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Up-regulated miR-548k promotes esophageal squamous cell carcinoma progression via targeting long noncoding RNA-LET

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ABSTRACT

Dysregulated noncoding RNAs have been observed in diverse cancers. *MIR458K* is frequently amplified in esophageal squamous cell carcinoma (ESCC). However, the expression, clinical significances, and action mechanisms of miR-548k in ESCC are still unclear. In this study, we found that miR-548k is significantly up-regulated in ESCC tissues and cell lines. Up-regulated miR-548k expression is significantly correlated with advanced invasion depth, lymph node metastasis, advanced TNM stage, and poor overall survival. Gain-of- and loss-of-function assays demonstrated that miR-548k promotes the proliferation and migration of ESCC cells *in vitro* and tumor growth *in vivo*. Mechanistically, we found that miR-548k directly targets and represses the expression of long noncoding RNA-LET (lncRNA-LET), and further down-regulates p53 and up-regulates NF90. In addition, we found that lncRNA-LET is down-regulated and inversely correlated with miR-548k in ESCC. Down-regulated lncRNA-LET also indicated poor overall survival of ESCC patients. Functional assays demonstrated that lncRNA-LET inhibits the proliferation and migration of ESCC cells, and the effects of miR-548k on ESCC are dependent on the negative regulation of lncRNA-LET. In summary, our data revealed the critical roles of miR-548k-lncRNA-LET regulation axis in ESCC and suggested that the miR-548k-lncRNA-LET regulation axis may be promising prognostic biomarkers and therapeutic targets for ESCC.

1. Introduction

Esophageal cancer is the seventh most common cancer and sixth leading cause of cancer-related death worldwide [1]. Esophageal squamous cell carcinoma (ESCC) is the major histopathological form and accounts for over 90% of esophageal cancer [2,3]. Despite advances in diagnostic and surgical techniques, most ESCC patients are still diagnosed in advanced stages and not eligible for surgical resection, resulting in a five-year survival rate of about 10% [4,5]. Therefore, identifying the detailed molecular mechanisms and genomic events contributing to the pathogenesis and progression of ESCC and developing more efficient therapeutic strategies are urgent [6,7].

A previous comprehensive genomic analysis of ESCC identified frequently amplified chromosome region 11q13.3–13.4 [4]. *MIR548K*, encoding a microRNA, is located in this region. microRNAs (miRNAs) are small noncoding RNAs with about 22 nucleotides in length [8–10]. Increasing evidences demonstrated that miRNAs are frequently dysregulated in many diseases and play critical roles in many pathophysiological processes [11–15]. Mechanistically, miRNAs mainly act as post-transcriptional regulators via directly binding to complementary sequences of target mRNAs [16,17]. However, the expression, clinical and therapeutic significances of miR-548k, which is encoded by *MIR548K*, in ESCC are still unknown. Furthermore, how miR-548k exerts its role is also unknown.

Long noncoding RNA (lncRNA) is another class of noncoding RNA with more than 200 nucleotides in length [18]. Accumulating evidences revealed that lncRNAs are implicated in diverse pathophysiological processes, particular in cancers [19–21]. Aberrant expression of lncRNAs are reported to be associated with cancer initiation and progression [22–24]. Due to the similar structure between mRNA and lncRNA, lncRNAs could also be target of miRNAs, such as lincRNA-UFC1, a target of miR-34a in hepatocellular carcinoma (HCC), and MEG3, a target of miR-29 in HCC [25,26]. However, the regulation of lncRNAs by miRNAs in ESCC remains unknown.

Although several lncRNAs have been shown to function as oncogenes or tumor suppressors in ESCC, such as CCAT1, HNF1A-AS1, HOTAIR, MALAT1, and AFAP1-AS1, thousands of lncRNAs that have been annotated are not functionally characterized [27–31]. lncRNA-LET, also known as NPTN intronic transcript 1, is located in chromosome 15q24.1 and has 2606 nucleotides in length. As a recently

Abbreviations: ESCC, esophageal squamous cell carcinoma; lncRNA, long noncoding RNA; miRNA, microRNA; HCC, hepatocellular carcinoma; qPCR, quantitative polymerase chain reaction; NC, negative control; EdU, Ethynyl deoxyuridine; WT, wild type; NF90, nuclear factor 90

