

Original Article

Folic acid receptor α facilitates nasopharyngeal carcinoma via ERK1/2/NF- κ B signaling pathway

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Abstract: Nasopharyngeal carcinoma (NPC) is an epithelial malignancy with high prevalence in Southeast Asia. Concurrent chemoradiotherapy is the most popular therapeutic method. NPC is mainly characterized by insidious onset and deep lesion site, and thus is difficult to treat. In clinical, the lack of targeted drugs could further affect the therapeutic efficacy for NPC. Folic acid receptor α (FR α) is an abnormally expressed molecule in various tumor tissues, and is involved in the occurrence and progression of these tumors. However, the potential role and mechanism of FR α in NPC remains unclarified. In this study, we detected overexpression of FR α in human NPC cell line CNE2 compared with normal nasopharyngeal epithelial cell line NP69 ($P < 0.05$). Further, CNE2 cells were randomly divided into control, scrambled siRNA (negative control) and FR α siRNA groups. FR α expression was determined by Western blot and real-time PCR analysis. Cell proliferation, invasion and apoptosis were detected by MTT assay, transwell assay, and flow cytometry, respectively. It was found that down-regulation of FR α by siRNA significantly inhibited cell proliferation and invasion, but markedly induced cell apoptosis of CNE2 cells compared with control group (all $P < 0.05$). Further mechanism study showed that pERK1/2 and NF- κ B expression was significantly decreased when compared to control group ($P < 0.05$). In conclusion, our study suggests that FR α might facilitate NPC via the ERK1/2/NF- κ B signaling pathway. FR α might be an effective biomarker of NPC and might be useful for the diagnosis and targeted treatment for the malignant disease.

Keywords: Folic acid receptor α , nasopharyngeal carcinoma, ERK1/2/NF- κ B signal pathway, cell proliferation, tumor invasion

Introduction

Nasopharyngeal carcinoma (NPC) is an epithelial malignancy in the ear-nose-throat system with a high prevalence [1]. Epidemiology of NPC has revealed susceptibility in specific ethnic groups and regions, such as the seaside area in southern China [2, 3]. Due to the complex structure of nasopharyngeal regions, the insidious disease onset, and the lack of targeted drugs, the overall efficacy of NPC treatment remains unsatisfactory [4, 5]. Therapeutic efficacy and outcome often varies in each individual patient due to different degrees of clinical manifestation, disease stage, and sensitivity to chemo- or radio-therapy [6, 7]. NPC is characterized by insidious onset site and slow progression in early stages, and thus is frequently mis- or mis-diagnosed. Although previous studies have reported various factors associated with the

pathogenesis of NPC, including infection of EB virus, genetic factor, dietary habit, environmental factors, etc [8, 9], the molecular pathology of NPC has not been fully clarified. Therefore, it is essential to elucidate the molecular pathology of NPC in order to identify specific molecular markers, especially in early stages of NPC, that may be useful for early diagnosis of NPC [10].

Water-soluble vitamin B9, also known as folic acid, is essential for a variety of biological processes in the body, including DNA synthesis, DNA repair, protein methylation, methionine cycle, and cell division [11-13]. Folic acid is transferred into cells upon binding to the folate receptor (FR) anchors on the plasma membrane [14]. Folate receptor α (FR α), the most important subunit of FR, is overexpressed in breast and ovarian cancers compared to normal epithelial cells [15, 16]. In this study, we

aimed to analyze the role and mechanism of FR α in NPC cells in order to elucidate the pathological mechanism of NPC.

