

Extracellular vesicles promote esophageal cancer progression by delivering lncZEB1-AS1 between cells

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Abstract. – OBJECTIVE: To explore the expression of extracellular vesicle-derived lncZEB1-AS1 in esophageal cancer and its role in esophageal cancer progression.

PATIENTS AND METHODS: The extracellular vesicles (EVs) from esophageal cancer patients (n = 26) and normal subjects (n = 26) were isolated by differential centrifugation. The expression of lncZEB1-AS1 in EVs was detected by Real-time PCR (polymerase chain reaction). The clinical data of normal subjects and patients were analyzed. In addition, the concentration of EVs and lncZEB1-AS1 in blood samples from normal subjects and esophageal cancer patients were assessed. After co-culture of esophageal cancer cell line EC109 and EVs with or without lncZEB1-AS1 knockdown, cell proliferation was detected by CCK-8 assay. The possible target microRNAs of lncZEB1-AS1 in cytoplasm were predicted with miRcode, followed by correlation analysis of lncZEB1-AS1 and miR-214. Through literature review, lncZEB1-AS1 positively regulates ZEB1 expression, which was consistent with our result.

RESULTS: Quantitative Real-time PCR showed that the serum levels of EVs and the content of lncZEB1-AS1 in EVs from esophageal cancer patients were significantly higher than those in normal controls. lncZEB1-AS1 was over-expressed in esophageal cancer cells co-cultured with EVs of esophageal cancer patients. CCK-8 results indicated that EC109 cells co-cultured with EVs of esophageal cancer patients had stronger proliferative capacity. miRcode showed that miR-214 ranked the first of microRNAs that lncZEB1-AS1 might target, and miR-214 expression was significantly increased after lncZEB1-AS1 knockdown in EC109. After over-expressing lncZEB1-AS1 in EC109 or co-culturing EVs of esophageal cancer patients with EC109 cells, we found that lncZEB1-AS1 positively regulates ZEB1. In contrast, interfering with the expression of lncZEB1-AS1 in esophageal cancer

cell lines can effectively reduce the expression of ZEB1.

CONCLUSIONS: EVs in the peripheral blood from esophageal cancer patients promote esophageal cancer progression by delivering lncZEB1-AS1 to esophageal cancer cells and targeting miR-214.

Key Words:

Extracellular vesicles (EVs), lncZEB1-AS1, Esophageal cancer, miR-214.

Introduction

Esophageal cancer is one of the most common malignant tumors in the world. Its five-year survival rate is less than 30%¹. Esophageal cancer is divided into two categories by histopathological classification²: squamous cell carcinoma and adenocarcinoma. Most of esophageal carcinomas are squamous cell carcinoma, accounting for almost 90% of all esophageal carcinomas. The majority of squamous cell carcinoma cases are found in Asian countries, including Kazakhstan, Iran, Central and Northern China. Esophageal adenocarcinoma cases are mainly found in the United States, Australia and Western European countries³. Esophageal squamous cell carcinoma is the most common pathological type in China, accounting for more than 90% of the total number of patients⁴ and has poor prognosis as well. Notwithstanding, one of the key strategies in reducing esophageal cancer mortality is early diagnosis and treatment due to the large number of deaths each year⁵. The risks of esophageal cancer include environmental and genetic factors. Current research indicates that people with

unhealthy eating and sedentary lifestyle habits are more likely to develop esophageal cancer⁶. In recent years, despite increasing advances in the treatment of esophageal cancer, the overall prognosis of patients with esophageal squamous cell carcinoma is still not optimistic. Early detection and intervention are of great significance to improve the prognosis of esophageal cancer.

The extracellular vesicles (EVs) are globular membrane vesicles formed by lipid bilayers and are subcellular components released by cells under spontaneous or certain conditions. They are essentially a group of nano-sized particles including apoptotic bodies, membrane particles, and microvesicles⁷.

Almost all cells can produce EVs. These bilayer vesicles contain lipid, protein, nucleic acid (DNA, mRNA, microRNA, lncRNA, circRNA, non-coding RNA) and other bioactive ingredients derived from the parent cell, which are packaged and carried on to recipient cells to modulate their functions⁸. They are involved in the inflammatory immune response, cell signaling, cell survival and apoptosis, angiogenesis, thrombosis, autophagy and other cellular functions. More importantly, EVs could maintain physiological stage and is involved in the progress of some diseases. Specific types of extracellular vesicles are expected to become new molecular markers that will aid in the diagnosis and prognosis of diseases as well as have broad prospects in anti-tumor therapy, regenerative medicine and immunomodulation⁹. This will open up new avenues for stem cells treatment in non-cellular pathways and revolutionize clinical treatment as a natural vector for vaccines or drugs.

