Long non-coding RNA DLX6-AS1 aggravates hepatocellular carcinoma carcinogenesis by modulating miR-203a/MMP-2 pathway

Lei Zhang a, Xiaowei He b, Ting Jin c, Gang Li d,⁎⁎, Zhenlin Jin e,⁎

a Department of Infection and Liver Diseases, Liver Research Center, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province 325000, China
b The Eye Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province 325000, China
c Operating Room, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province 325000, China
d Department of Chemoradiation Oncology, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province 325000, China

A R T I C L E   I N F O

Keyword:
- Hepatocellular carcinoma
- Long non-coding RNA
- DLX6-AS1
- miR-203a
- MMP-2

A B S T R A C T

Long non-coding RNAs (lncRNAs) have been wildly verified to modulate multiple tumorigenesis, especially hepatocellular carcinoma (HCC). In present study, our team aims to investigate the role of lncRNA DLX6-AS1 in the HCC carcinogenesis. Results of early-stage experiments found that DLX6-AS1 expression level was up-regulated in 60 cases of HCC tissue samples compared with adjacent normal tissue. Moreover, the aberrant overexpression of DLX6-AS1 indicated the poor prognosis of HCC patients. Loss-of-function experiments revealed that DLX6-AS1 knockdown inhibited the proliferation, migration and invasion of HCC cells in vitro, and decreased the tumor growth in vivo. Bioinformatics analysis predicted that miR-203a potentially targeted DLX6-AS1 3′-UTR, suggesting the interaction between miR-203a and DLX6-AS1. Furthermore, miR-203a also targeted MMP-2 mRNA 3′-UTR, which was validated by luciferase reporter assay. Taken together, our study discovered the oncogenic role of DLX6-AS1 in clinical specimens and cellular experiments, showing the potential DLX6-AS1/miR-203a/MMP-2 pathway. This results and findings provide a novel insight for HCC tumorigenesis.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common hepatic malignant neoplasm in primary liver malignancies cancer [1,2]. Besides, HCC is one of the most significant causes of cancer-related deaths in developing country. Although the therapeutic methods for HCC are developing, including surgery, chemotherapy and radiotherapy, the prognosis of HCC patients still remains poor with 15%-40% of five-year survival rate [3,4]. The in-depth pathogenesis of HCC is complex and the diagnostic approaches need to be investigate for early stage detection and favorable prognosis [5]. Therefore, new diagnostic markers and new targets of clinical treatments are urgent required.

With the increasing progress of high throughput sequencing and microarray analysis, more and more newfound non-coding RNAs (ncRNAs) have been reported. At molecular level, ncRNAs are nucleotide sequences transcribed from chromatin without proteins encoded capacity [6]. Long noncoding RNAs (lncRNAs) are type of ncRNAs with more than 200 nucleotides length [7]. Increasing researches have illustrated that lncRNAs regulate the tumors progression in multiple. For example, lncRNA SNHG1 exacerbated HCC cell proliferation, invasion, and migration in vitro through the inhibition of miR-195 [8].

MicroRNA (miRNA) is another type of ncRNA with 18–22 nucleotides length [9,10]. MiRNAs could target functional protein mRNA 3′-UTR to regulate the protein expression [11]. As well-known, hundreds of defined miRNAs have verified to function as regulator in tumors [12]. For example, miR-33b acts as target of sal-like protein 4 (SALL4) to suppresses the HCC cells proliferation and metastasis [13]. Emerging evidences have verified the interaction between lncRNAs and miRNAs during the tumorigenic process, annotating competing endogenous RNAs (ceRNAs) mechanism [14]. In series of oncogenesis, IncRNAs and miRNAs collectively participate in the tumorous occurrence, proliferation and metastasis. For instance, lncRNA Unigene56159 acts as a ceRNA of miR-140-5p to promote epithelial-mesenchymal transition in hepatocellular carcinoma cells [15].

