

Original Article

miR-210 promotes progression of endometrial carcinoma by regulating the expression of NFIX

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Abstract: microRNA-210 (miR-210) plays an important role in human disease, but its function in endometrial cancer (EC) is still unclear. Similarly, the nuclear factor I/X (NFIX) plays an important role in various biological functions of cells, but its function in EC is not yet known. In this study, we detected the expression of miR-210 from 66 EC patient tissues and 29 normal endometrium (NU) tissues by quantitative real-time PCR (RT-qPCR), as well as the expression of NFIX protein by western blot. We found that the expression of miR-210 in EC tissues was up-regulated and NFIX protein was down-regulated which was negatively correlated with NU tissues. The luciferase gene reporter system confirmed that miR-210 targeted inhibition of NFIX expression in HEC-1A cells. Up-regulation of miR-210 expression by transfection of miR-210-inhibitor could promote the proliferation, migration, and invasion of HEC-1A/HEC-1B cells. Taken together, we demonstrated that miR-210 could promote proliferation, migration and invasion by the negative regulation of NFIX expression *in vitro*, and that miR-210 promoted the progression of endometrial carcinoma by negative regulation NFIX expression.

Keywords: miR-210, NFIX, endometrial cancer, progression

Introduction

Endometrial cancer (EC) is one of the most common malignancies in the female reproductive tract. According to the American Cancer Society, there were 60,050 new cases of endometrial cancer in the United States and 10,470 deaths in 2015 [1]. In China, the incidence of endometrial cancer has also increased year by year, and is the second most common female reproductive system malignancies after cervical cancer [2]. microRNA (miRNA) is a group of short non-coding RNAs that do not encode proteins and are widely present in eukaryotes. They are approximately 19-25 nt in length, and their synthesis is an extremely complex biological process which mainly occur in the cytoplasm and nucleus [3]. miRNAs are incompletely complementarily paired with the 3'UTR region of the target gene mRNA through their "seed" sequence; this leads to the degradation of the target gene mRNA or inhibition of the expression of regulatory genes in the translation process, thereby regulating cell survival, differentiation, and response to the external envi-

ronment [4]. In the process of tumor development and progression, miRNAs mainly play a role in two ways: one is that miRNAs directly regulate proliferation and apoptosis of cancer cells; the other way is to regulate through down-regulation of oncogene and tumor suppressor gene expression [5, 6]. Finally, miRNA therapy is also being increasingly focused on cancer and various types of human diseases [7, 8].

miR-210 was found to have abnormal expression in breast cancer [9], hepatocellular carcinoma [10], pancreatic cancer [11] and renal cancer [12], and is related to the progress and prognosis of various cancers [13], but its function in endometrial cancer (EC) remains unclear. Similarly, NFIX had also been found to be abnormally expressed in a variety of tumors [13], but there are no data on EC. In this study, we detected the expression of miR-210 and NFIX protein in 66 cases EC tissues and 29 cases normal endometrium (NU) tissues, and found that miR-210 promoted the progression of endometrial carcinoma through negative regulation the expression of NFIX.

miR-210 targeting NFIX in endometrial carcinoma

Table 1. Relationship between miR-210 or NFIX expression and clinical characteristics of EC patients

Characteristic	n	miR-210 expression			NFIX protein expression		
		High	Low	P	High	Low	P
Age (years)							
<50	21	15	6	0.06	10	11	0.942
≥50	45	21	24		21	24	
Family history of tumor							
Negative	55	28	27	0.185	25	30	0.581
Positive	11	8	3		6	5	
Menopausal status							
Premenopausal	37	23	14	0.160	20	17	0.193
Postmenopausal	29	13	16		11	18	
Complications							
No	45	28	17	0.067	20	25	0.574
Yes	21	8	13		11	10	
FIGO stage							
I	49	19	30	<0.001	28	21	0.014
II	7	7	0		2	5	
III	10	10	0		1	9	
Histological Grade							
G1	28	4	24	<0.001	22	6	0.000
G2	28	22	6		8	20	
G3	10	10	0		1	9	
Lymph node metastasis							
No	56	26	30	0.002	30	26	0.011
Yes	10	10	0		1	9	

Materials and methods

Patients tissue samples

Sixty-six cases of endometrial carcinoma tissues were collected at Tangshan Workers' Hospital from patients with an average age of 47-73 years. According to the new revised standards of the International Obstetrics and Gynecology Union in 2009, there were 49 cases in FIGO I stage, 7 in II stage and 10 in III stage in this cohort. The other basic clinicodemographic information of these EC patients is shown in **Table 1**. EC patients who met the following conditions were excluded from this study: receiving treatment such as chemotherapy, radiotherapy, sex hormone therapy or immunotherapy before surgery; other comorbidities from cancer, infectious diseases or severe cardiovascular and cerebrovascular diseases; liver and kidney dysfunction; and incomplete clinical data.

