European Review for Medical and Pharmacological Sciences 2019; 23: 5657-5666

MicroRNA-93-5p/IFNAR1 axis accelerates metastasis of endometrial carcinoma by activating the STAT3 pathway

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Abstract. – OBJECTIVE: The aim of this study was to elucidate whether microRNA-93-5p/IFNAR1 axis promoted proliferative and metastatic changes of endometrial carcinoma (EC) cells by regulating the STAT3 signaling pathway.

PATIENTS AND METHODS: The expression of microRNA-93-5p in EC tissues and cells was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Western blot was conducted to detect the protein expression of IFNAR1 in EC cells. Chi-square test was used to analyze the relationship between microRNA-93-5p expression and pathological characteristics of EC patients. The negative control, microRNA-93-5p mimics, microRNA-93-5p inhibitor or IFNAR1 vector were transfected into EC cells. Moreover, the proliferative and migratory potentials of EC cells were determined by cell counting kit-8 (CCK-8) and transwell assay, respectively.

RESULTS: MicroRNA-93-5p expression was significantly upregulated in EC tissues and cells. High expression of microRNA-93-5p indicated poor survival of EC patients. The microRNA-93-5p expression level was correlated with FIGO stage and lymph node metastasis of EC. Meanwhile, a negative correlation was found between microRNA-93-5p and IFNR1 in EC tissues. Furthermore, the overexpression of microRNA-93-5p remarkably increased the viability and migratory rate of EC cells, which were reversed by IFNR1 up-regulation.

CONCLUSIONS: MicroRNA-93-5p/IFNAR1 axis accelerates metastasis of EC through the STAT3 pathway.

Key Words:
Endometrial carcinoma (EC), MicroRNA-93-5p, IFNAR1, STAT3.

Introduction

Endometrial carcinoma (EC) is one of the three major malignancies in the female reproductive system, ranking fourth in all female malignancies. Recently, the incidence of EC is on the rise[1-4]. So far, surgery is still the main therapeutic method for EC. Radiotherapy, chemotherapy and hormone drugs are usually applied in advanced or recurrent EC patients[5,6]. Recent data have shown a much younger onset of EC. Although the 5-year survival of early-stage EC is over 90%, it remains less than 20% in EC patients with distant metastasis[7]. The prognosis of advanced, poorly differentiated or specific types of EC is extremely poor. Hence, it is of great significance to elucidate the pathogenesis of EC, thereby developing a novel therapeutic target and improving clinical outcomes.

MicroRNAs are a type of non-coding, small RNAs with about 19-25 nucleotides in length. It is known to all that microRNAs are mainly distributed in eukaryotes. RNA-induced silencing complex regulates the expression of target mRNAs at the post-transcriptional level, thereafter degrading mRNAs or inhibiting mRNA translation[8]. At present, microRNAs are found to be closely related to tumorigenesis. Studies have demonstrated that they are involved in the processes of tumor cell differentiation, proliferation and apoptosis[9]. Due to aberrant expressions of miRNAs in various types of tumors, microRNAs can be served as diagnostic and prognostic markers. Typical microRNA expression profiles can accurately classify tumors. These tumor-specific microRNAs participate in tumor development, acting as oncogenes or tumor-suppressor genes[10]. Furthermore, they are involved in the regulation of tumor-associated signaling pathways, including JAK/STAT3 pathway[11], NF-KB pathway[12], and MAPK/ERK pathway[13]. MicroRNAs relative to EC have been widely concerned as well. For example, microRNA-200 family[14] and microRNA-205[15] are highly expressed in EC tissues. Moreover, they are capable of accelerating the occurrence and development of EC.

MicroRNA-93-5p belongs to the microRNA-106b-25 family, which is located on chromosome 11q22.1. It is relatively conserved in mammals. MicroRNA-93 promotes the proliferation, invasion and migration of HCC cells by directly

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targeting PTEN and CDKN1A, thus activating the c-Met/PI3K/Akt pathway[16]. As an oncogene, microRNA-93 stimulates the occurrence and development of liver cancer and gastric cancer by targeting PDCD4[17,18]. Besides, microRNA-93-5p is involved in the carcinogenesis of colorectal cancer[19], esophageal cancer[20], and lung cancer[21]. Nowadays, the biological function of microRNA-93-5p has been widely explored in multiple malignancies. However, its underlying mechanism in EC development is still unclear.

