European Review for Medical and Pharmacological Sciences 2019; 23: 6516-6523

Long noncoding RNA ZFAS1 acts as an oncogene by targeting miR-193a-3p in human non-small cell lung cancer

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Abstract. – OBJECTIVE: Recent researches have proved the important role of long noncoding RNAs (lncRNAs) in many diseases. In this study, the potential function of lncRNA ZFAS1 in the development of non-small cell lung cancer (NSCLC) was mainly explored.

PATIENTS AND METHODS: ZFAS1 expression in NSCLC patients was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Cell counting kit-8 (CCK-8) assay, colony formation assay and ethynyl deoxyuridine (EdU) incorporation assay were conducted to evaluate the regulatory effects of ZFAS1 on cellular behaviors of the NSCLC cells. Furthermore, the interaction between ZFAS1 and miR-193a-3p in mediating the progression of NSCLC was elucidated.

RESULTS: ZFAS1 expression was significantly higher in NSCLC samples relative to adjacent tissues. The proliferation of NSCLC cells was inhibited by silence of ZFAS1, and conversely, ZFAS2 overexpression promoted the proliferative ability. Further experiments showed that miR-193a-3p was directly targeted by ZFAS1.

CONCLUSIONS: ZFAS1 could enhance cell growth of NSCLC by targeting miR-193a-3p, suggesting that ZFAS1 may be a potential therapeutic target for NSCLC.

Keywords:
Long noncoding RNA, ZFAS1, Non-small cell lung cancer, NSCLC, miR-193a-3p.

Introduction

Lung cancer is one of the leading causes of cancer-related death globally and remains a public health threat to the society. Approximately 224,390 cases in America were diagnosed a lung cancer diagnosis in 2016[2]. Surgical resection is the preferred intervention for non-small cell lung cancer (NSCLC) patients diagnosed in early stage. However, most of NSCLC cases are diagnosed at an advanced stage and unfortunately, they lose the best surgical opportunity. Therefore, it is crucial to clarify the progression mechanism of NSCLC and improve the poor prognosis of affected patients. With the rapid development of technologies, 90% of the mammalian genome has been found to be transcribed to noncoding RNAs. Long noncoding RNAs (lncRNAs) are known as the transcriptions longer than 200 nt which could barely encode proteins. Some studies have uncovered its vital regulatory role in cellular biological processes. For instance, modulating the signal transducer and activator of transcription 1 (STAT1)-mitogen-activated protein kinase (MAPK) signal pathway, a downregulation of lncRNA P7 facilitates cell proliferation in hepatocellular carcinoma and is associated with unfavorable prognosis. Through the regulation of the epithelial-mesenchymal transition, lncRNA LINC00261 promotes cell migration in gastric cancer[3]. In addition, regulating miR-34c expression and targeting MUC2, lncRNA AF147447 represses proliferation and invasion in gastric cancer infected with the *Helicobacter pylori*. A knockdown of lncRNA MALAT1 suppresses migration in esophageal squamous cell carcinoma cells[4].

MicroRNAs (miRNAs), with 19-22 nucleotide nt in length, are small noncoding RNAs. They

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have been reported to participate in many diseases including cancers. By targeting FMNL2, miRNA-613 functions as a tumor suppressor in the progression of colorectal cancer[5]. Through a downregulation of the transcription factor FOXO1, overexpression of miRNA-370 promotes cell proliferation in human prostate cancer[6]. Previous researches have suggested that lncRNA ZFAS1 exerts an important role in tumor biology and development. However, the function of lncRNA ZFAS1 in NSCLC and the potential molecular mechanism has not been fully elucidated.

Patients and Methods

Tissue Samples

The NSCLC tissues and adjacent tissues were obtained from 55 NSCLC patients who underwent surgery at the Third Affiliated Hospital of Nanjing University of Chinese Medicine. No radiotherapy or chemotherapy was performed before the surgery. All fresh tissues surgically resected were immediately stored at –80°C. This study was approved by the Ethics Committee of Nanjing University of Traditional Chinese Medicine. The signed written informed consents were obtained from all the participants before the study.