Twenty-nine cases of normal endometrial tissues were also collected at Tangshan Workers'

Hospital, these NU tissues were all obtained from patients undergoing total hysterectomy for hysteromyoma. These patients were 45-69 years old and they also did not receive any sex hormone therapy before surgery. In addition, all patients participating in this study provided informed consent and were approved by the Tangshan Workers' Hospital Ethics Association.

Quantitative real-time PCR

Quantitative Real-time PCR (RT-qPCR) was used to detect the expression of GAPDH mRNA, U6 and miR-210 in tissues and cells. Trizol was used to extract total RNA from the tissue or cell. The extracted RNA was reverse-transcribed into cDNA by using PrimeScript™RT Master Mix reverse transcription kit (RR036B, Takara, Kusatsu, Japan). PCR parameters were set at: 37°C/60 minutes, 85°C/5 seconds. Then

a 20 µl Real-time fluorescence quantitative PCR (RT-qPCR) system was prepared according to the SYBR Green qPCR Master Mix kit instructions (638320, TakaRa, Kusatsu, Japan) and amplified using ABI 7500 fluorescence quantitative PCR instrument (Applied Biosystems, Maryland, USA). PCR parameters set: 95°C/30 s, [90°C/5 s, 65°C/30 s] -40 cycles.

PCR primers were as follows, U6-F: 5'-AUAAA-UCCUUUACACCUCTT-3', U6-F: 5'-AAUAAUCC-CUUUACACCUCTT-3'; GAPDH-F: 5'-CTGGGCTAC-ACTGAGCACC-3', GAPDH-R: 5'-AAGTGGTCGTTG-AGGGCAATG-3'; miR-210-F: 5'-ACACTCCAGCTGGAGCCCCTGCCACCGC-3', miR-210-R: 5'-TG-GTGTCTGGAGTCG-3.

Western blot

Tissue or cell lysates were separated by SDS-page and then transferred to a PVDF membrane. The following primary antibodies were used: anti-cyclin D1 (ab1013415, 1:2000, ABCAM, Cambridge, UK), or anti-GAPDH (ab9-484, 1:3000, ABCAM, Cambridge, UK). A sec-

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ond antibody was selected as follows: goat anti-rabbit (ab150077, 1:1000, ABCAM, Cambridge, UK), or goat anti-rat (ab150117, 1:1000, ABCAM, Cambridge, UK). Membranes were incubated with the primary antibody overnight at 4°C and the secondary antibody was incubated for 1 hour at room temperature.

Cells and cell transfection

HEC-1A (HTB-112, ATCC, VA, USA) and HEC-1B (HTB-113, ATCC, VA, USA) were cultured with DMEM medium (12491-15, ThermoFisher, CA, USA) which had 10% fetal bovine serum (10100-147, ThermoFisher, CA, USA) and 1% penicillin streptomycin (15640055, ThermoFisher, CA, USA).

miR-210-NC, miR-210-mimic and miR-210-inhibitor were designed and synthesized by Shenggong Bioengineering Co., Ltd. (Shanghai, China). These molecules were directly transfected into cells by Lipofectamine™ 2000 transfection reagent (11668019, Invitrogen, CA, USA). For wild type or mutation mRNA 3'-UTR of NFIX, they were first connected to pISCHECK2 (Promega, WI, USA) and then were directly transferred into cells by Lipofectamine™ 2000 transfection reagent.

Cell cloning assay

Finally, 4×10^3 cells/2 mL cells were inoculated into 6-well plates, and the culture medium was changed once every three day cells. After 3 weeks, the culture was stopped, the supernatant was removed, 4% formaldehyde was added for 15 minute, and then the cells were dyed with 0.25% crystal violet d for 25 minutes. At last, the culture plate was placed in a sterile, clean bench for drying and was imaged for cell counting.

