Research Communication

LAMC1 mRNA Promotes Malignancy of Hepatocellular Carcinoma Cells by Competing for MicroRNA-124 Binding with CD151

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Abstract

Specific RNAs can function as sinks for endogenous miRNAs, known as competing endogenous RNAs (ceRNAs). Here, we confirm a miR-124 mediated ceRNA crosstalk between LAMC1 and CD151 in hepatocellular carcinoma (HCC). miR-124 negatively regulates LAMC1 expression through two miRNA binding sites within its 3’ untranslated region (3’UTR) and suppresses migration and invasion of HCC cells through regulating LAMC1. The wild type LAMC1 miRNA response elements (MREs) facilitate expression of CD151, and this regulation is miR-124 dependent. In clinical hepatic tissues, LAMC1 and CD151 mRNAs exhibit positive correlation. Importantly, LAMC1 MREs promote HCC malignancy by absorbing miR-124 and by assisting CD151 expression. We conclude that LAMC1 mRNA acts as a trans regulator to stimulate CD151 expression by competing for miR-124 binding in HCC cells.

Keywords: microRNA; hepatocellular carcinoma; competing endogenous RNA; CD151; LAMC1

Introduction

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer and one of the leading causes of cancer deaths worldwide (1,2). There are approximately 630,000 new HCC cases globally with more than half of these cases occurred in China (3). Even after surgical resection, HCC patients have a poor survival rate of 20%–30% mainly due to high aggressiveness and frequent recurrence of HCC cells (4). A growing effort has been addressed toward the study of molecular mechanisms of liver carcinogenesis and therefore identifying novel therapeutic targets to improve the clinical management of HCC patients. However, the molecular mechanisms underlying HCC malignant characteristics still require better elucidation.

Recently, emerging evidence indicated that noncoding RNAs (ncRNAs) play a key role in hepatocarcinogenesis and tumor progression (5). The ncRNAs, known as the RNA transcripts that do not translate to proteins, exert essential functions in HCC cells by interacting with protein-coding genes to affect their expression and functions (6). MicroRNAs (miRNAs) are a class of single-stranded ncRNAs that bind to miRNA
response elements (MREs) on target RNA transcripts through sequence complementarity and result in degradation or translation repression of the transcript (7). A set of miRNAs have been confirmed to involve in the regulation of HCC malignant phenotypes (8,9). The wide range participation of miRNAs in HCC biology arises a phenomenon that the RNA transcripts containing same MREs can crosstalk with each other by competing for a limited pool of miRNAs. These RNAs bearing a same set of MREs are known as competing endogenous RNAs (ceRNAs) (10–14). Several studies have revealed ceRNA mechanisms in HCC cells. For example, long non-coding RNA (IncRNA) SNHG6–003 promotes proliferation and induces drug resistance of HCC cells by acting as a sponge of miR-26a/b and facilitating expression of transforming growth factor-β-activated kinase 1 (TAK1) (15). Another IncRNA HULC can sequester miR-200a-3p and raise expression level of ZEB1, a functional target of miR-200a-3p. The upregulated HULC promotes tumor invasion and metastasis of HCC cells via acting as a ceRNA (16).

In a previous study, we found that mRNA 3' untranslated region (3'UTR) of the oncogene PIK3C2A bears three effective miR-124 binding sites. These MREs confer a ceRNA capability on PIK3C2A mRNA to facilitate expression of CD151, another oncogene that enhances proliferation and metastasis in various cancers, by competing for miR-124 binding in HCC cells (17). According to a fact that miRNAs regulate a wide range of RNA transcripts (18,19), we presumed that other RNA transcripts may also crosstalk with CD151 via similar mechanism. In this study, by bioinformatics method, we predicted that 3'UTR of laminin γ1 (LAMC1), a member of extracellular matrix glycoproteins, contains two miR-124 binding sites. We revealed that LAMC1 is a bona fide target of miR-124. LAMC1 mRNA not only promotes HCC cell proliferation and metastasis by coding LAMC1 protein but also acts as a ceRNA to sequester miR-124 and supports CD151 expression. The collaboration of LAMC1 and CD151 may be implicated in HCC development.

