miR-9-5p Promotes β-Catenin-Activated Epithelial-To-Mesenchymal Transition of Triple-Negative Breast Cancer Via Targeting LZTS2

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Abstract

Background: Leucine zipper tumor suppressor 2 (LZTS2), an emerging tumor-suppressor, is attenuated in multiple cancers including prostate, lung and colon cancer. However, its expression and upstream regulatory mechanisms in triple negative breast cancer (TNBC) still remain unknown.

Materials and methods: The expression of LZTS2 in TNBC and matched para-carcinoma tissues was detected with immunohistochemistry. The correlations between LZTS2 expression and clinicopathological parameters were analyzed. Kaplan-Meier analysis was performed to determine the prognostic role of LZTS2 for TNBC patients. CCK-8, wound healing and transwell assay were used to detect the effect of LZTS2 overexpression on the proliferation, migration and invasion ability, respectively. The bioinformation algorithms were used to reveal the potential upstream regulatory miRNA. Then, dual-luciferase reporter assay was performed to confirm the regulatory effect of the chosen miRNA on the expression of LZTS2. miR-9-5p inhibitor was used to determine the effect of miR-9-5p on the subcellular localization of β-catenin. Then, western blotting was performed to reveal the effect of miR-9-5p on EMT-related proteins in TNBC cells. Xenograft tumor model was established to reveal the effect of miR-9-5p on TNBC progression in vivo.

Results: Low expression of LZTS2 was observed in 62 of 95 cases of TNBC tissue. Low expression of LZTS2 was correlated with poor postoperative DFS and OS of TNBC patients. LZTS2 could inhibit the proliferation, migration and invasion ability of TNBC cells. LZTS2 could be downregulated by miR-9-5p in TNBC, and the nuclear export of β-catenin was suppressed. Consequently, miR-9-5p inhibitor downregulated E-cadherin and upregulated N-cadherin, Twist and Vimentin in TNBC cells. Xenograft tumor model showed that miR-9-5p inhibitor could upregulate the expression of LZTS2 and induce nuclear export of β-catenin in TNBC.

Conclusions: miR-9-5p contributes to β-catenin-activated EMT via downregulating LZTS2, and thus promotes TNBC progression.

Introduction

Breast cancer ranks first in the female malignant tumor, threatening women's health worldwide [1]. Breast cancer can be classified into four subtypes including Luminal A, Luminal B, HER-2 overexpression and triple-negative, according to the expressions of ER, PR, ki-67 and HER-2 [2]. Among these four subtypes, triple-negative breast cancer (TNBC) still hold the poorest prognosis. Despite the improvement of treating approaches including surgery, chemotherapy, radiotherapy and immunotherapy, the overall survival of TNBC patients remains unsatisfactory. Aberrant silence of some tumor suppressor genes plays crucial roles in the progression of TNBC [3]. Taken above, deepening comprehension on the mechanisms of aberrant tumor suppressor gene expression is necessary for establishing novel treatment strategies for improving the prognosis of TNBC patients.
Leucine zipper tumor suppressor 2 (LZTS2) belongs to LZTS family which is related to transcription and cell cycle regulation [4]. LZTS2 is located at human 10q24.3, which is proximate to the site of the classical tumor suppressor gene PTEN, indicating that LZTS2 might exert tumor suppressing effects [5]. The expression of LZTS2 is attenuated or even lost in in numerous malignant tumors, such as laryngeal squamous cell carcinoma, prostate cancer, non-small cell lung cancer (NSCLC) and colon cancer [5–8]. As far as we know, the expression, function, and clinical significance of LZTS2 in TNBC still remains elusive.

Encyclopedia of DNA Elements (ENCODE) project has confirmed that the most human transcripts are non-coding RNAs including microRNAs (miRNAs) [9]. miRNAs are evolutionarily conserved single-stranded RNAs that consist of about 21–24 nucleotides [10]. miRNAs can bind to 3’-untranslated regions (UTRs) of target mRNAs to trigger their degradation or translation inhibition [11]. miR-9-5p is a recognized miRNA correlated with cancers, but its concrete function varies due to the differences of target mRNAs. For instance, miR-9-5p could suppress tumor proliferation and migration in gastric cancer by targeting neuropilin-1 [12], but it acted as a oncogene promoting angiogenesis and invasion by inhibiting SOCS5 in cervical cancer [13]. As for TNBC, miR-9-5p was identified as an upregulated miRNA related to regulation of transcription [14]. However, the mechanisms of miR-9-5p in promoting TNBC are still not clear.

In this study, we evaluated the expression and clinical significance of LZTS2 in TNBC. Then, for elucidating the mechanisms of LZTS2 attenuation in TNBC, we identified the effect of miR-9-5p on LZTS2 expression in TNBC cell lines. The results showed that miR-9-5p contributed to the progression of TNBC via downregulating LZTS2 expression in vitro and in vivo. Therefore, we speculated that miR-9-5p might be potential biomarker for the diagnosis and treatment of TNBC.