Cell Culture

The human NSCLC cell lines (A549, SPCA1 and H1299) and the normal human bronchial epithelial cell (16HBE) were provided by the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% of fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% of penicillin. They were cultured in an incubator containing 5% of CO_2 at 37°C.

Cell Transfection

cDNA oligonucleotides, specifically targeting ZFAS1 (si-ZFAS1) and lentivirus against ZFAS1 were synthesized by GenePharma (Shanghai, China) and inserted into the shRNA expression vector pGPH1/Neo. sh-ZFAS1 was transfected in H1299 cells, and ZFAS1 lentivirus was transfected in A549 cells, using Lipofectamine 2000. 48 h later, quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to verify the transfection efficiency.

Cell Counting Kit-8 (CCK-8) Assay

Following the protocol of CCK-8 assay (Dojindo Laboratories, Kumamoto, Japan), the cell growth ability of transfected cells in 96-well plates was assessed at 24, 48, and 72 h. A microplate photometer (Thermo Fisher Scientific, Waltham, MA, USA) was utilized to measure the absorbance at 450 nm.

Colony Formation Assay

The transfected H1299 and A549 cells were placed in a 6-well plate for 10 days. The formed colonies were fixed in 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet for 5 min. The Image-Pro Plus (Silver Springs, MD, USA) was used for data analysis.

Ethynyl Deoxyuridine (EdU) Incorporation Assay

According to the manufacturer's manual, an EdU Kit (Roche, Mannheim, Germany) was utilized to monitor the cell proliferation of transfected cells. The Zeiss Axiophot Photomicroscope (Carl Zeiss, Oberkochen, Germany) was performed to take the representative images.

RNA Extraction and qRT-PCR

Trizol reagent (Invitrogen, Carlsbad, CA, USA) was utilized to extract the total RNA. Through the reverse Transcription Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China), the total RNA was reversely transcribed to complementary deoxyribose nucleic acids (cDNAs). The primers used for qRT-PCR were as follows: ZFAS1, forward: 5′-CTATTGTCCTGCCCGTTAGAG-3′, reverse: 5′-GTCAGGAGATCGAAGGTTGTAG-3′; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward: 5′-CCAAAATCAGATGGGGCAATGCTGG-3′ and reverse 5′-TGATGGCATGGACTGTGGTCATTCA-3′. Thermal cycle was as follows: 30 s at 95°C, 5 s at 95°C and 35 s at 60°C, for a total of 40 cycles.

Dual-Luciferase Reporter Gene Assay

3′-Untranslated Region (3′-UTR) of ZFAS1 was cloned into the pGL3 vector (Promega, Madison, WI, USA), which was identified as wild-type (WT) 3′-UTR. The quick-change site-directed mutagenesis kit (Stratagene, Cedar Creek, USA) was used for site-directed mutagenesis of the miR-193a-3p binding site in ZFAS1 3′-UTR, which was named as mutant (MUT) 3′-UTR. Cells were co-transfected with WT-3′-UTR/MUT-3′-UTR and miR-ctrl/miR-193a-3p mimics for 48 h. Dual-luciferase reporter

assay system (Promega, Madison, WI, USA) was used for determining luciferase activity.

Statistical Analysis

Statistical analysis was conducted by the Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA). The Student's *t*-test was performed to compare intergroup differences. Data were presented as mean ± SD (Standard Deviation). $p<0.05$ was considered of statistical significance.

Results

ZFAS1 Expression in NSCLC Tissues and Cells

Firstly, the ZFAS1 expression was detected *via* qRT-PCR in NSCLC tissues and cell lines. Results showed that ZFAS1 was significantly upregulated in tumor tissue samples relative to adjacent ones (Figure 1A). Identically, the ZFAS1 expression was higher in NSCLC cells than that of in 16HBE cells (Figure 1B).

Overexpression of ZFAS1 Promoted Growth Ability of NSCLC Cells

In this study, we chose H1299 cell line for the silence of ZFAS1. The transfection efficacy of sh-ZFAS1 was detected by qRT-PCR (Figure 2A). The CCK-8 assay showed that the silence of ZFAS1 inhibited growth ability of H1299 cells (Figure 2B). Meanwhile, A549 cells were selected for the overexpression of ZFAS1. The transfection efficacy of ZFAS1 lentivirus was detected by qRT-PCR (Figure 2C). The CCK-8 assay showed that the overexpression of ZFAS1 enhanced growth ability of A549 cells (Figure 2D).