The molecular mechanism of EVs secretion and their role in modulating biological functions of specific components and signaling pathways by targeting cells are still under studied. However, in recent years, lncRNAs and circRNAs have begun to gain a massive research attention. Long non-coding RNAs (lncRNAs) are a class of RNA molecules with over 200 nt in length. Functionally, lncRNAs do not encode proteins. However, studies¹⁰ have pointed out that lncRNAs could regulate gene expressions at epigenetic, transcriptional and post-transcriptional levels. lncRNA was initially considered as a noise of genomic transcription and by-product of RNA polymerase II transcription without any biological function. However, recent studies have shown that lncRNAs are involved in many important regulatory processes such as X-chromosome si-

lencing, genomic imprinting, chromatin modification, transcriptional activation, transcriptional interference, and nuclear transport, thus, gaining widespread attention. In mammalian genomic sequences, 4% to 9% of the transcripts are lncRNA (corresponding protein-coding RNA ratio is 1%). Although the research on lncRNA has progressed rapidly in recent years, the function of most lncRNAs remains unclear¹¹. Numerous studies have shown that in tumor cells, the expression of certain specific lncRNAs may be altered. This change in expression level can serve as a marker for cancer diagnosis and potential drug targets. Currently, there are few lncRNAs that have been found to be clinically valuable in esophageal cancers with most of the current literature researching on HOTAIR, LOC285194, linc-POU3F3 and CCAT2¹². Extensive studies are therefore needed to elucidate the relationship between esophageal cancer and long-chain non-coding RNAs.

lncZEB1-AS1 is a lncRNA (ZEB1-AS1) derived from the ZEB1 promoter region¹³. Until now, little is known about its expression, role and mechanism. In this present analysis, we explored the expression of EV-derived lncZEB1-AS1 in esophageal cancer and its role in esophageal cancer progression.

Patients and Methods

Patients

A total of 21 patients who were histopathologically diagnosed as esophageal cancer from September 2015 to January 2017 were selected as the experimental group and 21 healthy people were selected as the control group. Blood sample of each subject was collected for EVs isolation. This study was approved by the Ethics Committee of China-Japan Union Hospital of Jilin University. Signed written informed consents were obtained from the patients and/or guardians.

Cell Culture

The esophageal cancer cell line EC109 was cultured with RPMI-1640 (Roswell Park Memorial Institute-1640) medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) in a 37°C, 5% CO₂ saturated humidity incubator. EC109 was provided by the American Type Culture Collection (ATCC, Manassas, VA, USA) and was passaged at a cell confluence of 80% at a 1:2 seeding density.

Mice Xenograft Model

EC109 cells were typsinized and collected by centrifugation. Cell viability was confirmed to be above 95% based on trypan blue staining. The cells (2×10^6) in 50% Matrigel (BD Biosciences, San Jose, CA, USA) were inoculated subcutaneously (2×10^6) into the right flank of 4- to 6-week-old female mice (The Animal Center, Nanjing Medical University, Jiangsu, China).

EVs Isolation and Co-Cultured with Esophageal Cancer Cells

EVs isolation was performed using conventional differential ultra-centrifugation at 4°C with TL-100 ultracentrifuge (Beckman Coulter, Miami, FL, USA). The EVs were collected as pellets and resuspended in RPMI-1640 medium without FBS (fetal bovine serum). EC109 cells were seeded in 12-well plates overnight after which 200 µg of EVs were added to each well. After 24 hours of incubation, EC109 cells were collected for qRT-PCR and quantitative protein assays.

siRNA and pcDNA Transfection

The cells were seeded in 6-well plates and transfected at a confluence of 60% with 10 µL lipofectamine 2000 (pcDNA with Entranster-R4000) supplemented with 10 µL 20 nM siRNA-ZEB1 suspended in 500 µL serum-free suspension and 1.5 mL 1640 medium (Gibco, Rockville, MD, USA). The control group of cells was treated with the same amount of lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and siRNA-control as the experimental group. The medium was changed 6 h after transfection. Si-ZEB1: 5'-GCUGAAA-GUCAAGCAAGCAdTdT-3', 5'-UGCUUGCU-UGACUUUCAGCdTdT-3'.

qRT-PCR

Total RNAs were extracted from tissue according to TRIzol protocol. The qRT-PCR is progressed in 50 µL reaction system, using the following conditions: reverse transcription reaction at 50°C for 30 min and denatured reverse transcriptase reaction at 92°C for 3 min. PCR was performed as the follows: denaturation at 92°C for 10 s, annealing at 55°C for 20 s and extension at 68°C for 20 s, for a total of 40 cycles. U6 and β-actin were used as internal controls for EVs and cells respectively. The $2^{-\Delta\Delta CT}$ method is used to calculate the relative expression. β-actin primer sequence: upstream primer: 5'-CTC-CATCCTGGCCTCGCT-GT-3', downstream primer: 5'-GCTGTCACCTTCACCGT-TCC-3'.