**Materials And Methods**

**Patients and Specimens**

The specimens of TNBC tissues and matched para-carcinoma tissues were collected from 95 patients who underwent radical operation at the Fourth Hospital of Hebei Medical University (Shijiazhuang, China) between Oct. 2015 and Mar. 2016. The median patient age at the time of surgery was 50 years (range: 29 - 74 years). None of the TNBC patients received preoperative radiotherapy, chemotherapy or immunotherapy. The clinical stage and histological tumor type were determined according to the International Union Against Cancer (UICC) Classification of 2009 (seventh edition). Patient's clinical information including age, TNM stage, invasion range, lymph node metastasis, was collected and stored in a database. All participant information was updated every 3 months by telephone follow-up. Complete follow-up was updated until death or Oct. 2020. Carcinoma tissue and para-carcinoma tissue specimens were collected and treated promptly after surgery. This research was approved by the ethic committee of the Hebei Medical University. All informed consents were signed by patients.

**Reagents and materials**
Antibody to LZTS2 (PA5-60871) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibodies to β-catenin (ab22656), E-cadherin (ab212059), N-cadherin (ab76011), Twist (ab50581), Vimentin (ab92547), and GAPDH (ab181602) were purchased from Abcam (Cambridge, UK). GoTaq qPCR Master Mix was purchased from Promega (Madison, WI, US). Revert Aid First Strand cDNA Synthesis Kits were purchased from Thermo Fisher Scientific.

Cell lines

The human TNBC cell lines (MDA-MB-468, HCC-1937, MDA-MB-231, MDA-MB-436, MDA-MB-453) and human normal mammary epithelial cell line (MCF-10A) were obtained from the Research Center of Hebei Medical University (Shijiazhuang, China). All of the employed cell lines were routinely cultured in medium DMEM (Thermo Fisher Scientific) containing 10% fetal bovine serum (FBS) (Gibco, New York, NY, USA) with 1% Pen/Strep and 1% non-essential amino acids (Solarbio, Beijing, China), at 37 ºC in the humidified atmosphere of the 5% CO₂ incubator.

Immunohistochemistry assay

Immunohistochemistry (IHC) was performed by using streptavidin-peroxidase (SP) method. Briefly, sections (5-μm thick) were pretreated using sodium citrate buffer (pH 6.0; 0.01 mol/l; Solarbio) at 98°C for 5 min to retrieve cell antigens, then blocked with goat serum at room temperature for 20 min. After incubated with antibody to LZTS2 at a dilution of 1:100 overnight at 4°C, the sections were biotinylated secondary antibody and streptavidin-biotinylated horseradish peroxidase complex (Zsbio, Beijing, China). Diaminobezidin (DAB, Zsbio) was used as color developer. In each experiment, negative control sections were treated similarly with phosphate-buffered saline (PBS) instead of the primary antibody. The expression was visualized and classified based on the percentage of positive cells and the intensity of staining according to the 0 - 4 semi-quantitative system [15].

quantitative real-time PCR

To quantify LZTS2 mRNA expression in TNBC and normal mammary epithelial cells, total RNA was extracted from cells with TRIlol reagent (Invitrogen), and cDNA was synthesized using TaqMan MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. Quantitative real-time PCR (qRT-PCR) analysis was performed using an ABI Prism 7900-HT Sequence Detection System (96-well, Applied Biosystems). LZTS2 Forward: 5’-TACCATCTGAGTTGCTGATTGC-3’, Reverse: 5’-AGAGAGGAAGGAATGGGAGATC-3’; GAPDH Forward: 5’-AGCCACATCGCTCACACAC-3’, Reverse: 5’-GCCCAATACGACCAAATCC-3’. U6 Forward: 5’-CTGCTTCGCGCACGATA-3’, Reverse: 5’-AACGCTTCACGAATTTCG-3’. The relative expression of LZTS2 and miR-9-5p was normalized to GAPDH expression. LZTS2 mRNA expression levels of TNBC cell lines were compared with that of MCF-10A cells. Experiments were performed in triplicate.

Western blotting
TNBC cells were lysed with 250 µl of lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, PH 8.0, 0.2 mM Na3VO4, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% NP-40). The lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were electrotransferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membranes were incubated in PBS containing 5% bovine serum albumin for 2 h at room temperature, followed by overnight incubation at 4°C with different dilutions of the primary antibodies, including antibodies to LZTS2 (1:1000), β-catenin (1:1000), E-cadherin (1:2000), N-cadherin (1:1500), Twist (1:2000), Vimentin (1:2000), and GAPDH (1:2000). The membranes were developed with the Odyssey infrared imaging system according to the manufacturer's instruction. The results presented are the mean band intensity relative to the control (GAPDH) intensity. Each experiment contained triplicate wells of each sample, and all experiments were repeated at least 3 times.

