRIG1 transcriptional inactivation in colorectal and cervical cancer [50, 51]. Furthermore,
previous studies have reported that DNA methylation and histone methylation could function synergistically in various cancers [52–55]. Thus, we hypothesized that LINC00152 might modulate LRIG1 expression by binding to EZH2 and then mediating H3K27me3 in the promoter regions of LRIG1 to facilitate CCA progress. Indeed, sufficient enrichment of endogenous LINC00152 was validated in the anti-EZH2 RNA immunoprecipitation (RIP) fraction associated with the input in comparison with the nonspecific IgG control fraction (Fig. 6A), which proved the bond of EZH2 and LINC00152. Additionally, the qRT-PCR and western blots results showed that knockdown of LINC00152 have no effect on the expression of EZH2 (Fig. 6B and C) which proved that LINC00152 could bind to EZH2 but have no effect on EZH2 expression. Moreover, EZH2 activation was reported to be closely related to cancer progression in various human malignant tumors, including CCA [56–58]. We inhibited the expression of EZH2 with efficient siRNAs (Fig. 6D). The results confirmed that knockdown of EZH2 could significantly inhibit cell proliferation and migration (Fig. 6E, F and G). Next, to investigate whether EZH2 could act as a negative regulator of transcription via H3K27me3, we further performed the following assays. The qRT-PCR and Western Blot results showed that the mRNA and protein levels of LRIG1 exhibited an increase in the EZH2-knockdown HuCCT1 and RBE cell lines (Fig. 6H and I). Then we conducted chromatin immunoprecipitation (ChIP) assays to analyze the enrichment of EZH2 and H3K27me3 in the promoter region of LRIG1. As shown in Fig. 6J, knockdown of LINC00152 markedly reduced the EZH2 enrichment and H3K27me3 levels in the promoter region of LRIG1 genes. Above results validated that LINC00152 could motivate CCA cells proliferation and migration partially through downregulating LRIG1 by binding to EZH2.

**LRIG1 overexpression inhibits CCA cell proliferation and metastasis**

Several studies have shown that LRIG1 could exert tumor-suppressive effects in various cancers [35, 59–61]. However, the function of LRIG1 in CCA has not been reported until now. To assess the expression of LRIG1 in CCA tissues, we analyzed the dataset TCGA and GSE76297, detecting the down-regulation of LRIG1 in CCA compared to that in normal tissue (Fig. 7A and B). Indeed, by qRT-PCR, the measure of LRIG1 expression in 17 pairs of CCA tumor tissues and the adjacent tissues showed the similar results (Fig. 7C). Moreover, overexpression of LRIG1 could evidently suppress proliferation and migration of HuCCT1 and RBE cell lines. In addition, LRIG1 could also partially reverse the LINC00152-mediated growth promotion (Fig. 7D-F). Taken together, our study demonstrated that LINC00152 could bind to EZH2 and then epigenetically mediate H3K27me3 in the LRIG1 promoter regions to inhibit LRIG1 expression, thus promoting CCA progress.

**Discussion**

Recently, more and more studies have implied the important roles of IncRNAs in the diagnosis, prognostic and therapeutic evaluation of cancers. In our present study, publicly available data and qRT-PCR results both uncovered that LINC00152 expression in CCA tissues was observably higher than that in corresponding non-tumor tissues, which implied the important role of LINC00152 in CCA progression. LINC00152 was reported upregulated in various cancers, promoting cancer cell proliferation and migration [20–32]. Nevertheless, the possible role of LINC00152 in CCA is still undocumented.
As the results indicated in our study, LINC00152 could regulate the proliferation and migration of CCA cells both \textit{in vitro} and \textit{in vivo}. Although the oncogenic feature of LINC00152 have been clarified in various cancers, the pathway and downstream genes correlated with LINC00152 in CCA remains uncertain. Therefore, we conducted RNA transcriptome sequencing and gene ontology analysis. The results implied that gene expression profiles were primarily associated with cell proliferation, adhesion and apoptosis and inducted us to estimate several target genes regulated by LINC00152. We observed a noticeable elevation of the suppressor LRIG1 expression in mRNA and protein levels and conducted further mechanism studies.