LncRNA-ZEB1-AS1 primer sequence: forward: 5'-ATTGTTAGGAAAGGTTATAAAAATTT-3'; reverse: 5'-ACCCAAACTATATAAAAAATTA-CAC-3'.

CCK8 Assay for Cell Proliferation

The transfection time point was 0 h. The control and treatment groups were inoculated into 96-well plates, each containing 5×10^3 cells and replicated in 6 wells. Five 96-well plates were inoculated repeatedly. After 6 hours, the activity of adherent cells was measured (0 h). Afterwards, 20 µL of cell counting kit-8 (CCK8, Dojindo, Kumamoto, Japan) solution was added to each well at 24 h, 48 h, 72 h and 96 h and then placed in 37°C and 5% CO₂ incubator for 2-3 h. This was followed by measuring the optical density (OD) using a microplate reader at a wavelength of 450 nm. Only CCK8 solution and media (without cells) were added into the control wells.

Western Blot

RIPA was used for cell lysate. Total proteins of the cells were extracted. The concentration of each protein sample was determined by a BCA kit (Beyotime, Shanghai, China). Briefly, 50 µg of total protein were separated by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) under denaturing conditions and transferred to PVDF (polyvinylidene difluoride) membranes. Membranes were blocked with 5% skimmed milk, followed by the incubation of specific primary antibodies (Cell Signaling Technology, Danvers, MA, USA) at a dilution of 1:1000 overnight. After washed with PBS for three times, membranes were then incubated with the HRP (horseradish peroxidase) labeled sheep anti-rabbit secondary antibody (1:5000) at room temperature for 1 h. Immunoreactive bands were exposed by enhanced chemiluminescence (ECL) method.

Statistical Analysis

Statistical Product and Service Solutions (SPSS22.0, IBM Corp., Armonk, NY, USA) and GraphPad Prism 6.0 (La Jolla, CA, USA) were used for data analysis and image editing respectively. *t*-test was used for the comparison of measurement data. Measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$). Classification data was measured by χ^2 -test. $p < 0.05$ was considered as statistically significant; * $p < 0.05$, ** $p < 0.01$.

Results

The Number of Serum Derived EVs and Extracellular lncZEB1-AS1 in Esophageal Cancer Patients Were Higher Than That of the Control Group

We selected the 21 patients who were diagnosed of esophageal cancer by histopathological examinations as the experimental group and 21 healthy people as the control group from September 2015 to January 2017. First, we extracted

the EVs from 100, 200 and 400 μ L of plasma from the healthy controls and measured the EV concentration, showing that the plasma volume was roughly in linear with the EVs in it (Figure 1A). The plasma of esophageal cancer experimental group and control group was separated and the concentrations were determined. The results showed that the concentration of plasma-derived EVs in esophageal cancer patients was significantly higher than that in healthy controls (Figure 1B).