⁎ Corresponding author at: Department of Hematology, The First Affiliated Hospital of Wenzhou Medical University, Nanbaixiang Street, Ouhai District, Wenzhou, Zhejiang Province 325000, China.
⁎⁎ Corresponding author at: Department of Chemoradiation Oncology, The First Affiliated Hospital of Wenzhou Medical University, Nanbaixiang Street, Ouhai District, Wenzhou, Zhejiang Province 325000, China.
E-mail addresses: andrewlee0923@163.com (L. Gang), jinzhenlin_beauty@126.com (Z. Jin).

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In our study, we found that lncRNA DLX6-AS1 expression was upregulated in HCC tissue compared to adjacent normal tissue. Moreover, in vivo and in vitro assay showed that DLX6-AS1 knockdown inhibited the proliferation, migration, invasion and tumor growth of HCC cells in vitro and vivo. This results and findings reveal the potential DLX6-AS1/miR-203a/MMP-2 pathway, providing a novel insight for HCC tumorigenesis.

2. Materials and methods

2.1. Patients and samples

The samples (60) of HCC tissue and matched adjacent normal tissue were enrolled at the First Affiliated Hospital of Wenzhou Medical University between Jun 2015 and Feb 2017. All tissue were excised during the surgery and immediately snap-frozen in liquid nitrogen until using. All samples were diagnosed by two pathologists to determine the clinicopathological parameters were recorded and classified during the research. All the study and protocols have been approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University. All patients have written the informed consent.

2.2. Cell lines and culture

Human HCC cell lines (MHCC97L, HCCLM3, HepG2, Hep3B, Huh7) and normal liver cell lines (LO2) were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). HCC cells were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% FBS (Gibco, USA), 100 U/mL penicillin and 100 μg/mL streptomycin. Normal liver cell lines were cultured with Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco, USA) containing 10% fetal bovine serum (FBS, Hyclone, USA) and 1% penicillin/streptomycin. Every 3 days, cells were passaged using 0.25% trypsin.

2.3. Cell transfection and reagents

Small interfering RNAs were synthesized and provided by GenePharma Co., Ltd. (Shanghai, China). HCC cells (HepG2, HCCLM3) were cultured with 60–70% confluency and then transfected with Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, Calif, USA) according to the manufacturer’s instruction. Small interfering RNAs contain si-DLX6-AS1, 5′-AAUAAAGACACUUACACUACUG-3′; miR-203a inhibitor, 5′-GCGCAAAAAAAGCGCCAGAG-3′.

2.4. Quantitative real time polymerase chain reaction (RT-PCR)

Total RNA was extracted from HCC tissues and cells using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. cDNA was synthesized using Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, Rockford, IL, USA). RT-PCR was performed with TaqMan® PCR Universal Mastermix (Applied Biosystems, Foster, Calif., USA). Primers were as follows: DLX6-AS1, forward: 5′-CCA AATGCTCATTCCGACCC-3′, reverse: 5′-TCTGGCTTCCCTTACACAAACAA-3′; miR-203a, forward: 5′-GCCGCTGGCTATTAACCTTG-3′, reverse, 5′-GTCGGGGCAGATAAGCGGTT-3′; GAPDH, forward, 5′-CGACACTTTATCATGGCTA-3′; reverse, 5′-TCTGTGCGATCATCAGAATG-3′. Relative levels of gene expression was expressed relative to GAPDH and calculated using the 2^{-ΔΔCt} method. All procedures were performed in triplicate.

2.5. Proliferation assay

The proliferation status of HCC cells was assessed by MTT cell proliferation and colony formation assay. For MTT assay, HCC cells transfected with siRNAs were seeded in 96-well plates at the density of 3 × 10^3 per well and then cultured for 24 h. Then, 20 μL of MTT (5 mg/mL solution was and incubated at 37 °C for 2 h. The optical density (OD) values were read at 570 nm by a microplate reader (Tecan Sunrise, Männedorf, Switzerland). For colony formation assay, HCC cells were seeded in 6-well plates at the density of 500/well for 2 weeks. After 14 days, colonies were fixed with 4% paraformaldehyde for 5 min and stained with 1% crystal violet for 10 min. The assays were repeatedly performed in triplicate.