Type I interferon receptor 1 (IFNAR) is a heterogeneous dimer composed of IFNAR1 and IFNAR2. IFNAR is located on the cell membrane, which forms a high-affinity complex by tightly binding to IFNAR1 and IFNAR2. IFNAR1 binds to and activates Tyk2 in the cytoplasmic domain. Meanwhile, it phosphorylates several proteins, mainly including STAT1 and STAT2. In addition to the classical JAK-STAT pathway, IFNAR1 negatively regulates STAT3 pathway after binding to IFN1[22-25]. STAT3 plays a protective role in proliferative, migratory and invasive potentials of EC cells, whereas inhibits cell apoptosis[26,27]. Bhattacharya et al[28] have suggested that IFNAR1 deficiency stimulates tumor development. However, microRNA-93-5p and IFNAR1 in EC are rarely reported.

This work explored the regulatory roles of microRNA-93-5p and IFNAR1 in the proliferation and migration of EC cells. We aimed to provide novel ideas and effective therapeutic targets for EC.

Patients and Methods

Tissue Samples and Clinical Data

EC tissues and normal endometrial tissues were surgically resected from 50 EC patients. After removal during surgery, all tissues were immediately preserved in liquid nitrogen for subsequent use. Clinical data of 50 EC patients were collected, including age, myometrial invasion, FIGO stage, menopausal status and lymph node metastasis (Table I). This study was approved by the Ethics Committee of Zhenhai People's Hospital. Signed informed consents were obtained from all participants before the study.

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

Total RNAs were extracted from EC tissues or cells according to the instructions of the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using the reverse transcription kit (TaKaRa, Otsu, Shiga, Japan). QRT-PCR was performed in strict accordance with SYBR Premix Ex Taq (TaKaRa, Otsu, Shiga, Japan). The relative gene expression was normalized to U6. Primer sequences used in this study were as follows: microRNA-93-5p, F: 5'-CTTGTGTAAACATCCTCGACTG-3', R: 5'-AGGCGTGTCGTGGAGTCG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3'.

Table I. Correlation between miR-93 expression and clinicopathologic features in EC patients.

Clinicopathologic features	Number of cases	miR-93 expression		*p*-value
		Low (n=25)	High (n=25)	
Age (years)				0.564
<55	30	14	16	
≥55	20	11	9	
Myometrial invasion				0.382
<1/2	19	11	8	
≥1/2	31	14	17	
FIGO stage				0.023*
I-II	28	18	10	
III-IV	22	7	15	
Lymph node metastasis				0.037*
Negative	17	12	5	
Positive	33	13	20	
Menopausal status				
Pre-/Perimenopausal	27	15	12	0.395
Postmenopausal	23	10	13	

Cell Culture and Transfection

EC cell lines Ishikawa and HEC-1B were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) and Dulbecco's Modified Eagle's Medium (DMEM; HyClone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin, respectively. All cells were maintained in a 37°C, 5% CO_2 incubator. The negative control, microRNA-93-5p mimics, microRNA-93-5p inhibitor or IFNAR1 vector were transfected into EC cells. Transfected cells for 48 h were harvested for Western blot and qRT-PCR experiments. Meanwhile, cells transfected for 24 h were collected for cell counting kit-8 (CCK-8) and transwell assay.

Western Blot

Total protein in transfected EC cells was lysed with radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China). The concentration of extracted protein was quantified using the bicinchoninic acid (BCA) protein assay kit (Pierce, Waltham, MA, USA). Briefly, gel electrophoresis was performed to separate the proteins with different molecular weight, which were then transferred onto polyvinylidene difluoride membranes. These membranes were incubated with anti-IFNAR, anti-STAT3, anti-p-STAT3, anti-MMP-9 (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. After incubating with these primary antibodies, the membranes were washed in Tris-Buffered Saline and Tween 20 (TBST; Beyotime, Shanghai, China) and then incubated with the HRP-conjugated secondary antibody at room temperature for another 2 h. Western blot detection kit and Image J software (NIH) were used to measure the blot signal and density.

Cell Proliferation Assay

EC cells were first inoculated into 96-well plates and cultured at 80% confluence. 6 replicates were set for each sample. Briefly, 10 μL of CCK-8 (Dojindo Molecular Technologies, Kumamoto, Japan) solution was added to each well, followed by incubation at 37°C for 2 h in the dark. The absorbance of each well at 450 nm was recorded by a microplate reader.