Cell transfection

A eukaryotic expression plasmid of the human LZTS2 gene was constructed using a pCDH vector (Invitrogen, Carlsbad, USA). We constructed LZTS2 cDNA expression vector on the basis of the sequence in NCBI (Accession number CH471066.2). The empty vector was used as negative control. HCC-1937 and MDA-MB-231 cells were cultured in six-well plates. When cells reached 80 - 90% confluence, transient transfections were performed by using Lipofectamine 2000 (Invitrogen) according to the manufacturer instructions. At 48 h after transfection, gene expression was confirmed by using Western blotting analysis or qRT-PCR.

CCK-8 assay

Cell proliferation was measured by cell-counting kit-8 (CCK-8) assay according to the manufacturer's protocol. Briefly, about 2 × 10^3 cells were seeded into a 96-well plate and incubated overnight. When cells were well adhered, 10 µl of CCK-8 (Abcam) was added to each well and incubated for 2 h in a 37 ºC. A microplate reader spectrophotometer () was used to measure the optical density (OD) at 450 nm. Proliferation rates were determined at 0, 24, 48, 72, 96 h after transfection. Experiments were performed in triplicate.

Wound-healing experiments

5 × 10^5 TNBC cells were seeded in 24-well plates. After scraping the cell monolayer with a sterile micropipette tip, the wells were washed with serum-free medium three times. The first image of each scratch was acquired at time zero. After 24 h, each scratch was examined and captured at the same location and the healed area was measured.

Transwell assay

Tumor cell migration assay was performed in a 24-well transwell chamber (Corning, NY, US), which contained an 8 µm pore size polycarbonate membrane filter and was precoated with 100 µg Matrigel for
invasion assay (Becton-Dickinson, Bedford, USA). Briefly, the cells were seeded in the upper chambers and incubated in 500 µl RPMI 1640 medium without FBS, while 500 µl medium with 10% FBS was placed in the lower chambers. The plates were incubated for 24 h in a 5% CO₂ humidified incubator at 37 °C. Cells on the upper side of the filters were removed by cotton-tipped swabs, and the filters were washed with PBS. Then the cells on the lower side were fixed in 4% formaldehyde and stained with 1% crystal violet in PBS for 5 min at room temperature. The cells on the lower side of the filters were defined as migration cells and counted at ×200 magnification in 5 random fields of each filter.

**Dual-luciferase reporter assay**

Plasmids containing the firefly luciferase reporter were constructed with LZTS2-3′-UTR-wildtype (LZTS2-3′-UTR-WT) and LZTS2-3′-UTR-mutation (LZTS2-3′-UTR-M). About 5 × 10⁴ cells were seeded in 24-well plates and allowed to settle overnight. On the next day, the cells were transfected with recombinant plasmids or an empty plasmid encoding the firefly luciferase reporter with Lipofectamine 2000. Renilla luciferase reporter pRL-CMV (Promega) was determined as an internal control and used for normalization. After 48 h, the reporter luciferase activity was measured with the Dual-luciferase Reporter assay system (Promega) according to the manufacturer’s instructions. All transfection assays were carried out in triplicate.

**Immunofluorescence**

HCC-1937 and MDA-MB-231 cells harvested and incubated with rabbit to human LZTS2, mouse to human β-catenin mAb at 4°C overnight. The cells were then stained by FITC conjugated goat anti-mouse and PE conjugated goat anti-rabbit antibody, followed by DAPI staining of the nucleus. The fluorescence was observed and analyzed with a fluorescence microscope at high magnification (× 200).

**In vivo tumor growth assay**

15 BALB/c nude mice were used for establishing xenograft tumor model. Approximately 0.5 × 10⁷ HCC-1937 cells were injected into the BALB/c nude mice subcutaneously. Tumor growth was recorded to measure the width or length, and the volume and weight of the tumor was calculated at indicated times. The mice were divided randomly to two groups: control group, miRNA inhibitor NC group and miR-9-5p inhibitor group. These mice were sacrificed and the cancer tissues were harvested at the thirtieth day. The experiments involving animals were approved by the Ethics Committee for the Use and Care of Animals of Hebei Medical University.

**Statistical analysis**

All statistical analyses were performed with SPSS statistics software, version 25.0 (SPSS, Chicago, IL, USA). Data were presented as mean ± SD. All P values were two-tailed and a P value < 0.05 was considered as statistically significant. Data were obtained from at least three independent experiments with a similar pattern.
Results

Low expression of LZTS2 in tumor tissues was correlated with poor postoperative prognosis of TNBC patients