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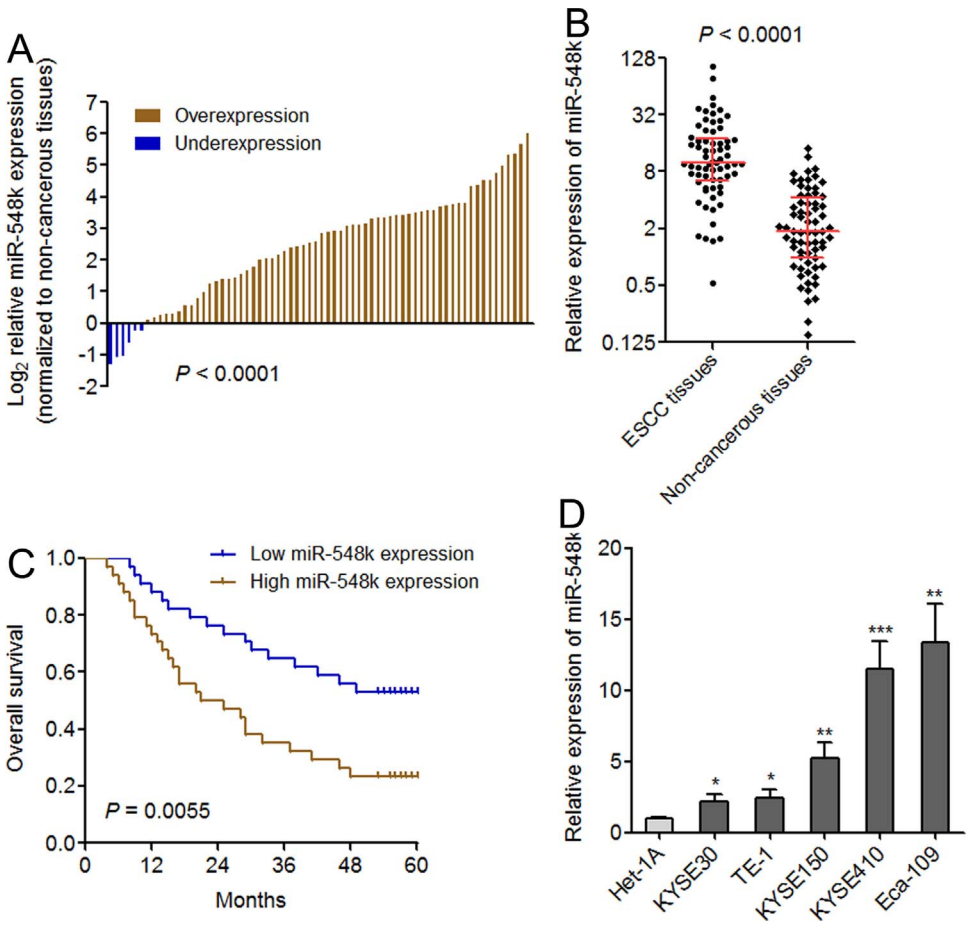

Fig. 1. *The expression pattern of miR-548k in ESCC and its clinical significances.* (A) miR-548k expression in 68 paired ESCC tissues and adjacent non-cancerous esophageal epithelial tissues was measured by qPCR and normalized to U6. The results are shown as $\log_2$ fold change of the expression values in ESCC to that in normal tissues. $P < 0.0001$ by Wilcoxon signed-rank test. (B) miR-548k expression in the same ESCC tissues and adjacent non-cancerous tissues as in A. The results are shown as median with interquartile range. $P < 0.0001$ by Wilcoxon signed-rank test. (C) Kaplan–Meier survival analysis of the correlation between miR-548k expression and overall survival of ESCC patients. The median expression level of miR-548k was used as the cut-off. $P = 0.0055$ by Log-rank test. (D) miR-548k expression in immortalized normal esophageal epithelial cell line Het-1A and ESCC cell lines KYSE30, TE-1, KYSE150, KYSE410, and Eca-109 was measured by qPCR and normalized to U6. The results are shown as mean ± S.D. (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by Student's *t*-test.

identified lncRNA, lncRNA-LET is low expressed and functions as a tumor suppressor in several cancers [32–34].

In this study, we investigated the expressions, clinical significances, and biological roles of miR-548k and lncRNA-LET in ESCC. The combination of bioinformatics analysis and experimental verification found that lncRNA-LET is a direct target of miR-548k and mediates the oncogenic roles of miR-548k in ESCC. Our data suggested that the miR-548k-lncRNA-LET regulation axis may serve as potential biomarkers and therapeutic targets for ESCC.

2. Materials and methods

2.1. Tissues specimens

Sixty-eight paired ESCC tissues and adjacent non-cancerous esophageal epithelial tissues were obtained from ESCC patients who underwent resection of the ESCC at the Second Affiliated Hospital of Fujian Medical University (Quanzhou, Fujian, China). None of the patients received chemotherapy or radiotherapy before surgery. The freshly resected tissues specimens were snap-frozen in liquid nitrogen and stored at −80 °C until use. The diagnosis of all tissues specimens was verified by histopathological examination. The use of these tissues specimens was conducted in accordance with protocols approved by the Review Board of the Second Affiliated Hospital of Fujian Medical University. All patients signed informed consent.

2.2. Cell lines and cell culture

The human immortalized normal esophageal epithelial cell line Het-1A was obtained from American Type Culture Collection (ATCC). ESCC cell lines KYSE30, TE-1, KYSE150, KYSE410, and Eca-109 were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Het-1A was cultured in BEBM (Lonza, Basel, Switzerland), and the ESCC cell lines KYSE30, TE-1, KYSE150, KYSE410, and Eca-109 were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA). All cells were cultured in medium supplemented with 10% fetal bovine serum (Invitrogen) in a humidified incubator containing 5% CO_2 at 37 °C.

2.3. RNA extraction, reverse transcription, and real time quantitative polymerase chain reaction (qPCR)

Total RNA was extracted using TRIzol regent (Invitrogen) in accordance with the manufacturer's instructions. After being treated with DNase I to remove DNA, the RNA was reverse transcribed to produce the first strand cDNA using the M-MLV Reverse Transcriptase (Invitrogen) in accordance with the manufacturer's instructions. qPCR was carried out on ABI StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). lncRNA-LET expression was measured by SYBR® Premix Ex Taq™ II (Takara, Dalian, China) in accordance with the manufacturer's instructions and normalized to GAPDH. miR-548k expression was measured by TaqMan MicroRNA Assays (Applied Biosystems) in accordance with the manufacturer's instructions and normalized to U6. The primer sequences are as follows: for lncRNA-LET, 5'-TGAGATGCTGGAATGATG-3' (forward) and 5'-GGCTAAAGAAGGAAAAGG-3' (reverse); for GAPDH,5'-GGAGCGAGATCCCTCCAAAAT-3' (forward) and 5'-GGCTGTTGTCATACTTCTCATGG-3' (reverse). The expression of RNAs was calculated by the comparative Ct method.

2.4. Plasmids construction and transfection

The cDNA coding lncRNA-LET was PCR-amplified using the *PfuUltra*