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MiR-940 inhibits the progression of NSCLC by targeting FAM83F

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Abstract. – OBJECTIVE: We investigate whether miR-940 could target family sequence similarity 83 member F (FAM83F) and further inhibit the progression of non-small cell lung cancer (NSCLC).

PATIENTS AND METHODS: The expression levels of miR-940 and FAM83F in tumor tissues and paracancerous tissues of 72 NSCLC patients were detected through quantitative real time-polymerase chain reaction (qRT-PCR). The relationship between their expression levels, tumor size, and prognosis of NSCLC was analyzed. Transfection plasmids were constructed to knockdown or overexpress miR-940 in H1299 cells (inhibitor group) and SK-MES-1 cells (mimic group). The viability of H1299 cells and SK-MES-1 cells was evaluated using cell counting kit-8 (CCK-8) assay after transfection. The combination of miR-940 and Ago2 was confirmed by RNA immunoprecipitation (RIP) experiment. The binding condition of miR-940 in FAM83F-WT and FAM83F-MUT groups was verified by luciferase reporter gene assay.

RESULTS: MiR-940 expression was noticeably decreased, while FAM83F expression was distinctly upregulated in NSCLC tissues than that of paracancerous tissues. The overall survival rate of NSCLC patients with highly-expressed miR-940 was significantly higher than those with lowly-expressed miR-940. Besides, miR-940 level was negatively correlated with tumor stage and size of NSCLC patients. Knockdown of miR-940 evidently enhanced the activity of H1299 cells, while overexpression of miR-940 decreased the viability of SK-MES-1 cells. In addition, miR-940 was confirmed to combine with FAM83F. Luciferase activity of cells co-transfected with FAM83F-WT and miR-940 mimic was significantly decreased.

CONCLUSIONS: MiR-940 inhibited the proliferation of cancer cells by targeting FAM83F and further restrained the progression of NSCLC.

Key Words:
MiR-940, FAM83F, NSCLC, Proliferation.

Introduction

Lung cancer is a malignant tumor with a high incidence and mortality rate, which seriously threatens human life and health[1,2]. Lung cancer is pathologically divided into two types including small cell lung cancer and NSCLC (non-small cell lung cancer)[3]. NSCLC accounts for about 85-90% of lung cancer cases, which is the main pathological type in clinical practice. NSCLC can be subdivided into three subtypes, including squamous cell carcinoma (25-30%), adenocarcinoma (40%), and large cell (undifferentiated) carcinoma (10-15%). Compared with small cell lung cancer, NSCLC exerts slow growth and late metastasis. However, the majority of NSCLC cases have been developed to middle or advanced stage when the clinical symptoms are apparent. Besides, only less than 20% of patients can be operated due to tumor metastasis and recurrence. More seriously, NSCLC patients that cannot undergo surgery are insensitive to radiotherapy and chemotherapy. Therefore, the prognosis of NSCLC patients so far is unsatisfactory. Recently, family sequence similarity 83 member F (FAM83F) has been found to present carcinogenic characteristics and high expression in various tumors[4]. Additionally, studies have shown that highly expressed FAM83F is related to tumor stage and survival rate of patients, suggesting its essential role in tumor occurrence and development[5,6]. FAM83F knockdown in human breast cancer cells can inhibit tumor formation[7] and affect the proliferation of cancer cells by regulating EGFR/RAS pathway[8]. At the same time, FMA83F can serve as a microRNA sponge to inhibit the proliferation and invasion of esophageal cancer cells[9,10]. MiRNA is an endogenous conserved non-coding RNA with a length of about 22 nucleotides. The first discovered miRNAs are lin-4 and let-7, which regulate the deve-