Materials and methods

Main equipments and reagents

Human NPC cell line CNE2 and normal nasopharyngeal epithelial cell line NP69 were purchased from ATCC cell bank (Manassas, VA, USA). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS) and streptomycin-penicillin were purchased from Hyclone (Logan, UT, USA). DMSO and MTT were purchased from Gibco (Carlsbad, CA, USA). Trypsin-EDTA lysis buffer was purchased from Sigma (St. Louis, MO, USA). PVDF membrane was purchased from Pall Life Sciences (East Hills, NY, USA). EDTA was purchased from Hyclone (Waltham, MA, USA). Western blot reagents were purchased from Beyotime (Shanghai, China). ECL reagent was purchased from Amersham Biosciences (Pittsburg, PA, USA). Rabbit anti-human FR α polyclonal antibody (1:2000), rabbit anti-human ERK1/2 monoclonal antibody (1:3000), rabbit anti-human pERK1/2 monoclonal antibody (1:3000), rabbit anti-human NF- κ B monoclonal antibody (1:4000), and mouse anti-rabbit or horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG were purchased from Cell Signaling (Danvers, MA, USA). Transwell chamber was purchased from Corning (Corning, NY, USA). RNA extraction kit and reverse transcription kit were purchased from Axygen (SF, CA, USA). Labsystem Version 1.3.1 microplate reader was purchased from Bio-rad (Hercules, CA, USA). Annexin V-FITC apoptotic assay kit was purchased from BD (San Jose, CA, USA).

Cell culture and grouping

CNE2 and NP69 cells were seeded at the density of 1×10^5 cells/cm² in 6-well plate containing DMEM medium, 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin. Cells at exponential phase were randomly divided into control group, scramble siRNA group (negative control) and FR α siRNA group.

Liposome transfection of FR α siRNA into CNE2 cells

FR α siRNA (Sense: 5'-GGA CUG AGC UUC UCA AUG UTT-3'; and anti-sense: 5'-ACA UUG AGA

AGC UCA GUC-3') or scrambled siRNA (Sense: 5'-GGA CUG AGC UUC UCA AUG UTT-3'; and anti-sense: 5'-ACA UUG AGA AGC UCA GUC-3') were respectively transfected into CNE2 cells. Briefly, FR α siRNA or scrambled siRNA were respectively incubated with liposome in 200 μ L of serum-free medium at room temperature for 15 min. FR α siRNA or scrambled siRNA dilutions was further treated with Lipo2000 reagent at room temperature for 30 min. Cells at 70%~80% confluence were incubated in serum-free medium supplemented with the transfection mixture at 37°C, 5% CO₂ for 6 h. The medium was replaced with regular medium and cells were incubated for an additional 48 h for further experiments.

Real-time PCR for FR α expression

Total RNA was extracted using Trizol reagent and reverse transcribed using the reverse transcription kit according to the manufacturer's instruction. The following primers were designed using Primer 6.0 and synthesized by Invitrogen (Shanghai, China): FR α -forward: 5'-TCTC-GACTCCACACAGT-3', reverse: 5'-GCCGGGTCA-TTAGCTATATT-3'; GAPDH-forward: 5'-ACCAGGT-ATCTTGGTTG-3'; and reverse: 5'-TAACCATGT-CAGCGTGGT-3'. Real-time PCR was performed using the following conditions: 92°C for 30 s, 35 cycles of 58°C for 45 s and 72°C for 35 s. Standard curve was firstly plotted using CT values of standards. Data was analyzed using the 2- Δ Ct method. Each sample was measured in triplicate. The relative expression of FR α was calculated using GAPDH as the internal reference.

MTT assay for cell proliferation

CNE2 cells at exponential phase were seeded in 96-well plate at a density of 3,000 cells/well, and randomly divided into control, scrambled siRNA and FR α siRNA groups (n=5 for each group). The cells were cultured for 48 h and treated with 20 μ L of MTT solution (final concentration of 5 mg/mL) for 4 h. Cells were rinsed and incubated with 150 μ L of DMSO for 10 min until the crystal violet was completely resolved. The absorbance value of each well was measured using a micro-plate reader at the wavelength of 570 nm. The experiment was performed in triplicate, and the proliferation rate of each group was calculated.