Previous studies have determined that a significant number of lncRNAs could function in cooperation with chromatin modifying enzymes to involved in promoting of epigenetic activation or silencing of gene expression [11, 42, 62]. Thereinto, LINC00152 was reported to have the capital to inactive target gene expression epigenetically through interaction with EZH2 in lung adenocarcinoma and gastric cancer [20, 26]. EZH2, an well-known oncogenic gene in various human malignant tumors, was reported to be capable of interacting with IncRNAs to catalyze the trimethylation of lysine residues 27 of histone 3 (H3K27me3) in the promoter regions of the target gene, thereby mediating transcriptional silencing [44, 63, 64]. Further, our resulting data revealed that LINC00152 was capable to bind to EZH2 but have no effect on EZH2 expression, silencing target genes expression in CCA cells as well.

LRIG1 have been showed to function as the tumor suppressor in various types of cancers. However, the role of LRIG1 in CCA tumorigenesis remains unclear. It is reported that hypermethylation of the suppressor gene LRIG1 promoter region leads to the LRIG1 transcription suppression in colorectal cancer and cervical cancer [50, 51]. Meanwhile, our results demonstrated that histone methylation (H3K27me3) induced by LINC00152 could also account for the LRIG1 downregulation in cancer. Furthermore, it was reported that histone methylation usually cooperate with DNA methylation to inactive target gene expression heritably [52, 53, 65]. As discovered by our findings, LRIG1, the tumor suppressor, could be affected by LINC00152-EZH2 complex through promoter H3K27me3 in CCA.

**Conclusion**

In summary, our study showed that upregulated LINC00152 could facilitate CCA progress through silencing the target tumor suppressor gene LRIG1 transcription at the epigenetic level, which suggested that LINC00152 could provide a theoretical basis for clinical diagnosis and treatment strategies in CCA.

**Abbreviations**

CCA: Cholangiocarcinoma; CCK-8: cell counting kit-8; ChIP: chromatin immunoprecipitation; EED: embryonic ectoderm development; ENCODE: Encyclopedia of DNA Elements; FBS: fetal bovine serum; FITC: fluorescein isothiocyanate; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GEO: Gene Expression Omnibus; H3K27me3: trimethylation of lysine residue 27 of histone 3; LINC00152: Long intergenic non-coding RNA 152; lncRNAs: long non-coding RNAs; LRIG1: leucine rich repeats and
Declarations

Ethics approval and consent to participate

This study was approved by the Research Ethics Committee of Nanjing Medical University (Nanjing, Jiangsu, People's Republic of China). Written informed consent was obtained from all participants.

Consent for publication

All authors have agreed to publish this manuscript.

Availability of data and materials

The data generated or analyzed during this study are included in this article, or if absent are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no conflict of interest.

Funding

This study was supported by the Project of Standard Diagnosis and Treatment of Key Disease of Jiangsu Province (BE2015722); Project of the peak of the six talents of Jiangsu Province (WSN-018); and Scientific Research Foundation for Health of Jiangsu Province (H201408).

Authors' contributions

Conceptualization, LM, QL and NW; Methodology, NW. and YY; Implementation, NW, YY, CZ and BX; Software, BX and JL; Writing – Original Draft, NW; Writing – Review and Editing, LM, QL, LD and XH; Funding Acquisition, LM; Supervision, LM and QL.

Acknowledgements

Not applicable.

