The long noncoding RNA linc00858 promotes progress of lung cancer through miR-3182/MMP2 axis

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Introduction

Cancer is a major public health problem worldwide. The American Cancer Society estimated new cases of 10 leading cancer types both in male and female in United States in 2019 and found lung cancer is the second most popular malignant tumour worldwide, leading the most cases of death [1].

Long non-coding RNAs (lncRNAs) are a class of ncRNAs of >200 nucleotides in length and no protein-coding function [2]. It has been reported that lncRNA could participate in the initiating and the progress of diseases by interfering with transcription, translation, epigenetic inheritance and some basic process of cell life activities [3]. Previous researches have demonstrated that IncRNAs play a significant role in various types of tumour [4–7], especially in lung adenocarcinoma [8–10]. For example, White and his colleagues characterized the IncRNA landscape in lung cancer and discovered 111 differentially expressed IncRNAs, which were termed as lung cancer-associated lncRNAs (LCALs). By integrating the exome sequencing data, they found that the expression levels of many LCALs have significant associations with the mutational status of key oncogenes in lung cancer. Liu and his colleagues found 624 lncRNAs altered > 1% and 36 lncRNAs altered > 10%. Among these lncRNAs, 20 lncRNAs were found altered > 10% in both LUSC and LUAD. It seems lncRNAs could act as oncogenes and provide novel targets for anti-lung cancer therapeutics [11]. Chen searched the human lung cancer mRNA expression datasets which were downloaded from GEO database and found the dysregulated IncRNAs reflected more comprehensive impairment in functional enrichment than dysregulated mRNAs [12]. In the current research, we identify the IncRNA 00858 which has been reported by Xu and his colleagues who found that linc00858 is highly expressed in lung adenocarcinoma. Here, we used the DIANA-LncBase to predict the target genes of linc00858 and identify miR-3182 which could down-regulate MMP2 in Osteosarcoma by targeting 3' UTR of MMP2 mRNA. In lung adenocarcinoma, 625 IncRNAs altered > 1% and 36 lncRNAs altered > 10%. Among these lncRNAs, 20 lncRNAs were found altered > 10% in both LUSC and LUAD.

While in lung adenocarcinoma, the functional role of linc00858 was verified by dual-luciferase reporter assay and western blot. The epithelial-mesenchymal transition (EMT) was examined by CCK8, transwell assay and western blot to evaluate the function of linc00858. Dual-luciferase reporter assay was used to identify the potential target of linc00858. Over-expression of linc00858 significantly promoted cell proliferation, invasion. We also found that linc00858 facilitated the EMT process. Dual-luciferase reporter assay revealed that linc00858 can bind with miR-3182 directly. Linc00858 can negatively regulate the expression of miR-3182. Further experiments demonstrated that MMP2 was the direct target of miR-3182. Rescue experiments revealed that linc00858 functioned through miR-3182/MMP2 axis. Taken together, we verified the role of an unknown linc00858 in lung cancer and provided its mechanism. Mechanistically, linc00858 acted as a competitive endogenous RNA to sponged miR-3182 and regulate MMP2 in lung cancer. Our study provided new clues for understanding the mechanism of lung cancer.

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organ fibrosis and the initiation or the metastasis in cancer progression [15–17]. Recent researches have revealed that EMT plays a critical role in the progression of lung cancer. For example, Yuan et al. demonstrated that Notch signalling promotes the progression of non-small cell lung cancer through enhancing EMT by cross-talks with several transcriptional factors [18]. Perumal et al. found PTEN inactivation induced EMT and metastasis in non-small cell lung carcinoma cells [19,20]. Zhang et al. revealed that hypoxic BMSC-derived exosomal miRNAs could promote STAT3-induced EMT in lung cancer cells, and yet, promote metastasis of lung cancer [21]. The association between no-coding RNAs and the process of EMT has been widely studied, though the underlying mechanism remains unknown. Guo concluded the multidimensional communication of microRNAs and lncRNAs in lung cancer and mentioned that miRNA–lncRNA pathways may have the common end point of EMT. For example, the miR-200 family were found could inhibit EMT by targeting mRNA of ZEB1 and ZEB2 who are the repressors of E-cadherin [22]. Respectively, Li et al. and Zhang et al. found that the lncRNA MALTA1 and lncRNA H19 with their targets miR-204 and miR-484 played an active role in the process of EMT in lung cancer [23]. In our research, we unmasked the association between the process of EMT and miR-3182 through targeting 3'-UTR of MMP2 mRNA which has been well studied in various cancers. Obviously, the current research provided new insights for understanding the mechanism of lung cancer and enriched the original literatures.