Folic acid receptor in NPC

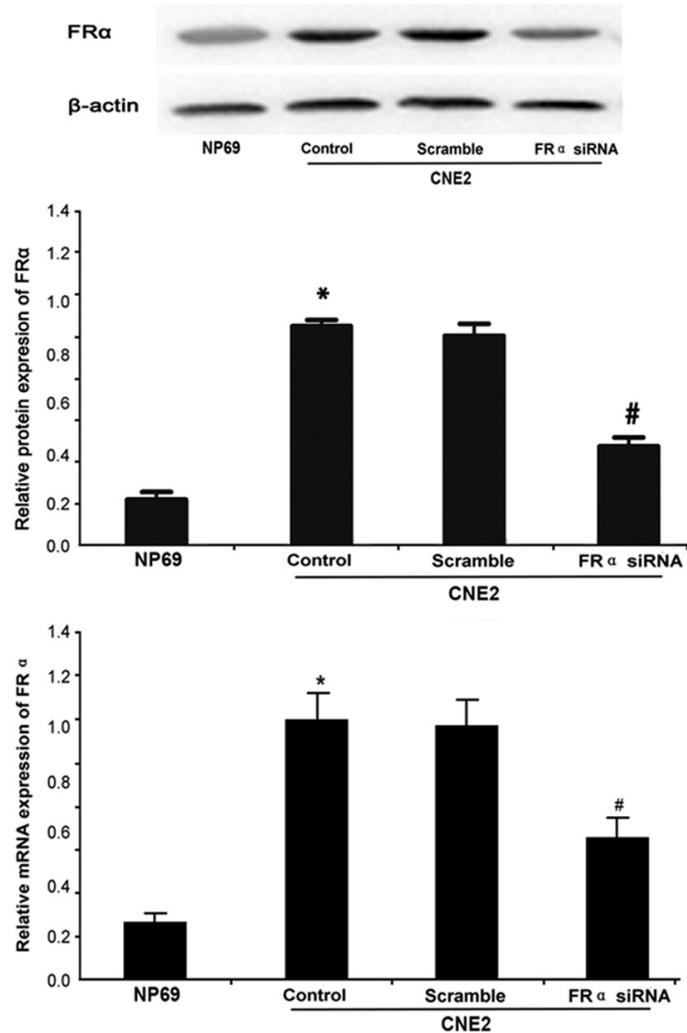


Figure 1. Comparison of FR α expression in NP69 and CNE2 cells with or without siRNA transfection after 48 incubation. *, $P < 0.05$ compared to NP69 cells. (Top) a representative image of Western blot analysis. (Middle) quantitative analysis of FR α protein expression. (Bottom) quantitative analysis of FR α mRNA expression. #, $P < 0.05$ compared to CNE2 control group without transfection.

Flow cytometric detection of cell apoptosis

CNE2 cells were seeded into 50-mL flasks at a density of 5×10^5 /mL, and randomly assigned into control, scrambled siRNA and FR α siRNA groups. At 48 h after transfection, cells were collected, washed in PBS, and centrifuged at 1000 r/min for 5 min. Cells were fixed in 75% cold ethanol overnight at 4°C. Cells were washed in PBS, and collected by centrifugation at 1000 r/min for 5 min, and resuspended in 0.8 mL of PBS containing 1% BSA. Cells were then treated with 100 μ g/mL PI staining dye (3.8% sodium citrate, pH 7.0) at 37°C in the

dark for 30 min. Cell apoptosis was detected using a flow cytometry and analyzed by FCS Express 3.0 software.

