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The negative feedback loop FAM129A/CXCL14 aggravates the progression of esophageal cancer

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Abstract. – OBJECTIVE: To explore the molecular mechanisms of FAM129A in regulating the progression of esophageal cancer and its prognosis.

PATIENTS AND METHODS: FAM129A levels in esophageal cancer tissues and paracancerous ones were detected by quantitative real-time polymerase chain reaction (qRT-PCR). Its influences on clinical features and prognosis in esophageal cancer patients were analyzed. Changes in proliferation and apoptosis in esophageal cancer cells after knockdown of FAM129A were examined by cell counting kit-8 (CCK-8), 5-Ethynyl-2'- deoxyuridine (EdU) assay and flow cytometry, respectively. The feedback loop FAM129A/CXCL14 was finally assessed.

RESULTS: FAM129A was upregulated in esophageal cancer tissues. High level of FAM129A predicted advanced tumor staging, large tumor size and poor prognosis in esophageal cancer patients. Knockdown of FAM129A inhibited proliferative ability and induced apoptosis in OE19 and OE33 cells. In addition, knockdown of FAM129A upregulated protein level of CXCL14 in esophageal cancer cells. CXCL14 was downregulated in esophageal cancer tissues and negatively correlated to FAM129A level. The negative feedback loop FAM129A/CXCL14 was responsible for aggravating the malignant phenotypes of esophageal cancer cells.

CONCLUSIONS: FAM129A is upregulated in esophageal cancer samples, and it is linked to tumor staging, tumor size and poor prognosis. FAM129A aggravates the progression of esophageal cancer by negatively regulating CXCL14 level.

Key Words:
FAM129A, CXCL14, Esophageal cancer, Proliferation.

Introduction

As a globally prevalent malignant tumor, the incidence of esophageal cancer is very high in our country, and it is the fourth most-common cause of cancer death[1-5]. Esophageal cancer involved multiple factors: poor dietary habits, carcinogens, diseases, tobacco, alcohol addiction, genetic predisposition, age, gender, etc[3-5]. The detective rate of middle or late stage esophageal cancer remains high because of atypical symptoms in the early stage[5]. Seriously, the 5-year survival of esophageal cancer hovers a low rate since chemotherapy and/or radiotherapy response is unsatisfactory[6-8]. Therefore, screening high-risk population and popularization of esophageal endoscopy contribute to improve the clinical outcomes of esophageal cancer patients[9,10]. So far, targeted therapy has shown promising applications in esophageal cancer treatment[11,12]. We need to search effective and sensitive hallmarks that can be used for diagnosis and treatment of esophageal cancer[13,14].

FAM129A was screened from Eker rats with TSC2 deficiency by amplified fragment length polymorphism of complementary deoxyribose nucleic acids (cDNAs)[15,16]. Its abnormal expression is associated with many types of tumors and precancerous lesions. Overexpression of FAM129A triggers thyroid tumor invasion and thyroid nodule hyperplasia, which is a potential hallmark to differentiate benign and malignant thyroid nodules[17,18]. FAM129A has been identified to participate in the development of many types of tumors[19-21]. Bioinformatics analysis showed that CXCL14 binds FAM129A. The chemokine CXCL14 is able to regulate angiogenesis, inflammatory response, immune response and other aspects during tumor progression[22,23]. The deficiency of CXCL14 has been detected in tumor tissues, thus leading to tumor cell growth[24,25].

55 cases of esophageal cancer and paracancerous tissues were collected to analysis FAM129A level and clinical features in esophageal cancer patients. Meanwhile, its regulatory effects on the malignant progression of esophageal cancer and the underlying mechanisms were explored. Our findings may provide a novel hallmark for screening and detection of esophageal cancer.