Silence of ZFAS1 Suppressed Proliferation of NSCLC Cells

Colony formation assay revealed that the number of colonies remarkably decreased after ZFAS1 was silenced in the H1299 cells (Figure 3A). Conversely, the number of colonies remarkably increased after ZFAS1 was overexpressed in A549 cells (Figure 3B). Moreover, the EdU-positive cells also revealed that the percentage of EdU-positive cells reduced after silence of ZFAS1 in the H1299 cells (Figure 3C), while the percentage of EdU-positive cells increased after overexpression of ZFAS1 in A549 cells (Figure 3D).

The Interaction Between MiR-193a-3p and ZFAS1 in NSCLC

DIANA LncBase Predicted v.2 (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php%2Fr%3Dlncbasev2%2Findex-predicted) was used to search for the miRNAs which contained complementary base with ZFAS1. Since miR-193a-3p was a tumor suppressor and is able to suppress cancer cell proliferation, we focused on miR-193a-3p among these predicted miRNAs, which was interacted with ZFAS1 (Figure 4A). The qRT-PCR assay showed that the expression of miR-193a-3p was upregulated after the transfection of sh-ZFAS1 (Figure 4B). Conversely, miR-193a-3p was down-regulated after the transfection

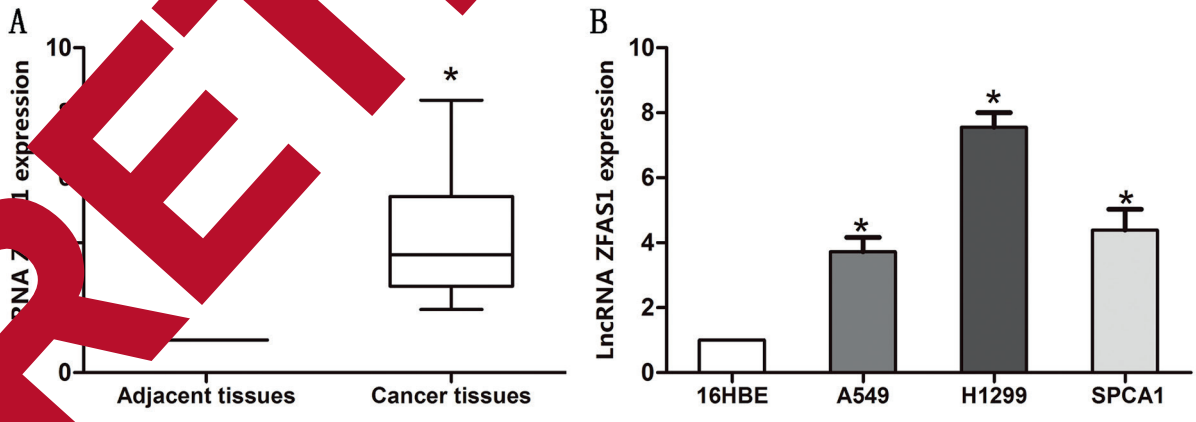


Figure 1. Expression levels of ZFAS1 increased in NSCLC tissues and cell lines. ***A,*** ZFAS1 expression significantly increased in NSCLC tissues compared with adjacent tissues. ***B,*** Expression levels of ZFAS1 relative to GAPDH were determined in the human NSCLC cell lines and 16HBE (normal human bronchial epithelial cell) by qRT-PCR. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