Western blot analyses

The expression of ERK1, ERK2 and NF- κ B protein in control, scrambled siRNA and FR α siRNA groups was determined by Western blot at 48 h after transfection. Briefly, total proteins in CNE2 cells were extracted using cell lysis buffer. The supernatant was collected by centrifugation at $10,000 \times g$, 4°C for 15 min. Total protein was separated by 10% SDS-PAGE electrophoresis and transferred to PVDF membrane using the semi-dry method. The membrane was treated in 5% skim milk at room temperature for 2 h, followed by incubation with rabbit anti-human ERK1 (extracellular signal-regulated kinase) monoclonal antibody (1:1000 dilution), rabbit anti-human ERK2 monoclonal antibody (1:2000), rabbit anti-human NF- κ B monoclonal antibody (1:1500) or rabbit anti-human β -actin monoclonal antibody (1:2000) overnight at 4°C. The membrane was rinsed with PBST, and incubated with secondary antibody at 37°C for 30 min. The membrane was treated in ECL reagent for 1 min for color development, followed by X-ray exposure. X-ray films were scanned using protein imaging analysis system and band density was

analyzed by Quantity One software. Each experiment was repeated 4 times.

Transwell assay for cell invasion

The cell invasion of control, scrambled siRNA and FR α siRNA groups was compared by transwell assay at 48 h after transfection. Briefly, Transwell chamber was pre-coated using 1:5 50 mg/L Matrigel dilutions on the bottom and upper layer of the membrane. CNE2 cells in different groups were cultured in serum-free medium for 24 h and cells were digested and added into the upper chamber of transwell. DM-

Folic acid receptor in NPC

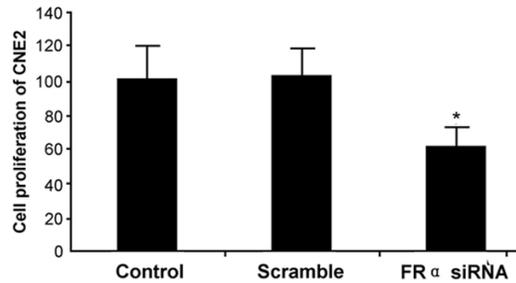


Figure 2. Effect of FR α down-regulation on proliferation of CNE2 cells. *, $P < 0.05$ compared to control group.

EM culture medium containing 10% FBS was added into the lower chamber. After 24 h, transmembrane cells were washed, fixed in cold ethanol and stained with crystal violet. The cells in five randomly selected visual fields of each well were counted under an inverted microscopy. The experiment was performed in triplicate.

Statistical analysis

Measurement data were presented as mean \pm standard deviation (SD) and analyze by SPSS19.0 (SPSS Inc, Chicago, IL, USA). One-way analysis of variance was used to compare difference among multiple groups, followed by LSD test in case of significant difference. $P < 0.05$ was considered statistically significant.

Results

Increased expression of FR α in CNE2 cells

FR α mRNA and protein expression in CNE2 and NP69 cells was compared by real-time PCR and Western blot, respectively. The results showed that only trace amounts of FR α in normal NP69 cell. The FR α mRNA and protein expression was significantly up-regulated in NPC cell CNE2 compared to NP69 cells ($P < 0.05$, **Figure 1**).

siRNA transfection reduced FR α expression in CNE2 cells

We further examine the effect of siRNA interference on the FR α expression in CNE2 cells. Real-time PCR and Western blot results showed that the FR α mRNA and protein level after siRNA transfection was significantly decreased compared to the control or scramble group ($P < 0.05$, **Figure 1**).

FR α siRNA reduced the proliferation of CNE2 cells

MTT assay was employed to evaluate the effect of FR α gene silencing on the proliferation on the cells. The results showed that the cell proliferation in siRNA group was lower was lower significantly compared to the control group ($P < 0.05$, **Figure 2**), which suggests that FR α siRNA could effectively inhibit the CNE2 cell proliferation. These results suggested that the knockdown of FR α gene expression in CNE2 cells benefits the inhibition of tumor proliferation.