Materials and Methods

Cell Lines, Transfection and Clinical Tissue Samples

Human HCC cell lines QGY-7703 and SMMC7721 were obtained from the Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The two HCC cell lines were maintained in RPMI-1640 medium (Solarbio, Beijing, China) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA) at 37 °C in a humidified chamber supplemented with 5% CO2. Transfection of plasmids was performed using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA).

Twenty pairs of human HCC tissues and paired normal hepatic tissues were obtained from the Biobank of Tianjin First Center Hospital with the patients’ informed consent. The normal hepatic tissues were the distal end of the operative excision far away from the tumor. The tissue samples were snap-frozen in liquid nitrogen immediately after surgical resection and then stored at −80 °C until use. This study was performed in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and was approved by the Ethics Committee of Tianjin First Central Hospital.

Bioinformatics

The ceRDB database (20) (http://www.oncomir.umn.edu/cefinder/basic_search.php) was used to predict CD151’s potential ceRNAs. TargetScanHuman Release 7.1 (http://www.targetscan.org/vert_71/) (21) was used to search the miRNA binding sites within the target miRNAs.

Vectors and Oligonucleotides

The miR-124 overexpression vector pcDNA3/pri-124, miR-124 suppression vector pSH1-H1-puro/TuD-124, CD151 full-length coding sequence (CDS) expression vector pcDNA3/CD151, CD151 small hairpin RNA (shRNA) expression vector pSilencer/shR-CD151 and the enhanced green fluorescent protein (EGFP) reporter vectors pcDNA3/EGFP-CD151-MRE-wt and -MRE-mut were constructed as previously described (17).

To construct the EGFP reporter vectors with LAMC1 MREs, the MRE sequences were first obtained by annealing the synthesized top and bottom oligonucleotides. The double-strand DNAs (dsDNAs) were then cloned into the pcDNA3/EGFP vector at the BamHI and EcoRI sites downstream of EGFP CDS. Also, the wild type or mutated LAMC1 MREs expression vector pcDNA3/LAMC1-MRE-wt or -MRE1/2-mut was constructed by inserting the annealed wild type or mutated LAMC1 MREs into a pcDNA3.1(+)- vector without the EGFP CDS.

To construct LAMC1 shRNA expression vector, a 66 bp dsDNA fragment (shR-LAMC1) was obtained via an annealing reaction. The fragment was then cloned into a pSilencer 2.1 neo vector (Ambion, Austin, TX) at the BamHI and HindIII sites.

Besides TuD vector, a 2'-O methyl modified miR-124 antisense oligonucleotide (ASO), synthesized by GenePharma (Shanghai, China), was used to suppress miR-124 in HCC cells. The other oligonucleotides used in this study were ordered from AuGCT (Beijing, China), and their sequences are shown in Supporting Information Table 1. All the constructed plasmids were validated using DNA sequencing performed by AuGCT.

Extraction of RNA and Protein

The large (>200 nt) and small (<200 nt) RNAs were extracted using mirVana miRNA Isolation Kit (Ambion) according to the manufacturer’s protocol. To extract protein from HCC cell lines, the cells were lysed using radio immunoprecipitation assay (RIPA) lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.2, 1% Triton X-100 and 0.1% sodium dodecyl sulfate (SDS)) with Pierce protease inhibitor (ThermoFisher, Waltham, MA). After centrifugation, the undissolved cell components were removed, and the total proteins were obtained.
Quantitative reverse transcription polymerase chain reaction (qRT-PCR)
Quantification of miR-124 and an endogenous control U6 small nuclear RNA (snRNA) was performed using stem-loop RT-PCR assay (22). Briefly, small RNA was reverse transcribed into cDNA using a PrimeScript II 1st Strand complementary DNA (cDNA) Synthesis Kit (TaKaRa) with miR-124-RT or U6-RT primers. The cDNAs were then used for amplification by PCR.

For quantification of protein-coding genes CD151 and LAMC1, large RNA was reverse transcribed into cDNA using oligo dT primers. The cDNAs were then used for amplification of target RNAs and an endogenous control β-actin.

All the quantitative PCR reactions were performed using the SYBR Premix Ex Taq II (TaKaRa) on a LightCycler 96 Real-Time PCR System (Roche, Basel, Switzerland). Gene expression was analyzed using the LightCycler 96 software (V1.1. Roche). In relative quantification, 2^ΔΔCt method was applied to calculate the RNA level normalized to the control group. In absolute quantification, a standard curve was made by measuring the standard DNA template in gradient dilution, and sample RNA amount was calculated according to the generated formula.