2.6. Transwell migration and invasion assay

The migration and invasion assays were performed using Transwell chambers (Millipore, Billerica, MA) on HCC cells (HepG2, HCCLM3). For the migration, cells were placed into 24-well plate, 1 × 10^5 cells were resuspended in 200 μL serum-free medium on upper chambers. 600 μL medium containing 10% FBS was added into lower chambers. Then, cells were incubated at 37 °C with 5% CO_2. For invasion assay, HCC cells (1 × 10^5) were resuspended in 200 μL serum-free medium and placed on upper chambers and 600 μL medium containing 10% FBS was added into lower chambers. After 48 h of incubation, cells were fixed with polyoxymethylene. Finally, the migrated and invaded cells were counted from five randomly fields.

2.7. Xenograft nude mouse model

The xenograft mice assay was approved by the Committee on Animal Welfare of First Affiliated Hospital of Wenzhou Medical University. Total twenty male BALB/c nude mice (about six weeks) were maintained in clean conditions. HepG2 and HCCLM3 cells (4 × 10^5 per 100 mL) were respectively transfected with shRNAs of DLX6-AS1 or controls. The transfected cells were subcutaneously injected into back of nude mice. Tumour size was measured every 4 days using length × width^2 × 0.5. Tumor weight was measured after sacrifice of mice.

2.8. Dual-luciferase reporter gene assay

Luciferase assays were performed using the Dual Luciferase Reporter assay system (Promega, Madison, WI, USA) according to the manufacturer instructions. The pGL3 plasmid was generated using a mammalian genomic DNA miniprep kit (Sigma, Louis, MO, USA). cDNA were subcloned into the luciferase reporter vector pGL3 (Promega, Madison, WI, USA). Renilla luciferase vector pGL3-DLX6T-AS1 was co-transfected as an internal control. The luciferase activity of Firefly luciferase compared to Renilla luciferase was measured 24 h after transfection according to the manufacturer’s protocol.

2.9. Western blot

Proteins were dissected and homogenized on ice in Radio Immunoprecipitation Assay (RIPA) buffer (Beyotime, Nanjin, China). Total protein was loaded onto 10% gels and transferred to polyvinylidene fluoride (PVDF) membrane. Then, PVDF membrane was blocked with 5% non-fat milk for 2 h at room temperature in TBST, and incubated with primary antibody (MMP-2, 1:1000, Cell Signaling) at 4 °C overnight. After that, it was incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. The PVDF membrane was visualized using Immuno Star LD (Wako Pure Chemical, Osaka, Japan), and then measured using LAS-4000 Mini (Fuji Film, Tokyo, Japan). Relative protein expression was normalized according to GAPDH.

2.10. Statistical analysis

All data are represented as mean ± standard deviation (SD). Statistical analysis was calculated with the SPSS (SPSS, Inc., Chicago, IL, USA) and GraphPad. One way ANOVA and student’s t-test were used.
for intra-group comparison. Kaplan-Meier’s method and the log-rank test were performed for survival rate. \( P < 0.05 \) was identified as significant difference.

3. Results

3.1. DLX6-AS1 was up-regulated in HCC tissue and correlated with clinical prognosis

In the initial stage of study, we measured the expression level of DLX6-AS1 in 60 cases of HCC tissue samples and paired adjacent normal tissue. The clinicopathologic data was presented in Table 1. Results showed that DLX6-AS1 expression was significantly up-regulated in HCC tumor tissue compared to normal tissue (Fig. 1). Moreover, DLX6-AS1 expression was higher in HCC patients with advanced clinical stage (III–IV phase) than that patients with early stage (I–II phase) (Fig. 1B). Based on the median value of DLX6-AS1, HCC patients (60 cases) were divided into high DLX6-AS1 expression group (n = 33 cases) and low DLX6-AS1 expression group (n = 27 cases) (Fig. 1C).

For HCC patients’ prognosis, Kaplan-Meier and log-rank test revealed that the higher DLX6-AS1 expression predicted poor prognosis that low DLX6-AS1 expression (Fig. 1D). Therefore, data and results demonstrated that DLX6-AS1 was up-regulated in HCC tissue and correlated with poor prognosis for HCC patients.