References
1. Razumilava N, Gores GJ. Cholangiocarcinoma. Lancet. 2014; doi:10.1016/S0140-6736(13)61903-0.
2. Rizvi S, Gores GJ. Pathogenesis, diagnosis, and management of cholangiocarcinoma. Gastroenterology. 2013; doi:10.1053/j.gastro.2013.10.013.
3. Everhart JE, Ruhl CE. Burden of digestive diseases in the United States Part III: Liver, biliary tract, and pancreas. Gastroenterology. 2009; doi:10.1053/j.gastro.2009.02.038.
4. Loosen SH, Roderburg C, Kauertz KL, Pombeiro I, Leyh C, Benz F, et al. Elevated levels of circulating osteopontin are associated with a poor survival after resection of cholangiocarcinoma. J Hepatol. 2017; doi:10.1016/j.jhep.2017.06.020.
5. Djebali S, Davis CA, Merkel A, Dobin A, Lassmann T, Mortazavi A, et al. Landscape of transcription in human cells. Nature. 2012; doi:10.1038/nature11233.
6. Harrow J, Frankish A, Gonzalez JM, Tapanari E, Diekhans M, Kokocinski F, et al. GENCODE: the reference human genome annotation for The ENCODE Project. Genome Res. 2012; doi:10.1101/gr.135350.111.
7. Carninci P, Kasukawa T, Katayama S, Gough J, Frith MC, Maeda N, et al. The transcriptional landscape of the mammalian genome. Science. 2005; doi:10.1126/science.1112014.
8. Guttman M, Rinn JL. Modular regulatory principles of large non-coding RNAs. Nature. 2012; doi:10.1038/nature10887.
9. Batista PJ, Chang HY. Long noncoding RNAs: cellular address codes in development and disease. Cell. 2013; doi:10.1016/j.cell.2013.02.012.
10. Hung T, Wang Y, Lin MF, Koegel AK, Kotake Y, Grant GD, et al. Extensive and coordinated transcription of noncoding RNAs within cell-cycle promoters. Nat Genet. 2011; doi:10.1038/ng.848.
11. Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, et al. Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature. 2010; doi:10.1038/nature08975.
12. Guttman M, Donaghey J, Carey BW, Garber M, Grenier JK, Munson G, et al. lincRNAs act in the circuitry controlling pluripotency and differentiation. Nature. 2011; doi:10.1038/nature10398.
13. Yildirim E, Kirby JE, Brown DE, Mercier FE, Sadreyev RI, Scadden DT, et al. Xist RNA is a potent suppressor of hematologic cancer in mice. Cell. 2013; doi:10.1016/j.cell.2013.01.034.
14. Zhang D, Li H, Xie J, Jiang D, Cao L, Yang X, et al. Long noncoding RNA LINC01296 promotes tumor growth and progression by sponging miR-5095 in human cholangiocarcinoma. Int J Oncol. 2018; doi:10.3892/ijo.2018.4362.
15. Wilusz JE, Sunwoo H, Spector DL. Long noncoding RNAs: functional surprises from the RNA world. Genes & development. 2009; doi:10.1101/gad.1800909.
16. Hu X, Feng Y, Zhang D, Zhao SD, Hu Z, Greshock J, et al. A functional genomic approach identifies FAL1 as an oncogenic long noncoding RNA that associates with BMI1 and represses p21 expression in cancer. Cancer Cell. 2014; doi:10.1016/j.ccr.2014.07.009.
17. Zhang E, He X, Zhang C, Su J, Lu X, Si X, et al. A novel long noncoding RNA HOXC-AS3 mediates tumorigenesis of gastric cancer by binding to YBX1. Genome Biol. 2018; doi:10.1186/s13059-018-
18. Yuan JH, Yang F, Wang F, Ma JZ, Guo YJ, Tao QF, et al. A long noncoding RNA activated by TGF-β promotes the invasion-metastasis cascade in hepatocellular carcinoma. Cancer Cell. 2014; doi:10.1016/j.ccr.2014.03.010.

19. Neumann O, Kesselmeier M, Geffers R, Pellegrino R, Radlwimmer B, Hoffmann K, et al. Methylome analysis and integrative profiling of human HCCs identify novel protumorigenic factors. Hepatology. 2012; doi:10.1002/hep.25870.

20. Chen WM, Huang MD, Sun DP, Kong R, Xu TP, Xia R, et al. Long intergenic non-coding RNA 00152 promotes tumor cell cycle progression by binding to EZH2 and repressing p15 and p21 in gastric cancer. Oncotarget. 2016; doi:10.18632/oncotarget.6949.

21. Ji J, Tang J, Deng L, Xie Y, Jiang R, Li G, et al. LINC00152 promotes proliferation in hepatocellular carcinoma by targeting EpCAM via the mTOR signaling pathway. Oncotarget. 2015; doi:10.18632/oncotarget.5970.

22. Yue B, Cai D, Liu C, Fang C, Yan D. Linc00152 Functions as a Competing Endogenous RNA to Confer Oxaliplatin Resistance and Holds Prognostic Values in Colon Cancer. Molecular therapy : the journal of the American Society of Gene Therapy. 2016; doi:10.1038/mt.2016.180.

23. Cai Q, Wang ZQ, Wang SH, Li C, Zhu ZG, QuanZW, et al. Upregulation of long non-coding RNA LINC00152 by SP1 contributes to gallbladder cancer cell growth and tumor metastasis via PI3K/AKT pathway. Am J Transl Res. 2016;8:4068-4081.