Materials and methods

Cell culture and samples

Human lung cancer cell lines A549 and H1299, H226, H446 and human bronchial epithelial cells BEAS-2B were stored in our laboratory or obtained from the Shanghai cell bank of the Chinese Academy of Sciences. Cells were cultured in DMEM medium or in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Life Technologies, Waltham, MA) in 5% CO2 incubator at 37°C. Lung cancer tissues were obtained from lung cancer patients who received pulmonary lobe resection in our hospital, while the normal lung tissues were obtained from the tissues adjacent to cancer. The liver, brain, spleen, pancreas, kidney and heart tissues were obtained from the C57/BL6 mouse. This study was approved by the Institute Research Ethics Committee of Henan provincial chest hospital and every patient was agreed and signed the informed consent. These tissues were stored in -80°C for further research once being resected.

Transfection

Linc00858 over-expressed vector and MMP2 over-expressed vector was constructed by GenePharma (Shanghai, China). The 3'-untranslated region (UTR) of MMP2, with wild-type (WT) or mutant (mut) binding sites for miR-3182, was amplified and cloned into the pGL3 vector (Promega, Madison, WI) to generate the plasmid pGL3-WT-MMP2-3'-UTR or pGL3-mut-MMP2-3'-UTR. The miR-3182 mimics, NC mimics were purchased from Shanghai GenePharma (Shanghai, China). Briefly, cell lines A549 and H1299 were infected with vector, miR-3182 mimics and NC mimics by lipofectamine reagent (Invitrogen) according to the manufacturer’s protocol when cell confluence reached 30%. Cells were incubated for 48 h before further researches.

CCK-8 assay

The proliferation rates of A549 and H1299 cell lines were tested by CCK-8 kit (Dojindo, Kumamoto Japan). And 3.0 × 10^3 linc00858 over-expressed vector transfected cells and empty vector transfected cells in 100 μL were incubated in 96-well plates. At 0, 24, 48, 72 and 96 h, 10 μL CCK-8 reagent was added to each well and incubated at 37°C for 2 h. The optical density was measured at 450 nm.

Transwell invasion assay

A549 and H1299 cell lines’ invasion ability was tested by transwell invasion assay. Briefly, transwell insert chamber coated with Matrigel (BD Biosciences, San Jose, CA) was used according to the manufacturer’s instruction. linc00858 over-expressed vector transfected cells and empty vector transfected cells were incubated in insert chambers for 24 h and invaded cells were dyed with crystal violet and observed under a microscope.

Dual-luciferase reporter assay

A549 and H1299 cell lines were co-transfected with plasmid pGL3-WT-MMP2-3'-UTR or pGL3-mut- MMP2-3'-UTR and miR-3182 mimics or NC mimics. Dual-luciferase reporter assay was conducted using Dual Luciferase Reporter Assay System (Promega) following the manufacturer’s protocol. Firefly luciferase activity and Rinilla luciferase activity were measured with Multiskan Spectrum (Thermo Fisher, Waltham, MA).

Real-time PCR

Total RNA was extracted and lysed by TRIzol reagent (Thermo Fisher). RNA reverse transcription was performed using a PrimeScript™ RT reagent Kit with gDNA eraser (Takara, Osaka, Japan) according to the manufacturer’s instructions, and cDNA was performed by qRT-PCR using SYBR® Premix Ex Taq™ (Takara). The data were normalized using β-actin levels and further analysed by the 2 – ΔΔCT method.

Western blot

A549 and H1299 cell lines were harvested and lysed by RIPA lysis buffer containing proteinase inhibitor (Roche, Branchburg, NJ). Total protein was quantified using BCA protein assay kit (Pierce, Waltham, MA). Protein samples were resolved by 10% SDS-PAGE gel (120 V, 120 min) and transferred to polyvinylidene difluoride (PVDF) membrane (300 mA, 90 min). And membranes were blocked with 5%..
pure milk for 1 h. Then they were incubated with primary antibodies against β-catenin (1:1000, CST, USA), E-cadherin (1:1000, CST, USA), N-cadherin (1:1000, CST, USA), vimentin (1:1000, CST, USA), GAPDH (1:1000, CST, USA), β-actin (1:1000, CST, USA) at 4°C overnight, followed by incubation with a peroxidase-conjugated goat anti-rabbit (or mouse) IgG antibody. Immunopositive bands were analysed using a FluorChem M system (ProteinSimple, San Jose, CA).

Data analysis

We used SPSS version 21.0 (SPSS, Chicago, IL) to calculate the values (means ± standard error of the mean (SEM)). And statistical analyses were analysed using two-sided Student’s t-test or one-way ANOVA. The statistical significance was \( p < .05 \).