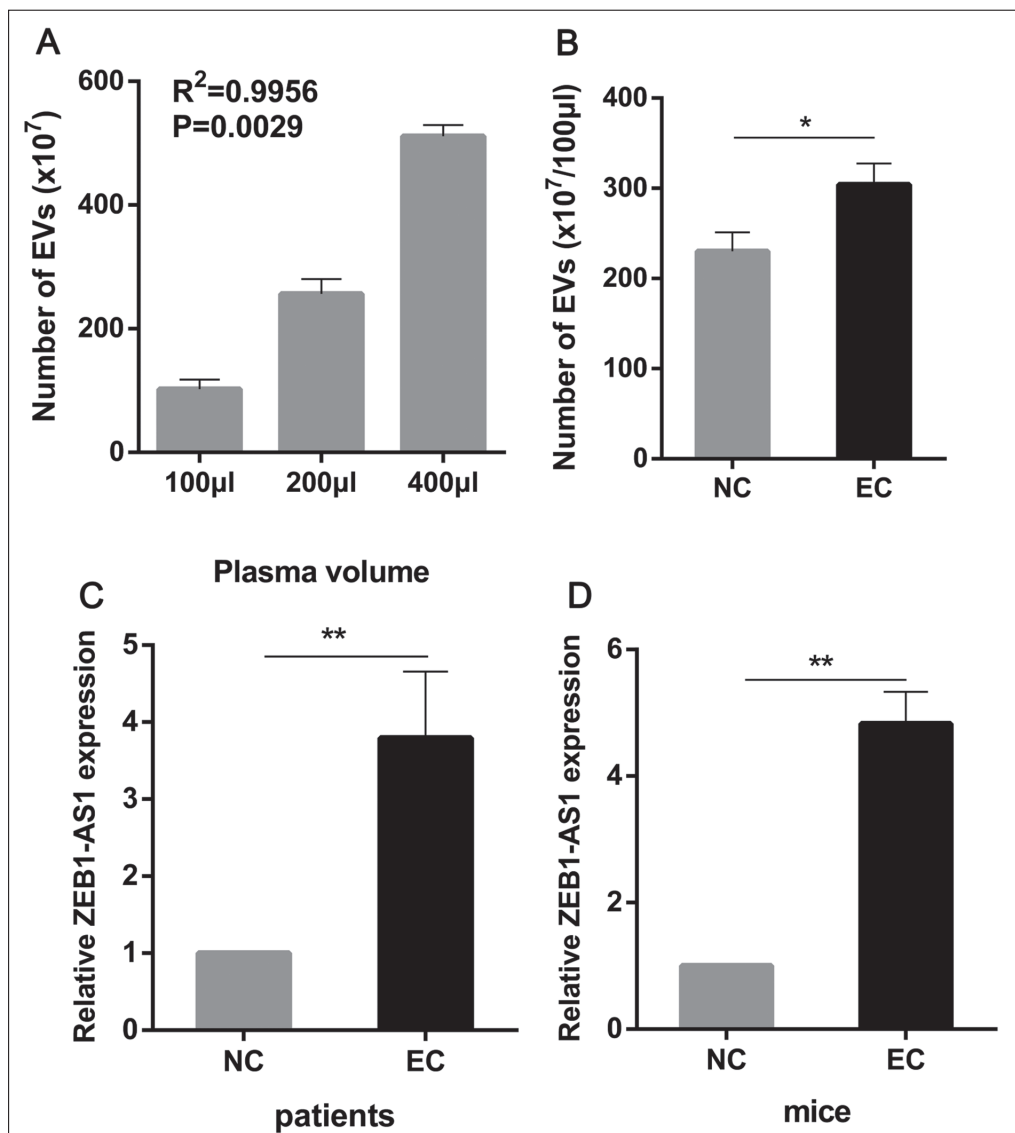


Figure 1. The number of serum derived EVs and extracellular lncZEB1-AS1 in esophageal cancer patients and healthy donors. **A**, The number of EVs isolated from 100, 200, 400 μ L of plasma from healthy donors are dose-dependent ($R = 0.9956$; $p = 0.0029$). **B**, The concentration of EVs isolated from the plasma of esophageal cancer patients was significantly higher than that of the normal control group ($n = 21$). **C**, The expression of EV-derived lncZEB1-AS1 in plasma of esophageal cancer patients was significantly higher than that of the normal control group ($n = 21$). **D**, The expression of lncZEB1-AS1 in plasma-derived extracellular vesicles of mice bearing subcutaneous tumor of esophageal cancer cell was significantly higher than that of normal control group ($n = 6$).

We next extracted the RNA from EVs and results showed a higher concentration of lncZEB1-AS1 in plasma-derived EVs of esophageal cancer patients than that of healthy controls (Figure 1C). Experiments carried out in animals showed the same result. We subcutaneously inoculated mice with EC109 esophageal cancer cell lines, followed by isolating EVs from 500 μ L of blood from mice. Quantitative PCR results showed that the expression of lncZEB1-AS1 in plasma-derived EVs of mice bearing esophageal cancer cell lines was significantly higher than that of the normal control group (Figure 1D). Overall, the EVs concentration in plasma of patients with esophageal cancer and lncZEB1-AS1 in plasma-derived EVs were higher than that of the normal control group.

EVs Can Transmit lncZEB1 to Esophageal Cancer Cells

To further explore the effect of EVs on esophageal cancer cells *in vitro*, we first examined the relative expression of lncZEB1-AS1 in esophageal cancer cells, plasma-derived EVs of esophageal cancer patients and healthy human subjects (GAPDH and U6 were used as internal control for esophageal cancer cells and EVs respectively). Quantitative PCR results showed that the relative expression of lncZEB1-AS1 in esophageal cancer cells and normal plasma-derived EVs was lower than that in plasma-derived EVs from esophageal cancer patients (Figure 2A). Additionally, we observed a significant increase in lncZEB1-AS1 expression in esophageal cancer cells after co-culturing EC109 with plasma-derived EVs