Transwell Assay

30 μL diluted Matrigel (1:10) was coated in the upper transwell chamber (BD Biosciences, Franklin Lakes, NJ, USA). Subsequently, 200 μL serum-free medium containing 5×10^4 cells and 600 μL complete medium were added in the upper and lower chamber, respectively. After 48 h of culture, cells were fixed in formaldehyde for 30 min. Cells and medium in the upper chamber were carefully cleaned. Meanwhile, cells in the lower chamber were stained with violet crystal. Cells in the bottom were captured using the Olympus fluorescent microscope and calculated.

Statistical Analysis

GraphPad Prism 6 (La Jolla, CA, USA) was used for all statistical analysis. Experimental data were expressed as mean ± SD ($\overline{x}\pm s$). Chi-square test was used to compare the differences between the two groups. $p<0.05$ was considered statistically significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Results

MicroRNA-93-5p Was Differentially Expressed in Normal Endometrial Tissues and EC Tissues

A total of 25 normal endometrial tissues and 50 EC tissues were collected. QRT-PCR data showed the expression of microRNA-93-5p in EC tissues was markedly higher than that of normal endometrial tissues ($p<0.001$, Figure 1A). Furthermore, microRNA-93-5p expression in EC tissues with lymph node metastasis or FIGO stage of III+IV was significantly higher than those with negative metastasis or stage I+II, respectively ($p<0.001$, Figure 1B and 1C). The Kaplan-Meier survival analysis was used to predict the relationship between microRNA-93-5p expression and prognosis of EC patients. The results indicated that EC patients with higher expression of microRNA-93-5p exhibited remarkably lower survival than those with lower expression ($p=0.0312$, Figure 1D). These results confirmed that the expression level of microRNA-93-5p was correlated with poor prognosis of EC.

MicroRNA-93-5p Accelerated Migratory and Proliferative Potentials of EC Cells

EC cell lines Ishikawa and HEC-1B were utilized for total RNAs extraction. QRT-PCR data showed significantly higher expression of microRNA-93-5p in EC cell lines than normal cells. This indicated that microRNA-93-5p was highly expressed in EC cells (Figure 2A). The transwell assay revealed that microRNA-93-5p mimics transfection in Ishikawa cells markedly elevated migratory potential (Figure 2B). On the contrary,