3.2. DLX6-AS1 knockdown inhibited the proliferation of HCC cells in vitro

The abundance of DLX6-AS1 in HCC tissue was markedly higher than that in adjacent normal tissue. To verify the biological role of DLX6-AS1 in HCC cells, functional confirming experiments in vitro were performed. In HCC cell lines (MHCC97L, HCCLM3, HepG2, Hep3B,

Table 1

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*AFP, alpha-fetoprotein; TNM, tumor-node-metastasis.

Data and results from experiments are presented in the following figures:

Fig. 1. DLX6-AS1 overexpression in HCC tissue and clinical significance. (A) RT-PCR showed that DLX6-AS1 expression was up-regulated in 60 cases of HCC tissue compared to normal tissue. (B) DLX6-AS1 expression in advanced stage HCC patients (III–IV phase) and early stage (I–II phase). (C) Distinction of high DLX6-AS1 expression group (n = 33 cases) and low DLX6-AS1 expression group (n = 27 cases). (D) Five year’s survival rate of HCC patients with high/low DLX6-AS1 expression calculated by Kaplan-Meier and log-rank test.
Huh7), DLX6-AS1 expression was significantly up-regulated compared with normal liver cell line (LO2) (Fig. 2A). Then, in HepG2 and HCCLM3 transfected with si-DLX6-AS1, DLX6-AS1 expression levels were obviously decreased compared to control group (Fig. 2B). For the proliferation capacity, MTT assay and colony formation assay togetherly demonstrated that DLX6-AS1 knockdown induced by si-DLX6-AS1 inhibited the proliferation vitality of HCC cells (Fig. 2C–E). In summary, results indicated that DLX6-AS1 expression was significantly up-regulated in HCC cell lines, and DLX6-AS1 knockdown inhibited the proliferation of HCC cells in vitro.

3.3. DLX6-AS1 knockdown inhibited the migration, invasion of HCC cells in vitro

Fig. 2. DLX6-AS1 knockdown inhibited the proliferation of HCC cells in vitro. (A) DLX6-AS1 expression in HCC cell lines (MHCC97L, HCCLM3, HepG2, Hep3B, Huh7) and normal liver cell line (LO2). (B) DLX6-AS1 expression in HepG2 and HCCLM3 transfected with si-DLX6-AS1. (C) MTT assay showed the absorbance of HepG2 and HCCLM3 cells measured at 570 nm. (D and E) Colony formation assay showed the clone number in HepG2 and HCCLM3 respectively. Data are presented as the mean ± SD. *P < 0.05, **P < 0.01 compare to control group.

Huh7), DLX6-AS1 expression was significantly up-regulated compared with normal liver cell lines (LO2) (Fig. 2A). Then, in HepG2 and HCCLM3 transfected with si-DLX6-AS1, DLX6-AS1 expression levels were obviously decreased compared to control group (Fig. 2B). For the proliferation capacity, MTT assay and colony formation assay togetherly demonstrated that DLX6-AS1 knockdown induced by si-DLX6-AS1 inhibited the proliferation vitality of HCC cells (Fig. 2C–E). In summary, results indicated that DLX6-AS1 expression was significantly up-regulated in HCC cell lines, and DLX6-AS1 knockdown inhibited the proliferation of HCC cells in vitro.

3.3. DLX6-AS1 knockdown inhibited the migration, invasion of HCC cells in vitro

It had been proved that DLX6-AS1 knockdown inhibited the proliferation of HCC cells in vitro. In this part, Transwell assay was performed to measure the migration and invasion of HCC cells. Results showed that the relative migrative and invasive cells number of DLX6-AS1 knockdown in HepG2 cell line were significantly lower than that in control group (Fig. 3A and B). Moreover, the same assay was performed in HCCLM3 cell line, showing the similar results according with prior (Fig. 3C and D). Therefore, these data revealed that DLX6-AS1 knockdown inhibited the migration, invasion of HCC cells in vitro.