Western Blotting
Cellular total proteins were resolved on an SDS denaturing polyacrylamide gel and then transferred onto a nitrocellulose membrane. Antibodies to CD151 (Abcam, Cambridge, MA), to LAMC1 (Santa Cruz, Dallas, TX) or an endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH), (Signalway Antibody, College Park, MD) were incubated with the membranes overnight at 4 °C. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse or goat anti-rabbit IgG, Abbkine, Redlands, CA). Protein expression was evaluated by enhanced chemiluminescence substrate (Boster, Wuhan, China), and the bands were captured by a FluorChem FC2 Imaging System (Alpha Innotech, Kasendorf, Germany). The band intensity was analyzed by an AlphaView SA software V3.4.0 (ProteinSimple, San Jose, CA).

EGFP Reporter Assay
EGFP reporter vector was transfected into HCC cells along with other plasmids as required in each group. Identical amount of red fluorescent protein (RFP) expression vector pDsRed2-N1 (Clontech) was cotransfected in each group to serve as the loading control. After 48 h, the cells were lysed with RIPA lysis buffer, and EGFP and RFP intensities were measured by an EnSpire Multilabel Reader (PerkinElmer, Waltham, MA).

RNA Immunoprecipitation Assay
RNAs binding with AGO2 protein were enriched and detected by using a MagnaRIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA) following the manufacturer’s instruction. The AGO2 antibody was purchased from Abcam (Cat# ab57113). miR-124, LAMC1 miRNA and CD151 mRNA in the immunoprecipitated RNAs were analyzed via quantitative RT-PCR.

miRNA-MRE Affinity Tests
An extracellular RNA–RNA pull-down assay was performed to test the affinity between miR-124 and MRE. Briefly, single strand RNA of LAMC1 MRE and CD151 MRE were synthesized with biotin labeled at the 3’ end. The RNAs were conjugated to the streptavidin magnetic beads (ThermoFisher), which were then applied for binding with excessive amount of synthesized miR-124 in hybridization buffer (6× saline sodium citrate (SSC), 0.2% SDS, 10× Denhardt’s). The captured miR-124 by the RNA-conjugated beads was detected by using quantitative RT-PCR (qRT-PCR). The sequences of the synthesized RNAs (ordered from Sangon, Shanghai, China) are available in Supporting Information Table 1.

The miR-124-MRE affinity was also detected by miRNA neutralization and target derepression experiments. A synthesized locked nucleic acid (LNA) oligonucleotide complementary to the miR-124 seed was applied to neutralize endogenous miR-124 in HCC cells. This is a 8-mer tiny LNA oligonucleotide (tnLNA-124, 5’ GTGCCCTTA 3’, ordered from Sangon Biotech, Shanghai, China) for miRNA loss-of-function studies (23). The tnLNA-124 was transfected into HCC cells at a gradient concentration from 0.01 nM to 120 nM, and LAMC1 and CD151 mRNA levels in the transfected cells were detected by qRT-PCR. The correlation between mRNA level and tnLNA-124 concentration was analyzed by nonlinear regression, and the concentration of tnLNA-124 required for half-maximal release of the mRNAs (IC50) was calculated according to the fitted curve.

Cell Counting Kit-8 Cell Viability Assay
Transfected HCC cells were seeded in 96-well plates. At 48 h after seeding, cell counting kit 8 (CCK-8) (Dojindo, Kumamoto, Japan) was added to the cells, and absorbance of the cell culture at 450 nm (A450) was measured using an EnSpire Multilabel Reader (PerkinElmer).

Cell Scratch Healing Assay
HCC cells were transfected in 12-well plates. When the cells yielded confluent monolayers at about 24 h after transfection, a straight wound was made in each well using a sterile 200-μL pipette tip. The culture medium was replaced immediately to remove the suspended cells. Photographs were taken at 0 h and 24 h after scratching, and two straight lines were drawn at the border of the wound. The width of each wound was measured at midpoints of the lines, and healing rate was calculated as follows: (wound width at 0 h – wound width at 24 h)/wound width at 0 h.

