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LncRNA FAM83A-AS1 aggravates the malignant development of esophageal cancer by binding to miR-495-3p

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Abstract. – OBJECTIVE: It is of significance to screen out differentially expressed long non-coding RNAs (lncRNAs) that can be utilized as tumor biomarkers in esophageal cancer. This study aims to uncover the effect of lncRNA FAM83A-AS1 on regulating migratory potential in esophageal cancer and the underlying mechanism.

PATIENTS AND METHODS: Tumor tissues and adjacent normal ones were collected from 62 esophageal cancer patients for detecting FAM83A-AS1 levels. Correlations of FAM83A-AS1 with clinical indexes and overall survival of esophageal cancer patients were analyzed. Thereafter, regulatory effects of FAM83A-AS1 on migratory potential in OE19 and OE33 cells were examined by transwell and wound healing assay. Then, the target genes of FAM83A-AS1 were predicted and functionally analyzed, and a protein interaction network was constructed. Finally, the mechanism of FAM83A-AS1 in regulating the downstream gene miR-495-3p was analyzed through Luciferase assay and rescue experiments.

RESULTS: It was found that FAM83A-AS1 was upregulated in esophageal cancer tissues and cell lines. Higher rates of lymphatic and distant metastasis and worse survival were observed in esophageal cancer patients expressing higher level of FAM83A-AS1. Besides, the knockdown of FAM83A-AS1 suppressed migratory potential in OE19 cells, while the overexpression of FAM83A-AS1 yielded the opposite trend in OE33 cells. Moreover, miR-495-3p was indicated to be the target gene binding FAM83A-AS1, and it was lowly expressed in esophageal cancer and negatively regulated by FAM83A-AS1. Furthermore, the overexpression of miR-495-3p partially abolished the regulatory effect of FAM83A-AS1 on migratory potential in esophageal cancer.

CONCLUSIONS: FAM83A-AS1 is upregulated in esophageal cancer, and it stimulates migratory potential in esophageal cancer by negatively regulating miR-495-3p.

Key Words:
FAM83A-AS1, MiR-495-3p, Esophageal cancer, Migration.

Introduction

Esophageal cancer is the eighth most popular malignancy in the world[1,2]. China has the highest incidence of esophageal cancer, and esophageal squamous cell carcinoma (ESCC) is the prevalent subtype[3,4]. Because ESCC is the prevalent subtype, ESCC patients were selected in this study.

Despite great advances have been achieved in the treatment of esophageal cancer, the 5-year survival is only about 15% to 20%[5,6]. In fact, most esophageal cancer patients are diagnosed in the middle or advanced stage because effective and sensitive diagnostic approaches are lacked[5,7]. Both environmental and genetic factors attribute to the development of esophageal cancer. In particular, people with special diets and lifestyles, such as cured foods, hot foods, long-term drinking, and heavy smoking, are more likely to develop esophageal cancer [8,9]. Currently, the role of genetic factors in the pathogenesis of esophageal cancer has been gradually recognized[9]. Higher susceptibility to tumors is observed in people carrying certain genetic mutations associated with malignant metabolism and tumor cell phenotypes[9-11]. It is of significance to seek for specific diagnostic and therapeutic targets for esophageal cancer in the early stage[12,13].

Long non-coding RNAs (lncRNAs) are non-coding RNAs with over 200 nucleotides long. They cannot encode proteins because open reading frames are lacked[14,15]. It is reported that over 60% genomes in mammals can be transcribed, and most of transcripts are lncRNAs[15,16]. Ln-

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cRNAs are involved in life activities[17,18]. In tumor diseases, dysregulated lncRNAs are capable of influencing tumor development[18,19]. Upregulated lncRNA H19 in colorectal cancer stimulates tumor growth by recruiting and binding to eIF4A3, thus leading to a poor prognosis[20].

FAM83A-AS1 is a classic tumor-associated lncRNA, serving as an oncogene in some types of tumors[21-23]. The aim of this paper was to uncover the role of FAM83A-AS1 in regulating esophageal cancer and the underlying mechanism.