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Patients and Methods

Esophageal Cancer Samples

Esophageal cancer tissues and paracancerous ones were collected from 55 patients between June 2017 and December 2019, pathologically confirmed by hematoxylin and eosin (H&E) staining and stored at -80°C. Clinical and follow-up data of the 55 esophageal cancer patients were completely recorded. Tumor node metastasis (TNM) staging and histological classification of esophageal cancer were defined according to the criteria proposed by UICC/AJCC. Inclusion criteria: patients with no severe diseases in other organs, and none of patients had preoperative chemotherapy or molecular targeted therapy. Exclusion criteria: patients with distant metastasis, those complicated with other malignancies, those with mental disease, those complicated with myocardial infarction, heart failure or other chronic diseases, or those previously exposed to radioactive rays. This investigation was approved by the Ethics Committee of Shangqiu First People's Hospital and it was conducted after informed consent of each subject.

Cell Culture

Esophageal cancer cell lines (OE19, OE33, TE-1, KYSE30 and EC-109) and the 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) in a 5% CO_2 incubator at 37°C. 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin were applied in culture medium.

Transfection

Cells were inoculated in 6-well plates and cultured to 60% confluence. They were transfected with plasmids constructed by GenePharma (Shanghai, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfection efficacy was tested by quantitative real-time polymerase chain reaction (qRT-PCR) at 48 h.

Cell Counting Kit-8 (CCK-8) Assay

Cells were inoculated in a 96-well plate with 2×10^3 cells per well. At the appointed time points, absorbance value at 490 nm of each sample was recorded using the CCK-8 kit (RIBOBIO, Guangzhou, China) for plotting the viability curves.

5-Ethynyl-2'-Deoxyuridine (EdU) Assay

Cells were pre-inoculated in a 24-well plate (2×10^4 cells/well). They were incubated in 4% methanol for 30 min, followed by 10-min permeabilization in 0.5% TritonX-100 (Solarbio, Beijing, China), and 30-min reaction in 400 μL of 1×ApollorR. Afterwards, cells were dyed in 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA) for another 30 min. Positive EdU-stained cells were counted for calculating EdU-positive rate.

Flow Cytometry

Cells were suspended in 0.5 mL of pre-cold 1×binding buffer and incubated with 1.25 μL of Annexin V-FITC (fluorescein isothiocyanate) in the dark for 15 min. After centrifugation at 1000×g for 5 min, the precipitant was re-suspended in 0.5 mL of 1×binding buffer and incubated with 10 μL of Propidium Iodide (PI) in the dark. Distributions of living cells, necrosis cells and apoptosis cells were determined by flow cytometry (FACSCalibur; BD Biosciences, Detroit, MI, USA). Apoptosis rate was calculated as the ratio of apoptosis cell number to the total cell number.

QRT-PCR

Extracted RNAs by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I treatment, and reversely transcribed into cDNAs using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). Primers used in qRT-PCR were synthesized using Primer 5.0 software. The obtained cDNAs underwent qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Each sample was performed in triplicate. Relative level was calculated by $2^{-\Delta\Delta Ct}$. FAM129A: forward: 5'-AGAAGGGTCACTACGGCTCA-3', reverse: 5'-CTCCAGGAGTCCAAACCAGG-3'; CXCL14: forward: 5'-ATCATCACCACCAAGAGCGT-3', reverse: 5'-TCTTCGTAGACCCTGCGCTT-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward: 5'-CAGAGCTCCTCGTCTTGCC-3', reverse: 5'-GTCGCCACCATGAGAGAC-3'.

Western Blot

Cells were lysed for isolating proteins and electrophoresed. Protein samples were loaded on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 hours. Primary and secondary antibodies were applied for indicated time. Band exposure and analyses were finally conducted.