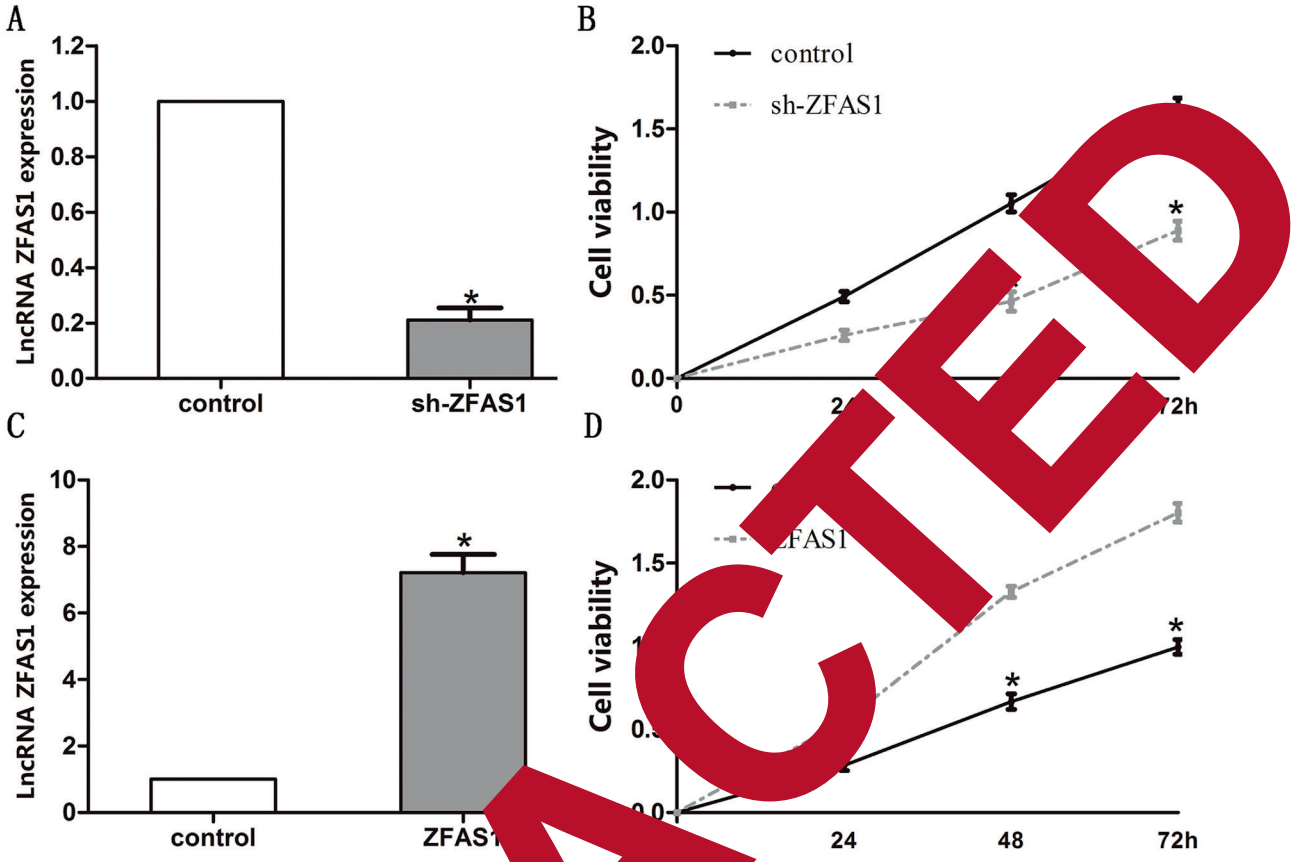