First, we analyzed the TNBC cohort in The Cancer Genome Atlas (TCGA) database to realize the expression of LZTS2 in TNBC. The results showed that LZTS2 expression in TNBC tissues was lower than that in normal breast tissues (Fig. 1a; P = 0.033). Next, aiming at validating the above results, we used IHC to detect LZTS2 expression in 95 pairs of TNBC. As shown in Fig. 1b, the staining of LZTS2 existed in the plasma membrane and cytosol of breast cancer cells. Among the 95 TNBC tissues, 62 cases (65.26%) showed low expression of LZTS2. The patients harboring high expression of LZTS2 tended to have high TNM stage, wide invasion range and positive lymph node metastasis. Then, for elucidating the prognostic role of LZTS2 in TNBC, we analyzed its correlation with the post-operational disease-free survival (DFS) and overall survival (OS) of TNBC patients. Kaplan–Meier analysis indicated that low expression of LZTS2 was correlated with shorter DFS (Fig. 1c) (log-rank test: P < 0.001). The median DFS for the patients with high and low LZTS2 expression were 36.0 and 23.0 months, respectively. Meanwhile, Kaplan–Meier analysis demonstrated that low expression of LZTS2 was correlated with shorter OS (Fig. 1d) (log-rank test: P = 0.025). Then, the COX proportional hazard regression analysis was performed to further determine the prognostic significance of LZTS2 expression in TNBC. The covariates involved in the COX proportional hazard model were gender, TNM stage, invasion range, lymph node metastasis and LZTS2 expression. As shown in Fig. 1e, TNM stage and LZTS2 expression were correlated with postoperative DFS and OS. The unadjusted hazard ratios (HRs) of LZTS2 expression for DFS and OS of TNBC patients were 0.425 (95% CI: 0.246–0.736, P = 0.002) and 0.426 (95% CI: 0.211–0.859, P = 0.017) for DFS and OS, respectively. Meanwhile, the HRs of TNM stage for DFS and OS of TNBC patients were 2.090 (95% CI: 1.205–3.623, P = 0.009) and 5.068 (95% CI: 2.102–12.216, P < 0.001) for DFS and OS, respectively. In addition, the median OS for patients with low LZTS2 expression was 42.0 months, while the median OS for patients with high LZTS2 expression had not reached. These results indicated that the TNBC patients with low LZTS2 expression had worse prognosis than those with high expression.

LZTS2 overexpression suppresses the proliferation, migration, and invasion ability of HCC-1937 and MDA-MB-231 cells

Aiming at further studying the function of LZTS2 in TNBC, Western blotting was used to determine LZTS2 expression in human normal mammary epithelial cell line (MCF-10A) and TNBCC cell lines (MDA-MB-468, HCC-1937, MDA-MB-231, MDA-MB-436, MDA-MB-453). The results demonstrated that the expressions of LZTS2 were low in all chosen TNBC cell lines, compared to that of MCF-10A cells (Fig. 2a) (all P < 0.001). Among these TNBC cell lines, the HCC-1937 and MDA-MB-231 cells showed the lowest expression of LZTS2, so these two cell lines were chosen for next experiments. For revealing the biological role of LZTS2 in TNBC, we performed plasmid transfection to overexpress LZTS2 in HCC-1937 and MDA-MB-231 cells. First, we used CCK-8 method to evaluate the effect of LZTS2 on the proliferation
ability of HCC-1937 and MDA-MB-231 cells. The results showed that LZTS2 overexpression significantly decreased the proliferation rate of HCC-1937 and MDA-MB-231 cells (Fig. 2b). Next, we assessed the effect of LZTS2 overexpression on the migration and invasion ability with wound healing and transwell experiments. As Fig. 2c showed, LZTS2 overexpression significantly inhibited the migration ability of HCC-1937 and MDA-MB-231 cells, compared with the corresponding control cells (53.25 ± 1.45 µm vs. 75.68 ± 1.79 µm; 54.82 ± 1.65 µm vs. 77.04 ± 1.57 µm; both P < 0.001). Similarly, LZTS2 overexpression also significantly inhibited the invasion ability of HCC-1937 and MDA-MB-231 cells, compared with the corresponding control cells (155.17 ± 2.95 cells/field vs. 76.64 ± 3.17 cells/field; 145.41 ± 2.97 cells/field vs. 72.89 ± 1.60 cells/field; P < 0.001; Fig. 2d). Taken above, LZTS2 overexpression inhibited proliferation, migration, and invasion ability of HCC-1937 and MDA-MB-231 cells, which indicated that LZTS2 played important roles in managing the malignant behaviors of TNBC cells.