Transwell Migration and Invasion Assays
In migration assay, 2 × 10^4 QGY-7703 or SMMC-7721 cells within 200 μL serum-free medium were added to the upper chamber of Transwells (Corning, Corning, NY, Cat#3422), and the lower chamber was filled with complete medium with 10% FBS. The cells were allowed to penetrate at 37 °C, 5% CO2 for 12 h (QGY-7703) or 24 h (SMMC-7721). Then, the penetrated cells attached to the lower surface were stained with 2% crystal violet and observed under a Nikon Ni-U microscope. The
stained cells in five random view fields at $\times 100$ magnification were counted.

In invasion assay, the upper chambers of the Transwells were precoated with Matrigel (BD, Franklin Lakes, NJ), and $4 \times 10^4$ cells were added. The other protocol was same with migration assay.

**In Vivo Xenograft Tumor Studies**

First, HCC cells stably expressing wild type or mutated LAMC1 MREs were prepared. Briefly, QGY-7703 cells were transfected with pcDNA3/LAMC1-MRE-wt or -MRE1/2-mut, followed by selection for 20 days in complete medium supplemented with $300 \mu g/mL$ of Geneticin (Gibco). Effective overexpression of the MREs in the selected cells were confirmed by qRT-PCR.

An amount of $6 \times 10^6$ selected QGY-7703 cells in 200 $\mu L$ medium were inoculated into right axillary fossa of male athymic BALB/c nude mice aged 4–5 weeks. Size of the xenograft tumors was monitored by measuring length ($L$) and width ($W$) with calipers, and tumor volume was calculated as ($L^3 W^2$) $\times 0.5$. At the 17th day after injection, the mice were sacrificed, and the tumors were isolated and photograph was taken. The mice used in this study were handled in accordance with the EU Directive 2010/63/EU, and this work was approved by the Ethics Committee of Tianjin First Central Hospital.

**Statistical Analysis**

All the data were recorded as mean $\pm$ standard deviation (SD) collected from three independent experiments with three technical replicates (five in CCK-8 assays) in each experiment. The hypothesis test for significance between two groups used Student’s $t$ test. For three or more groups, a one-way analysis of variance (ANOVA) was used, followed by Student–Newman–Keuls $q$ test for comparing each two groups. The statistical significance was set at $P \leq 0.05$. Data processing and figure making were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA).

**Results**

**mir-124 Directly Regulates LAMC1 Expression**

First, by using bioinformatics tool, two potential mir-124 binding sites were predicted within LAMC1 3’UTR (Fig. 1A). We then constructed four EGFP reporter vectors with wild type or mutated MREs downstream of EGFP coding sequence (Fig. 1A). Also, mir-124 level was artificially raised by an ectopic expression vector and was reduced by a structured TuD inhibitor expressed by plasmid (24) or a synthesized ASO (25) in QGY-7703 and SMMC-7721 cells (Fig. 1B). In the reporter assays, mir-124 negatively regulated EGFP intensity of the vector with wild type MREs. Each of the MRE in LAMC1 3’UTR contributed to mir-124-mediated reaction, because EGFP intensity was also responsive to mir-124 alteration when either of the MRE was mutated. However, when both of the MREs were mutated, mir-124 aborted to affect EGFP expression (Fig. 1C). It was found that mir-124 inhibited endogenous LAMC1 expression in mRNA (Fig. 1D) and protein (Fig. 1E) levels. qPCR showed that mir-124 level was lower, while LAMC1 mRNA level was higher in the HCC tissues than in the normal hepatic tissues (Fig. 1F). In these tissue samples, mir-124 and LAMC1 mRNA exhibited negative correlation (Fig. 1G). These data suggested that mir-124 directly binds to the MREs in LAMC1 3’UTR and negatively regulates LAMC1 expression.

The canonical model of miRNA-mediated mRNA degradation occurred in the RNA-induced silencing complex, in which AGO2 protein was a key factor in recognition of miRNA-mRNA targeting and in mRNA cleavage (7,26). We found that AGO2 antibody was able to pull-down mir-124 and its targets CD151 and LAMC1 in the RIP assays (Fig. 1H). This result highlighted regulation of CD151 and LAMC1 miRNAs by mir-124.