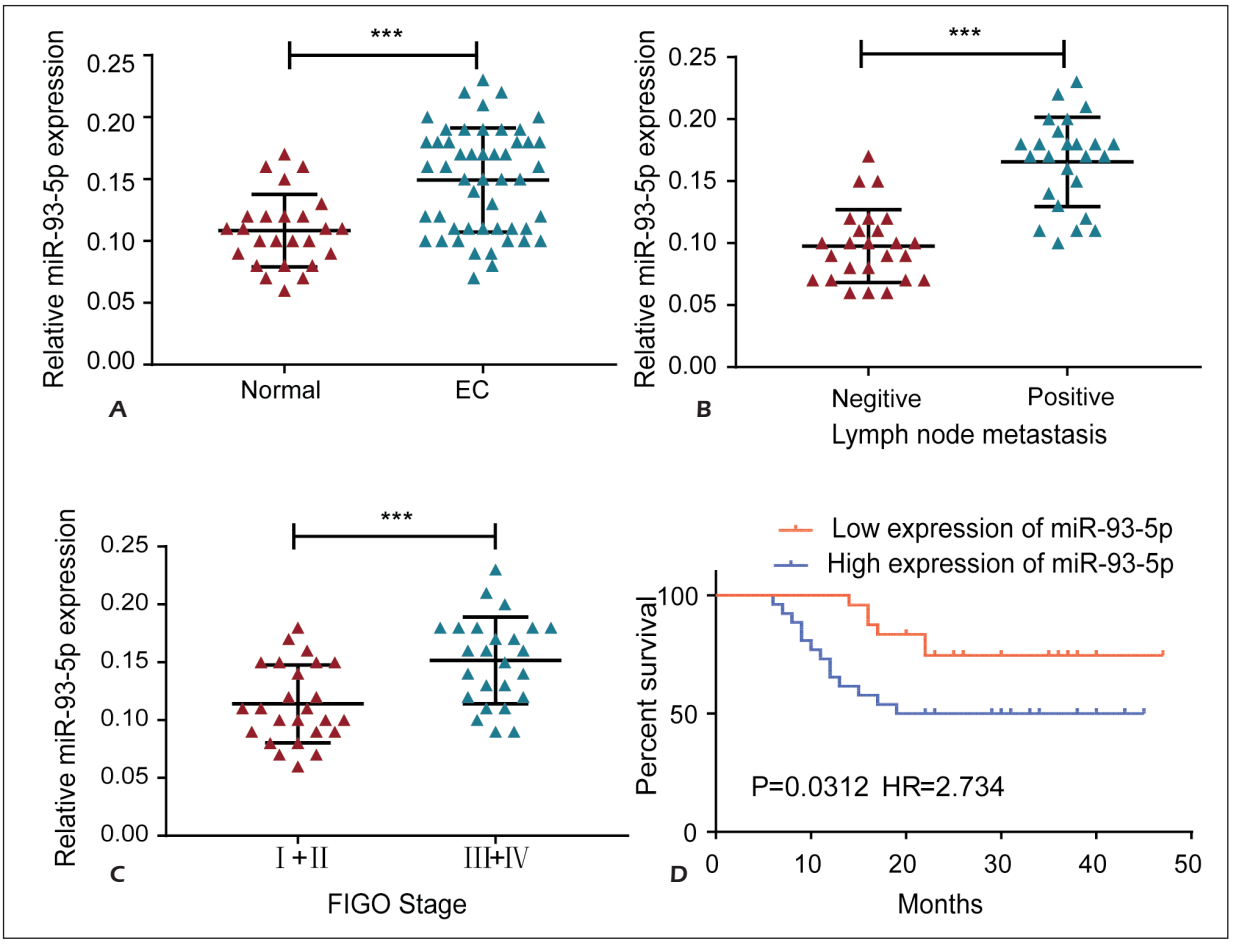

Figure 1. MicroRNA-93-5p was differentially expressed in normal endometrial tissues and EC tissues. **A**, QRT-PCR data showed significantly higher expression of microRNA-93-5p in EC tissues when compared with normal endometrial tissues. **B**, MicroRNA-93-5p expression in EC tissues with lymph node metastasis was markedly higher than those with negative metastasis. **C**, MicroRNA-93-5p expression in EC tissues with FIGO stage of III+IV was remarkably higher than those with stage I+II. **D**, The Kaplan-Meier survival analysis showed that EC patients with higher expression of microRNA-93-5p had lower survival than those with lower expression (p=0.0312).

the transfection of microRNA-93-5p inhibitor in HEC-1B inhibited cell migration (Figure 2C). The CCK-8 assay indicated that microRNA-93-5p overexpression in Ishikawa cells enhanced cell viability (Figure 2D). However, microRNA-93-5p knockdown markedly inhibited the viability of HEC-1B cells (Figure 2E). Therefore, we proposed that microRNA-93-5p deficiency suppressed viability and migration of EC cells.

IFNAR1 Was Lowly Expressed in EC and Was Regulated by MicroRNA-93-5p

Subsequently, we analyzed the relationship between microRNA-93-5p and IFNAR1 expressions in EC tissues. The Pearson correlation analysis showed that microRNA-93-5p was negatively correlated with IFNAR1 in EC samples (Figure 3A). Western blot analysis identically revealed significantly lower protein expression of IFNAR1 in EC tissues than normal endometrial tissues (Figure 3B). Furthermore, the protein expression of IFNAR1 in EC cells was determined after altering microRNA-93-5p expression. Ishikawa cells overexpressing microRNA-93-5p presented markedly lower IFNAR1 expression than controls (Figure 3C, left). On the contrary, the transfection of microRNA-93-5p inhibitor in HEC-1B cells remarkably downregulated IFNAR1 expression (Figure 3C, right). To elucidate whether IFNAR1 was involved in behavioral changes of EC cells regulated by microRNA-93-5p, IFNAR1 expression interfered. The transwell assay showed that IFNAR1 reduced the promotive effect of microRNA-93-5p on the migration of Ishikawa cells (Figure 3D). Be-