miR-9-5p targets the 3′-UTR of LZTS2 mRNA in HCC-1937 and MDA-MB-231 cells

For studying the upstream regulatory mechanisms, we used bioinformation algorithm TargetScan (http://www.targetscan.org/vert_72/) and miRanda (http://www.microrna.org) to predicting the potential miRNA regulating the expression of LZTS2. Overlap analysis indicated that LZTS2 might be regulated by miR-9-5p, which had been proved to be a miRNA upregulated in TNBC [14]. As the Fig. 3a showed, there was one hypothetic miR-9-5p binding sites in the 3′-UTR of LZTS2 mRNA. For further studying the inhibitory function of miR-9-5p on LZTS2, we then detected the expression of miR-9-5p in TNBC cells. Consequently, we found that miR-9-5p was highly expressed in all chosen TNBC cell lines, compared to that of MCF-10A cells (Fig. 3b) (all P < 0.01). Meanwhile, HCC-1937 and MDA-MB-231 cells showed the highest expression of miR-9-5p among these TNBC cell lines. Thus, we performed reporter assays with a luciferase plasmid harboring the 3′-UTR sequence of LZTS2 containing the predicted site for binding miR-9-5p. Furthermore, we built mutant reporter vectors containing one mutation in the miR-9-5p binding site on LZTS2 3′-UTR (LZTS2-3′-UTR-M). These plasmids were transfected into HCC-1937 and MDA-MB-231 cells with miR-9-5p inhibitor. As shown in Fig. 3c, miR-9-5p inhibitor increased luciferase activity in HCC-1937 and MDA-MB-231 cells which were transfected with LZTS2-3′-UTR-WT (0.36 ± 0.02 vs. 0.99 ± 0.03, 0.35 ± 0.02 vs. 1.02 ± 0.04, both P < 0.001), but not in those with LZTS2-3′-UTR-M (1.02 ± 0.03 vs. 1.01 ± 0.04, P = 0.909; 1.02 ± 0.03 vs. 1.01 ± 0.03, P = 0.723). This result suggested that miR-9-5p specifically targeted the binding site in the 3′-UTR of LZTS2 mRNA. Meanwhile, LZTS2 protein expressions were increased after addition of miR-9-5p inhibitor in HCC-1937 and MDA-MB-231 cells (0.22 ± 0.03 vs. 1.28 ± 0.03, 0.23 ± 0.01 vs. 1.28 ± 0.05, both P < 0.001), which were consistent with the result of luciferase reporter assay (Fig. 3d). These results demonstrated that miR-9-5p could downregulate LZTS2 expression in TNBC through binding its 3′-UTR.

miR-9-5p inhibition-induced LZTS2 upregulation suppresses epithelium-to-mesenchymal transition of HCC-1937 and MDA-MB-231 cells by deactivating β-catenin

Considering that miR-9-5p could bind the 3′-UTR of LZTS2 mRNA to inhibit its expression, we next evaluated the effect of miR-9-5p on the malignant behaviors of TNBC cells, thus we performed CCK-8,
wound healing and transwell experiments. CCK-8 results showed that miR-9-5p inhibitor reduced the proliferation ability of HCC-1937 and MDA-MB-231 cells, compared to corresponding control and miRNA inhibitor NC group (Fig. 4a, all P < 0.05). Meanwhile, the migration ability of HCC-1937 and MDA-MB-231 cells could be significantly suppressed by miR-9-5p inhibitor, compared to the corresponding groups treated with miRNA inhibitor NC (0.63 ± 0.02 vs. 0.83 ± 0.02, 0.64 ± 0.02 vs. 0.81 ± 0.02, both P < 0.001B; Fig. 4b). Similarly, the invasion ability of HCC-1937 and MDA-MB-231 cells could also be significantly suppressed by miR-9-5p inhibitor, compared to the corresponding groups treated with miRNA inhibitor NC (0.36 ± 0.02 vs. 1.02 ± 0.04, 0.35 ± 0.02 vs. 1.02 ± 0.04, both P < 0.001; Fig. 4c). Since LZTS2 was reported to inhibit the function of β-catenin by suppressing its expression or promoting its nuclear export in prostate and colon cancer, respectively [6, 16], we then detected its expression and subcellular location in TNBC HCC-1937 and MDA-MB-231 cells. As Fig. 4d showed, miR-9-5p inhibitor significantly reduced the nucleocytoplasmic ratio of β-catenin in HCC-1937 and MDA-MB-231 cells, compared to corresponding miRNA inhibitor NC-treated group, respectively (0.270 ± 0.018 vs. 2.349 ± 0.157, 0.260 ± 0.022 vs. 2.654 ± 0.220, both P < 0.001). Activation of Wnt/β-catenin pathway is crucial for epithelium-to-mesenchymal transition (EMT) initiation in many cancers, thus we next detected the expression of EMT-related proteins in TNBC cells after treatment of miR-9-5p. The results showed that miR-9-5p inhibitor significantly down-regulated the expression of E-cadherin and up-regulated the expression of N-cadherin, Twist and Vimentin, while si-LZTS2 rescued this phenomenon (Fig. 4e). Above results demonstrated that miR-9-5p could inhibit the EMT process of TNBC cells via upregulating LZTS2.

Inhibition of miR-9-5p upregulated LZTS2 expression and suppressed tumor growth of cervical cancer in vivo

In order to investigate whether miR-9-5p acted as an oncogene in vivo, we established a xenograft tumor mouse model. The results showed that the size and weight of tumors in miR-9-5p inhibitor group were significantly reduced, compared to the control and miRNA inhibitor NC group (Fig. 5a, b, P < 0.01). Moreover, the tumor tissues of miR-9-5p inhibitor group showed high expression of LZTS2 while the control group showed low expression of LZTS2 (Fig. 5c). Consistent to the results in vitro, the cytoplasmic β-catenin was significantly increased in miR-9-5p inhibitor group, compared to control group (Fig. 5d). Taken above, these results suggested that miR-9-5p could promote the progression of TNBC in vivo by suppressing the expression of LZTS2.