24. Wu Y, Tan C, Weng WW, Deng Y, Zhang QY, Yang XQ, et al. Long non-coding RNA Linc00152 is a positive prognostic factor for and demonstrates malignant biological behavior in clear cell renal cell carcinoma. Am J Cancer Res. 2016;6:285-99.

25. Yu J, Liu Y, Guo C, Zhang S, Gong Z, Tang Y, et al. Upregulated long non-coding RNA LINC00152 expression is associated with progression and poor prognosis of tongue squamous cell carcinoma. J Cancer. 2017; doi:10.7150/jca.17510.

26. Chen QN, Chen X, Chen ZY, Nie FQ, Wei CC, Ma HW, et al. Long intergenic non-coding RNA 00152 promotes lung adenocarcinoma proliferation via interacting with EZH2 and repressing IL24 expression. Mol Cancer. 2017; doi:10.1186/s12943-017-0581-3.

27. Sun Z, Guo X, Zang M, Wang P, Xue S, Chen G. Long non-coding RNA LINC00152 promotes cell growth and invasion of papillary thyroid carcinoma by regulating the miR-497/BDNF axis. J Cell Physiol. 2019; doi:10.1002/jcp.26928.

28. Cai J, Zhang J, Wu P, Yang W, Ye Q, Chen Q, et al. Blocking LINC00152 suppresses glioblastoma malignancy by impairing mesenchymal phenotype through the miR-612/AKT2/NF-κB pathway. J Neurooncol. 2018; doi:10.1007/s11060-018-2951-0.

29. Chen P, Fang X, Xia B, Zhao Y, Li Q, Wu X. Long noncoding RNA LINC00152 promotes cell proliferation through competitively binding endogenous miR-125b with MCL-1 by regulating mitochondrial apoptosis pathways in ovarian cancer. Cancer Med. 2018; doi:10.1002/cam4.1547.
30. Reon BJ, Takao Real Karia B, Kiran M, Dutta A. LINC00152 Promotes Invasion through a 3'-Hairpin Structure and Associates with Prognosis in Glioblastoma. Molecular cancer research : MCR. 2018; doi:10.1158/1541-7786.

31. Li N, Feng XB, Tan Q, Luo P, Jing W, Zhu M, et al. Identification of Circulating Long Noncoding RNA Linc00152 as a Novel Biomarker for Diagnosis and Monitoring of Non-Small-Cell Lung Cancer. Dis Markers. 2017; doi:10.1155/2017/7439698.

32. Wu J, Shuang Z, Zhao J, Tang H, Liu P, Zhang L, et al. Linc00152 promotes tumorigenesis by regulating DNMTs in triple-negative breast cancer. Biomed Pharmacother. 2018; doi:10.1016/j.biopha.2017.11.055.

33. Chaisaingmongkol J, Budhu A, Dang H, Rabibhadana S, Pupacdi B, Kwon SM, et al. Common Molecular Subtypes Among Asian Hepatocellular Carcinoma and Cholangiocarcinoma. Cancer Cell. 2017; doi:10.1016/j.ccell.2017.05.009.

34. Suzuki Y, Sato N, Tohyama M, Wanaka A, Takagi T. cDNA cloning of a novel membrane glycoprotein that is expressed specifically in glial cells in the mouse brain. LIG-1, a protein with leucine-rich repeats and immunoglobulin-like domains. The Journal of biological chemistry. 1996;271:22522-22527.

35. Powell AE, Wang Y, Li Y, Poulin EJ, Means AL, Washington MK, et al. The pan-ErbB negative regulator Lrig1 is an intestinal stem cell marker that functions as a tumor suppressor. Cell. 2012; doi:10.1016/j.cell.2012.02.042.

36. Hedman H, Henriksson R. LRIG inhibitors of growth factor signalling - double-edged swords in human cancer? European journal of cancer. 2007; doi:10.1016/j.ejca.2006.10.021.

37. Ljuslinder I, Golovleva I, Palmqvist R, Oberg A, Stenling R, Jonsson Y, et al. LRIG1 expression in colorectal cancer. Acta Oncol. 2007; doi:10.1080/02841860701426823.

38. Miller JK, Shattuck DL, Ingalla EQ, Yen L, Borowsky AD, Young LJ, et al. Suppression of the negative regulator LRIG1 contributes to ErbB2 overexpression in breast cancer. Cancer Res. 2008; doi:10.1158/0008-5472.CAN-07-6316.