Results

Biological features of linc00858

The aim of this study is to investigate the function and mechanism of lincRNA00858 in lung cancer. To verified the role of linc00858 in lung cancer, first, we used qPCR to examine the expression of linc00858 in lung cancer tissues. The expression of linc00858 was significantly increased in lung cancer tissues compared with normal tissues (Figure 1(A)). Next, multi-tissues analysis revealed that linc00858 mainly expressed in lung tissues, suggesting that linc00858 may possessed important role in lung cancer (Figure 1(B)). Moreover, we verified the expression of linc00858 in lung cancer cell lines. And the expression of linc00858 was significantly increased in lung cancer cell lines, such as A549 and H1299 (Figure 1(C)).

Functional analysis of linc00858 in lung cancer

To verify the role of linc00858 in lung cancer, first, we over-expressed linc00858. The expression of linc00858 was increased about 6-fold times (Figure 2(A)). Next, CCK-8 was used to evaluate the proliferation of linc00858 over-expressed cells. Over-expression of linc00858 significantly promoted the proliferation of A549 and H1299 cells (Figure 2(B)). And upregulation of linc00858 significantly facilitated the EMT process in A549 and H1299 cells.

Considering EMT has been reported to play an important role in metastasis, we detected the EMT markers in protein level and mRNA level. The expression of epithelial marker E-cadherin was decreased in A549 and H1299 cells, whereas the expression of Vimentin and N-cadherin was manifestly increased in A549 and H1299 cells via western blotting (Figure 2(C)). Similar results can be achieved via real-time PCR in two different cell lines (Figure 2(D)). In addition, we also performed transwell assay to evaluate the invasion of linc00858 (Figure 2(E)). Our results suggested that overexpression of linc00858 could distinctly promoted the cell invasion in A549 and H1299 cells, respectively. Thus, our results demonstrated that linc00858 can promote the cell proliferation, EMT process and cell invasion in lung cancer.

miR-3182 is the direct target of linc00858

To study the possible mechanism of linc00858, we used bioinformatics software to predict the potential binding miRNAs. Our results suggested that miR-3182 possessed the potential binding ability. The potential binding sequence was showed in Figure 3(A). Next, we used dual-luciferase reporter assay to evaluate whether miR-3182 can bind with linc00858. Our results suggested that miR-3182 can directly bind with wt linc00858, whereas no significant difference was observed in mut linc00858 group (Figure 3(B)). The expression of miR-3182 was manifestly decreased in linc00858 over-expression group, suggesting that linc00858 can negatively regulate the expression of miR-3182 (Figure 3(C)). Next, we explored the function of miR-3182 in lung cancer. Over-expression of miR-3182 significantly inhibited the proliferation of A549 and H1299 cells (Figure 3(D)). And over-expression of miR-3182 can inhibited the EMT process via qPCR and western blotting assay (Figure 3(E,F)). In addition, the cell invasion was decreased in miR-3182 over-expressed group (Figure 3(G)). Thus, our results suggested that linc00858 can negatively regulate the expression of miR-3182.

linc00858 promote lung cancer development through negatively regulate miR-3182

To further prove that miR-3182 was the direct target of linc00858, we performed rescue experiment. The expression of miR-3182 was significantly down-regulated in lung cancer cell lines (Figure 4(A)). Over-expression of miR-3182 partially

Figure 1. Biological features of linc00858. (A) The expression of linc00858 was upregulated in lung cancer tissues. (B) Multi-tissue analysis revealed that linc00858 mainly expressed in lung tissue. (C) The expression of linc00858 was upregulated in lung cancer cell lines.
rescued the pro-proliferation effect of linc00858 in A549 and H1299 cell lines (Figure 4(B)). Over-expression of miR-3182 can up-regulated the expression of EMT markers, E-cadherin compared with co-transfection group, whereas Vimentin and N-cadherin were significantly decreased in co-transfection group (Figure 4(C)). Rescue experiment revealed that miR-3182 down-regulated the cell invasion compared with linc00858 group (Figure 4(D)). Thus, our results suggested that linc00858 promote lung cancer development through negatively regulate miR-3182.
MMP2 is the direct target of miR-3182

To further explore the potential mechanism of miR-3182 involved in lung cancer, we studied the potential target of miR-3182, MMP2. Firstly, the potential binding sequences were showed (Figure 5(A)). Dual-luciferase activity assay revealed that MMP2 is the direct target of miR-3182, and no significant difference was observed in mut MMP2 group (Figure 5(B)). Over-expression of miR-3182 could inhibited the expression of MMP2 via qPCR, suggesting that miR-3182 can negatively regulate the expression of MMP2 through real-time PCR (Figure 5(C)). Similar results can be achieved through western blotting (Figure 5(D)). However, over-expression of linc00858 could up-regulated the expression of MMP2 via qPCR and western blotting (Figure 5(E,F)), suggesting that...
linc00858 can positively regulate the expression of MMP2. Thus, our results revealed that linc00858 may functioned through regulating MMP2 in lung cancer.