FR α siRNA stimulated the apoptosis of CNE2 cells

Flow cytometry assay was used to evaluate the effect of FR α down-regulation on apoptosis of CNE2 cells. The results indicated that decreased FR α expression remarkably facilitates the cell apoptosis, and the cell apoptosis of which was also significantly lower compared to the control group ($P < 0.05$, **Figure 3**). These results suggested that the knockdown of FR α gene expression in CNE2 cells could accelerate the tumor cell apoptosis.

FR α siRNA inhibited the invasion ability of CNE2 cells

Transwell chamber assay was used to evaluate the effect of FR α down-regulation on invasion ability of CNE2 cells. The results showed that the tumor invasion in siRNA transfected CNE2 cells was significantly inhibited compared to control group ($P < 0.05$, **Figure 4**). These results suggest that the altered changes of FR α in NPC cells might affect the tumor invasion.

FR α regulated CNE2 cells via ERK1/2/NF- κ B signaling pathway

Western blot was used to evaluate the effect of FR α on ERK1/2/NF- κ B signal pathway in CNE2 cells. The results showed that the ERK1/2 or NF- κ B expression was significantly inhibited in siRNA interference group (inhibition of FR α expression) compared to control group ($P < 0.05$ **Figure 5**). These results suggest that the down-regulation of FR α could inhibit phosphorylation of ERK1/2 and further inhibit the ERK1/2/NF- κ B signaling pathway to modulate the occurrence and progression of NPC.