**mir-124 Suppresses HCC Malignant Phenotypes via Negatively Regulating LAMC1**

Next, we explored the effects of mir-124 on HCC cells. shRNA expression vectors were constructed to depress endogenous LAMC1 in HCC cell lines (Fig. 2A). In the previous study, we have confirmed that mir-124 reduced proliferation of HCC cells by targeting CD151 (17). Here we further validated that suppression of mir-124 enhanced HCC cell proliferation. However, this effect could not be eliminated by subsequent LAMC1 silencing (Fig. 2B), suggesting that LAMC1 was not involved in regulation of HCC proliferation. Then, in the scratch healing assay, overexpression of mir-124 retarded healing rate of the scratch artificially made on the in vitro cultured HCC cell lines, and this effect could be eliminated by sequentially overexpressing CD151. Furthermore, inhibition of mir-124 enhanced healing rate, which was alleviated by suppressing any of the mir-124’s target, CD151 or LAMC1 (Fig. 2C). Our previous study validated that mir-124 retarded migration and invasion of HCC cells, in which CD151 is involved (17). This study further confirmed that a low mir-124 level led to high migration and invasion activities in HCC cells, and silencing of LAMC1 restored these effects (Fig. 2D). These data confirmed LAMC1 as mir-124’s functional target in HCC.

**LAMC1 MREs Promote CD151 Expression through Sequestering mir-124 from CD151 3’UTR**

Given that LAMC1 and CD151 miRNAs share mir-124 binding site, we presumed that a ceRNA regulation mode exists between these two genes. To validate this, we constructed another reporter vector with CD151 MRE downstream the EGFP coding region (Fig. 3A). In the two HCC cell lines, exact overexpression of the wild type LAMC1 MREs elevated EGFP intensity, and this effect could be restored by increasing mir-124. Also, shRNA mediated suppression of LAMC1 MREs led to a depressed EGFP intensity, which could be partly saved by sequentially inhibiting mir-124. The influence of EGFP by LAMC1 MREs depended on the CD151 MRE within the reporter vector, because mutation of this site aborted LAMC1 MREs induced EGFP alteration (Fig. 3B). We next detected the effects of LAMC1 MREs on endogenous CD151 expression. As was expected, the wild type but not the mutated LAMC1 MREs elevated CD151 expression, and
miR-124 directly targets LAMC1 through two MREs within its mRNA. (A) The wild type sequences of the two MREs (LAMC1-MRE-wt) within LAMC1 3’ UTR were cloned into a fluorescent reporter vector pcDNA/EGFP downstream of the EGFP CDS. Other three reporter vectors with both of the two MREs mutated (LAMC1-MRE1/2-mut) or with either of the MREs mutated (LAMC1-MRE1-mut or LAMC1-MRE2-mut, the mutated nucleotides are marked red) were also constructed. (B) miR-124 was overexpressed by pcDNA3/pri-124 vector or suppressed by miR-124 TuD inhibitor vector or synthesized miR-124 ASO in HCC cell lines, and miR-124 level was detected by quantitative RT-PCR. U6 snRNA was regarded as the endogenous normalizer. (C) In the EGFP reporter assay, a reporter vector was transfected into HCC cell lines along with miR-124 gain- or loss-of-function plasmids. A RFP expression vector pDsRed2-N1 was cotransfected as a normalize. At 48 h after transfection, cells were lysed, and EGFP and RFP activities were detected. The histogram shows normalized EGFP intensity (EGFP/RFP). (D, E) miR-124 level was artificially altered in HCC cell lines, and LAMC1 mRNA (D) and protein (E) levels were detected by qRT-PCR and Western blot assays, respectively. β-actin mRNA and GAPDH protein were regarded as the endogenous normalizers. (F) miR-124 and LAMC1 mRNA levels in 20 pairs of HCC (Ca) and normal hepatic (N) tissues were detected by qRT-PCR. (G) Correlation between miR-124 and LAMC1 mRNA levels in the clinical tissues were analyzed. (H) RIP assay was performed in HCC cell lysate using magnetic beads conjugated AGO2 antibody. miR-124, LAMC1 mRNA and CD151 mRNA in the immunoprecipitated RNAs were detected by RT-PCR. Normal mouse IgG was used as isotype control. (*P < 0.05; **P < 0.01; n.s., not significant).