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lopment of the larvae of *Caenorhabditis elegans* in the gene regulatory network[11,12]. MiRNA has many different functions, such as mRNA degradation, translation inhibition, histone modification and DNA methylation, thus playing an important role in gene regulation. Research has shown that miRNA is almost involved in the whole process of tumor occurrence and development. MiRNA expression is not only specific in different tumor tissues, but also in the different stages[13]. Overexpression or inhibition of miRNA expression may be related to the occurrence of malignant tumors. For example, miRNA blocks programmed cell death and mediates tumor pathogenesis by regulating tumor cell growth or acting on transcription factors[14]. Many studies have found that the miRNA expression in tumor cells and normal tissues are significantly different, indicating that miRNA is vital in the formation and development of tumors. Researches have shown that miR-451 acts directly on CAB39 and promotes tumor growth and invasion by inhibiting CAB39 expression[15]. In esophageal cancer tumor cells, miR-30b inhibits tumor growth, metastasis and invasion by directly acting on HOXA1[16]. In NSCLC patients, miR-205 inhibits the expression of SMAD4 and promotes the growth of NSCLC[17]. MiR-374a is highly expressed in NSCLC tissues than that in normal tissues[18]. *In vivo* and *in vitro* functional experiments revealed that miR-374 could directly target Wnt5a as an oncogene, thereby regulating the proliferation, apoptosis, EMT (epithelial-mesenchymal transition), invasion and metastasis of NSCLC[19]. The above studies demonstrate that miRNA are involved in the occurrence and development of many tumors through regulating target genes and their signaling pathways, thus promoting or inhibiting cancer progression. Further study on the specific mechanism of miRNA can provide new directions and targets for the diagnosis and treatment of NSCLC.

Patients and Methods

Patients

NSCLC tissues and paracancerous tissues of 72 NSCLC patients were surgically resected. All patients did not receive preoperative tumor treatments, such as chemoradiotherapy and biological immunotherapy. This study was approved by the medical Ethics Committee of the Affiliated Tumor Hospital of Xinjiang Medical University, and all patients have signed informed consents. The obtained specimen tissues were stored in liquid nitrogen within 10 min after surgical excision for future use. The detailed descriptions of clinical and tumor characteristics were shown in Table I.

Cell Culture

NSCLC cell lines (H1299, H1755, SK-MES-1, A549) were purchased from ATCC (Manassas, VA, USA). The cells were cultured in DMEM-F12 (Dulbecco's modified eagle medium –F12) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) at 37°C and 5% CO_2 incubator. The growth status of cells was daily observed. Fresh culture medium was replaced every 1-2 days. The cells were passaged when they reach 80% confluence.

Cell Transfection

The cells in logarithmic phase were seeded into 6-well plates. When the adherent cells reached 50-80% of confluence, the transfection was carried out according to the instructions of the Lipofectamine 2000 transfection kit (Invitrogen, Carlsbad, CA, USA). 50 nmol/L of miR-940 mimic or miR-940 inhibitor was transfected in each well.

RNA Extraction and Quantitative Real Time Polymerase Chain Reaction (Qrt-PCR)

1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) was added to the cell or tissue samples for extracting total RNA. The RNA concentration was measured by NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The reverse transcriptase kit was used to reverse RNA to cDNA. The reverse transcriptase system contained 1 μL 10 mmol/L Oligo (dT), 8 μL mRNA and 3 μL Diethyl pyrocarbonate (DEPC) treated water (Beyotime, Shanghai, China). After centrifugation and gentle mix, the mRNA expression level was detected by ABI7500 (Applied Biosystems, Foster City, CA, USA) fluorescence quantitative PCR. PCR reaction system was carried out in accordance with the instructions of the kit (miScript SYBR Green PCR kit) (TaKaRa, Otsu, Shiga, Japan) at 95°C for 5 min, 40 cycles of 95°C for 15 s, 58°C 30 s and 74°C 30 s. Finally, computed tomography (CT) values were obtained. Each sample was retested for 3 times. At the end of the reaction, all the CT values were recorded for statistical analysis of data. Primers were as

Table I. Correlation of miR-940 expression and clinicopathologic features of NSCLC.