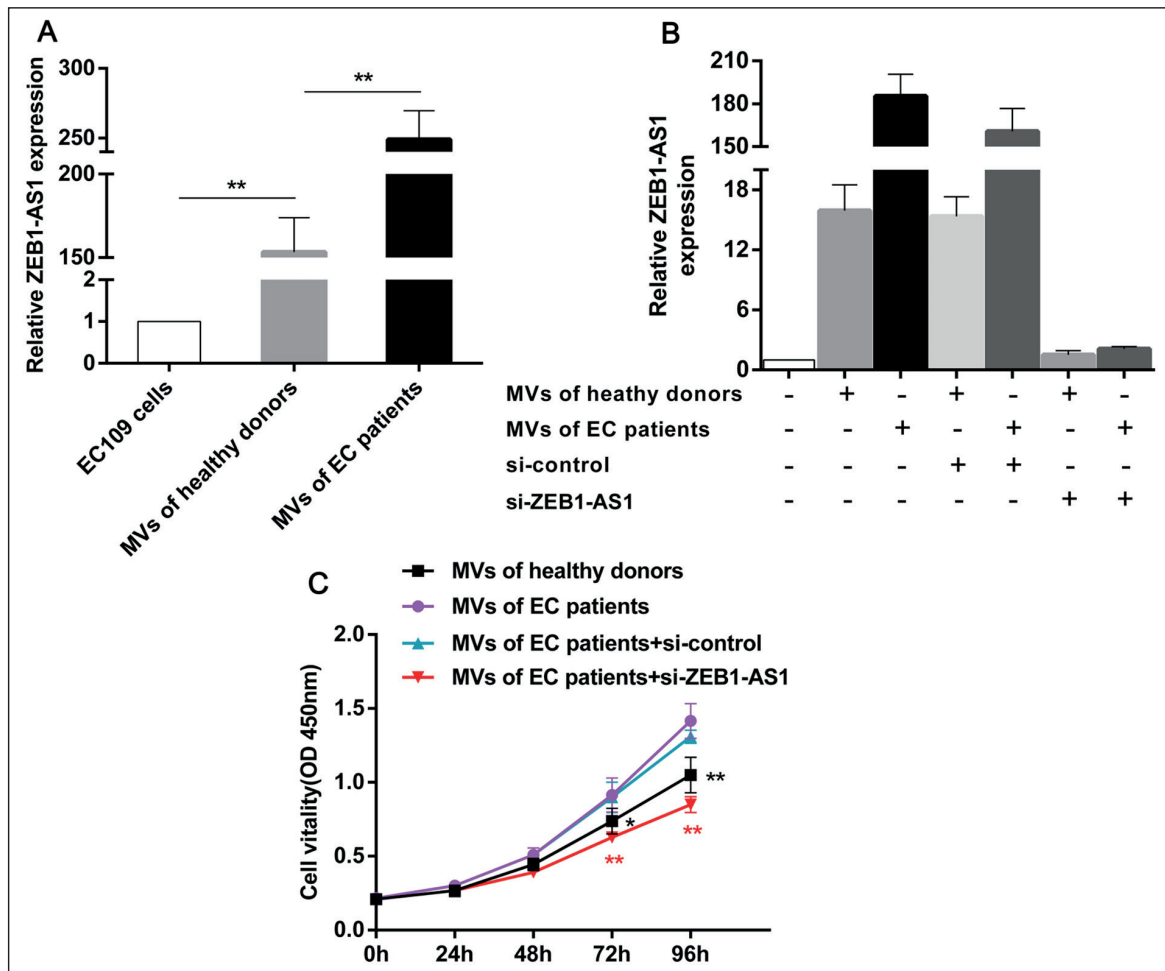


Figure 2. Transmission of lncZEB1 by EVs to esophageal cancer cells. **A**, The expression of EV-derived lncZEB1-AS1 in esophageal cancer cells and normal human plasma were lower than that in plasma of esophageal cancer patients. **B**, The expression of lncZEB1-AS1 in esophageal cancer cells significantly increased, when esophageal cancer cells were co-cultured with plasma-derived EVs. This effect can be reversed by transfecting si-ZEB1 into esophageal cancer cells. **C**, The results of CCK-8 showed that the proliferation of esophageal cancer cells was significantly promoted when esophageal cancer cells were co-cultured with plasma-derived EVs from esophageal cancer patients.

from esophageal cancer patients. This increase, however, was reversed when esophageal cancer cells were transfected with si-ZEB1 (Figure 2B). CCK-8 results showed an increased proliferation of esophageal cancer cell after co-cultured with EVs extracted from esophageal cancer patients (Figure 2C). In summary, EVs can transmit lncZEB1 to esophageal cancer cells, promoting esophageal cancer cell proliferation.