Discussion

TNBC is the most aggressive subtype of breast cancer, which is not sensitive to endocrinotherapy or anti-HER2 agents[17]. However, the elusive comprehension about the dysregulated molecules involved in TNBC limits the improvement of clinical therapeutic efficacy. Attenuation of tumor suppressing proteins is a frequent cancer hallmark promoting TNBC progression. Herein, studying the roles of the tumor suppressing proteins and the upstream regulatory factors might contribute to establish novel treatment strategy for improving the prognosis of TNBC patients.
LZTS2, also known as LAPSER1, is a lately identified potential tumor suppressor [5]. Anergy of LZTS2 has been proven to be correlated with enhanced malignant behaviors of multiple cancer cells, such as lung, colon and prostate cancer [7, 8]. Johnson et al. confirmed that the presence of LZTS2 was crucial for preventing spontaneous tumor development, indicating that LZTS2 exhibited tumor suppressing effects even in tumor initial phase [18]. Loss of LZTS2 can be a candidate biomarker for diagnosis and prognosis of laryngeal squamous cell carcinoma [5]. In present study, we first investigated the expression in TNBC and its clinical significance. The results showed that LZTS2 presented low expression in TNBC tissues. Low expression of LZTS2 was significantly correlated with higher TNM stage, wider invasion range and positive lymph node metastasis. Then, we evaluated the role of LZTS2 in predicting the prognosis of TNBC patients. Kaplan-Meier analysis demonstrated that low expression of LZTS2 was correlated with shorter DFS and OS of TNBC patients. Above results indicated that LZTS2 might contribute to the progression of TNBC.

Next, we attempted to explore the upstream factor downregulating LZTS2 expression in TNBC. Emerging studies have revealed that miRNAs participated in the pathogenesis of TNBC by affecting cellular processes including cell proliferation, migration, invasion and metastasis, etc. [19] For instance, miR-301a can promote the proliferation and invasion ability of TNBC cells by upregulating Cip2a [20]. Herein, we used bioinformation algorithms TargetScan and miRanDa to predict the possible miRNA regulating the expression of LZTS2. By overlap analyzing the results of different bioinformation algorithms, we found that miR-9-5p was a potential miRNA which might bind to the 3'-UTR of LZTS2 mRNA. Our results indicated that miR-9-5p was highly expressed in the TNBC cells, compared with MCF-10A. Luciferase report result showed that miR-9-5p could downregulate LZTS2 by interacting with its 3'-UTR of LZTS2 mRNA. Inhibiting miR-9-5p expression could upregulate LZTS2 of TNBC cells. Consequently, the proliferation, migration and invasion ability of TNBC cells, indicating a tumor promoting function of miR-9-5p.

EMT refers to the process that epithelial cancer cells acquire mesenchymal phenotype [21]. EMT is important for some cancer cells to exhibit malignant behaviors such as invading, metastasizing, immune escape etc. [22]. Activation of Wnt/β-catenin pathway is crucial for EMT initiation in many kinds of cancers including TNBC [23]. Thyssen et al. reported that LZTS2 could interact with β-catenin and promoted its nuclear export [16]. Nuclear location is essential for β-catenin to exhibit its function, thus nuclear export attenuated the function of β-catenin, which led to deactivation of Wnt/β-catenin pathway [24]. We then detected the effect of miR-9-5p/LZTS2 axis on the expression of EMT-related proteins. The results showed that inhibition of miR-9-5p upregulated the expression of E-cadherin while downregulated the expressions of N-cadherin, Twist and Vimentin. E-cadherin is necessary for epithelial cells to maintain cell-cell adhesion, thus attenuation of E-cadherin is regarded as a hallmark of EMT [25]. Along with the loss of E-cadherin, upregulation of N-cadherin, which is a downstream regulatory target of β-catenin, is another marker of EMT process of cancers [26]. When the epithelial cancer cells acquire mesenchymal features, some transcription factors (e.g. Twist, Slug and Snail) are activated to promote cell invasion and metastasis [27]. The present study demonstrated that inhibiting miR-9-5p could upregulate LZTS2 to suppress the function of β-catenin in TNBC cells, thereby to upregulate E-cadherin and downregulate N-
cadherin, Twist and Vimentin. These results provided some evidence that miR-9-5p could promote Wnt/β-catenin-mediated EMT process of TNBC.

**Conclusion**

In summary, we confirmed the low expression status and tumor-suppressing function of LZTS2 in TNBC. Meanwhile, we identified that the expression of LZTS2 was negatively correlated with miR-9-5p in TNBC. Addition of miR-9-5p inhibitor could significantly suppress the malignant behaviors of TNBC cells by upregulating LZTS2, thereby to inhibit the activation of β-catenin. Consequently, miR-9-5p inhibitor suppressed the EMT process of TNBC cells. Taken together, our results revealed that miR-9-5p mediated LZTS2 attenuation played pivotal roles in TNBC, which demonstrated that the treatment targeting miR-9-5p might have potentials in improving the prognosis of TNBC patients.