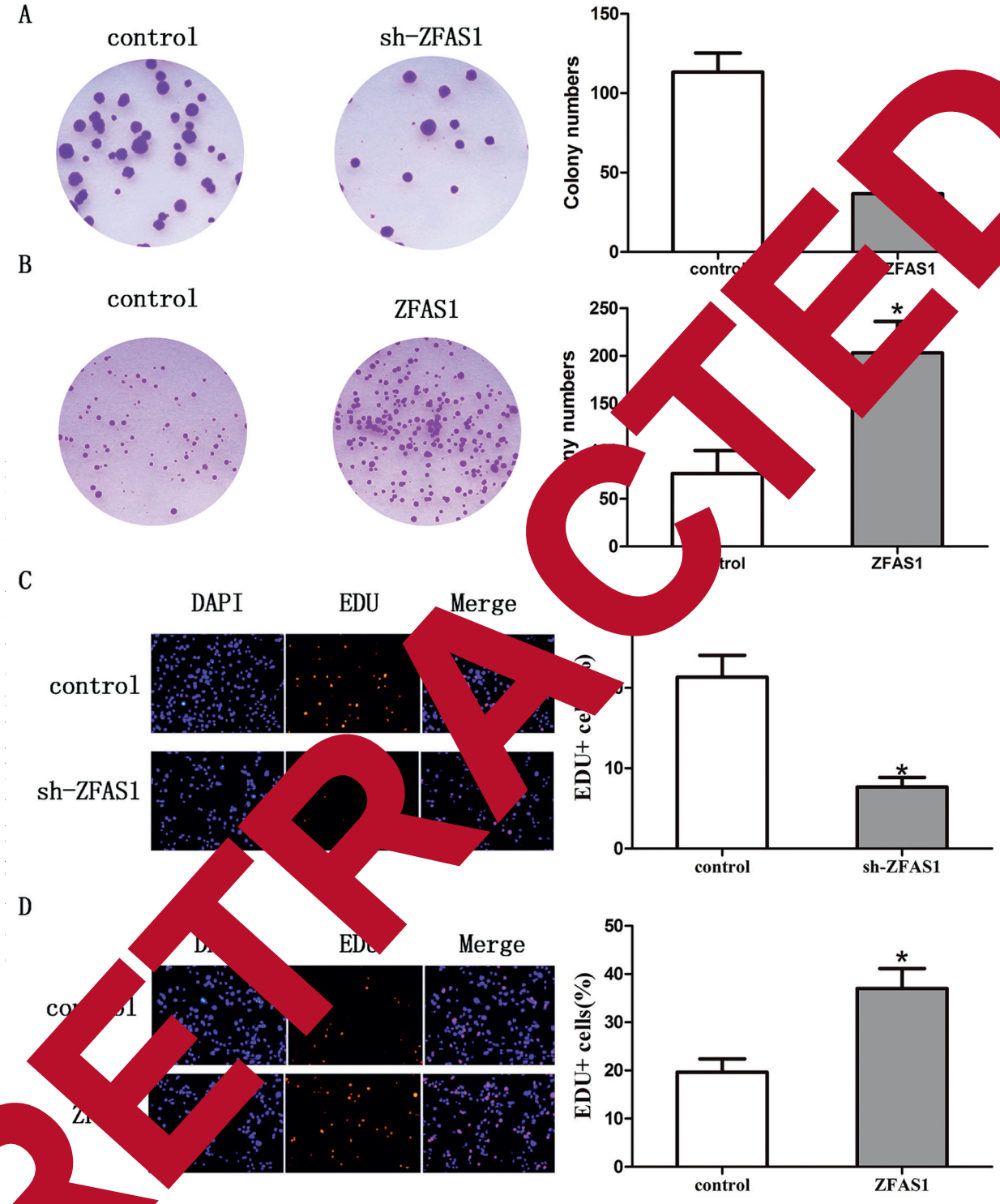