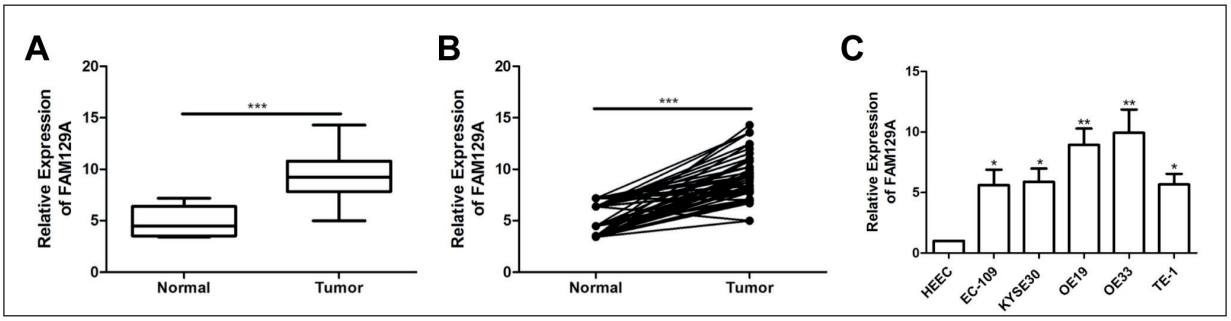

Figure 1. FAM129A was highly expressed in esophageal cancer tissues and cell lines. **A**, **B**, FAM129A levels in esophageal cancer tissues and paracancerous ones. **C**, FAM129A levels in esophageal cancer cell lines. Data were expressed as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Differences between groups were analyzed by the *t*-test. $p<0.05$ was considered as statistically significant.

Results

FAM129A Was Highly Expressed in Esophageal Cancer Tissues and Cell Lines

QRT-PCR data revealed higher abundance of FAM129A in esophageal cancer tissues than paracancerous ones (Figure 1A, 1B). As expected, FAM129A was upregulated in esophageal cancer cell lines (Figure 1C). OE19 and OE33 cells expressed the highest level of FAM129A among the five tested esophageal cancer cell lines, and they were utilized in the following experiments.

FAM129A Expression Was Correlated with Tumor Staging, Tumor Size and Overall Survival in Esophageal Cancer Patients

Recruited esophageal cancer patients were divided into high and low expression groups using the median level of FAM129A in their tumor tissues as the cut-off value. Chi-square analysis obtained the conclusion that FAM129A level was correlated to tumor staging and tumor size in esophageal cancer patients (Table I). In addition, the AUC value of 0.815 (95% CI=0.711-0.892) by the ROC curve was obtained, suggesting the diagnostic value of FAM129A in patients with esophageal cancer.

Table I. General information for the enrolled esophageal cancer patients with low and high expression of FAM129A.

Parameters	Number of cases	FAM129A expression		*p*-value
		Low (n=34)	High (n=21)	
Age (years)				0.821
<60	22	14	8	
≥60	33	20	13	
Tumor size				0.017
<2 cm	27	21	6	
≥2 cm	28	13	15	
T stage				0.018
T1-T2	32	24	8	
T3-T4	23	10	13	
Lymph node metastasis				0.173
No	35	24	11	
Yes	20	10	10	
Distance metastasis				0.052
No	35	25	10	
Yes	20	9	11	