lncZEB1 Delivered by EVs May Target Downstream miR-214

To explore the regulatory mechanisms of lncZEB1-AS1 in target cells, we used miRcode.org to predict the possible target miRNAs of lncZEB1. miRcode website showed miR-214 as

the highest microRNA candidate that could be targeted by lncZEB1-AS1 (Figure 3A), and miR-214 expression was significantly increased after interference with lncZEB1-AS1 in EC109 (Figure 3B). This data suggest that, lncZEB1 delivered by EVs may act on esophageal cancer cells by targeting miR-214.

lncZEB1 Can Promote the Proliferation of Esophageal Cancer Cells by Upregulating the Expression of Downstream ZEB1

In previous studies, lncZEB1-AS1 has been found to positively regulate the expression of ZEB1. As a result, the change of ZEB1 expression in EC109 cells after co-culturing EVs from

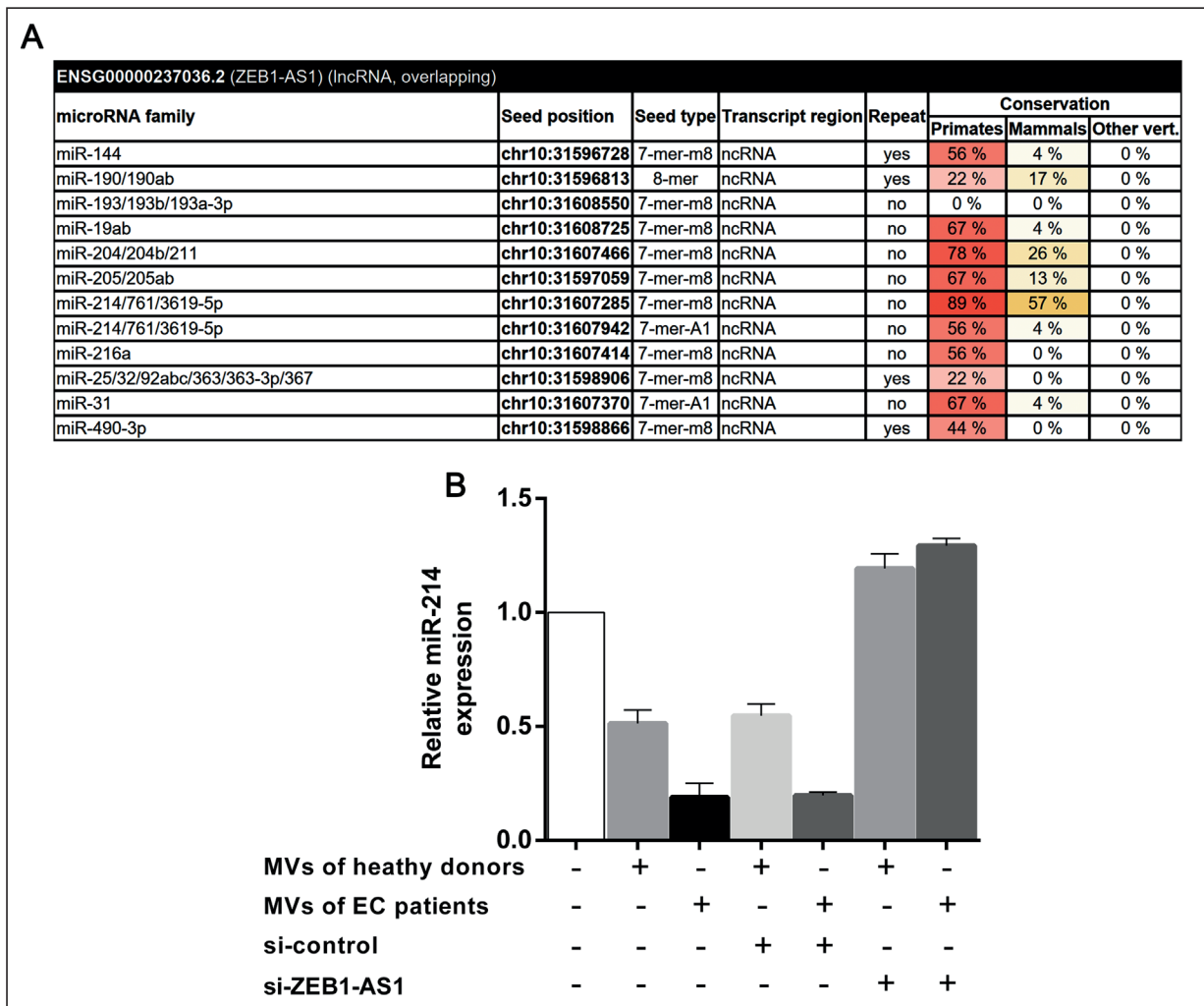


Figure 3. lncZEB1 delivered by EVs may target downstream miR-214. **A**, miRcode predicted possible target miRNAs of lncZEB1-AS1. **B**, Contents of miR-214 in esophageal cancer cells significantly decreased when esophageal cancer cells were co-cultured with plasma-derived EVs from esophageal cancer patients. This effect can be reversed by transfecting si-ZEB1 into esophageal cancer cells.