Further forced expression of miR-124 alleviated this change. On the other hand, low LAMC1 MREs caused depressed CD151 expression, which could be rescued by miR-124 inhibition (Fig. 3C,D). These data supported that LAMC1 mRNA acted as a ceRNA to positively regulate CD151 expression by sequestering endogenous miR-124.
We then evaluate whether an opposite regulation, that is, CD151 MRE induced LAMC1 elevation, existed in HCC cells. By using the EGFP-LAMC1-MRE-wt reporter vector (Fig. 1A), we found that CD151 MRE failed to alter fluorescent intensity (Fig. 3E). Also, CD151 MRE did not affect endogenous LAMC1 expression (Fig. 3F,G). These data validated that the ceRNA modulation between LAMC1 and CD151 mRNAs is monodirectional. The underlying reason may be explained by the miRNA–MRE affinity test, which indicated that affinity between miR-124 and LAMC1 MREs was stronger than that between miR-124 and CD151 MRE (Fig. 3H). Also, miRNA neutralization and target release experiments suggested that higher
LAMC1 MREs facilitated CD151 expression by absorbing miR-124. (A) Two EGFP reporter vectors with wild type (wt) or mutated (mut) CD151 MRE downstream of EGFP CDS were constructed. (B) EGFP reporter assay was performed by using the pcDNA3/EGFP-CD151-MRE-wt or -mut reporter vectors in HCC cells. (C,D) The LAMC1 MREs were artificially expressed or inhibited in HCC cells, followed by alteration of miR-124 level. CD151 mRNA (C) and protein (D) levels were detected by qRT-PCR and Western blot assays, respectively. (E–G) Effect of CD151 MRE on LAMC1 expression was detected by EGFP reporter (E), qRT-PCR (F) and Western blot (G) assays, respectively. (H) In the miR-124-MRE affinity test, biotin-labeled LAMC1 MREs or CD151 MREs conjugated with streptavidin magnetic beads were served as bait RNAs to capture miR-124 prey RNAs. The captured miR-124 level was measured by qRT-PCR. (I) A gradient concentration of miR-124 neutralizer tnLNA-124 was transfected into HCC cells, and LAMC1 and CD151 mRNAs were detected by qRT-PCR. The tnLNA-124 concentration required for half-maximal release of the mRNAs (IC50) was calculated by the fitted curve. (J) Correlation between LAMC1 and CD151 mRNA levels in the clinical HCC and normal hepatic tissues were analyzed. (K) The copy number of LAMC1 MREs, CD151 MREs and miR-124 in HCC cell lines was detected by absolute qRT-PCR. (wt, wild type; mut, mutated; *P < 0.05; **P < 0.01; n.s., not significant).
concentration of miR-124 neutralizer was needed to achieve a half-maximal release of LAMC1 than that for CD151 in HCC cells (Fig. 3I), highlighting the stabler binding of miR-124 with LAMC1 MREs.

In the clinical HCC and normal hepatic tissues, CD151 and LAMC1 mRNAs exhibited positive correlation (Fig. 3I), further supporting the miR-124-involved ceRNA pathway on CD151 by LAMC1 mRNA.

It has been indicated that the relative levels of miRNAs and targets will determine the strength of ceRNA activity (27,28). By absolute qRT-PCR, we found that miR-124 and the two MREs exhibited comparable levels in HCC cell lines even though they are not in exactly equimolar amounts (Fig. 3K). This may ensure an active cross-talk between the ceRNAs.

**LAMC1 MREs Enhance HCC Malignant Phenotypes by Sequestering miR-124 and Upregulating CD151**

To illustrate effects of LAMC1 MREs on HCC cell malignancy, we detected cellular viability in the two HCC cell lines. As a result, forced expression of the wild type LAMC1 MREs led to an enhancement of HCC cell viability, which could be restored by either expressing miR-124 or suppressing CD151, supporting that miR-124 involved ceRNA pathway may contribute to this process (Fig. 4A). Importantly, mutated LAMC1 MREs failed to enhance HCC cell viability (Fig. 4A), suggesting that the LAMC1 MREs’ oncogenic activity was associated with miR-124 and its functional target, i.e. CD151. Moreover, the wild type, but not the mutated LAMC1 MREs promoted migration, invasion and wound healing activities of HCC cell lines and, as expected, these effects could be alleviated by enhanced miR-124 or depressed CD151 (Fig. 4B,C). In the in vivo studies, tumors generated by QGY-7703 cells stably expressing wild type LAMC1 MREs exhibited a higher proliferation activity than the control cells, and mutated LAMC1 MREs failed to produce tumors with advanced growth ability (Fig. 4D). These results highlight that LAMC1 MREs play a oncogenic role in HCC cells by acting as a ceRNA to facilitate CD151 expression.