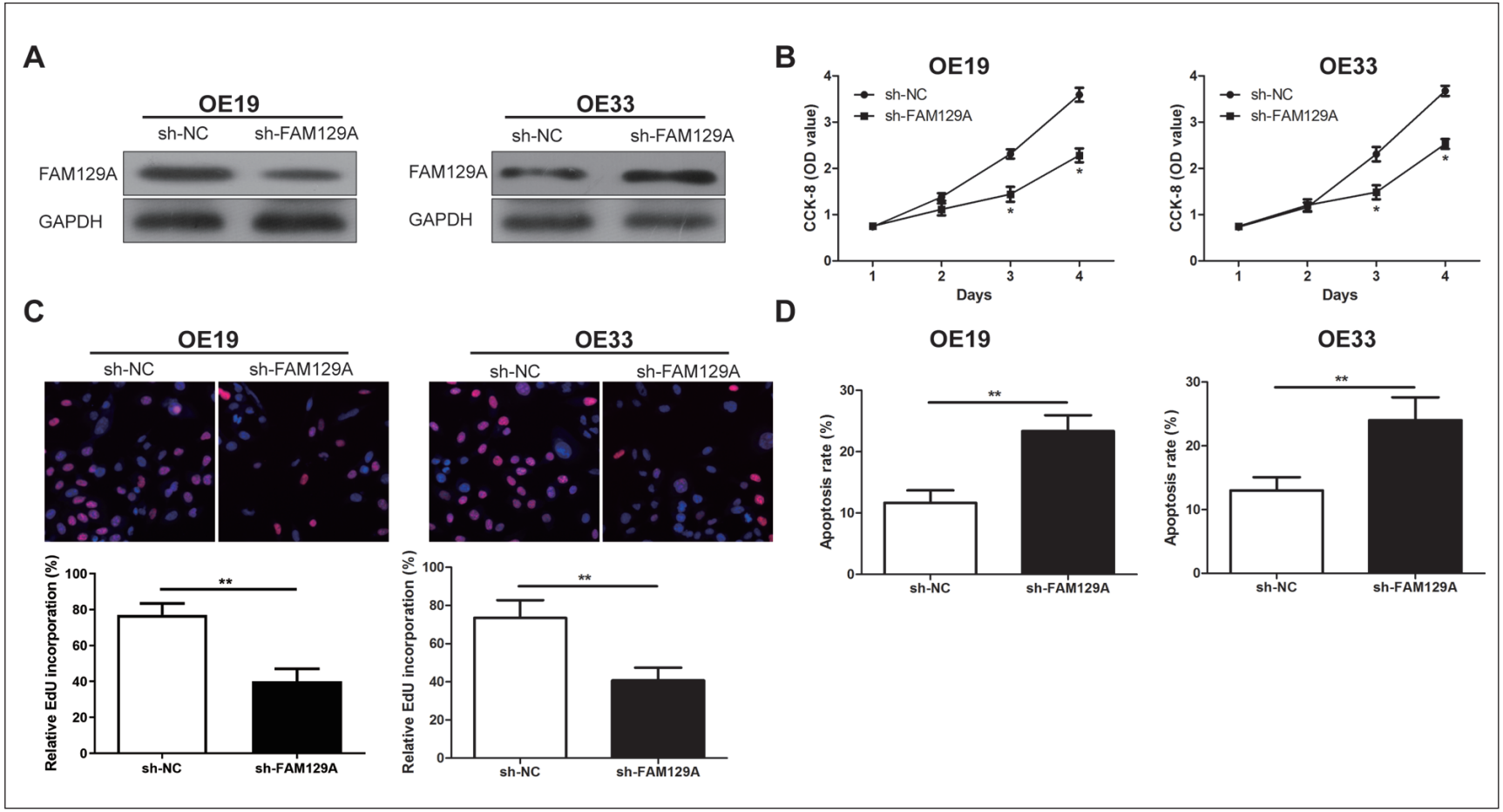

Figure 2. Knockdown of FAM129A inhibited proliferative ability and induced apoptosis in esophageal cancer. **A**, Protein level of FAM129A in OE19 and OE33 cells transfected with sh-NC or sh-FAM129A. **B**, Viability in OE19 and OE33 cells transfected with sh-NC or sh-FAM129A. **C**, EdU-positive rate in OE19 and OE33 cells transfected with sh-NC or sh-FAM129A, **D**, Apoptosis rate in OE19 and OE33 cells transfected with sh-NC or sh-FAM129A. Data were expressed as mean ± SD. *$p < 0.05$, **$p < 0.01$.