Clinicopathological features	Number	miR-940 expression		*p*-value
		Low (n=36)	High (n=36)	
GAge (years)				0.8130
≤ 56	33	17	16	
> 56	39	19	20	
Gender				0.3458
Male	36	20	16	
Female	36	16	20	
Tumor size				0.0003*
≤ 3 CM	45	15	30	
> 3 CM	27	21	6	
TNM stage				0.0001*
I-II	30	7	23	
III-IV	42	29	13	
Lymph node metastasis				0.0590
Absent	30	13	17	
Present	42	23	19	

*$p < 0.05$.

follows: U6 (forward): 5'-GTCCTGGCAGATATACACTAAACAT-3'; U6 (reverse): 5'-CTCACGCTTGAATTCATGCGGCTT-3'; miR-940 (forward): 5'-GCATCGTTCCTTCAAGCCGATCT-3'; miR-940 (reverse): 5'-TGGGTGAGTCGTTCGG-3; FAM83F (forward): 5'-ACAGAACGTAGAGCCCTTTGACA-3'; FAM83F (reverse): 5'-TCGAGCCACAGTGGAGGAGTA-3'

Cell Counting Kit-8 (CCK-8) Assay

Cells in good growth were treated with trypsin to prepare cell suspension, and the cell density was adjusted to 1.0×10^5/mL. 100 μL cell suspension was added in each well of 96-well plate and placed at 37°C and 5% CO_2 incubator. After cell culture for 6 h, 24 h, 48 h, 72 h, 96 h, respectively, 10 μL of CCK-8 solution (Dojindo, Kumamoto, Japan) was added. After incubate in 37°C for 4 h, the optical density (OD) at the wavelength of 450 nm was determined by Microplate Spectrophotometer (Biotek Winooski, VT, USA).

RNA Binding Protein Immunoprecipitation Assay

Cell sediment was collected by centrifugation for 5 min at 12000 r/min in 4°C, and was suspended by PBS after discarding the supernatant. Then, we repeated the above treatment twice. The collected cells were treated with a proper amount of RNA immunoprecipitation (RIP) washing buffer, protease inhibitor mixture, RNase inhibitor, and lysed by ultrasound instrument. The supernatant was obtained for immunoprecipitation by centrifugation for 10 min at 12000 r/min in 4°C, and then stored at -80°C. The magnetic beads were prepared. According to Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Abcam, Cambridge, MA, USA), the antibody against target protein was coated with magnetic beads and incubated with RNA extracted from the supernatant. The RNA was extracted by TRIzol, and qRT-PCR was used to detect the RNA enrichment.

Luciferase Reporter Gene Assay

24 h before transfection, cells were digested with trypsin and seeded into a 24-wells plate. On the day of transfection, the culture medium was removed and replaced with fresh antibiotic-free complete culture medium. The mixture of transfection reagent and different plasmids were prepared. FAM83F-WT plasmid, FAM83F-MUT plasmid, and basic medium were mixed in A tube, while the transfection reagent and basic medium were mixed in B tube. Then, B tube solution was added to each group of A tube. The transfection mixture was dripped to cells evenly, followed by cell culture in the incubator at 37°C. After transfection for 48 h, the cell transfection efficiency was measured by fluorescence microscopy. The cells transfected were grouped as follows: 1) FAM83F-WT and NC negative control; 2) FAM83F-WT and miR-940 mimic; 3) FAM83F-WT and miR-940 inhibitor; 4) FAM83F-MUT and NC negative control; 5) FAM83F-MUT and miR-940 mimic; 6) FAM83F-MUT and miR-940