39. Tanemura A, Nagasawa T, Inui S, Itami S. LRIG-1 provides a novel prognostic predictor in squamous cell carcinoma of the skin: immunohistochemical analysis for 38 cases. Dermatologic surgery : official publication for American Society for Dermatologic Surgery [et al.]. 2005;31:423-430.

40. Thomasson M, Hedman H, Guo D, Ljungberg B, Henriksson R. LRIG1 and epidermal growth factor receptor in renal cell carcinoma: a quantitative RT-PCR and immunohistochemical analysis. British journal of cancer. 2003; doi:10.1038/sj.bjc.6601208.

41. Ye F, Gao Q, Xu T, Zeng L, Ou Y, Mao F, et al. Upregulation of LRIG1 suppresses malignant glioma cell growth by attenuating EGFR activity. J Neurooncol. 2009; doi:10.1007/s11060-009-9836-1.

42. Marchese FP, Huarte M. Long non-coding RNAs and chromatin modifiers: their place in the epigenetic code. Epigenetics. 2014; doi:10.4161/epi.27472.

43. Shen X, Liu Y, Hsu YJ, Fujiwara Y, Kim J, Mao X, et al. EZH1 mediates methylation on histone H3 lysine 27 and complements EZH2 in maintaining stem cell identity and executing pluripotency. Mol
44. Cao R, Wang L, Wang H, Xia L, Erdjument-Bromage H, Tempst P, et al. Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science. 2002; doi:10.1126/science.1076997.

45. Khalil AM, Guttman M, Huarte M, Garber M, Raj A, Rivea Morales D, et al. Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. Proc Natl Acad Sci U S A. 2009; doi:10.1073/pnas.0904715106.

46. Qu D, Sun WW, Li L, Ma L, Sun L, Jin X, et al. Long noncoding RNA MALAT1 releases epigenetic silencing of HIV-1 replication by displacing the polycomb repressive complex 2 from binding to the LTR promoter. Nucleic Acids Res. 2019; doi:10.1093/nar/gkz117.

47. Li J, Wang X, Tang J, Jiang R, Zhang W, Ji J, et al. HULC and Linc00152 Act as Novel Biomarkers in Predicting Diagnosis of Hepatocellular Carcinoma. Cell Physiol Biochem. 2015; doi:10.1159/000430387.

48. Deng X, Zhao XF, Liang XQ, Chen R, Pan YF, Liang J. Linc00152 promotes cancer progression in hepatitis B virus-associated hepatocellular carcinoma. Biomed Pharmacother. 2017; doi:10.1016/j.biopha.2017.03.031.

49. Wang Y, Liu J, Bai H, Dang Y, Lv P, Wu S. Long intergenic non-coding RNA 00152 promotes renal cell carcinoma progression by epigenetically suppressing P16 and negatively regulates miR-205. Am J Cancer Res. 2017;7:312-322.

50. Kou C, Zhou T, Han X, Zhuang H, Qian H. LRIG1, a 3p tumor suppressor, represses EGFR signaling and is a novel epigenetic silenced gene in colorectal cancer. Biochemical and biophysical research communications. 2015; doi:10.1016/j.bbrc.2015.06.173.

51. Lando M, Fjeldbo CS, Wilting SM, C Snoek B, Aarnes EK, Forsberg MF, et al. Interplay between promoter methylation and chromosomal loss in gene silencing at 3p11-p14 in cervical cancer. Epigenetics. 2015; doi:10.1080/15592294.2015.1085140.

52. Viré E, Brenner C, Deplus R, Blanchon L, Fraga M, Didelot C, et al. The Polycomb group protein EZH2 directly controls DNA methylation. Nature. 2006; doi:10.1038/nature04431.

53. Takeshima H, Wakabayashi M, Hattori N, Yamashita S, Ushijima, T. Identification of coexistence of DNA methylation and H3K27me3 specifically in cancer cells as a promising target for epigenetic therapy. Carcinogenesis. 2015; doi:10.1093/carcin/bgu238.

54. Gao F, Ji G, Gao Z, Han X, Ye M, Yuan Z, et al. Direct ChIP-bisulfite sequencing reveals a role of H3K27me3 mediating aberrant hypermethylation of promoter CpG islands in cancer cells. Genomics. 2014; doi:10.1016/j.ygeno.2013.12.006.