Transwell

Corning BioCoat™ Matrigel transwell Invasion chamber (354480, Corning, USA) was used to evaluate the invasion ability of HEC-1A and HEC-1B. Briefly, 3×10^4 cells were added into the upper chamber with 200 μ l DMEM medium without FBS. At the same time, 700 μ l DMEM medium with 10% FBS was added into lower chamber. After 24 hours of incubation in a cell culture incubator, we removed the chamber, aspirated the medium, gently rinsed the chamber three times with 37°C pre-warmed PBS,

gently wiped the upper non-migrating cells with a cotton swab, and added 1 ml 14% formaldehyde to the 24-well plate. We then put in incubator for 25-30 minutes. Once we removed the chamber, it was washed three times with PBS and air dried. We added 800 μ l of 0.1% crystal violet to each well of the 24-well plate for staining. After 30 minutes, plates were placed in double distilled water and rinsed lightly. Afterwards, the plates were inverted and allowed to air dry. Five randomly selected fields were used for counting under a microscope at 200 \times .

Scratch test

Here, a concentration of 5×10^5 cells/2 mL was inoculated in 6-well plates. The next day, we scratched a horizontal line on the surface of the plate with a pipette tip and a ruler. The cells were softly washed three times with PBS, the cells were removed and serum-free medium was added. They were placed in one 37°C and 5% CO₂ incubator and incubated for 24 hours; then images were taken.

Statistical analysis

In this study, SPSS 20.0 was used for statistical analysis of data. Measurement data was thought to be represented as mean-standard \pm deviation, and count data was recorded as a percent. Differences between the two treatment groups were compared by chi-square test or student's t test. Pearson method was used to analyze the correlation between the two indicators. P<0.05 was considered a significant difference.

Results

miR-210 was up-regulated in EC tissues

RT-qPCR was used to detect the expression of miR-210 in 66 cases endometrial cancer (EC) tissues and 29 cases normal endometrium (NU) tissues. As shown in **Figure 1A**, the expression of miR-210 in EC tissues was higher than that in NU tissues (P<0.001). We further subdivided the 66 EC tissues, by comparing the expression of miR-210 in different groups. We found that the expression of miR-210 increased with the increase of FIGO stage (**Figure 1B**) and histological grade (**Figure 1C**). We observed miR-210 in EC patients with lymph node metas-

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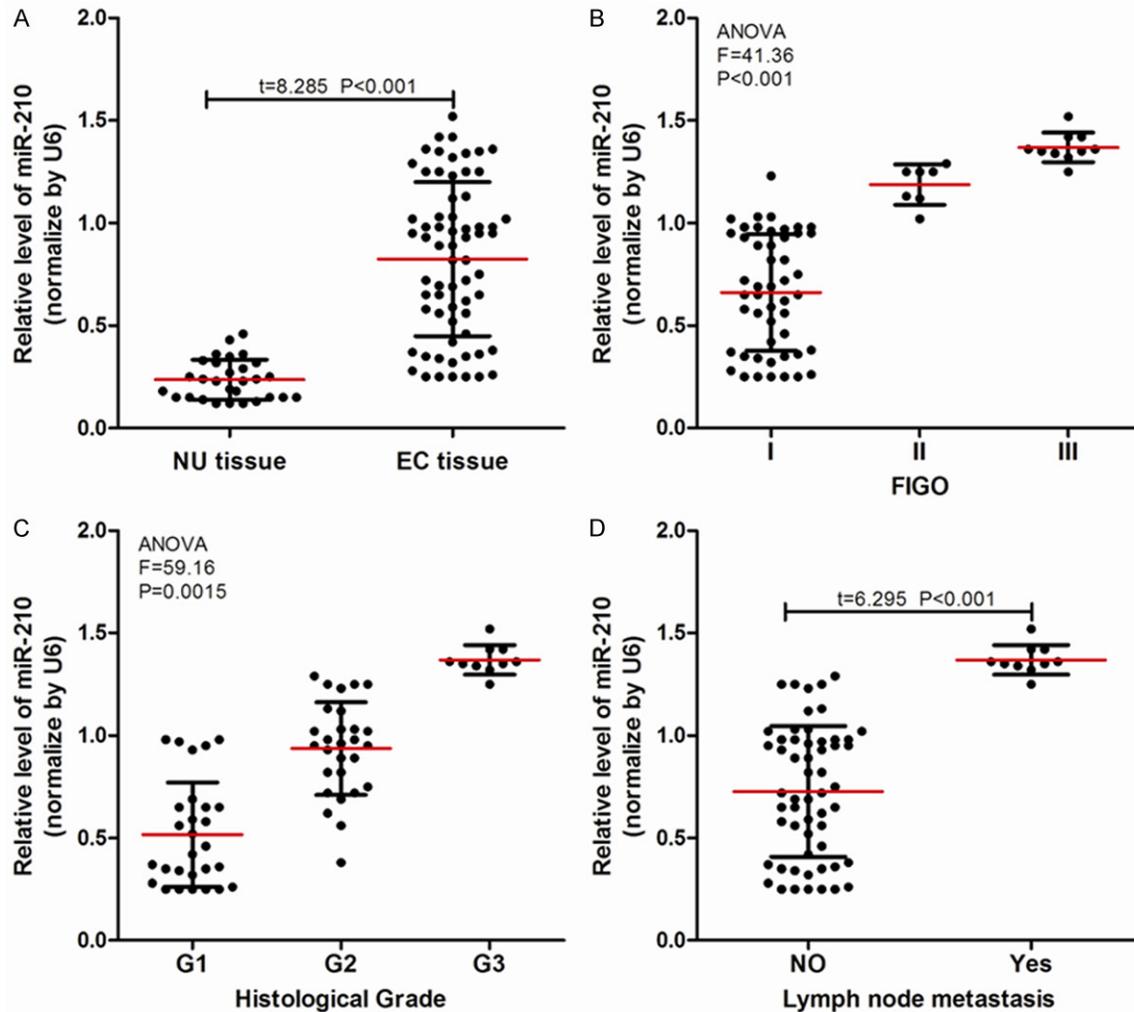