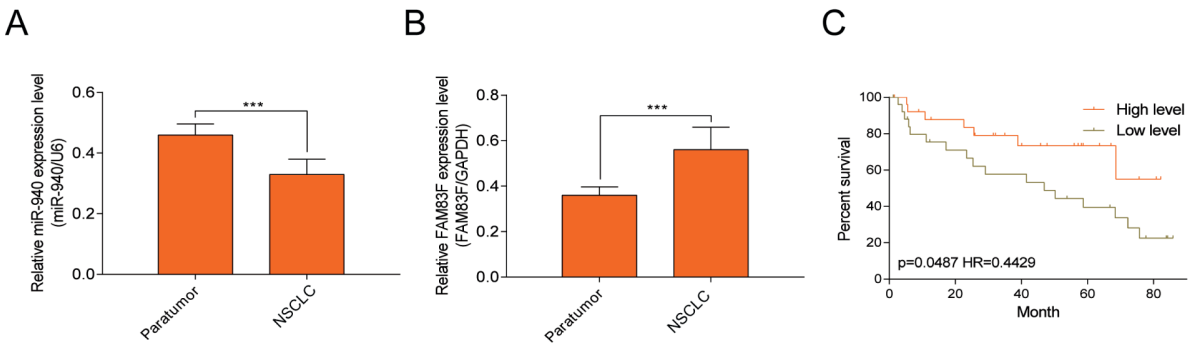

Figure 1. Low expression of miR-940 in NSCLC tissues. ***A,*** The expression level of miR-940 in tumor tissues was significantly higher than that in paracarcinoma tissues of 72 NSCLC patients. ***B,*** FAM83F expression in NSCLC tissues was significantly higher than that in paracarcinoma tissues. ***C,*** The survival rate of NSCLC patients with high miR-940 expression was significantly higher than that of low expression.

inhibitor. After transfection for 48 h, the cells were digested and the luciferase activity of each group was detected.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) was used for statistical analysis, and Graphpad Prism software (La Jolla, CA, USA) was used for image processing and analysis. The measurement data were expressed in the form of mean ± standard deviation ($\bar{x}\pm s$). Paired *t* test was used for measurement data analysis between two groups, and chi-square test was used for classification data. $p<0.05$ was considered statistically significant.

Results

MiR-940 was Lowly Expressed in NSCLC Tissues

QRT-PCR data indicated that the mRNA level of miR-940 in NSCLC samples was significantly higher than that in paracancerous samples (Figure 1A). At the same time, miR-940 expression was found negatively correlated with tumor stage and size in NSCLC patients (Table I). By bioinformatics prediction, FAM83F was screened out as the potential target gene of miR-940. In addition, it was found that FAM83F expression in NSCLC samples was significantly higher compared with the paracancerous samples (Figure 1B). The survival analyses showed that the overall survival of NSCLC patients with high miR-940 expression was significantly higher than those with low expression. (Figure 1C).

MiR-940 Inhibited the Proliferation of NSCLC Cells

To further confirm the effect of miR-940 on NSCLC cells, its expression in NSCLC cell lines was detected. The results indicated that miR-940 expression was the highest in H1299 cells and the lowest in SK-MES-1 cells (Figure 2A). Thus, H1299 and SK-MES-1 cells were selected for subsequent experiment. We transfected miR-940 inhibitor in H1299 cells and miR-940 mimic in SK-MES-1 cells, respectively. QRT-PCR was used to identify the transfection efficiency. As a result, miR-940 expression was significantly decreased in H1299 cells (Figure 2B) and was remarkably increased in SK-MES-1 cells (Figure 2C). The cell viability of cells after overexpression or knockdown of miR-940 was detected by CCK-8 assay. The results showed that the activity of H1299 cells increased significantly after miR-940 knockdown (Figure 2D), while the opposite result was observed after the overexpression of miR-940 in SK-MES-1 cells (Figure 2E).

MiR-940 Inhibited NSCLC Progression by Regulating Target Gene FAM83F

To further explore the interaction of miR-940 and FAM83F, we constructed the dual-luciferase reporter vectors of FAM83F wild-type (FAM83F-WT) and mutant (FAM83F-MUT). MiR-940 combined with the FAM83F-WT recombinant vector, but the binding site of miR-940 in FAM83F-MUT recombinant vector was missing (Figure 3A). Correlation analyses showed that miR-940 was negatively correlated to FAM83F (Figure 3B). Meanwhile, in the RIP

experiment, we verified that miR-940 was involved in the formation of silent complex RISC (Figure 3C&3D). The results of luciferase reporter gene experiment showed that the fluorescence values of FAM83F-WT and miR-940 mimic group were significantly decreased in H1299 and SK-MES-1 cells (Figure 3E-3F).