**Declarations**

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We want to thank all participants involved in this study.

**Authors’ contributions**

CG designed this study. JZ wrote the manuscript. LZ provided clinical specimens and follow-up data. JZ, JW and PH conducted experiments in vitro. JZ and XZ conducted experiments in vivo. JZ and CZ analyzed the data. All authors read and approved the final manuscript.

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**Availability of data and materials**

The authors declare that data used in the current study will be available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

This research was approved by the ethic committee of the Hebei Medical University. All informed consents were signed by patients.

**Consent for publication**

Not applicable.

**Competing interests**
The authors declare that they have no competing interests.

References


Figures
The results showed that LZTS2 expression in TNBC tissues was lower than that in normal breast tissues (Fig. 1a; P = 0.033). Next, aiming at validating the above results, we used IHC to detect LZTS2 expression in 95 pairs of TNBC. As shown in Fig. 1b, the staining of LZTS2 existed in the plasma membrane and cytosol of breast cancer cells. Among the 95 TNBC tissues, 62 cases (65.26%) showed low expression of LZTS2. The patients harboring high expression of LZTS2 tended to have high TNM stage, wide invasion range and positive lymph node metastasis. Then, for elucidating the prognostic role of LZTS2 in TNBC, we analyzed its correlation with the post-operative disease-free survival (DFS) and overall survival (OS) of TNBC patients. Kaplan–Meier analysis indicated that low expression of LZTS2 was correlated with shorter DFS (Fig. 1c) (log-rank test: P < 0.001). The median DFS for the patients with high and low LZTS2 expression were 36.0 and 23.0 months, respectively. Meanwhile, Kaplan–Meier analysis demonstrated that low expression of LZTS2 was correlated with shorter OS (Fig. 1d) (log-rank test: P = 0.025). Then, the COX proportional hazard regression analysis was performed to further determine the prognostic significance of LZTS2 expression in TNBC. The covariates involved in the COX proportional hazard model were gender, TNM stage, invasion range, lymph node metastasis and LZTS2 expression. As shown in Fig. 1e, TNM stage and LZTS2 expression were correlated with postoperative DFS and OS. The unadjusted hazard ratios (HRs) of LZTS2 expression for DFS and OS of TNBC patients were 0.425 (95% CI: 0.246 - 0.736, P = 0.002) and 0.426 (95% CI: 0.211 - 0.859, P = 0.017) for DFS and OS, respectively. Meanwhile, the HRs of TNM stage for DFS and OS of TNBC patients were 2.090 (95% CI: 1.205 - 3.623, P = 0.009) and 5.068 (95% CI: 2.102 - 12.216, P < 0.001) for DFS and OS, respectively. In addition, the median OS for patients with low LZTS2 expression was 42.0 months, while the median OS for patients with high LZTS2 expression had not reached. These results indicated that the TNBC patients with low LZTS2 expression had worse prognosis than those with high expression.
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The results demonstrated that the expressions of LZTS2 were low in all chosen TNBC cell lines, compared to that of MCF-10A cells (Fig. 2a) (all P < 0.001). Among these TNBC cell lines, the HCC-1937 and MDA-MB-231 cells showed the lowest expression of LZTS2, so these two cell lines were chosen for next experiments. For revealing the biological role of LZTS2 in TNBC, we performed plasmid transfection to overexpress LZTS2 in HCC-1937 and MDA-MB-231 cells. First, we used CCK-8 method to evaluate the
effect of LZTS2 on the proliferation ability of HCC-1937 and MDA-MB-231 cells. The results showed that LZTS2 overexpression significantly decreased the proliferation rate of HCC-1937 and MDA-MB-231 cells (Fig. 2b). Next, we assessed the effect of LZTS2 overexpression on the migration and invasion ability with wound healing and transwell experiments. As Fig. 2c showed, LZTS2 overexpression significantly inhibited the migration ability of HCC-1937 and MDA-MB-231 cells, compared with the corresponding control cells (53.25 ± 1.45 μm vs. 75.68 ± 1.79 μm; 54.82 ± 1.65 μm vs. 77.04 ± 1.57 μm; both P < 0.001). Similarly, LZTS2 overexpression also significantly inhibited the invasion ability of HCC-1937 and MDA-MB-231 cells, compared with the corresponding control cells (155.17 ± 2.95 cells/field vs. 76.64 ± 3.17 cells/field; 145.41 ± 2.97 cells/field vs. 72.89 ± 1.60 cells/field; P < 0.001; Fig. 2d). Taken above, LZTS2 overexpression inhibited proliferation, migration, and invasion ability of HCC-1937 and MDA-MB-231 cells, which indicated that LZTS2 played important roles in managing the malignant behaviors of TNBC cells.