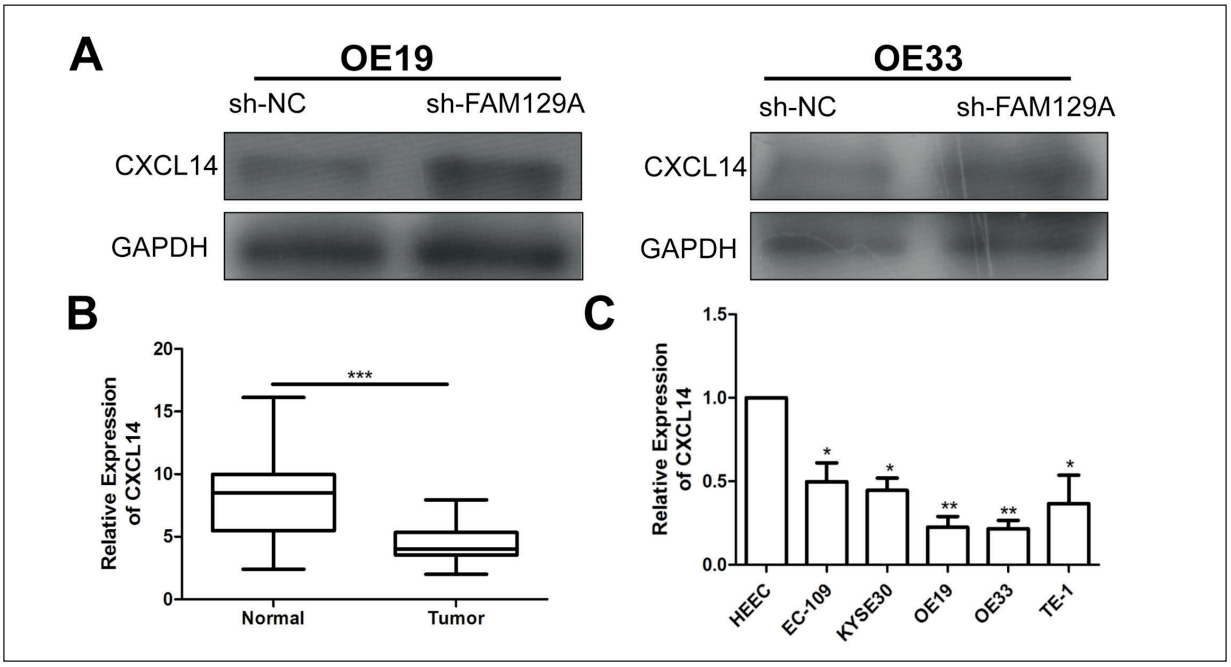

Figure 3. The negative interaction between FAM129A and CXCL14. **A**, Protein levels of CXCL14 in OE19 and OE33 cells transfected with sh-NC or sh-FAM129A. **B**, CXCL14 levels in esophageal cancer tissues and paracancerous ones. **C**, CXCL14 levels in esophageal cancer cell lines. Data were expressed as mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Knockdown of FAM129A Inhibited Proliferative Ability and Induced Apoptosis in Esophageal Cancer

Transfection of sh-FAM129A effectively downregulated FAM129A in OE19 and OE33 cells (Figure 2A). Knockdown of FAM129A greatly reduced viability and EdU-positive rate in esophageal cancer cells, suggesting the inhibited proliferative ability (Figure 2B, 2C). Subsequently, flow cytometry results illustrated higher apoptosis rate in OE19 and OE33 cells transfected with sh-FAM129A than those of controls (Figure 2D). Hence, FAM129A was able to promote proliferation and suppress cell apoptosis in esophageal cancer.

The Negative Interaction Between FAM129A and CXCL14

As Western blot analyses uncovered, knockdown of FAM129A upregulated protein level of CXCL14 in esophageal cancer cells (Figure 3A). CXCL14 was found to be downregulated in esophageal cancer tissues and negatively correlated to FAM129A level (Figure 3B). Similarly, CXCL14 was identically downregulated in esophageal cancer cells (Figure 3C). By analyzing follow-up data, lowly expressed CXCL14 was an unfavorable factor for the prognosis in esophageal cancer.