**linc00858/miR-3182/MMP2 axis in lung cancer**

To verify the role of linc00858/miR-3182/MMP2 axis in lung cancer, we performed rescue experiment. Over-expression of MMP2 can reversed the proliferation effect of linc00858 and miR-3182 in A549 and H1299 cell lines (Figure 6(A)). Moreover, transwell assay demonstrated that over-expression of MMP2 can increased the invaded cells compared with miR-3182 and linc00858 group (Figure 6(B)). The epithelial marker E-cadherin was decreased while the mesenchymal markers N-cadherin and vimentin were increased in co-transfection group when compared with lincRNA 00858 and miR-3182 group (Figure 6(C)). Over-expression of MMP2 can reversed the EMT process of linc00858 and miR-3182 in A549 and H1299 cell lines.

**Discussion**

Lung cancer is one of the most popular malignant tumours worldwide and leading the most cases of death in 2019 which cause a major public health problem worldwide [1]. In this research, we demonstrated that the long non coding RNA linc00858 inhibits progress of lung cancer through targeting/miR-3182/MMP2 axis. The current research is consisted with previous researches which have demonstrated that linc00858 playing a role in promoting tumour progression.

MiR-3182 is one of the families of microRNAs which are highly conserved set of single stranded non-coding RNAs (−19–23-nucleotide length). It is reported that miR-3182 is abnormally expressed in various human disease, especially in malignant tumours. For example, Zhu et al. found miR-3182 expression and function are inversely correlated with lnc ODRUL expression which is found Contributed to Osteosarcoma Progression and they found miR-3182 could inhibit the progress of osteosarcoma by targeting the 3'UTR of MMP2 mRNA which is exactly the same target in our research [24]. Ju et al. found hsa-miR-3182 was up-regulated in lung adenocarcinoma cell lines with EGFR exon 19 deletion (H1650 and PC9) than in wild-type (H1299 and A549) [25]. Razaviyan et al. found miR-3182 is up-regulated in breast cancer cell lines MDA-MB-231 than in normal breast cell line MCF-10A and miR-3182 may play a tumour-suppressive role by targeting mTOR and S6K1 Genes of mTOR signalling pathway in triple-negative breast cancer [26]. In this research, we revealed linc00858 was up-regulated especially in lung tissues and in lung cancer. And over-expressed linc00858 significantly increased the proliferation and invasion of A549 and H1299 cell lines, and inhibited the expression of miR-3182. When we over-expressed miR-3182 by co-transfected with miR-3182 mimics in A549 and H1299 cell lines transfected with LINC00858 over-expressed vector, the increased cell proliferation rates were rescued. Besides, we evaluated
the EMT markers such as β-catenin, E-cadherin, N-cadherin, vimentin by western blot and qPCR and found up-regulation of linc00858 significantly facilitated the EMT process in A549 and H1299 cells, while over-expression of miR-3182 inhibited the EMT process. By bioinformatics analysis we predicted that linc00858 may target miR-3182 and miR-3182 may target 3' UTR of MMP2 mRNA through complementary base pairing. Thus, we performed dual-luciferase reporter assay and demonstrated the direct binding of linc00858 and miR-3182, miR-3182 and 3'UTR of MMP2 mRNA. It seems linc00858 acted as a competitive endogenous RNA to sponged miR-3182 which can regulate the expression of MMP2 by targeting 3'UTR of MMP2 mRNA, and yet, promoted progress of lung cancer.

Our study provided new clues for understanding the mechanism of lung cancer and enriched the original literatures. Combined together the previous research and the current research, miR-3182/MMP2 axis may play an important role in various tumour types and further researches are urgently needed.

To sum up, we demonstrated that the long non coding RNA linc00858 could promote progress of lung cancer by competitively sponging miR-3182 which playing a tumour-suppressive role by targeting 3' UTR of MMP2 mRNA and therefore inhibiting EMT process. This research provided new clues for understanding the association between non-coding RNA and the development of lung cancer.

Disclosure statement

The authors declare no competing financial interests.

Author contribution

Wenguang Wang designed the research, Mingqiang Xue and Dongfeng Shi performed the experiments. Guanghui Xu analysed the data.

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