Patients and Methods

Esophageal Cancer Samples

A total of 62 paired esophageal species and adjacent normal ones were surgically resected and stored at -80°C. Clinical data of them were recorded. This study was approved by the Ethics Committee of China-Japan Union Hospital of Jilin University and conducted after informed consent was obtained from each subject.

Cell Culture

Esophageal cancer cell lines (OE19, OE33, TE-1, and EC-109) and an esophageal epithelial cell line (HEEC) were purchased from 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% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C. Cell passage was conducted using 1×tyrpsin + ethylenediaminetetraacetic acid (EDTA).

Transfection

Cells were cultured to 30-40% confluence in 6-well plates and transfected with plasmids constructed by GenePharma (Shanghai, China), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 48 hours later, the cells were collected for the following use.

Transwell Migration Assay

A total of 200 μL of suspension (5×10^5 cells/mL) were inoculated in the upper transwell chamber (Millipore, Billerica, MA, USA) inserted in a 24-well plate with 500 μL of medium containing 10% FBS in the bottom. After 48-h incubation, bottom cells reacted with 15-min methanol, 20-min crystal violet, and captured using a microscope. Migratory cells were counted in 10 random fields per sample.

Wound Healing Assay

Cells were inoculated in 6-well plates and grown to 90% confluence. After an artificial wound was created in cell monolayer, medium with 1% FBS was replaced. 24 hours later, wound closure was captured.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Extracted RNAs by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNAs underwent qRT-PCR using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were the internal references. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. FAM83A-AS1: forward: 5'-ACCTGAGTGGTTTGGTTGGG-3', reverse: 5'-CCCCAGAGCACTTCCTTAGC-3', GAPDH: forward: 5'-CTGGGCTACACTGAGCACC-3', reverse: 5'-AAGTGGTCGTTGAGGGCAATG-3', MiR-495-3p: forward: 5'-GCGGAAACAAACATGGTGCA-3', reverse: 5'-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACAAGAAG-3', U6: forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'.

Luciferase Assay

Cells inoculated in a 24-well plate were co-transfected with miR-495-3p mimic/NC mimic and FAM83A-AS1-WT/FAM83A-AS1-MUT, respectively using Lipofectamine 3000. Then, they were lysed for determining the relative Luciferase activity 48 h later.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM Corp., Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation (SD). The differences between groups were analyzed by the *t*-test. Chi-square test was used for analyzing the relationship between FAM83A-AS1 and clinical data of esophageal cancer patients. Besides, Pearson correlation test was applied for evalu-

ating the relationship between expression levels of miR-495-3p and FAM83A-AS1 in esophageal cancer species. Finally, Kaplan-Meier curves were depicted for survival analysis, followed by Log-rank test. $p<0.05$ was considered as statistically significant.

Results

FAM83A-AS1 Was Highly Expressed In Esophageal Cancer

Compared with that in adjacent normal tissues, FAM83A-AS1 was upregulated in esophageal cancer tissues (Figure 1A). Similarly, it was highly expressed in esophageal cancer cell lines (Figure 1B).

High Level of FAM83A-AS1 Was Unfavorable to Prognosis in Esophageal Cancer Patients

Clinical data of included esophageal cancer patients were collected. Based on the median level of FAM83A-AS1, patients were assigned into high and low FAM83A-AS1 expression groups. As analyzed, FAM83A-AS1 level was positively correlated with rates of lymphatic and distant metastasis, while its level was unrelated to age, gender, and tumor staging in esophageal cancer patients (Table I). In addition, Kaplan-Meier curves revealed that high level of FAM83A-AS1 was unfavorable to overall survival in esophageal cancer patients (Figure 1C). This suggests that FAM83A-AS1 may be a new biomarker in predicting the malignant development of esophageal cancer.