The Negative Feedback Loop FAM129A/CXCL14 Regulated Phenotypes of Esophageal Cancer

We thereafter focused on the involvement of FAM129A/CXCL14 axis in the malignant progression of esophageal cancer. Downregulated FAM129A in OE19 and OE33 cells with FAM129A knockdown was reversed by silence of CXCL14 (Figure 4A). Notably, higher viability and EdU-positive rate were found in cells with co-silence of FAM129A and CXCL14 than those with solely knockdown of FAM129A (Figure 4B, 4C). Flow cytometry data showed that knockdown of CXCL14 could abolish the regulatory effect of FAM129A on cell apoptosis in esophageal cancer (Figure 4D).

Discussion

The development of esophageal cancer is a complicated process involving gene mutations and signaling transduction[1-3]. Tumor hallmarks are beneficial to develop targeted therapy, displaying a vital clinical value[10-14]. High-expressed FAM129A has been identified in multiple types of tumors, which provide a favorable condition for malignant progression and differentiation of

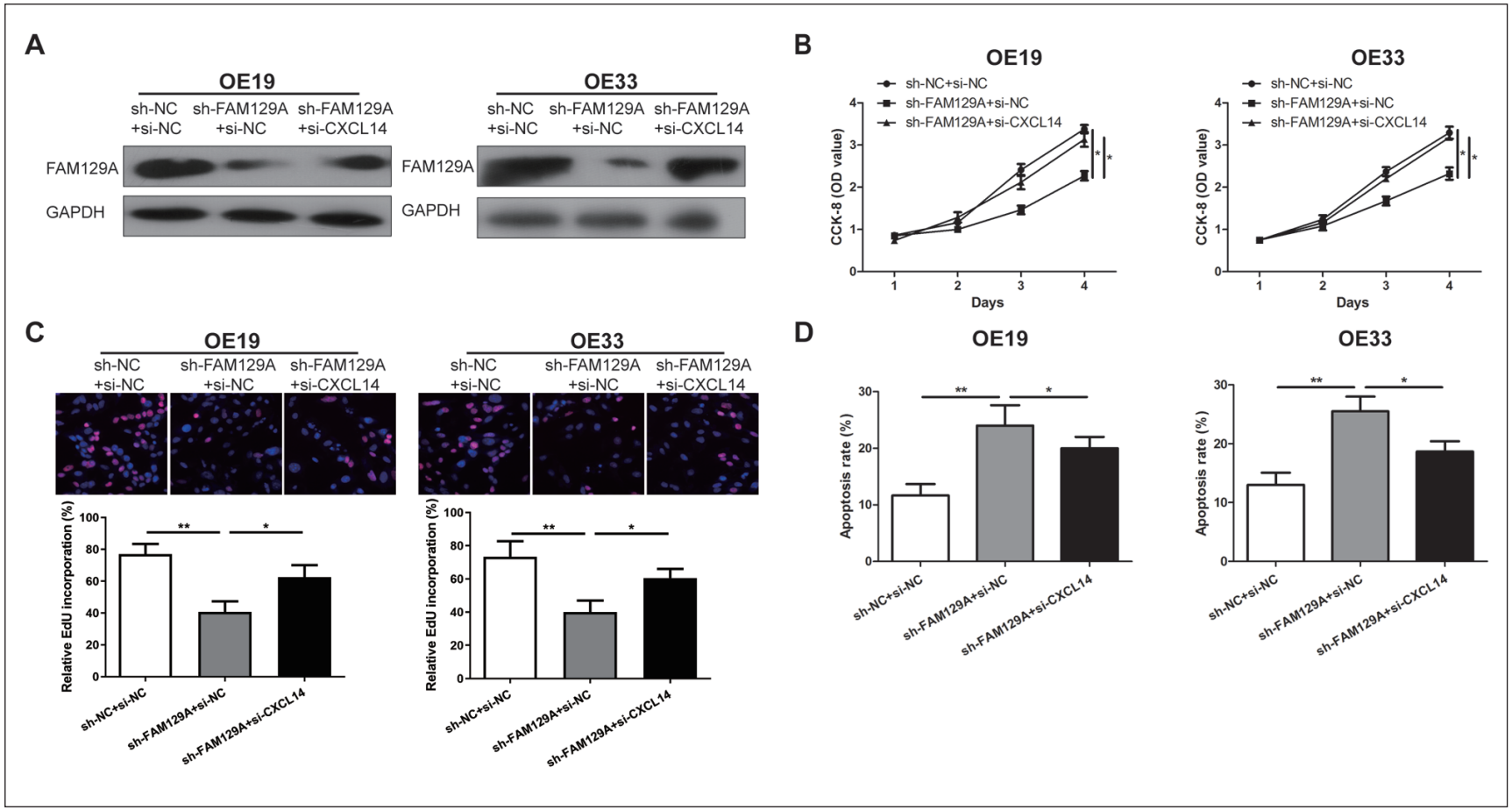

Figure 4. The negative feedback loop FAM129A/CXCL14 regulated phenotypes of esophageal cancer. **A**, Protein level of FAM129A in OE19 and OE33 cells co-transfected with sh-NC+si-NC, sh-FAM129A+si-NC or sh-FAM129A+si-CXCL14. **B**, Viability in OE19 and OE33 cells co-transfected with sh-NC+si-NC, sh-FAM129A+si-NC or sh-FAM129A+si-CXCL14. **C**, EdU-positive rate in OE19 and OE33 cells co-transfected with sh-NC+si-NC, sh-FAM129A+si-NC or sh-FAM129A+si-CXCL14. **D**, Apoptosis rate in OE19 and OE33 cells co-transfected with sh-NC+si-NC, sh-FAM129A+si-NC or sh-FAM129A+si-CXCL14. Data were expressed as mean ± SD. *$p < 0.05$, **$p < 0.01$.

tumor cells[20,21]. However, its biological functions in esophageal cancer are unclear. Here, we consistently found out that FAM129A was upregulated in esophageal cancer tissues. Moreover, it was correlated to tumor staging, tumor size and poor prognosis in esophageal cancer patients. FAM129A might exert cancer-promoting role in the progression of esophageal cancer.

