Biomedicine & Pharmacotherapy 108 (2018) 316–324

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

LINC00657 played oncogenic roles in esophageal squamous cell carcinoma by targeting miR-615-3p and JunB

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ARTICLE INFO

Keywords:
LINC00657
miR-615-3p
JunB
Esophageal squamous cell carcinoma
ceRNA

ABSTRACT

Background: The prognosis of esophageal squamous cell carcinoma (ESCC) is relatively poor due to the absence of efficient treatment. In this manuscript, we have investigated the specific roles and molecular mechanisms of LINC00657 to order to identify novel therapeutic targets for ESCC.

Method: The LINC00657 expression in ESCC tissues and cell lines were evaluated by quantitative real-time PCR. The expression of LINC00657 in ESCC cells was regulated by lentivirus transfection. Online bioinformatics analysis tools were used to predict the potential targets of LINC00657 and miR-615-3p. TCGA database was used to analyze the prognosis of ESCC patients. Transwell, wound healing assay and MTT were performed to investigate the ESCC cells' biological functions. JunB expression was evaluated by Western blot.

Result: LINC00657 was moderately increased in ESCC both in vivo and in vitro and up regulated by irradiation. LINC00657 knockdown could inhibit the migration and proliferation of ESCC cells. And downregulation of LINC00657 significantly enhanced the radio-sensitivity. Moreover, LINC00657 could act as a ceRNA to increase the expression of JunB by binding to miR-615-3p. Meanwhile, overexpression of miR-615-3p resulted in anti-tumor effects and led to the down-regulation of JunB. Survival analysis from TCGA indicated that ESCC patients with higher JunB expression had significant poorer prognosis.

Conclusion: LINC00657 might be involved in regulating ESCC's response to radiation; and it functioned as an oncogene in ESCC by targeting miR-615-3p and JunB, providing novel potential therapeutic targets.

1. Introduction

The incidence of esophageal squamous cell carcinoma(ESCC), which arises from the epithelia cells of the esophageal, is relatively high in developing countries [1]. According to data from SEER (Surveillance, Epidemiology, and End Results Program), the mean incidence of ESCC is around 0.5%; moreover, the 5-year survival rate of ESCC is about 18.8%, which is relatively low compared with other cancers, like breast cancer [2]. To date, the optimal treatment for ESCC is lacking: chemotherapy and surgery cannot significantly control the recurrence of ESCC [3]. Although, radiotherapy is a mainstay of ESCC treatment, some patients fail to benefit from radiotherapy because of the radioresistance. Therefore, it is urgent to investigate novel molecular mechanisms for ESCC initiation and progression to provide potential therapeutic targets for ESCC.

Long non-coding RNAs can be defined as transcripts which are longer than 200 nucleotides but cannot translated into proteins [4]. Increasing studies have reported that lncRNAs were involved in diverse biological functions, especially tumor progression, by modulating the abundance of interacting mRNAs and proteins [5]. In our previous

Abbreviations: ESCC, esophageal squamous cell carcinoma; SEER, Surveillance, Epidemiology, and End Results Program; TCGA, the cancer genome atlas; NORAD, Noncoding RNA Activated by DNA Damage; LINCRNA, long intergenic non-coding RNA; ceRNA, competing endogenous RNA; MTT, 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di- phenytetrazoliumromide; qRT-PCR, quantitative real time polymerase chain reaction; RIPA, Radioimmunoprecipitation assay; SDS-PAGE, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; ANOVA, analysis of variance

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https://doi.org/10.1016/j.biopha.2018.09.003
Received 27 May 2018; Received in revised form 2 September 2018; Accepted 3 September 2018

study, we have screened miR-615-3p as an oncogene in gastric cancer by microRNA microarray. In this manuscript, we have used StarbaseV2.0to predict LINC00657 as a potential upstream regulator for miR-615-3p and further investigated its role in ESCC cell linecut. LINC00657, also named as NORAD (Noncoding RNA Activated by DNA Damage), has been found associated with DNA damage-it can be up-regulated after DNA damage [6]. Recently, numerous studies have reported its oncogenic role in different cancers such as breast cancer [7], colorectal cancer [8] and hepatocellular carcinoma [9]. However, the specific role and molecular mechanisms of LINC00657 in ESCC is ambiguous.

In this study, we have found that LINC00657 was significantly increased in ESCC cells after irradiation treatment. Knockdown of LINC00657 could inhibit the invasion, migration and viability of ESCC cells. Furtherly, it could increase the expression of JunB by suppressing the expression of miR-615-3p. These findings revealed the oncogenic function of LINC00657 and its potential clinical significance.

2. Method

2.1. ESCC tissues samples

12 pairs of esophageal squamous cell carcinoma (ESCC) tissues and adjacent normal esophageal mucosa tissues were recruited from the 12 ESCC patients who were diagnosed at First Affiliated Hospital of Xi'an JiaoTong University. And all ESCC tissues were confirmed by pathology. Samples were stored at liquid nitrogen immediately after collection. This study has been approved by Ethnic Committee of Xi'an JiaoTong University.