Figure 1. miR-210 expression in normal tissue and EC tissue. (A) miR-210 was highly expressed in endometrial carcinoma (EC) tissues (n=66) vs. normal endometrium (NU) tissues (n=29); (B-D) The expression of miR-210 in (B) different FIGO stages, (C) histologic grades, and (D) lymph node status.

tasis was higher than that in EC patients without lymph node metastasis (**Figure 1D**).

In addition, we analyzed the relationship between miR-210 expression and the clinical characteristics of EC patients, and found that (**Table 1**) the expression of miR-210 in 66 cases EC patients had no significant correlation with age, family history of tumor, menopausal status or complications, but had a significant correlation with FIGO stage, histological grade and lymph node metastasis.

NFIX protein was down-regulated in EC tissues

Western blot was used to measure the expression of NFIX protein in 66 cases EC tissues and 29 cases of NU tissues, as shown in **Figure 2A**.

The expression of NFIX protein in EC tissues was lower than that in NU tissues (P<0.001). Further, we also found that the expression of NFIX protein decreased with the increase in FIGO stage (**Figure 2B**) and histological grade (**Figure 2C**). NFIX protein in EC patients with lymph node metastasis was higher than that in patients without lymph node metastasis (**Figure 2D**).

Just as with miR-210, we also found that the expression of NFIX protein in 66 cases EC patients had no significant correlation with age, family history of tumor, menopausal status, or complications, but had a significant correlation with FIGO stage, histological grade, and lymph node metastasis (**Table 1**).