Figure 2. ZFAS1 promoted (the) growth ability of NS ***A,*** The ZFAS1 expression in H1299 cells transfected with sh-ZFAS1 and the control vector was de d by qRT-P APDH was used as an internal control. ***B,*** The CCK-8 assay showed that silence of ZFAS1 signif ssed pro ion in H1299 cells. ***C,*** The ZFAS1 expression in A549 cells transfected with ZFAS1 lentivirus ne) c vector w ected by qRT-PCR. GAPDH was used as an internal control. ***D,*** The CCK-8 assay showed tha overexp on of ZFAS ignificantly enhanced proliferation in A549 cells. The results represent the average of three ndent (mean ± standard error of the mean). *p<0.05, as compared with the control cells.

of ZFAS1 len ure 4C). ermore, the dual-luci se rep gene assay revealed that co-tr ection of ZF VT and miR-193a-3p mim argely decreased uciferase activity, w the co sfection of ZFAS1-MUT and mi a-3p no effect on the luciferase activity (P e). Mea e, correlation analysis onst that -193a-3p expression level gativ ated to ZFAS1 expression in NS C tissue igure 4E). In summary, these da nstrated that miR-193a-3p was a direct t o 1.

Discussion

Over the past several decades, the morbidity of lung cancer has increased worldwide, especially in industrially advanced countries. Primary features of the NSCLC are the migration and invasion of neoplasms, which are responsible for the high mortality rate[7]. The median survival time of NSCLC patients at an advanced stage barely exceeds 18 months from diagnosis[8]. An increasing number of studies have explored the important regulatory effects of ncRNAs on mammalian genes. It has been reported that dysfunction of ncRNA is involved in epigenetic alterations, which contribute to tumorigenesis and metastasis. Thus, tumor-related ncRNAs may provide novel ideas for the diagnosis and treatment of tumors. Several ncRNAs have been reported to contribute to the malignancy of tumor cells in NSCLC. Through the inhibition of STAT3, miR-124 depresses tumor growth and promotes cell apoptosis induced by radiation in NSCLC[9]. Through regulating miR-377-3p-E2F3 signaling pathway,



ure 3. ZFAS1 promoted (the) proliferation of NSCLC cells. ***A,*** The colony formation assay showed that the number of lonies significantly decreased *via* silence of ZFAS1 in H1299 cells. ***B,*** The colony formation assay showed that the number lonies significantly increased *via* the overexpression of ZFAS1 in A549 cells. ***C,*** The EdU incorporation assay showed t number of EdU-positive cells significantly decreased *via* silence of ZFAS1 in H1299 cells. ***D,*** The EdU incorporation assa showed that the number of EdU-positive cells significantly increased *via* (the) overexpression of ZFAS1 in A549 cells. Results represent the average of three independent experiments (mean ± standard error of the mean). *$p<0.05$, as compared with the control cells.