2.2. Cell lines and cell culture

Human ESCC cell lines (Eca-109, TE-1, and KY-SE) and Human normal esophageal cell line (HEEC) were purchased from ATCC (American Type Culture Collection, Manassas, USA). Eca-109 was cultured in Dulbecco's modified Eagle's medium (high glucose) with 10% fetal bovine serum. KY-SE, TE-1 and HEEC were cultured in Roswell Park Memorial Institute 1640 medium with 10% fetal bovine serum. All cells were incubated at 37 ℃ in a 5% CO_2 incubator.

2.3. Cell transfection

Lentivirus particles, including LV-has-mir-615, LV-has-mir-615-3p inhibition, LV-sh-LINC00657, were designed and purchased from Genechem (Shanghai, China). Plasmids that contained LINC00657 3′UTR was designed and purchased from Genechem (Shanghai, China). To stably overexpress the miRNA-615-3p and knock down the miRNA-615-3p and LINC00657 in ESCC cells, KY-SE cells were infected with these lentivirals particles repectively. Puromycin (1.5 μg/mlor 2 μg/ml, MP, USA) was used to identify the stable cells after 48 h infection. Lipofectamine 2000 (Invitrogen, USA) was used to assist the transfection of plasmid. Transfection was performed according to the manufacturer's instructions. qRT-PCR analysis was performed to confirm the transfection efficiency.

2.4. Quantitative reverse transcription-PCR

Total RNA was extracted by using Trizol reagent which was purchased from Invitrogen (Carlsbad, USA). Next, RT reagent Kit, which was purchased from Takara, was used to synthesis the complementary DNA by reversely transcribing the RNA into DNA. SYBR Premix DimerEraser™ (Perfect Real Time) assay kits was used to perform the real-time PCR amplification reaction. The process was completed in the system of TL988-IV System (Tianlong, Xi'an, China). As for LINC00657, GAPDH was used as the quantitative control, as for miRNA-615-3p, U6 was used as the quantitative control. All PRC primers were shown as follows:

GAPDH-F 5′-GTCTCCTCTGACTTCAACAGCG-3′,
GAPDH-R 5′-ACCACCCTGTTGCTGTAGCCAA-3′
LINC00657-F 5′- TGATAGGATACATCTTGGACATGGA-3′
LINC00657-R 5′- AACCTAATGAACAAGTCCTGACATACA-3′
U6-F 5′- CTCGCTTCGGCAGCACATATACT-3′
U6-R 5′-ACGCTTCACGAATTTGCGTGTC-3′
miRNA-615-3p-F 5′-ACACTCCAGCTGGGTCCGAGCCTGGGTCTC-3′
miRNA-615-3p- R:5′-TGGTGTCGTGGAGTC-3′;

2.5. Western blotting

Total proteins were extracted by using RIPA (Radioimmunoprecipitation assay) and extraction buffer (Pioneer, China), and supplemented with phosphatase inhibitors (Roche, Switzerland) and protease inhibitors (Roche, Switzerland). SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) was used to separate the protein with different molecular weight. Then the proteins were transferred to polyvinylidene fluoride membranes (Millipore). The membranes were incubated with the primary antibody, including JunB primary antibody (1:1000) and β-tublin primary antibody (1:1000), at 4 ℃ overnight. And the next day, the membranes were incubated with the second antibody at room temperature for 1.5 h. The band was visualized by using chemiluminescence reagent (Millipore, USA) in ChemiDoc System (Bio-Rad, USA). The expression value of specific protein was quantified by Image-J software and the relative expression of protein was calculated by Specific protein/GAPDH.

2.6. Cell proliferation assay

To evaluate the cells proliferation abilities, ESCC cells were planted into 96-well plates at a density of 1.5 × 103 cells per well. The reagent MTT (3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di- phenytetrazoliumromide) (5 mg/ml 20ul) was added to each well at 24 h, 48 h, 72 h respectively. Cells proliferation abilities were revealed by measuring the absorbance at 490 nm at 24 h, 48 h, 72 h respectively. Furthermore, flow cytometric bromodeoxyuridine (BrdU) incorporation analysis was also performed to investigate the cell proliferation abilities. Cells were incubated with 30 μM BrdU labeling solution for 30 min at 37 ℃ in the CO2 incubator. Next, BrdU labeling solution from cells was removed and washed twice in PBS(phosphate- buffered saline). BrdU with PE-BrdU fluorescent antibodies has been stained for 30 min at 4 ℃ in the dark environment. The percentage of BrdU positive cells in each sample was analyzed by the FASC Calibur MT ow cytometer (BD Bioscience).

2.7. Transwell assay

24-Well Transwell Chamber was used to evaluate the migration abilities of ESCC cells. ESCC cells were cultured in serum-free medium for 24 h. Next, they were planted on the upper chambers (1×10^4 cells per well) for 36 h. Finally, the ESCC cells on the lower surface were fixed by methanol and stained by Giemsa. Images were captured under the microscopy.

2.8. Colony formation assay

The cells were plated in the 6-well plates with the concentration of 500 cells/well, 1000 cells/well, 2000 cells/well, 4000 cells/well, 8000 cells/well, which were respectively exposed to the graded doses X-ray (0, 2, 4, 6, 8 Gy). After 2 weeks, the medium was removed and the cells were washed by PBS. Cells were fixed by methanol and then cells were stained with 0.5% crystal violet for 15 min. Only the colony consisted of more than 50 cells could be counted. The survival fraction (SF) was calculated. And the survival lines were fitted by GraphPad Prism 6.0 by