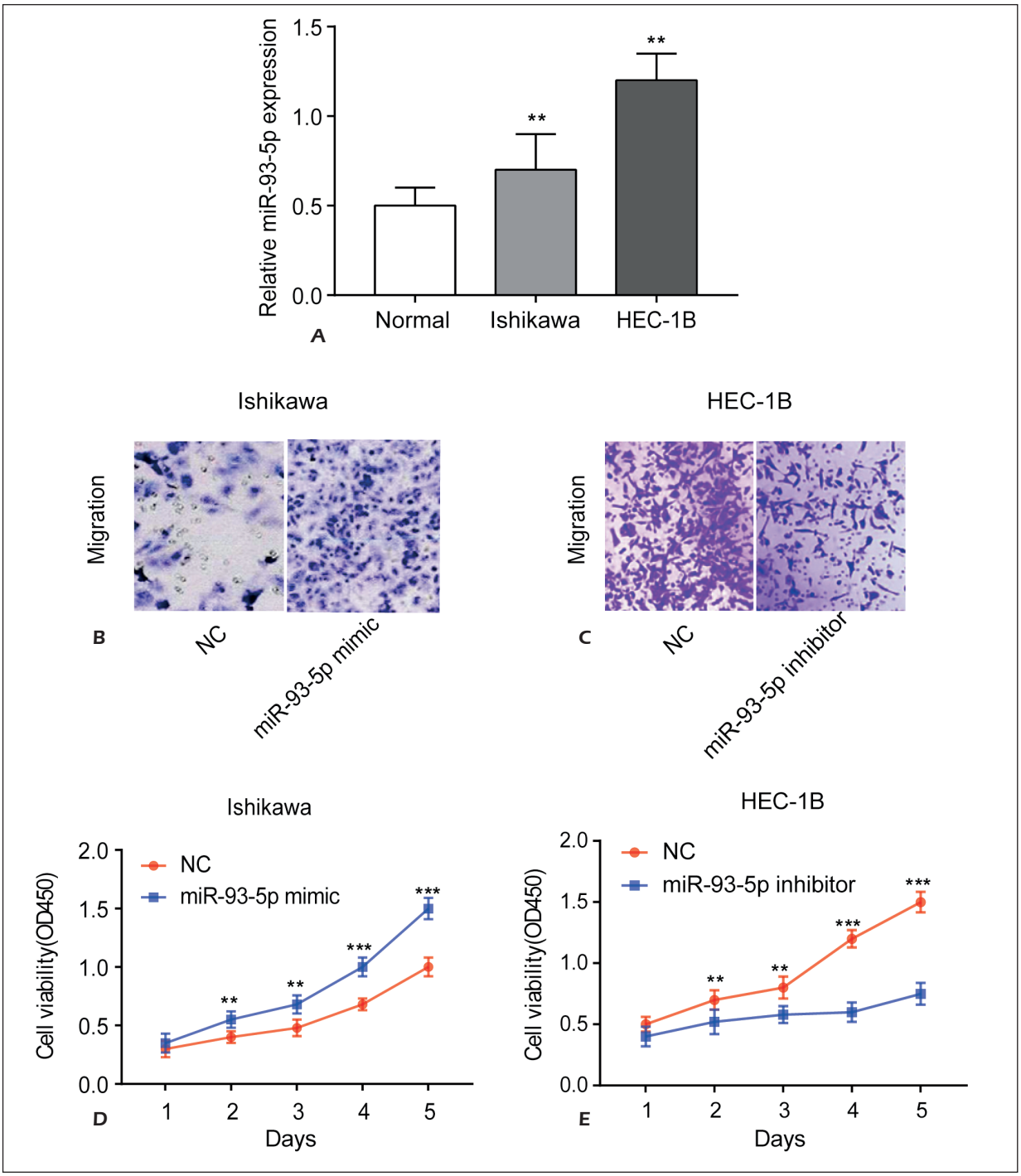

Figure 2. MicroRNA-93-5p promoted migration and proliferation of EC cells. **A**, QRT-PCR data showed significantly higher expression of microRNA-93-5p in EC cell lines than normal cells. **B**, The transwell assay revealed that transfection of microRNA-93-5p mimics in Ishikawa cells elevated migratory potential (magnification x 100). **C**, The transwell assay revealed that transfection of microRNA-93-5p inhibitor in HEC-1B inhibited cell migration. **D**, CCK-8 assay revealed that microRNA-93-5p overexpression in Ishikawa cells enhanced cell viability. **E**, The CCK-8 assay revealed that microRNA-93-5p knockdown in HEC-1B cells inhibited cell viability.

sides, CCK-8 data also indicated that IFNAR1 suppressed the promotive effect of microRNA-93-5p on the viability of Ishikawa cells (Figure 3E). The above results all proved that microRNA-93-5p promoted metastasis and proliferation of EC cells by downregulating IFNAR1 expression.