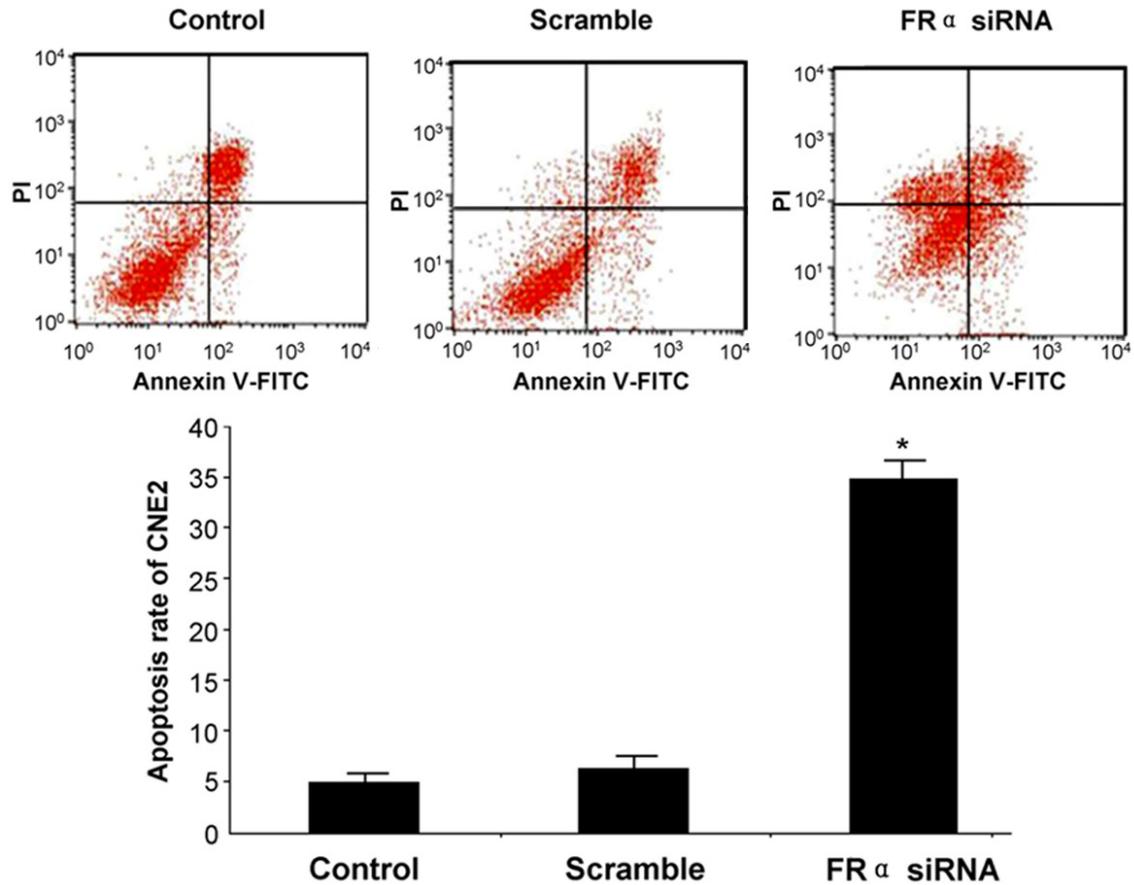


Figure 3. Effect of FR α down-regulation on the apoptosis of CNE2 cells. (Top) flow cytometry analysis of cell apoptosis in different groups. (Bottom) comparison of CNE2 apoptosis rate in different groups. *, P<0.05 compared to control group.

Discussion

Currently, most NPC patients are at terminal stages when diagnosed [9]. It is therefore important to identify reliable molecular markers of NPC. Moreover, the identification of effective molecular marker is useful for the evaluation of treatment efficacy in NPC patients. In addition, the identified molecular markers might also be good candidates for targeted therapy [10]. As the precursor of purine and pyrimidine synthesis, folic acid acts as the major donor of one-carbon unit, and regulates cell growth, division and survival [17]. FR α is up-regulated in various malignant tumors, including breast cancer, cervical carcinoma, and retinoblastoma, and the investigation of FR α expression in tumor tissues can benefit the diagnosis, treatment and prognosis of these tumors [18, 19]. Consistently, we only detected trace amounts of FR α in normal NP69 cells, whereas overexpression of FR α was observed in NPC CNE2 cells,

indicating that FR α might be associated with the occurrence and progression of NPC.

Further, we investigated the regulatory role of FR α in NPC by siRNA interference. It was found that siRNA targeting FR α significantly inhibited cell proliferation and invasion, but markedly induced cell apoptosis of CNE2 cells. The inhibitory effect of FR α -siRNA on CNE2 cell proliferation might be attributed to lower folic acid intake due to lower FR α expression, leading to abnormal intracellular DNA synthesis and chromosome breakage [20]. The ERK1/2 are key members belonging to the serine/threonine protein kinase family [21]. ERK1/2 can be activated by various stimuli, such as cell adhesion, cell stress response, and hormones. The activated ERK1/2 can regulate phosphorylation of other proteins such as transcriptional factors, and thus play an important role in the modulation of the transcription of related genes [22]. Continuous activation of ERK1/2 may lead to