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As the Fig. 3a showed, there was one hypothetic miR-9-5p binding sites in the 3'-UTR of LZTS2 mRNA. For further studying the inhibitory function of miR-9-5p on LZTS2, we then detected the expression of miR-9-5p in TNBC cells. Consequently, we found that miR-9-5p was highly expressed in all chosen TNBC cell lines, compared to that of MCF-10A cells (Fig. 3b) (all P < 0.01). Meanwhile, HCC-1937 and MDA-MB-231 cells showed the highest expression of miR-9-5p among these TNBC cell lines. Thus, we performed
reporter assays with a luciferase plasmid harboring the 3'-UTR sequence of LZTS2 containing the predicted site for binding miR-9-5p. Furthermore, we built mutant reporter vectors containing one mutation in the miR-9-5p binding site on LZTS2 3'-UTR (LZTS2-3'-UTR-M). These plasmids were transfected into HCC-1937 and MDA-MB-231 cells with miR-9-5p inhibitor. As shown in Fig. 3c, miR-9-5p inhibitor increased luciferase activity in HCC-1937 and MDA-MB-231 cells which were transfected with LZTS2-3'-UTR-WT (0.36 ± 0.02 vs. 0.99 ± 0.03, 0.35 ± 0.02 vs. 1.02 ± 0.04, both P < 0.001), but not in those with LZTS2-3'-UTR-M (1.02 ± 0.03 vs. 1.01 ± 0.04, P = 0.909; 1.02 ± 0.03 vs. 1.01 ± 0.03, P = 0.723). This result suggested that miR-9-5p specifically targeted the binding site in the 3'-UTR of LZTS2 mRNA. Meanwhile, LZTS2 protein expressions were increased after addition of miR-9-5p inhibitor in HCC-1937 and MDA-MB-231 cells (0.22 ± 0.03 vs. 1.28 ± 0.03, 0.23 ± 0.01 vs. 1.28 ± 0.05, both P < 0.001), which were consistent with the result of luciferase reporter assay (Fig. 3d). These results demonstrated that miR-9-5p could downregulate LZTS2 expression in TNBC through binding its 3'-UTR.
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Considering that miR-9-5p could bind the 3'-UTR of LZTS2 mRNA to inhibit its expression, we next evaluated the effect of miR-9-5p on the malignant behaviors of TNBC cells, thus we performed CCK-8, wound healing and transwell experiments. CCK-8 results showed that miR-9-5p inhibitor reduced the proliferation ability of HCC-1937 and MDA-MB-231 cells, compared to corresponding control and miRNA inhibitor NC group (Fig. 4a, all P < 0.05). Meanwhile, the migration ability of HCC-1937 and MDA-MB-231
cells could be significantly suppressed by miR-9-5p inhibitor, compared to the corresponding groups treated with miRNA inhibitor NC (0.63 ± 0.02 vs. 0.83 ± 0.02, 0.64 ± 0.02 vs. 0.81 ± 0.02, both P < 0.001; Fig. 4b). Similarly, the invasion ability of HCC-1937 and MDA-MB-231 cells could also be significantly suppressed by miR-9-5p inhibitor, compared to the corresponding groups treated with miRNA inhibitor NC (0.36 ± 0.02 vs. 1.02 ± 0.04, 0.35 ± 0.02 vs. 1.02 ± 0.04, both P < 0.001; Fig. 4c). Since LZTS2 was reported to inhibit the function of β-catenin by suppressing its expression or promoting its nuclear export in prostate and colon cancer, respectively [6, 16], we then detected its expression and subcellular location in TNBC HCC-1937 and MDA-MB-231 cells. As Fig. 4d showed, miR-9-5p inhibitor significantly reduced the nucleocytoplasmic ratio of β-catenin in HCC-1937 and MDA-MB-231 cells, compared to corresponding miRNA inhibitor NC-treated group, respectively (0.270 ± 0.018 vs. 2.349 ± 0.157, 0.260 ± 0.022 vs. 2.654 ± 0.220, both P < 0.001). Activation of Wnt/β-catenin pathway is crucial for epithelium-to-mesenchymal transition (EMT) initiation in many cancers, thus we next detected the expression of EMT-related proteins in TNBC cells after treatment of miR-9-5p. The results showed that miR-9-5p inhibitor significantly down-regulated the expression of E-cadherin and up-regulated the expression of N-cadherin, Twist and Vimentin, while si-LZTS2 rescued this phenomenon (Fig. 4e). Above results demonstrated that miR-9-5p could inhibit the EMT process of TNBC cells via upregulating LZTS2.
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In order to investigate whether miR-9-5p acted as an oncogene in vivo, we established a xenograft tumor mouse model. The results showed that the size and weight of tumors in miR-9-5p inhibitor group were significantly reduced, compared to the control and miRNA inhibitor NC group (Fig. 5a, b, P < 0.01). Moreover, the tumor tissues of miR-9-5p inhibitor group showed high expression of LZTS2 while the control group showed low expression of LZTS2 (Fig. 5c). Consistent to the results in vitro, the
cytoplasmic β-catenin was significantly increased in miR-9-5p inhibitor group, compared to control group (Fig. 5d). Taken above, these results suggested that miR-9-5p could promote the progression of TNBC in vivo by suppressing the expression of LZTS2.

Figure 5

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