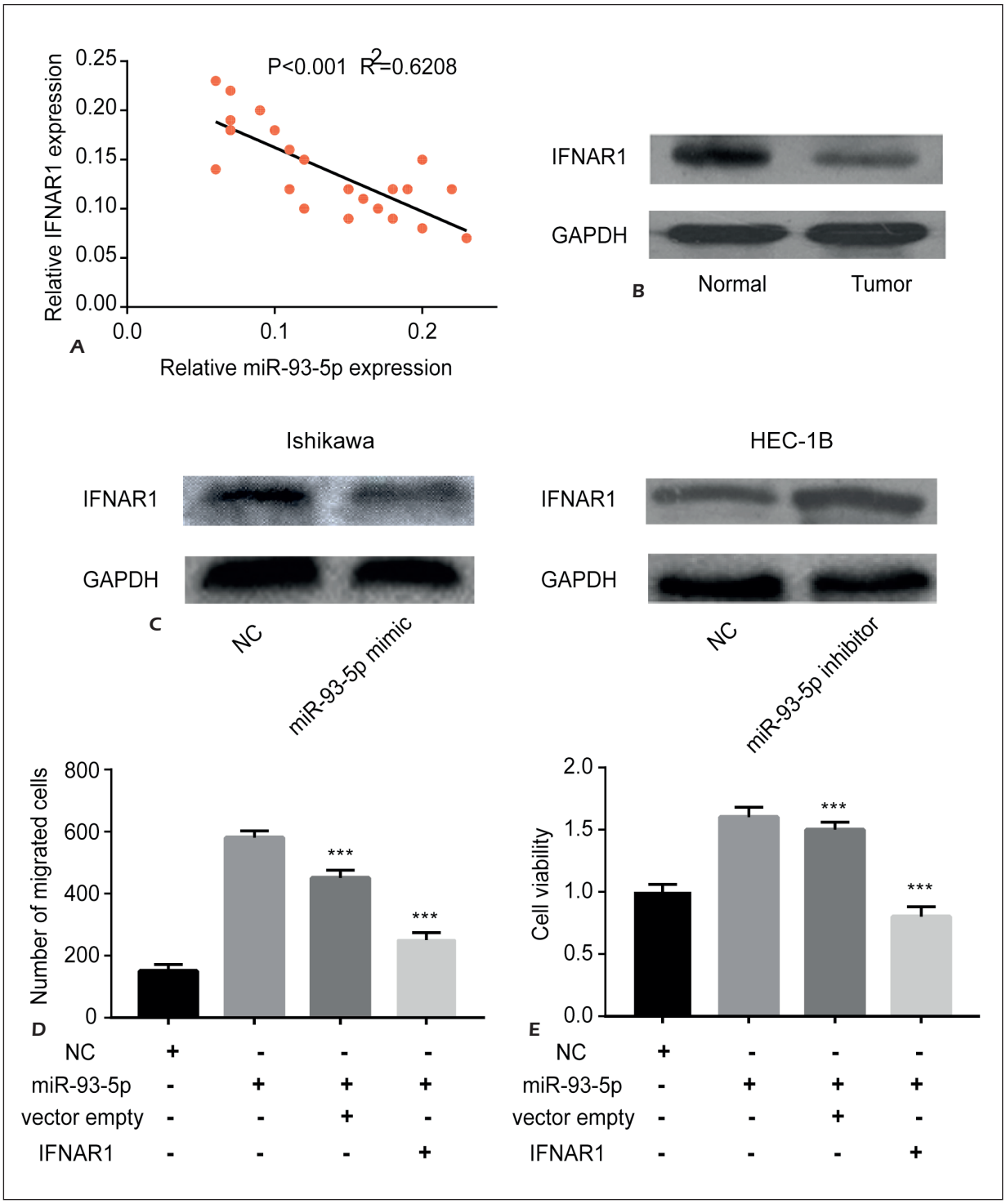

Figure 3. IFNAR1 was lowly expressed in EC and was regulated by microRNA-93-5p. **A**, Pearson correlation analysis showed a negative correlation between microRNA-93-5p and IFNAR1 in EC samples. **B**, Western blot analysis identically revealed lower expression of IFNAR1 in EC tissues than normal endometrial tissues. **C**, Ishikawa cells overexpressing microRNA-93-5p presented lower expression of IFNAR1 than controls. The transfection of microRNA-93-5p inhibitor in HEC-1B cells downregulated IFNAR1 expression. **D**, The transwell assay showed that IFNAR1 reduced the promotive effect of microRNA-93-5p on migration of Ishikawa cells. E, CCK-8 data indicated that IFNAR1 reduced the promotive effect of microRNA-93-5p on viability of Ishikawa cells.