miR-210 targeting NFIX in endometrial carcinoma

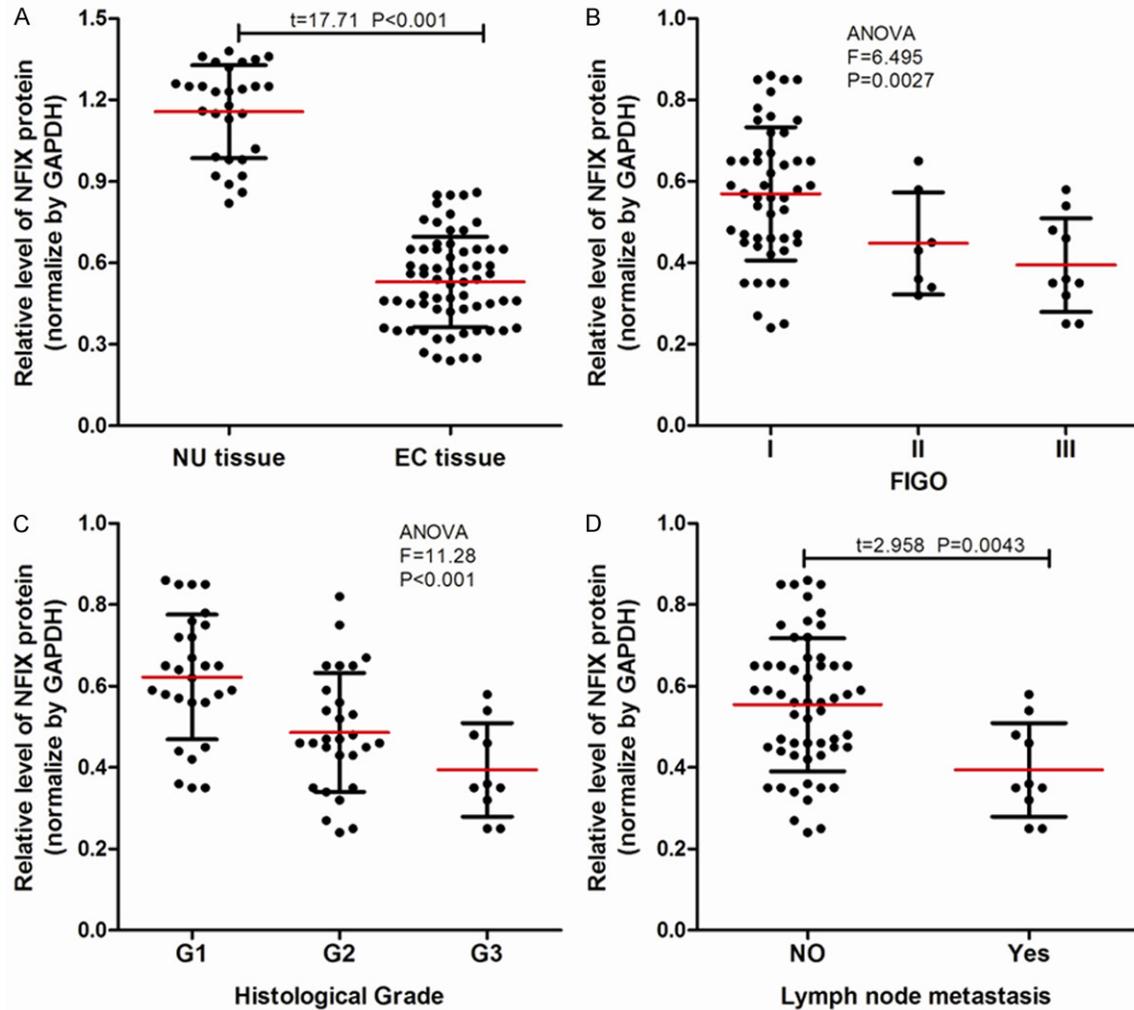


Figure 2. NIFX protein expression in normal tissue and EC tissue. (A) NIFX protein was minimally expressed in endometrial carcinoma (EC) tissues (n=66) vs. normal endometrium (NU) tissues (n=29); (B-D) The expression of NIFX protein in (B) different FIGO stages, (C) histologic grades, and (D) lymph node status.

miR-210 inhibited the expression of NFIX

We analyzed the relationship between miR-210 expression and NIFX protein expression in 66 EC patients, and found that there was a significant positive correlation between miR-210 expression and NIFX protein expression in 66 EC patients (**Figure 3A**).

Moreover, we found that there are mutual binding sequences between them by analyzing the sequences of NFIX and miR-210 (**Figure 3B**). In order to confirm that miR-210 can regulate NIFX expression by binding to the NFIX 3'-UTR, we used the luciferase gene reporter system. Results showed that the transfection of miR-210-mimic significantly decreased WT type

3'-UTR luciferase activity ($P<0.001$) in HEA-1A, but did not work in MUT.

Further, as shown in **Figure 3D, 3E**, miR-210 could regulate the expression of NIFX protein in HEC-1A/HEC-1B cell. These results suggest that miR-210 targeted inhibition the expression of NFIX in EC cells.

miR-210 regulated the proliferation of EC cells in vitro

Tumor size played an important role in many tumor staging guidelines, and the proliferation capacity of tumor cells affects the progress of cancer. In this study, we used the clone formation assay to evaluate the proliferation of EC cells *in vitro*, and found that up-regulation of