FAM83A-AS1 Stimulated Migratory Potential in Esophageal Cancer

To explore the biological function of FAM83A-AS1 in esophageal cancer, the knockdown and overexpression models of FAM83A-AS1 in OE19 and OE33 cells were constructed, respectively (Figure 1D). Transwell assay showed that the knockdown of FAM83A-AS1 reduced migratory OE19 cells, and conversely, the overexpression of FAM83A-AS1 elevated migratory OE33 cells (Figure 2A). Similarly, the knockdown of FAM83A-AS1 decreased wound closure percentage in OE19 cells, and overexpression of FAM83A-AS1 yielded the opposite result in OE33 cells (Figure 2B).

FAM83A-AS1 Negatively Regulated MiR-495-3p

Through prediction in miRDB (n=66), TargetScan (n=53), and StarBase (n=103), miR-495-3p was screened out to be the only miRNA analyzed in three databases (Figure 3A). MiR-495-3p level was negatively regulated by

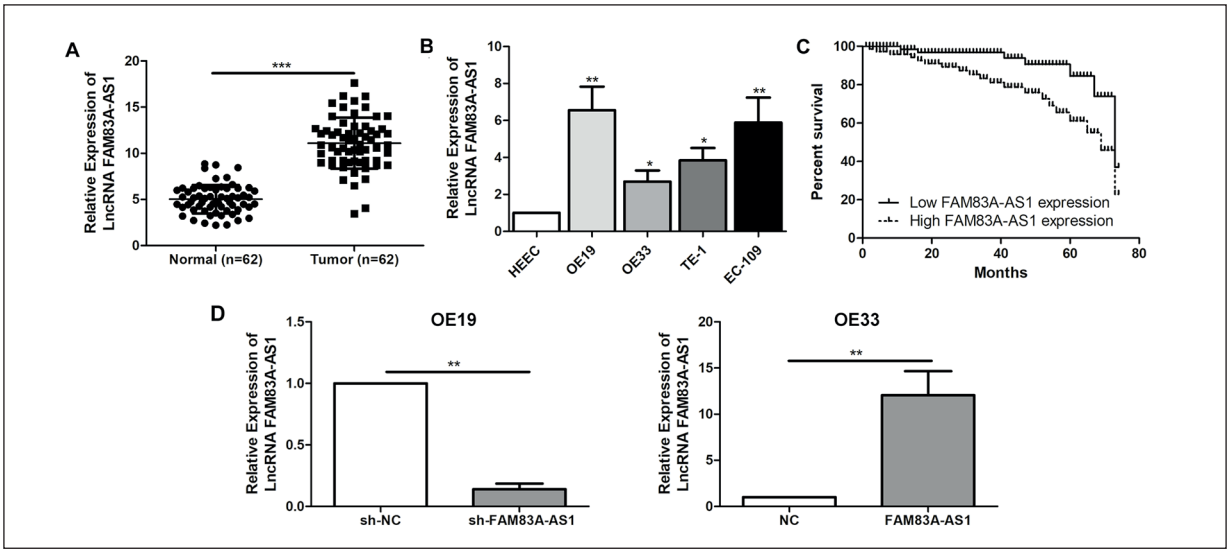

Figure 1. FAM83A-AS1 is highly expressed in esophageal cancer. **A,** FAM83A-AS1 levels in esophageal cancer tissues (n=62) and adjacent normal tissues (n=62). **B,** FAM83A-AS1 level in HEEC cells and esophageal cancer cells. **C,** Overall survival in esophageal cancer patients with high or low expression of FAM83A-AS1. **D,** Transfection efficacy of sh-FAM83A-AS1 and pcDNA-FAM83A-AS1 in OE19 and OE33 cells, respectively. Data are expressed as mean ± SD, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Table I. Association of LncRNA FAM83A-AS1 expression with clinicopathologic characteristics of esophageal cancer.