Discussion

Lung cancer is also known as bronchial tumor, referring to the malignant tumor originated from the bronchial mucosal epithelium. Its morbidity and mortality are in the forefront of all kinds of malignant tumors, which seriously threatens the

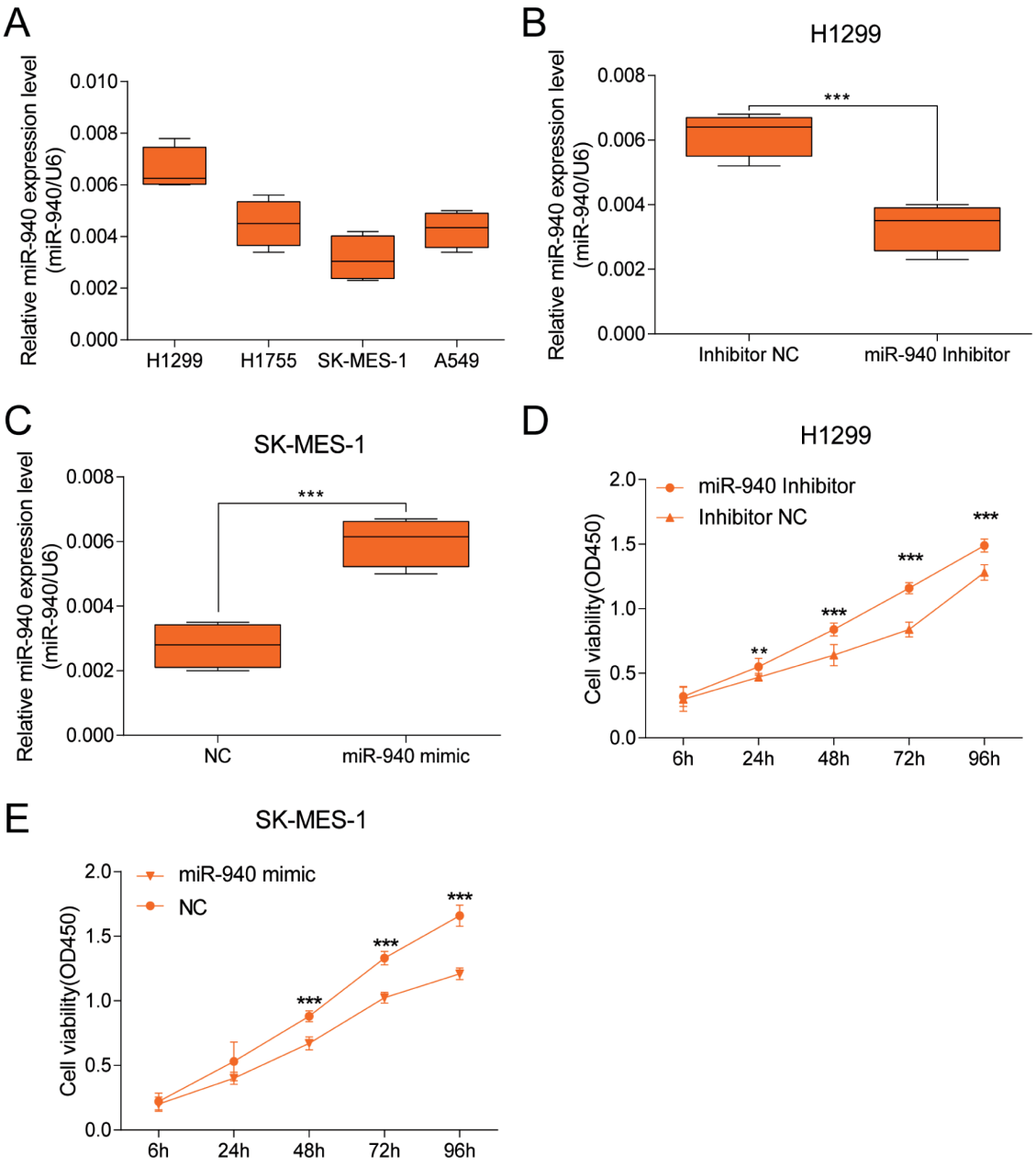

Figure 2. MiR-940 inhibited the proliferation of NSCLC cells. ***A,*** The expression of miR-940 was the highest in H1299 cells and the lowest in SK-MES-1 cells ***B,*** MiR-940 expression was significantly decreased in H1299 cells transfected with miR-940 inhibitor. ***C,*** MiR-940 expression was increased in SK-MES-1 cells transfected with miR-940 mimic. ***D,*** The activity of H1299 cells increased significantly after interfering with miR-940 expression. ***E,*** After the overexpression of miR-940 in SK-MES-1 cells, the cell activity was significantly decreased.

health of all mankind. Lung cancer poses great pressure and heavy burden on medical and health systems of various countries[20, 21]. The main pathological types of lung cancer include small-cell lung cancer and NSCLC, of which NSCLC accounts for 85-90% of total lung cancer cases[22]. The unremitting efforts of researchers in the field of oncogene, anti-oncogene and related signal transduction pathway for many years have greatly improved the efficacies of diagnosis and treatment of NSCLC. However, the 5-year survival rate of NSCLC patients is still not optimistic. Therefore, it is an important topic in current therapeutic field to deeply study the molecular mechanism of NSCLC and find new therapeutic targets. MiRNA is a class of small non-coding RNA and can regulate the expressions of target genes. MiRNA is differentially expressed in a wide va-