miR-210 targeting NFIX in endometrial carcinoma

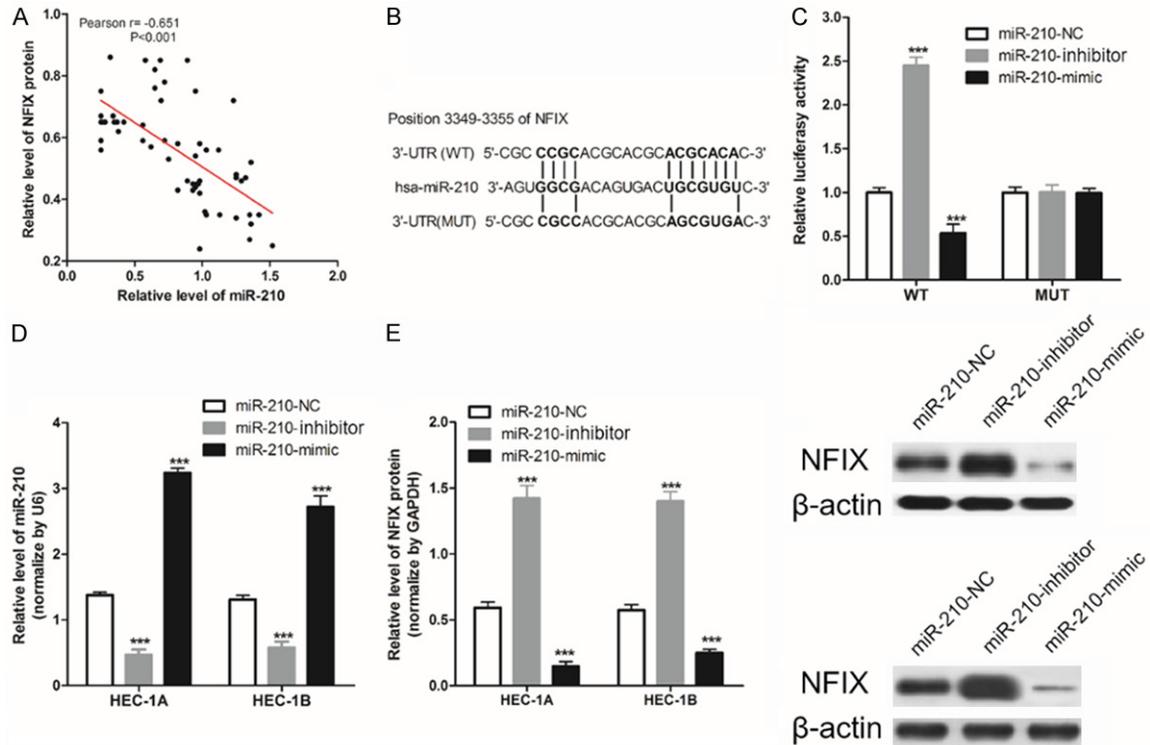


Figure 3. miR-210 targeted inhibition of NFIX expression in HEC-1A cells. A. The expression of NFIX protein was negatively related to the expression of miR-210 in 66 EC tissues; B. Construction of a WT-NFIX 3'UTR luciferase reporter vector, and a MUT-NFIX 3'UTR luciferase reporter vector with mutations on miR-210 binding sites of the NFIX 3'UTR; C. WT-NFIX/MUT-NFIX and miR-210-NC/miR-210-mimic/miR-210-inhibitor were transfected into HEC-1A cells, and luciferase activity was detected; D. RT-qPCR was used to measure the expression of miR-210 in HEC-1A/HEC-1B cells; E. Western blot was then used to detect the expression of NFIX protein in HEC-1A/HEC-1B cells. *** $P < 0.001$ vs. miR-210-NC.

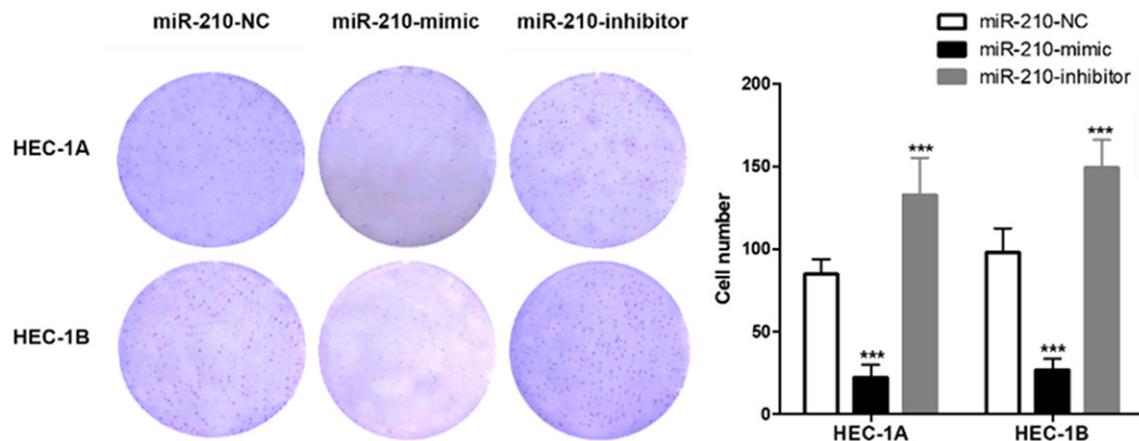


Figure 4. miR-210 regulated the proliferation of HEC-1A/HEC-1B cells *in vitro*.