55. Yu Y, Zhang M, Wang N, Li Q, Yang J, Yan S, et al. Epigenetic silencing of tumor suppressor gene CDKN1A by oncogenic long non-coding RNA SNHG1 in cholangiocarcinoma. Cell Death Dis. 2018; doi:10.1038/s41419-018-0786-6.

56. Guo J, Cai J, Yu L, Tang H, Chen C, Wang Z. EZH2 regulates expression of p57 and contributes to progression of ovarian cancer in vitro and in vivo. Cancer Sci. 2011; doi:10.1111/j.1349-7006.2010.01836.x.
57. Tang B, Du J, Li Y, Tang F, Wang Z, He S. EZH2 elevates the proliferation of human cholangiocarcinoma cells through the downregulation of RUNX3. Med Oncol. 2014; doi:10.1007/s12032-014-0271-6.

58. Li W, Zheng J, Deng J, You Y, Wu H, Li N, et al. Increased levels of the long intergenic non-protein coding RNA POU3F3 promote DNA methylation in esophageal squamous cell carcinoma cells. Gastroenterology. 2014; doi:10.1053/j.gastro.2014.03.002.

59. Torigoe H, Yamamoto H, Sakaguchi M, Youyi C, Namba K, Sato H, et al. Tumor-suppressive effect of LRIG1, a negative regulator of ErbB, in non-small cell lung cancer harboring mutant EGFR. Carcinogenesis. 2018; doi:10.1093/carcin/bgy044.

60. Mao F, Holmlund C, Faraz M, Wang W, Bergenheim T, Kvarn brink S, et al. Lrig1 is a haploinsufficient tumor suppressor gene in malignant glioma. Oncogenesis. 2018; doi:10.1038/s41389-017-0012-8.

61. Yokdang N, Hatakeyama J, Wald JH, Simion C, Tellez JD, Chang DZ, et al. LRIG1 opposes epithelial-to-mesenchymal transition and inhibits invasion of basal-like breast cancer cells. Oncogene. 2016; doi:10.1038/onc.2015.345.

62. Neumann P, Jaé N, Knau A, Glaser SF, Fouani Y, Rossbach O, et al. The IncRNA GATA6-AS epigenetically regulates endothelial gene expression via interaction with LOXL2. Nat Commun. 2018; doi:10.1038/s41467-017-02431-1.

63. Serresi M, Siteur B, Hulsman D, Company C, Schmitt MJ, Lieftink C, et al. Ezh2 inhibition in Kras-driven lung cancer amplifies inflammation and associated vulnerabilities. J Exp Med. 2018; doi:10.1084/jem.20180801.

64. Wang X, Zhao H, Lv L, Bao L, Wang X, Han S. Prognostic Significance of EZH2 Expression in Non-Small Cell Lung Cancer: A Meta-analysis. Sci Rep. 2016; doi:10.1038/srep19239.

65. Kim KH, Roberts CW. Targeting EZH2 in cancer. Nature medicine. 2016; doi:10.1038/nm.4036.

66. Fahrner JA, Eguchi S, Herman JG, Baylin SB. Dependence of histone modifications and gene expression on DNA hypermethylation in cancer. Cancer research. 2002; 62:7213-7218.

Figures
Relative expression of LINC00152 in cholangiocarcinoma (CCA) tissues (A) Hierarchical clustering analysis of lncRNAs that were differentially expressed (fold change > 2; P < 0.05) in CCA cancer and normal tissues from GEO datasets (GSE76297). (B) LINC00152 expression was increased in CCA using the GSE76297 database. (C) LINC00152 is overexpressed in CCA tissues (n = 36) compared with noncancerous tissues (n = 9) using TGCA database. (D) The relative expression of LINC00152 was...
measured in 17 pairs of CCA tissues by qRT-PCR. The levels of LINC00152 in CCA tissues were higher than those in non-tumorous tissues. The ΔCt value was determined by subtracting the GAPDH Ct value from the LINC00152 Ct value. A smaller ΔCt value indicates higher expression. All experiments were performed in biological triplicates. Error bars indicate means ± SD. **P < 0.01; ****P < 0.0001.