3.4. DLX6-AS1 silencing decreased the tumor growth of HCC cells in vivo

Despite of the in vitro assay, our team also performed xenograft mice experiments to measure the effects of DLX6-AS1 silencing on HCC cells tumor growth. HCC cells were transfected with sh-DLX6-AS1. Results showed that DLX6-AS1 silencing induced by sh-DLX6-AS1 could significantly decrease the tumor volume of HCC cells after subcutaneously injection, including HepG2 cells and HCCLM3 cells (Fig. 4). Thus, results showed that DLX6-AS1 silencing could suppress the tumor growth of HCC cells in vivo.

3.5. Bioinformatics tool predicted the interaction between DLX6-AS1 and miR-203a

The oncogenic role of DLX6-AS1 has been verified by a series of function validation experiments. Bioinformatics online tools were performed to predict the potential target miRNAs for DLX6-AS1, revealing the complementary binding sites with miR-203a at 3’-UTR (Fig. 5A).
DLX6-AS1 knockdown inhibited the migration, invasion of HCC cells in vitro. (A) The representative images of migrated and invasive cells in HepG2 cells. (B) The relative quantification (fold change) of cell number in HepG2 cells. (C) The representative images of migrated and invasive cells in HCCLM3 cells. (D) The relative quantification (fold change) of cell number in HCCLM3 cells. Data are presented as the mean ± SD. **P < 0.01 compare to control group.

DLX6-AS1 silencing decreased the tumor growth of HCC cells in vivo. (A) Representative images of neoplasm in subcutaneous of nude mice. (B and C) Tumor volume of neoplasm in mice injected with HCC cells measured by length × width² × 0.5. (D and E) Tumor weight of neoplasm in mice measured after sacrifice. Data are presented as the mean ± SD. *P < 0.05, **P < 0.01 compare to control group.
Fig. 5. miR-203 targeted DLX6-AS1 at 3′-UTR and negatively correlated with DLX6-AS1 expression. (A) Bioinformatics programs predicted the complementary binding sites with miR-203a and DLX6-AS1 3′-UTR. (B) Luciferase reporter assay validated the binding of miR-203a and DLX6-AS1. (C) miR-203a expression in HCC cell lines (MHCC97L, HCCLM3, HepG2, Hep3B, Huh7) and normal liver cell line (LO2). (D) miR-203a expression in HCC cells transfected with si-DLX6-AS1. (E) miR-203a expression in HCC tissue compared with adjacent normal tissue. Data are presented as the mean ± SD. **P < 0.01 compare to control group.

Fig. 6. MMP-2 acted as target gene of DLX6-AS1/miR-203a. (A) The matched binding base within DLX6-AS1 3′-UTR and miR-203a, including wild type and mutant type. (B) Luciferase reporter assay showed the interaction within miR-203a and MMP-2 mRNA 3′-UTR. (C) DLX6-AS1 and MMP-2 expression levels when transfected with miR-203a inhibitor. (D and E) Western blot showed MMP-2 protein expression in HepG2 cells when co-transfected with miR-203a inhibitor and/or si-DLX6-AS1. Data are presented as the mean ± SD. *P < 0.05, **P < 0.01 compare to control group.
Luciferase reporter assay validated that miR-203a targeted DLX6-AS1 3′-UTR at molecular level (Fig. 5B). In the meantime, miR-203a expression in HCC cell lines was significantly down-regulated compared with normal liver cell line (Fig. 5C). In HCC cells transfected with si-DLX6-AS1, miR-203a expression was increased (Fig. 5D). In HCC tissue samples, miR-203a expression was decreased compared with adjacent normal tissue (Fig. 5E). Therefore, results indicated that miR-203a targeted DLX6-AS1 at 3′-UTR and negatively correlated with DLX6-AS1 expression.