the expression of miR-210 by transfection of miR-210-mimic could increase the number of cell clones (Figure 4), and down-regulation of the expression of miR-210 by transfection of the miR-210-mimic could decrease the number of cell clones.

miR-210 regulated the migration/invasion of EC cells in vitro

One of the most important biological characteristics of cancer cells is their mobility and invasiveness, and the invasion and metastasis of

miR-210 targeting NFIX in endometrial carcinoma

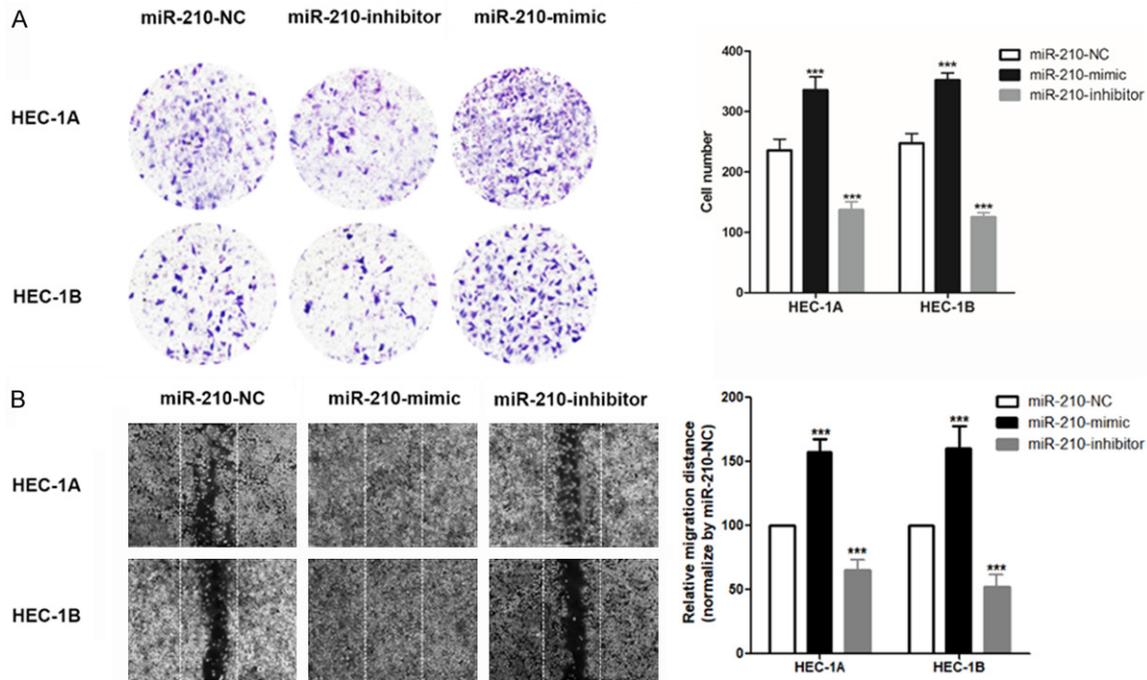


Figure 5. miR-210 regulated the migration/invasion of HEC-1A/HEC-1B cells in vitro. A. Transwell assay was used to evaluate the invasion ability of HEC-1A and HEC-1B cells; B. A scratch test was used to evaluate migration ability in HEC-1A and HEC-1B cells; ***P<0.001 vs. miR-210-NC.

cancer cells are also the most important factors leading to treatment failure and even death of cancer patients [14]. In this study, we found that miR-210-mimic could increase the number of invading HEC-1A/HEC-1B cells, and miR-210-inhibitor decreased it (Figure 5A). The same trend also occurs in the number of migrating HEC-1A/HEC-1B cells (Figure 5B).