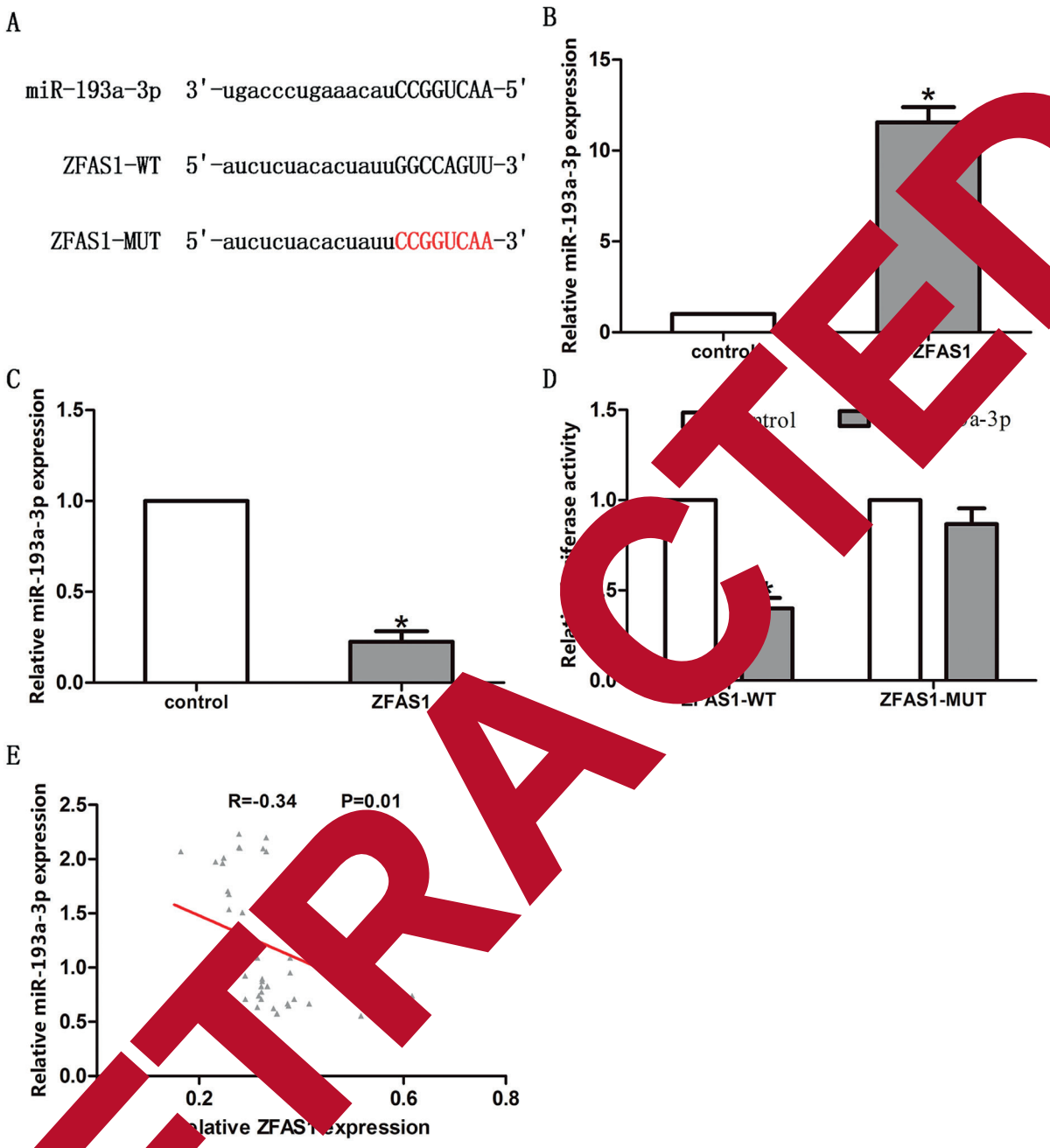


Fig ...Rec...l repres...n between ZFAS1 and miR-193a-3p. ***A,*** The binding sites of miR-193a-3p on ZFAS1. ***B,*** MiR-193a-3p ...n incre...n sh-ZFAS1 group compared with control group. ***C,*** MiR-193a-3p expression decreased in ...AS1 le... group ...pared with control group. ***D,*** The co-transfection of miR-193a-3p and ZFAS1-WT strongly de-...the lu... ...ity, while co-transfection of miR-control and ZFAS1-WT or miR-193a-3p and ZFAS1-MUT did not cha... ...he luci... ...tivity. ***E,*** The linear correlation between the expression level of miR-193a-3p and ZFAS1 in NSCLC tiss... The results ...present the average of three independent experiments. Data are presented as the mean ± standard error of... ...0.05.

...morigenesis of NSCLC and might act as an o... ...ne. Recently, many reports reveal that lncRNAs interact with microRNAs in disease progression. MiRNA expressions can be activated by lncRNAs. Moreover, lncRNAs regulate miRNA by targeting mRNA binding during tumorigenesis. For example, lncRNA CAMTA1 enhances cell mobility in breast cancer by targeting miR-20b [15]. A knockdown of lncRNA TUG1 depresses the cell proliferation and invasion in osteosarco-

ma *via,* sponging miR-153[16]. Many microRNAs are abnormally expressed in tumorigenesis. MiR-193a-3p functions as a tumor suppressor in many malignant tumors including NSCLC. For example, miR-193a-3p suppresses the progression of colorectal cancer by targeting KRAS[17]. The cell proliferation and metastasis of renal cell carcinoma are regulated by miR-193a-3p[18]. Recently, miR-193a-3p is identified as a tumor suppressor in lung cancer and inhibits its development of lung cancer *via,* targeting KRAS[19]. In this work, we firstly discovered the interaction between miR-193a-3p and lncRNA ZFAS1. We found that miR-193a-3p could directly bind to ZFAS1 through a dual-luciferase reporter gene assay. In addition, the miR-193a-3p expression could be suppressed by upregulating ZFAS1, while the down-regulated ZFAS1 induced a reverse outcome. Furthermore, the expression of miR-193a-3p was negatively correlated with ZFAS1 in NSCLC tissues. It is suggested that ZFAS1 might promote tumorigenesis of NSCLC through directly targeting miR-193a-3p.

Conclusions

We revealed that ZFAS1 was upregulated i NSCLC tissues and could facilitate cell proli ation in NSCLC through targeti R-193a-3 ZFAS1 may contribute to th eatment as a candidate target.

Conflict of Inter

The Authors decl have no confl interests.

Fundin knowledgeme

The s was gr d by the National Nature Science Fou n of C (No. 81473609).

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