MicroRNA-93-5p/IFNR1 Axis Regulated STAT3 Pathway and MMP9 Expression

To further elucidate whether the STAT3 signaling pathway was involved in the metastasis of EC cells regulated by microRNA-93-5p/IFNAR1 axis, the protein expressions of relative genes were determined by Western blot. MicroRNA-93-5p knockdown markedly increased IFNAR1 expression, whereas decreased expressions of STAT3, MMP-9, and p-STAT3 (Figure 4A). Opposite results were observed in Ishikawa cells overexpressing microRNA-93-5p (Figure 4B). These results showed that the microRNA-93-5p/IFNAR1 axis participated in EC development by regulating the STAT3 signaling pathway.

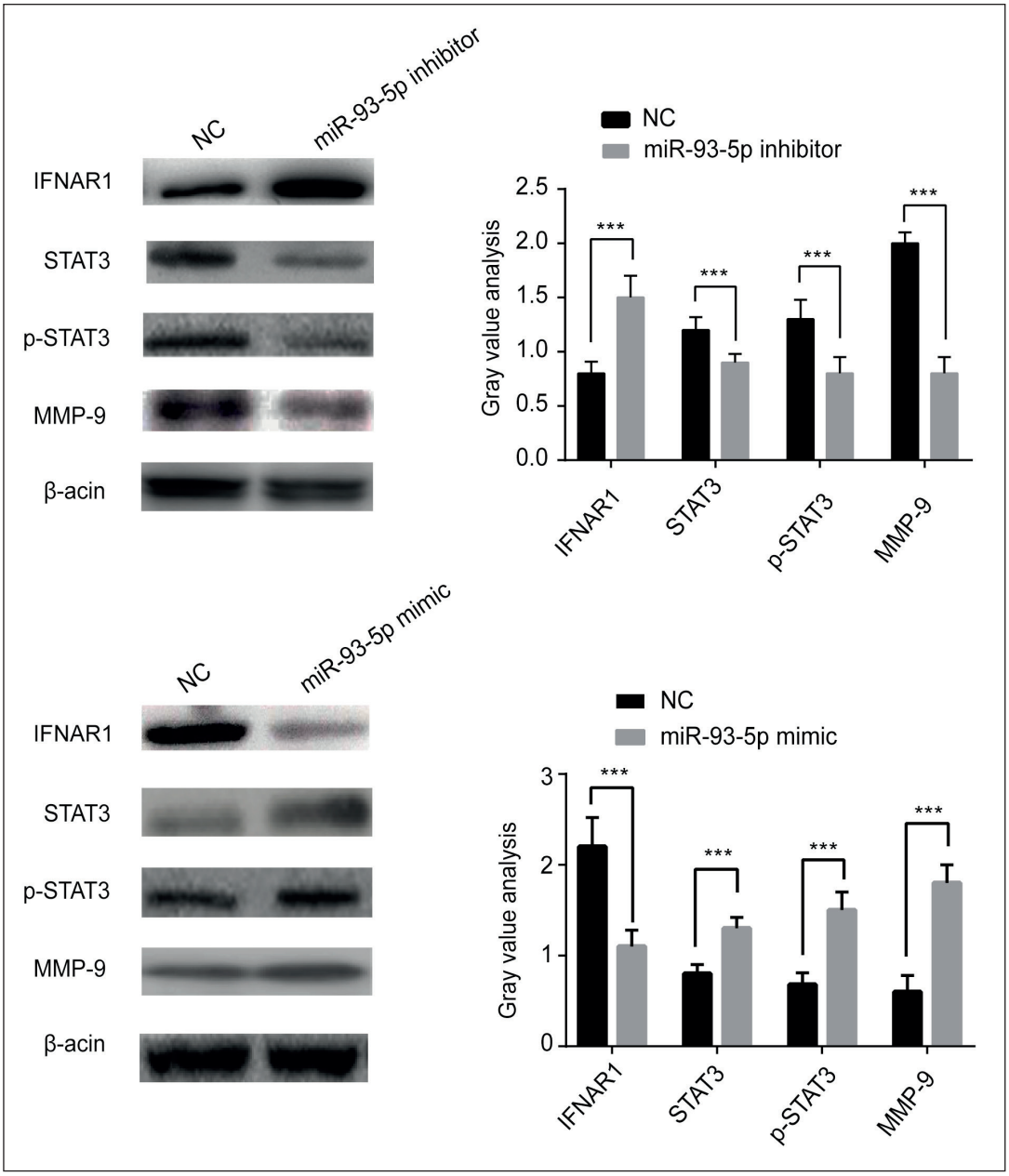

Figure 4. MicroRNA-93-5p/IFNR1 axis regulated STAT3 signaling pathway and MMP9 expression. **A**, Western blot analyses showed that microRNA-93-5p knockdown in HEC-1B cells markedly increased IFNAR1 expression, whereas decreased expressions of STAT3, MMP-9 and p-STAT3. **B**, Western blot analyses showed that microRNA-93-5p knockdown in Ishikawa cells markedly decreased IFNAR1 expression, whereas increased expressions of STAT3, MMP-9 and p-STAT3.

Discussion

MicroRNAs have been identified to be closely related to the development of various human tumors[29-31]. They can inhibit or promote tumorigenesis according to their different functions. Studies have found multiple differentially expressed microRNAs in different stages of EC. For example, miR-372, acting as a tumor suppressor, inhibits the occurrence and development of EC by targeting RhoC[32]. MiRNA-106b-93-25 is highly expressed in EC. Meanwhile, it antagonizes proliferation and accelerates apoptosis of EC cells by inhibiting p21 and Bim[33]. Hence, the determination of microRNAs relative to EC is of great clinical significance. However, the specific mechanism of microRNA-93-5p in EC remains unclear. Our study indicated that microRNA-93-5p was highly expressed in EC tissues compared with normal endometrial tissues. High expression of microRNA-93-5p was associated with poor survival of EC patients. This suggested that microRNA-93-5p might be a potential biomarker for evaluating the prognosis of EC. Biological experiments conducted *in vitro* showed that the upregulation of microRNA-93-5p significantly promoted the proliferative and migratory potentials of EC cells. Conversely, microRNA-93-5p knockdown obtained the opposite results. Therefore, we believed that microRNA-93-5p promoted the development of EC.