3.6. MMP-2 acted as target gene of DLX6-AS1/miR-203a

MiR-203a was the target miRNA of lncRNA DLX6-AS1. Furthermore, bioinformatics analysis predicted that MMP-2 mRNA 3′-UTR had the matched binding base with miR-203a (Fig. 6A). Luciferase reporter assay revealed that miR-203a directly targeted MMP-2 mRNA 3′-UTR (Fig. 6B). To verify the interaction of miR-203a within DLX6-AS1 and MMP-2, miR-203a inhibitor was transfected into HepG2 cells. Results showed that DLX6-AS1 and MMP-2 expression levels were both increased when transfected with miR-203a inhibitor (Fig. 6C). Western blot results showed that miR-203a inhibitor increased MMP-2 protein expression, and the co-transfection of miR-203a inhibitor and si-DLX6-AS1 decreased it (Fig. 6D and E). In summary, results concluded that DLX6-AS1/miR-203a exerted biological role via targeting MMP-2, suggesting the potential pathway for the HCC tumorigenesis.

4. Discussion

Emerging evidences have illustrated the more and more important role of long non-coding RNA (lncRNA) in carcinogenesis, including hepatocellular carcinoma (HCC), bladder cancer and glioma [16,17]. In our study, our team found that lncRNA DLX6-AS1 was up-regulated in HCC tissue and cell lines. Besides, we also found that DLX6-AS1 functioned as oncogenic molecular in the HCC progression. The carcinogenesis is a complex intermolecular interaction. LncRNAs are type of epigenetics regulatory mechanism, including transcriptional regulation and post-transcriptional regulation [18,19]. Li et al. reported that lncRNA DLX6-AS1 was up-regulated in lung adenocarcinoma tissues, and the high DLX6-AS1 expression levels were significantly associated with both histological differentiation and TNM stage [20]. In this study, our prior experiments found that tumorigeneses closely correlated with HCC patients’ poor prognosis. Besides, in vivo and vitro assay validated that DLX6-AS1 promoted the proliferation, migration and invasion of HCC cells. Therefore, the comprehensive data indicate the tumor promoting role of HCC cells, suggesting the oncogenic molecular in the HCC progression.

The pathogenesis progression of HCC is ambiguous and multiple factors are involved in the course [21,22]. Series of pathological process are closely correlated with it, including differentiation, proliferation, invasion and metastasis [23]. For example, lncRNA DGC5 is found to be down-regulated in HCC tissues and serum, which was correlated with a poor survival [24]. HOX Antisense lincRNA (HOXA-AS2) was increased in HCC tissues and HOXA-AS2 knockdown significantly inhibited HCC cell proliferation and invasion. LncRNA CCHE1 indicated a poor prognosis of HCC patients and promotes carcinogenesis through ERK/MAPK pathway activation [25]. From these perspectives, we could conclude that lncRNAs regulate the HCC tumorigenesis involving multiple molecular levels. Except for HCC tumors, lncRNAs also participate in other tumors, such as glioma, non-small cell lung cancer, gastric cancer and bladder cancer [26,27]. In renal cell carcinoma, lncRNA DLX6-AS1 has been verified to promote the progression via mir-26a/PTEN axis, acting as an oncogenic lncRNA for the genesis [28].

In present study, the overexpression of DLX6-AS1 indicates the poor prognosis of HCC patients. Due to the stable abundance and expression of DLX6-AS1, it might act as an effective diagnostic marker for HCC early diagnose. In 105 cases of tumor tissue and serum of HCC patients, lncRNA UCA1 was significantly higher in patients with HCC, acting as the vital discrimination of HCC from benign liver disease and healthy controls [29]. Another lncRNA CCAT1 and CCAT2 was significantly associated with a poor RFS and a poor OS, serving as important prognostic biomarkers in colorectal cancer [30]. In our study, we found that DLX6-AS1 was correlated with the 5-year survival rate of HCC patients, thus, lncRNA DLX6-AS1 could act as a diagnostic marker for HCC early detection.

In summary, our study investigates the deepening function of DLX6-AS1 in the HCC tumorigenesis, and validates its biologic mechanism via miR-203a/MMP-2 pathway. These finding could help us for better treatment of HCC.

Conflict of interest

All authors declare no conflicts of interest.

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