Discussion

In this study, we found that the expression of miR-210 in 66 sample EC tissues was higher than that in 29 samples of NU tissues. Additionally, this expression was significantly correlated with FIGO stage, histologic grade, and lymph node metastasis. miR-210 is located on the human chromosome 11q15.5, and the target gene of miR-210 has reached 50 [15]. miR-210 regulates the expression of these genes and is widely involved in various physiological and pathological metabolic processes *in vivo*, such as the development of a variety of adult stem cells, embryonic development, cell growth and apoptosis, cell differentiation, angiogenesis, the regulation of the immune system, and the occurrence and development of tumors [16]. Previous research has shown

that compared with normal tissues, the expression of miR-210 is increased in many tumors, such as melanoma [17], lung cancer [18], pancreatic cancer [11], breast cancer [9], and head and neck tumor [19], kidney tumors, etc. Such expression was also likely to be closely related to the occurrence and development of tumors. This suggested that the higher expression of miR-210 in EC tissue might be related to the occurrence and development of EC.

In addition, this study found that the expression of NFIX protein in 66 cases EC tissues were lower than in 29 cases NU tissues, and had a significant correlation with FIGO stage, histological grade and lymph node metastasis. Nuclear factor I (NFI) is a family of closely related transcription factors. They constitutively bind as dimers to specific sequences of DNA with high affinity, and so could be used as a transcription switch from embryonic to fetal muscle [20]. They also can regulate proliferation and migration during SVZ neural niche development [21]. NFIX is an important member of the NFI family and participated in the regulation of the proliferation and differentiation of radial glial cells in the embryonic forebrain, as well as the migration of neurons within

the postnatal cerebellum [22]. Recent studies in the field of cancer found that NFIX was down-regulated in various tumor tissues [23] such as lung cancer [24] and esophageal cancer [25]. Moreover, down-regulating the expression of NFIX in esophageal cancer cells did not only increase the proliferation capacity of esophageal cancer cells, but also increased its invasion and migration ability, indicating that NFIX may be involved in regulating biological functions of tumor cells.

Further research has found that the expression of miR-210 and NFIX protein in EC tissues had a negative correlation. Additionally, the luciferase gene reporter system confirmed that miR-210 could target inhibition of NFIX expression in EC cells. miR-210 played an important regulatory role under hypoxic conditions, and in many cell types, hypoxia could induce cell cycle arrest through HIF-1 α . One of its possible mechanisms was a change in the expression of miR-210, so as to regulate the progression of cell cycle by inhibiting the transcription factor E2F family member E2F3, myc antagonistic factor max network transcriptional repressor (MNT) to regulate the progression of the cell cycle [26]. Zhang *et al.* [27] pointed out that the myc antagonist MNT was a destroyer of myc/Max/Med network structure and could inhibit the expression of myc target gene, and miR-210 could also promote the progression of cell cycle by down-regulating MTT. In addition, Chen *et al.* [26] pointed out that miR-210 could positively or negatively regulate the myc pathway through the regulation of many key points in the cell cycle. This was facilitated through gene regulation of the c-myc signaling pathway, and thus participates in the proliferation and metabolism of tumor cells. In this study, we found up-regulation of the expression of miR-210 via transfection of a miR-210-mimic that could increase the number of EC cell clones, and down-regulated the expression of miR-210 by transfection of miR-210-mimic decreasing the number of cell clones. Previous studies have shown that down-regulating the expression of NFIX in tumor cells such as lung cancer or esophageal cancer can enhance the proliferation of cancer cells [24, 25]. Combining our research suggested that miR-210 could enhance EC cell proliferation by negatively regulating the expression of NFIX protein.

The invasion and migration of cancer cells from the primary site to other tissues and organs of the patient are the main causes of failure and death in cancer patients [14]. In this study, we found miR-210 could positively regulate the invasion and migration ability of EC cells. Similarly, Zhang *et al.* [28] showed that miR-210 was down-regulated in breast cancer tissues and cells. Inhibition of miR-210 expression in breast cancer cells could significantly reduce its invasion and migration ability. In addition, many previous studies have also demonstrated that inhibition of miR-210 expression *in vitro* can reduce the invasion and migration abilities of hepatoma [29], gastric cancer [30], breast cancer [31], and colorectal cancer [32] cells.

Conclusion

miR-210 was up-regulated and NFIX down-regulated in EC tissues. The expression of miR-210 and NFIX protein in EC tissues had a negative correlation. miR-210 promoted the proliferation, migration, and invasion of HEC-1A/HEC-1B cells and the progression of endometrial carcinoma by negative regulation of the expression of NFIX.

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Disclosure of conflict of interest

None.

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