**Discussion**

Emerging studies have revealed that miRNA involved ceRNA networks are implicated in cancer initiation and development (27,29). Disruption of ceRNA crosstalk can upset key physiological regulatory interactions and may support tumorigenesis (27,30). In this study, we demonstrated a ceRNA regulation mode in HCC cells, in which abnormally elevated LAMC1 mRNA facilitated CD151 expression by competing for endogenous miR-124 and promoted HCC malignancy. This conclusion is supported by the following results. First, the shared MREs within LAMC1 and CD151 mRNAs provide the possibility of these two mRNA transcripts to affect each other by vying for miR-124 binding. Second, miR-124 suppresses endogenous LAMC1 and CD151 expression, suggesting an effective regulation of miR-124 on the two target RNAs. Third, the MREs within LAMC1 mRNA facilitate CD151 expression depending on the miR-124 binding sequence within CD151 mRNA. LAMC1 and CD151 mRNAs exhibited positive correlation in clinical hepatic tissues, highlighting the ceRNA mode between these two mRNA transcripts. Fourth, both LAMC1 and CD151 proteins display oncogenic activities in HCC cells. Importantly, artificially expressed LAMC1 MREs, but not its protein-coding region, play oncogenic roles in HCC cells. LAMC1 MREs’ activities could be alleviated by either overexpressing miR-124 or suppressing CD151. Last, it has been suggested that the extent of ceRNA crossregulation depends on expression levels of individual components of the ceRNA network. Optimal ceRNA cross-talk occurs at a near-equimolar equilibrium of all ceRNAs and miRNAs within a network (28). In the two HCC cell lines, although the levels of miR-124 and the MREs within the two ceRNAs are not equal, the difference is not enormous with the highest molecules (CD151 MREs) being about 80-fold as much as the lowest ones (miR-124). This may provide a permissive molecular environment that allows for the evolution of ceRNA networks. Collectively, the above data illustrated a miR-124-mediated ceRNA network between LAMC1 and CD151 mRNAs. Here, we should note that in the miR-124 loss-of-function assay, we used both vector expressed TuD and synthesized ASO. These two kinds of miRNA decoys showed similar extent in miR-124 blockage (Fig. 1B). We chose TuD in the following studies because this kind of miRNA inhibitor contains optimal inherent structural features that support miRNA inhibition (31). Also, plasmid vector based TuD facilitated continuous expression of the inhibitor molecules, which may achieve long-term suppression of endogenous miR-124 (24).

Recent analyses of the human transcriptome have revealed that only 1%–2% of genome have protein-coding potential, even though over three quarters of the genome is transcribed (32,33). These noncoding sequences have been considered as “dark matter” or “junk DNA” due to the lack of systematic means to functionally annotate these elements. Recently, emerging studies have hinted at possible functions of the noncoding sequences (34,35), suggesting that the mammalian genome “carrying information” is significantly larger than previously expected. Beside the open reading frame, miRNAs also contain noncoding sequences known as 5’ or 3’-UTR. It has been known that the 5’ or 3’-UTR can form to special structure or bind to RNA binding proteins (RBPs) to control translation process (36–39). Except RNA–protein interaction, another key function of mRNA UTR is to bind miRNAs (RNA–RNA interaction) and to influence translation or stability of mRNA transcripts (7). miRNA-mediated mRNA control is common in mammalian cells, because mRNA transcripts are densely covered with MREs (18). The widespread MREs make the RNA transcripts possible to influence each others’ levels by ceRNA mechanism (10,11,13), which provides a novel regulatory function of mRNA 3′UTRs. Besides acting as cis regulatory elements to bind miRNAs and affect their own transcripts, 3′UTRs also act in trans to modulate other gene’s expression (10). In this study, we revealed that LAMC1 mRNA not only encodes protein to participate in downstream functions but also plays...
LAMC1 MREs enhanced malignancy of HCC cells by sequestering miR-124 from CD151 mRNA. (A) The expression vector of wild type LAMC1 MREs (LAMC1-MRE-wt) was transfected into HCC cells along with miR-124 expression vector (pri-124) or CD151 suppressor vector (shR-CD151) in the HCC cells. Also, the mutated LAMC1 MREs (LAMC1-MRE1/2-mut) were also overexpressed. Viability of the transfected HCC cells was measured using CCK-8 assay. (B) Migration and invasion activities of the transfected HCC cells were evaluated by Transwell experiments. (C) Migration activity of the transfected HCC cells were evaluated by cell scratch healing assay. (D) QGY-7703 stably expressing wild type or mutated LAMC1 MREs were prepared and inoculated into BALB/c nude mice to produce xenograft tumor models. Size of the xenograft tumors was monitored from the 8th day after inoculation every 3 days. At the 17th day after injection, the tumors were isolated and photograph was taken.