Figure 2

LINC00152 promotes CCA cell proliferation and migration in vitro. (A) CCK8 assays were implemented to determine the viability of si-LINC00152-treated or pcDNA3.1-LINC00152-treated CCA cells. (B) Representative results of colony formation of si-LINC00152-treated or pcDNA3.1-LINC00152-treated CCA cells. Colonies were counted and captured. (C) Proliferating CCA cells were labeled with EdU. The Click-it reaction revealed Edu staining (red). Cell nuclei were stained with DAPI (blue). (D) Transwell assays were used to investigate the migration of CCA cells after transfection, respectively. All experiments were performed in biological triplicates. Error bars indicate means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001.
Figure 3

Effects of LINC00152 on CCA apoptosis and cycle in vitro. (A) At 48 h after transfection, flow cytometry was used to measure the apoptotic rates of CCA cells. LL, dead cells; UL, viable cells; LR, early apoptotic cells; UR, terminal apoptotic cells. (B) At 48 h after transfection, cell cycle analysis by flow cytometry in CCA cells. The bar chart represents the percentage of cells in G1–G0, S, or G2–M phase, as indicated. All experiments were performed in biological triplicates. Error bars indicate means ± SD. *P < 0.05; **P < 0.01.
LINC00152 knockdown inhibits CCA cell tumor growth in vivo. (A and B) The HuCCT1 cells with stable knockdown of LINC00152 were injected into the nude mice. (C) Tumor volumes were calculated after injection every 2 days. Bars indicate S.D. (D) Tumor weights are represented as means of tumor weights ± S.D (standard deviation). *P < 0.05; **P < 0.01.
RNA-seq after LINC00152 knockdown in CCA cells. (A) Mean-centered, hierarchical clustering of gene transcripts altered (≥1.5-fold change) in si-NC-treated cells and siRNA LINC00152-treated cells with three repeats. (B) Gene ontology analysis for all genes with abnormal expression level after knockdown of LINC00152. (C) qRT-PCR analysis confirmed the altered mRNA levels of genes selectively in CCA cells after knockdown of LINC00152. (D) The altered protein levels of LRIG1 were selectively confirmed by
western blot after transfecting with si-LINC00152 into CCA cells. Error bars indicate means ± SD.*P < 0.05; **P < 0.01; ***P < 0.001.

**Figure 6**

LINC00152 binds to EZH2 to silence LRIG1 epigenetically. (A) RIP experiments for EZH2 were performed, and the co-precipitated RNA was subjected to qRT-PCR for LINC00152. The fold enrichment of LINC00152 in RIPS is relative to its matching IgG control RIP. (B and C) The qPCR and western blot assays detected the expression of EZH2 after knockdown of LINC00152. (D) The altered protein level of EZH2 following treatment of CCA cells with si-EZH2. (E) CCK8 assays were used to determine the viability of CCA cells treated with si-EZH2. (F) Representative results of colony formation of si-LINC00152-treated CCA cells. Colonies were counted and captured. (G) Transwell assays were used to investigate the migration of CCA cells after transfection. (H and I) Knockdown EZH2 triggered LRIG1 expression at the mRNA levels by
qPCR and protein levels by western blot. (J) The enrichment of EZH2 and H3K27me3 in the promoter region of LRIG1 was identified via ChIP assays, and this enrichment was decreased after LINC00152 knockdown in the CCA cell line. Enrichment was quantified relative to the amount of input controls. Antibody directed against immunoglobulin G (IgG) was used as a negative control. Error bars indicate means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001; n.s., not significant.

Figure 7
LRIG1 is a bona target of LINC00152, and counterbalance the activity of LINC00152. (A and B) Hierarchical clustering analysis of genes that were differentially expressed (fold change > 2; P < 0.05) in CCA cancer and normal tissues from TCGA database. Expression levels of LRIG1 in cholangiocarcinoma by analysis of TCGA and GSE76297 data. (C) The decreased expression of LRIG1 was detected in 17 pairs of CCA tissues by qRT-PCR. The ΔCt value was determined by subtracting the GAPDH Ct value from the LRIG1 Ct value. A smaller ΔCt value indicates higher expression. (D-F) CCK8 assays (D), colony formation (E) and transwell assays (F) were implemented to analyze cells transfected with vector/pcDNA-LINC00152/pcDNA-LRIG1 or co-transfected with pcDNA-LINC00152 and pcDNA-LRIG1. The results showed that overexpression of LINC00152 could promote cell proliferation and migration, and upregulated LRIG1 could reverse LINC00152-mediated growth and migration promotion.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTableS1.xlsx
- SupplementaryTableS2.xlsx
- SupplementaryTableS3.xlsx
- SupplementaryFigureS1.docx
- SupplementaryFigureS1.tif