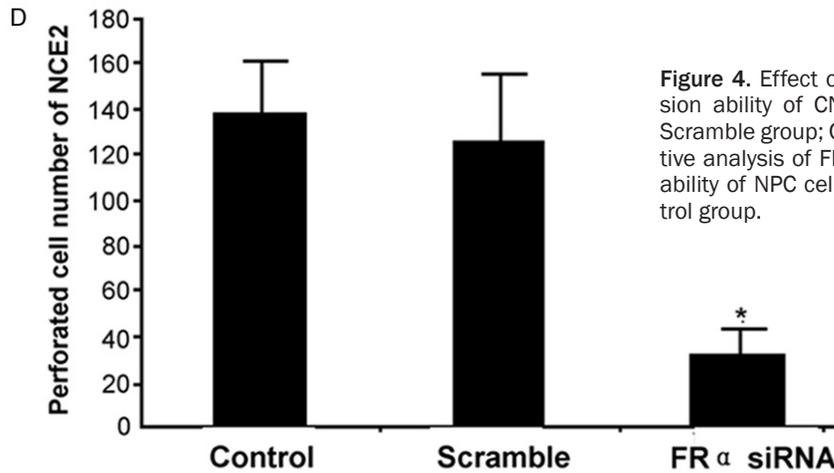
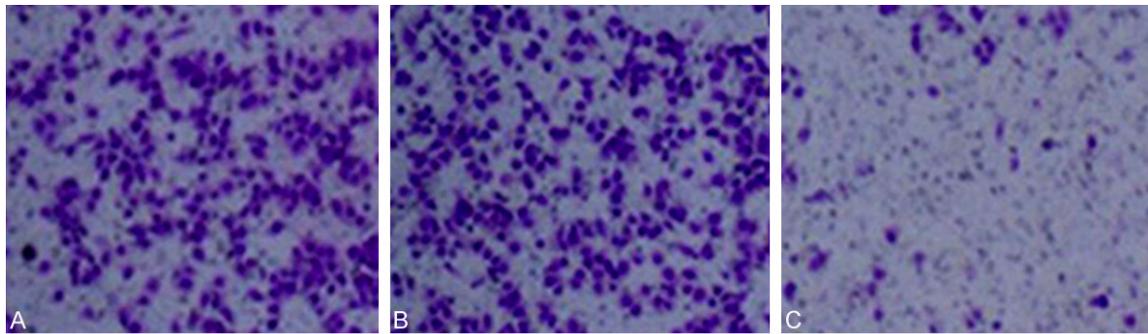


Figure 4. Effect of FR α down-regulation on invasion ability of CNE2 cells. A: Control group; B: Scramble group; C: FR α siRNA group. D: Quantitative analysis of FR α down-regulation on invasion ability of NPC cells. *, P<0.05 compared to control group.

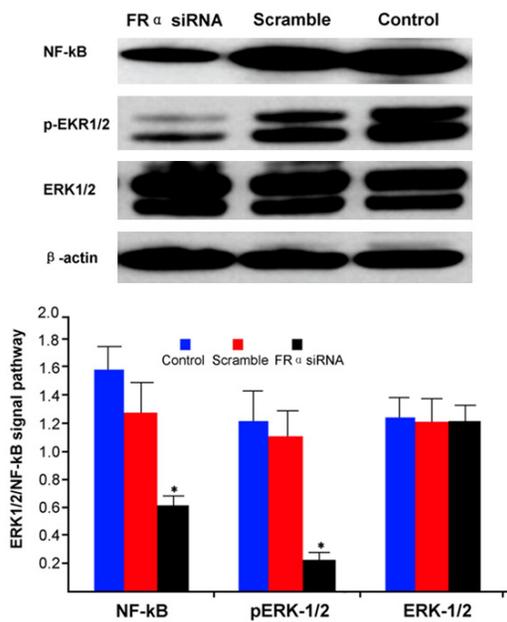


Figure 5. Modulatory effect of ERK1/2/NF- κ B signal pathway on CNE2 cells. (Top) representative image of Western blot. (Bottom) quantitative analysis of expression of ERK1/2, p-ERK1/2 and NF- κ B. *, P<0.05 compared to control group.

over-proliferation of cells and even malignant transformation [22]. Furthermore, overexpression of p-ERK1/2 can increase the expression of NF- κ B, a down-stream signaling protein, which activates various cytokines and cascade reactions, and ultimately leads to tumor occurrence [23]. In this study, we showed that down-regulation of FR α by siRNA reduced ERK1/2 phosphorylation and NF- κ B expression in NPC cells, suggesting that FR α might facilitate NPC via the ERK1/2/NF- κ B signaling pathway.

In conclusion, FR α expression was up-regulated in NPC CNE2 cells. Down-regulation of FR α by siRNA significantly inhibited cell proliferation and invasion, but markedly induced cell apoptosis of CNE2 cells, probably through modulating the ERK1/2/NF- κ B signaling pathway. FR α might be an effective biomarker of NPC and useful for the diagnosis and targeted treatment for the malignant disease.

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Disclosure of conflict of interest

None.

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Folic acid receptor in NPC

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