(*P < 0.05; **P < 0.01; n.s., not significant).

an additional non-protein-coding role depending on its 3’UTR to communicate indirectly with CD151 mRNA. ceRNA interactions can be either symmetrical, whereby two ceRNAs coregulate each other, or asymmetrical, whereby one ceRNA regulates expression of the other but not the reverse (17,40). In this study, we found that the ceRNA crosstalk between
LAMC1 and CD151 was asymmetrical. LAMC1 MREs positively affected CD151 expression, and CD151 MRE, however, was unable to cause a detectable alteration of LAMC1 level. One reason may be that LAMC1 mRNA bears two effective MREs, making this RNA a capacious miR-124 “sponge” to release CD151 from miR-124. This presumption was supported by a fact that forced expression of RNA fragments with three CD151 MREs in each RNA molecule also effectively enhanced LAMC1 level (17). Another possible reason is that LAMC1 MREs also displayed stronger affinity to miR-124 than CD151 MREs. This was verified by the RNA–RNA pull-down experiment, in which LAMC1 MREs bound about 20 times the number of miR-124 molecules bound by CD151 MREs when the two MREs were in equimolar amounts. Moreover, when endogenous miR-124 was neutralized by tRNA (23), CD151 mRNA was easier to be released from miR-124 binding than LAMC1 mRNA. In other words, a stronger affinity exists between miR-124 and LAMC1 MREs. These data indicates that in ceRNA model, the strength of a RNA to alter another one may depend on the number of effective MREs within these ceRNAs as well as the affinity between miRNA and MREs.

Laminins, a family of extracellular matrix glycoproteins, are indispensable building blocks for cellular networks physically bridging the intracellular and extracellular compartments and relaying signals critical for cellular behavior (41). As a member of laminin family, LAMC1 acts as an oncogene implicated in a wide variety of tumor phenotypes including cell adhesion, differentiation, signaling and metastasis (42–44). In HCC cells, we found that as a direct target of miR-124, LAMC1 mRNA not only promoted migration and invasion by protein coding but also sequestered miR-124 from CD151 mRNA, leading to an elevation of CD151 expression. Abnormally increased LAMC1 further make CD151 get rid of control by limited endogenous miR-124, and their synergetic relation may be a key role in HCC initiation and development.

This research revealed a ceRNA network between two protein-coding RNAs in HCC cells. It is noteworthy that ncRNAs, such as long ncRNAs (lncRNAs), pseudogenes and circRNAs, may behave more effectively as ceRNAs than do protein-coding RNAs, because they can absorb miRNAs without any interference from translation process (13,45). Thus, we look forward to further illumination of ncRNA transcripts involved ceRNA regulation in HCC cells in the following studies.

Collectively, this study demonstrated a ceRNA crosstalk between LAMC1 and CD151 mRNAs in HCC cells. Abnormally elevated LAMC1 mRNAs not only promoted HCC migration and invasion by translation of LAMC1 protein but also acted in trans to facilitate CD151 expression by absorbing miR-124. The aberration of this ceRNA crosstalk may serve as a key step in HCC development (Fig. 5). This finding carries implications for the development of novel therapeutic approaches of HCC by enhancing miR-124 to reconstruct an effectual control of the two oncogenes by miR-124.

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