Uncontrolled proliferation and apoptosis of tumor cells are fundamental events triggering tumor growth. Induction of tumor cell apoptosis is a novel research direction in anti-cancer treatment[26,27]. Our findings revealed that knockdown of FAM129A in esophageal cancer cells weakened proliferative ability and induced apoptosis. As a result, targeted regulation of FAM129A expression may be a new idea for the treatment of esophageal cancer.

Bioinformatics analysis suggested the interaction between FAM129A and CXCL14. Interestingly, knockdown of FAM129A upregulated protein level of CXCL14 in esophageal cancer cells. CXCL14 locates on human chromosome 5q31.1. Loss of heterozygosity (LOH) occurs in many cancers, and the deletion of genes at this site may be related to tumorigenesis. CXCL14 is highly expressed in normal tissues, and it is downregulated or even deficient in tumor samples[22,23]. As a member of the CXC chemokine family, CXCL14 is involved in many aspects of tumor progression[24,25]. In this study, esophageal cancer tissues were collected, and the qRT-PCR results showed CXCL14 was downregulated and negatively linked to FAM129A. Importantly, silenced CXCL14 was able to reverse the role of silenced FAM129A in regulating proliferative ability and apoptosis in esophageal cancer cells. Collectively, we have identified a negative feedback loop FAM129A/CXCL14, which was responsible for aggravating the malignant progression of esophageal cancer and became a promising biomarker for diagnosis and treatment for esophageal cancer.

Conclusions

FAM129A is upregulated in esophageal cancer samples, and it is linked to tumor staging, tumor size and poor prognosis. FAM129A aggravates the progression of esophageal cancer by negatively regulating CXCL14 level.

Conflict of Interest

The authors declare that they have no conflict of interests.

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