Parameters	No. of cases	FAM83A-AS1 expression		*p*-value
		High (%)	Low (%)	
Age (years)				0.426
< 60	26	14	12	
≥ 60	36	23	13	
Gender				0.502
Male	28	18	10	
Female	34	19	15	
T stage				0.065
T1-T2	36	25	11	
T3-T4	26	12	14	
Lymph node metastasis				0.005
No	38	28	10	
Yes	24	9	15	
Distance metastasis				0.006
No	42	30	12	
Yes	20	7	13	

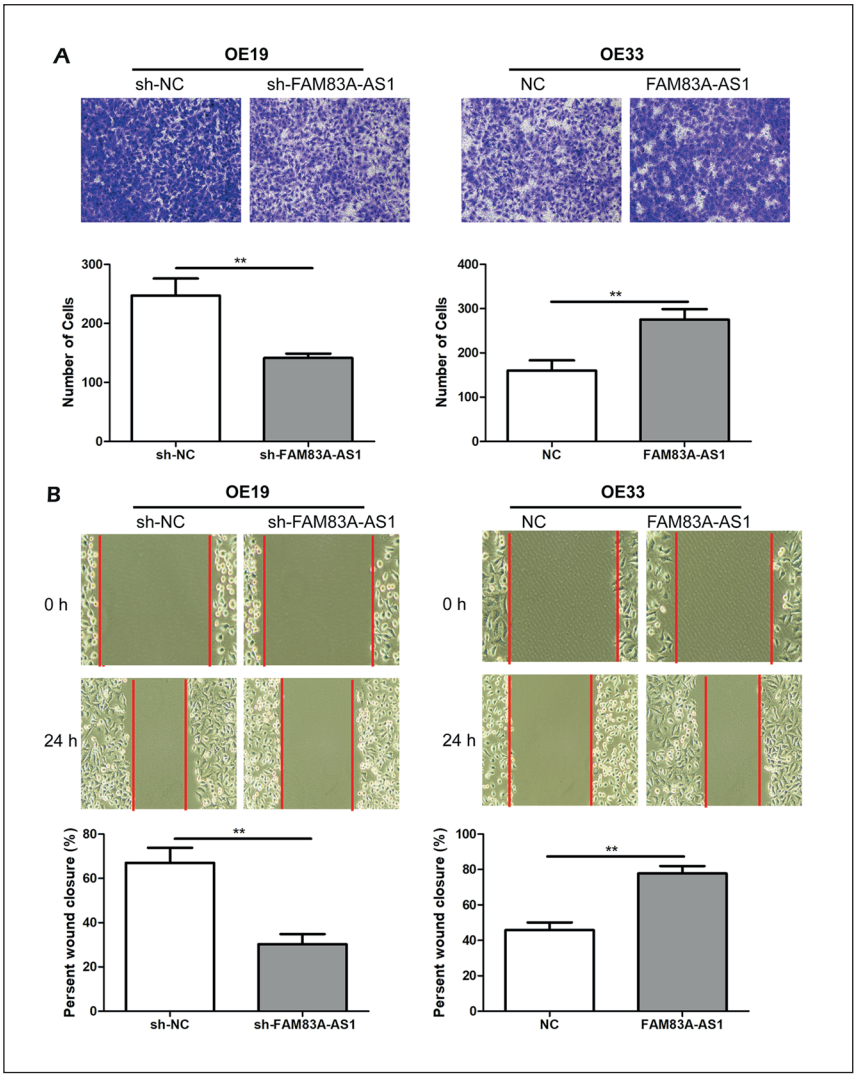

Figure 2. FAM83A-AS1 stimulates migratory potential in esophageal cancer. **A,** Migration in OE19 and OE33 cells influenced by FAM83A-AS1 (magnification: 40×). **B,** Wound healing percentage in OE19 and OE33 cells influenced by FAM83A-AS1 (magnification: 40×). Data are expressed as mean ± SD, $^{**}p < 0.01$.

FAM83A-AS1 in OE19 and OE33 cells (Figure 3B). Subsequently, Luciferase assay further verified that FAM83A-AS1 could specifically bind to miR-495-3p (Figure 3C). Compared with normal tissues and cell lines, miR-495-3p was lowly expressed in esophageal cancer species (Figure 3D) and cell lines (Figure 3F). Moreover, miR-495-3p level was found to be negatively correlated to FAM83A-AS1 level (Figure 3E).