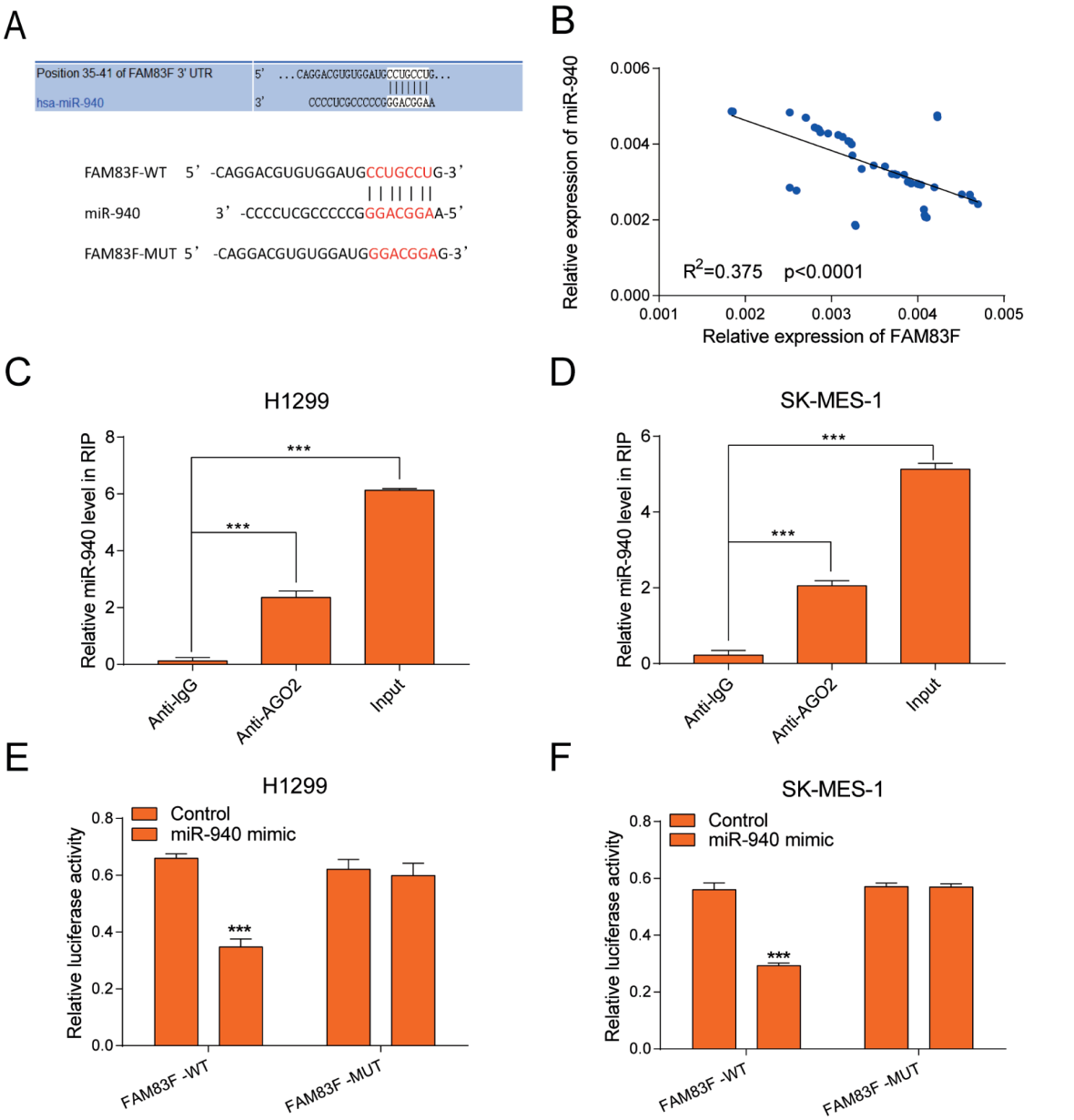

Figure 3. MiR-940 inhibited NSCLC progression by regulating target gene FAM83F. ***A,*** Construction FAM83F-WT and FAM83F-MUT sequence. ***B,*** MiR-940 was negatively correlated to FAM83F. ***C-D,*** In the RIP experiment, it is verified that miR-940 was involved in the formation of silent complex RISC. ***E-F,*** The results of luciferase reporter gene experiment showed that the fluorescence values of FAM83F-WT and miR-940 mimic group significantly decreased in H1299 and SK-MES-1 cells.

riety of tumors. Some miRNA families display functions similar to oncogenes or tumor-suppressor genes[23], and they mediate the expressions of target genes mainly by inhibiting translation or degradation of mRNA. Meanwhile, miRNA is involved in the biological processes of most mammals and functions in controlling various signaling pathways[24]. Studies have shown that miRNA mainly regulates key factors in development, differentiation, apoptosis and cell cycle[25]. MiRNA also plays a role in the occurrence and development of EMT (epithelial-mesenchymal transition)[26], which can affect the prognosis of tumor patients[27]. As a small RNA, miR-940 has become a hot research topic, especially in the research on the relationship between tumors, cell growth and cell differentiation. It is very likely to be an ideal target for gene therapy for malignant tumors. Studies have shown that miR-940 in plasma can be a new biological marker in gastric cancer[28]. Additionally, miR-940 was found to inhibit the growth of HCC cells and was associated with the prognosis of HCC patients[29]. Meanwhile, miR-940 could inhibit the growth and migration of triple-negative breast cancer cells[30]. By targeting PKC-δ, miR-940 overexpression can inhibit cell proliferation and induce apoptosis in OVCAR3 cells of ovarian cancer[31]. However, the role of miR-940 in NSCLC has been rarely reported. In this study, we explored the role of miR-940 in NSCLC by detecting its mRNA level in tumor tissues and paracarcinoma tissues of NSCLC patients. The relationship between miR-940 expression and the survival rate of NSCLC patients was analyzed as well. Viability of NSCLC cells was enhanced after miR-940 knockdown. At the same time, we found that FAM83F was highly expressed in the tumor tissues of NSCLC patients. Therefore, we suspected that miR-940 affected the occurrence and development of NSCLC by acting on FAM83F. The binding of miR-940 and FAM83F was further verified through the RIP experiment, indicating that miR-940 could target FAM83F and further inhibit the progress of NSCLC.

Conclusions

We showed that miR-940 inhibited the progress of NSCLC by targeting FAM83F.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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