IFNAR1 exerts a crucial role in tumor development. Bhattacharya et al[34] have shown that the downregulation of IFNAR1 directly attenuates the anti-proliferative, anti-migration and pro-apoptotic effects of IFNAR1 on tumor cells. However, the role of IFNAR1 in EC metastasis remains unclear. Here, we found that IFNAR1 was lowly expressed in EC cells. Moreover, it significantly promoted metastasis of EC cells. Further analysis revealed that the upregulation of IFNAR1 reversed the regulatory effect of microRNA-93-5p on enhanced metastasis and viability of EC cells. The above findings suggested that microRNA-93-5p promoted proliferative and metastatic potentials of EC cells by downregulating IFNAR1.

As a member of the STAT protein family, STAT3 is a receptor tyrosine kinase activated by cytokines (such as IL-6) or upstream receptor kinases (such as Janus-activated kinases (JAKs))[35]. Activated STAT3 (p-STAT3) upregulates downstream genes, including Cyclin D1, BCL-XL[36], VEGF[37], COX-2 and MMPs[38]. This can eventually promote proliferation, neovascularization and metastasis and inhibit apoptosis of tumor cells. At present, many studies have explored the relationship between STAT3 and cervical cancer development. Schroer et al[39] have found that p-STAT3 can induce co-expression of MMP-9 by inducing CCL2, thereby affecting the progression of cervical cancer. STAT3 directly binds to the promoter region of proto-oncogene SKp2, which also promotes the proliferation of cervical cancer cells[40]. Our work found that microRNA-93-5p knockdown markedly upregulated IFNAR1, whereas downregulated STAT3, MMP-9 and p-STAT3 in EC cells. These results indicated that microRNA-93-5p/IFNR1 axis regulated the STAT3 signaling pathway and MMP9 expression, thus participating in EC development.

Conclusions

We found that microRNA-93-5p was highly expressed in EC tissues and cells, which is markedly correlated with poor prognosis of EC patients. MicroRNA-93-5p/IFNAR1 axis promoted metastasis of EC by activating the STAT3 signaling pathway. Our results suggest that microRNA-93-5p/IFNAR1 axis can serve as novel biological hallmarks and therapeutic targets for EC.

Conflict of Interests

The authors declare that they have no conflict of interest.

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