MiR-495-3p Abolished Regulatory Effects of FAM83A-AS1 on Malignant Development of Esophageal Cancer

MiR-495-3p mimic and inhibitor were constructed to elucidate the involvement of miR-495-3p in the development of esophageal cancer. It was found that miR-495-3p could negatively regulate FAM83A-AS1 level in esophageal cancer cells (Figure 4A). Notably, decreased migratory number (Figure 4B) and wound closure percentage (Figure 4C) in OE19 cells with FAM83A-AS1 knockdown were partially reversed by co-silence of miR-495-3p. As expected, enhanced migratory potential in OE33 cells overexpressing FAM83A-AS1 was abolished by co-overexpression of miR-495-3p.

Discussion

Esophageal cancer is featured by high incidence and high mortality[1,2]. Conventional TNM staging is unable to predict risk stratification and estimate clinical outcomes[2-4]. Combined therapeutic strategies based on radiotherapy and chemotherapy display an unsatisfactory efficacy in esophageal cancer patients in middle or advanced stage[5-7]. Hence, it is necessary to develop novel biomarkers

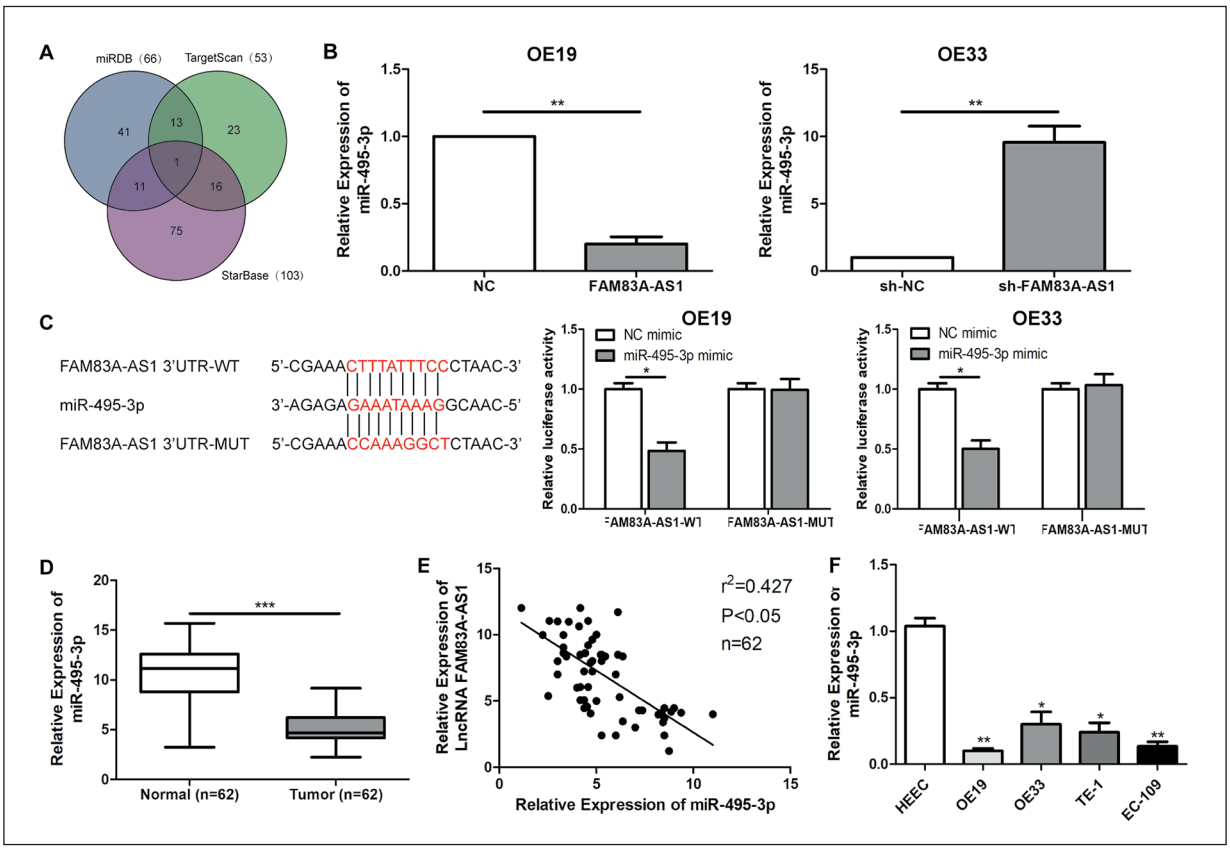

Figure 3. FAM83A-AS1 negatively regulates miR-495-3p. **A,** Potential target genes binding FAM83A-AS1. **B,** MiR-495-3p level in OE19 and OE33 cells influenced by FAM83A-AS1. **C,** Luciferase activity in co-transfected OE19 and OE33 cells. **D,** MiR-495-3p levels in esophageal cancer tissues (n=62) and adjacent normal tissues (n=62). **E,** A negative correlation between expression levels of FAM83A-AS1 and miR-495-3p in esophageal cancer species. **F,** MiR-495-3p level in HEEC cells and esophageal cancer cells. Data are expressed as mean ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