esophageal cancer patients with EC109 cells was measured. Western blot results showed that the expression of ZEB1 in EC109 cells was significantly upregulated after co-culturing EC109 with EVs from esophageal cancer patients (Figure 4A). The previous results indicated that ZEB1 was positively correlated with lncZEB1-AS1 expression. ZEB1 expression was interfered in EC109 cells co-cultured with EVs from esophageal cancer patients, and CCK-8 results showed a significant decrease in proliferation of esophageal cancer cells (Figure 4B). However, there was no significant change in lncZEB1-AS1 expression after ZEB1 inhibition (Figure 4C). In summary,

EVs-mediated delivery of lncZEB1 to esophageal cancer cells, promotes esophageal cancer cell proliferation by upregulating the expression of downstream ZEB1.

Discussion

Esophageal cancer is one of the most malignant tumors in the world, which seriously endangers people's health¹⁴. According to data released by World Health Organization, the incidence of esophageal cancer ranked the 9th of all malignant tumors in the world, about 7.0/100,000. The

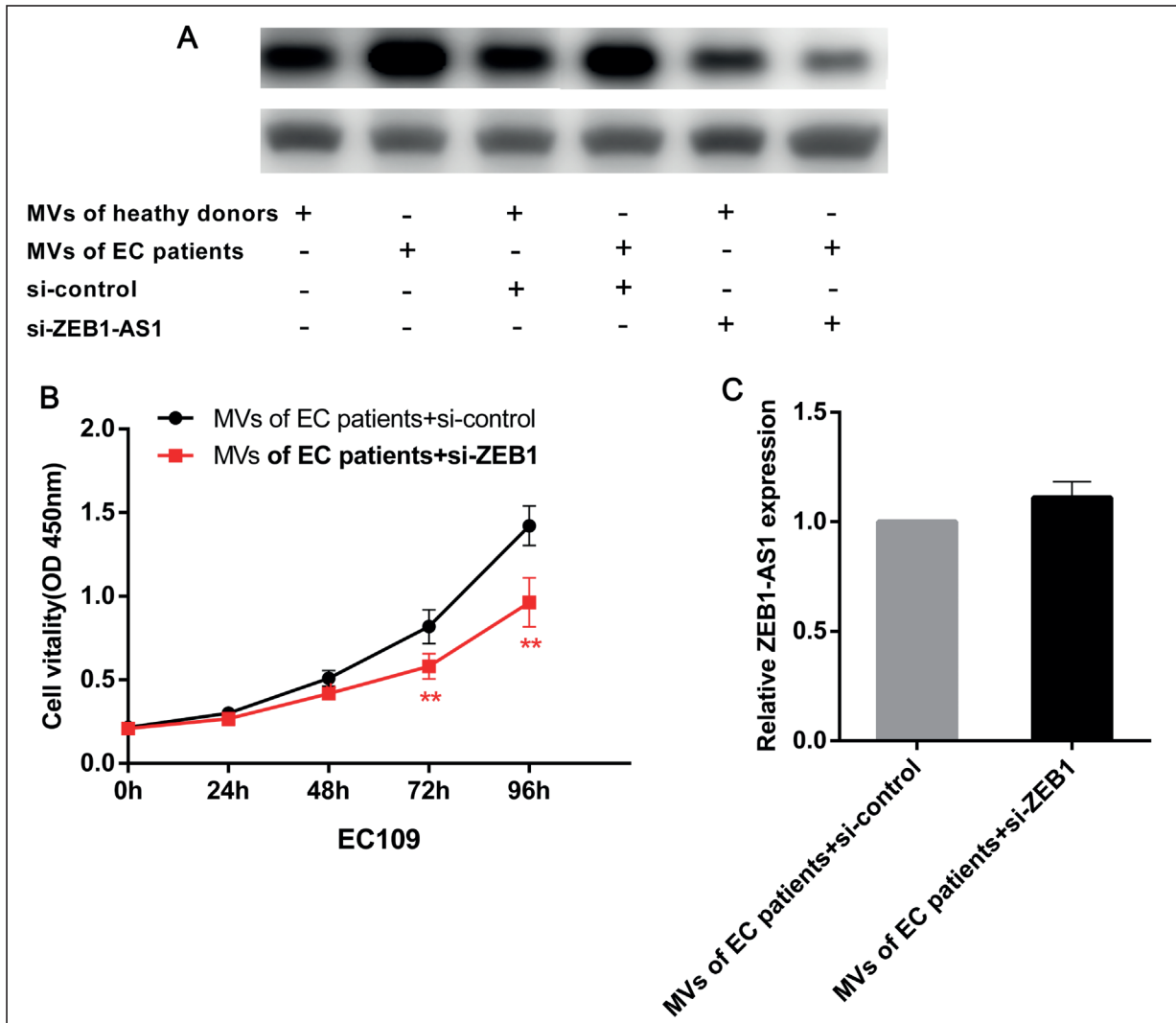


Figure 4. LncZEB1 promoted the proliferation of esophageal cancer cells by upregulating the expression of downstream ZEB1. **A**, The expression of ZEB1 in EC109 cells was significantly upregulated when co-cultured with plasma-derived EVs from esophageal cancer patients. **B**, CCK-8 assay results showed that the proliferation of esophageal cancer cells was significantly attenuated, after interference with ZEB1 in EC109 cells. **C**, There was no significant change in the expression of lncZEB1-AS1 after interference with ZEB1 in EC109 cells.

mortality rate of esophageal cancer ranked 8th in all malignant tumors, it is about 5.8/100,000¹⁵. In China, the incidence of esophageal cancer ranks 5th and the mortality rate ranks 4th in the country. There are more than 450,000 esophageal cancer patients worldwide and the incidence is on the rise¹⁶. However, most of patients were in advanced disease when first diagnosed due to the lacked effective diagnosis. The overall survival rate of esophageal cancer is still low, with the five-year survival rate of only 15-25%¹⁷. With the development of Translational Medicine, looking for specific tumor markers is key to early diagnosis and providing opportunities for radical surgery. At the same time, molecular targeted therapy¹⁸ is another option for cancer patients after surgery, radiotherapy and chemotherapy¹⁹, which specifically acts on tumor cells and inhibits oncogene function and thus inhibits the growth of tumor cells²⁰. Currently, targeted therapy has become vital in the treatment of cancer. Up-to-date, the exact mechanism of esophageal cancer is not yet clear, hence it is imperative to investigate and explore the value of esophageal cancer molecular markers and target molecules, so as to provide novel targets for the early diagnosis and individualized treatment.