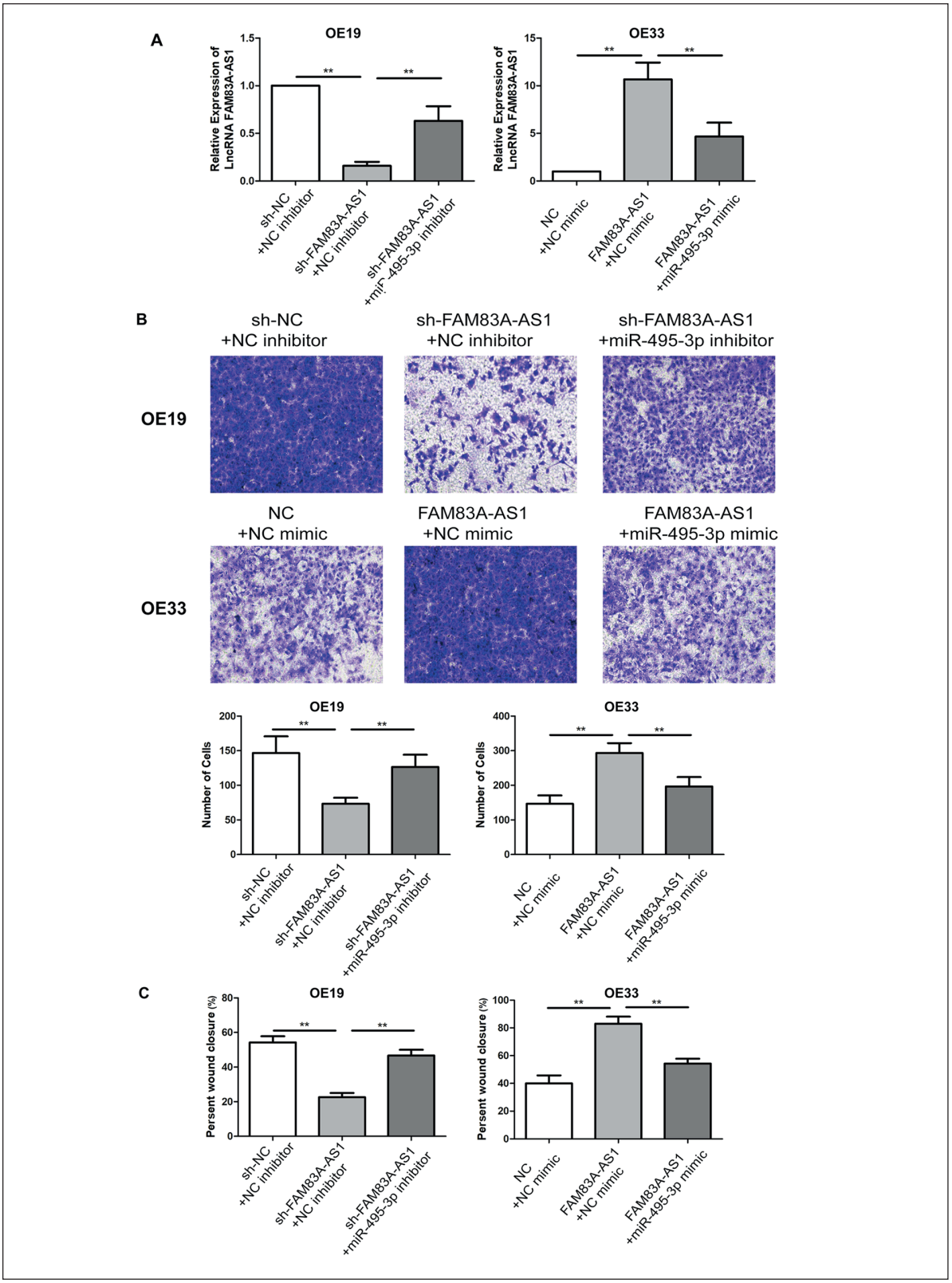

Figure 4. MiR-495-3p abolishes regulatory effects of FAM83A-AS1 on malignant development of esophageal cancer. **A,** FAM83A-AS1 level in OE19 and OE33 cells regulated by FAM83A-AS1 and miR-495-3p. **B,** Migration in OE19 and OE33 cells regulated by FAM83A-AS1 and miR-495-3p (magnification: 40×). **C,** Wound healing percentage in OE19 and OE33 cells regulated by FAM83A-AS1 and miR-495-3p (magnification: 40×). Data are expressed as mean ± SD, $^{**}p < 0.01$.

for esophageal cancer[12,13]. LncRNAs have been identified as vital regulators in life activities[14-16]. Through transcriptional, post-transcriptional or epigenetic regulations, lncRNAs are involved in tumor diseases[14-17]. Differentially expressed lncRNAs have been discovered in tumor species[17-20]. Generally speaking, tumor invasiveness is a key event that aggravates tumor development[23,24]. LncRNAs targeting tumor cell invasiveness improve clinical outcomes of affected people[18-20].

Owing to the tissue specificity, lncRNAs are considered to be linked to tumor development[16,17]. FAM83A-AS1 is upregulated in hepatocellular carcinoma, which is related to pathological features and prognosis[20-22]. Here, FAM83A-AS1 levels in esophageal cancer tissues and adjacent ones was determined. It was found that FAM83A-AS1 was upregulated in esophageal cancer tissues, and its level was positively correlated to metastasis rates. High level of FAM83A-AS1 was unfavorable to the prognosis in esophageal cancer patients. *In vitro* studies demonstrated the promotive effect of FAM83A-AS1 on migratory potential in esophageal cancer cells.

Abnormally expressed gene profiles (mRNAs and non-coding sequences) are closely related to human diseases including cancer[13,14]. LncRNA and circRNA can be used as competitive endogenous RNA (ceRNA) to regulate malignant tumor behaviors[23-25]. Through prediction in miRDB, TargetScan, and StarBase, downstream miRNAs that potentially bind to lncRNAs are analyzed[26]. After verification by Luciferase assay, miR-495-3p was detected to bind to FAM83A-AS1 and lowly expressed in esophageal cancer, and its level was negatively regulated by FAM83A-AS1. Interestingly, miR-495-3p was able to reverse the regulatory effects of FAM83A-AS1 on migratory potential in OE19 and OE33 cells. To sum up, a negative feedback loop FAM83A-AS1/miR-495-3p was responsible for the malignant development of esophageal cancer.

Conclusions

In short, FAM83A-AS1 is upregulated in esophageal cancer, and it stimulates migratory potential in esophageal cancer by negatively regulating miR-495-3p.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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