Extracellular vesicles refer to multifoamed bodies formed by endocytosis of body cells. These vesicles are released by cell membrane fusion and secreted to the extracellular environment at physiological and pathological conditions. According to the diameter of EVs, EVs can be divided into three groups: apoptotic bodies (>1000 nm), microvesicles (100-1000 nm) and exosomes (30-100 nm)²¹. Initially, EVs were only viewed as “trash bags” of cells to remove unwanted macromolecules, but now they are thought to be carriers of intercellular signals and can be used for intercellular communication. EVs surface protein signaling molecules recognize target cells and are uptaken through receptor ligand binding or endocytosis to alter the physiology and pathology of target cells²². A large number of studies have shown that abnormal cells also secrete EVs, and a variety of its contents significantly altered. Compared with traditional disease diagnostic markers, EVs can be stably present in body fluids and have a long half-life²³. Researches show that 2% of the human genome produces transcripts that encode RNA and the remaining 98% are non-coding RNAs that can't be translated into proteins, but are widely involved in human physiology and

pathology activities – tumor occurrence and development. Non-coding RNAs are divided into two major groups according to size: short-chain non-coding RNAs (e.g., miRNAs) and long non-coding RNAs (LncRNAs)²⁴. LncRNAs generally exceed 200 bp in length and lack a clear open reading frame, so they do not encode proteins and regulate gene expression levels in the form of RNA²⁵. LncRNA is widely involved in various biological processes²⁶, and abnormal expression of LncRNA is closely related to various diseases including cancer. Specific tumor-associated LncRNA has become a hot spot in the early diagnosis and treatment of tumors. Although lncRNAs have been implicated in many disease conditions, their mechanism in esophageal cancer development is not yet clear.

In summary, our study found that lncZEB1 is overexpressed in plasma-derived EVs from esophageal cancer patients and could be transmitted to esophageal cancer cells, promoting esophageal cancer progression.

Conclusions

We observed that EVs in the peripheral blood of esophageal cancer patients promote esophageal cancer progression by delivering lncZEB1-AS1 to esophageal cancer cells and targeting miR-214.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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