Folate receptor alpha promotes endometrial carcinoma cell proliferation and inhibits apoptosis by regulating the ERK signaling pathway

Jing Zhang, Yuzhi Li, Lihua Wang, Yuan Zhang, Qingsong Zhang, Jing Liu

Department of Gynecologic Oncology, The First Affiliated Hospital of Bengbu Medical College, Bengbu 233004, Anhui Province, P. R. China

Received August 28, 2017; Accepted January 11, 2019; Epub July 15, 2019; Published July 30, 2019

Abstract: About 90% of endometrial carcinoma cases present with folate receptor alpha (FRα) overexpression. Studies have shown that the acid liposome coupled receptor can phosphorylate ERK in cancer. A recent study found that FRα mediated folic acid and can activate the ERK signaling pathway through the non-receptor tyrosine kinase (c-Src). However, the roles of FRα and the ERK signaling pathway in the pathogenesis of endometrial carcinoma are still unclear. A total of 56 tumor tissues from patients with endometrial carcinoma were collected. Other normal endometrium tissues were gathered from hysteroscopy diagnosis and treatment. An in vitro study compared FRα, p-ERK1/2, and survivin expressions between normal endometrial epithelial cells and different differentiation degrees of endometrial cancer cells. KLE cells were cultured in vitro and divided into five groups, including a control group, an si-NC group, an si-FRα group, a PD98059 (ERK1/2 inhibitor) group, and a si-FRα + PD98059 group. FRα and survivin expressions, together with ERK signaling pathway activity, were measured. Ki-67 expression and cell apoptosis were determined by flow cytometry. Caspase-3 activity was evaluated by spectrophotometry. FRα, p-ERK1/2, and survivin expressions were significantly increased in endometrial carcinoma tissues compared to normal tissues. They were clearly elevated in the endometrial cancer cell line compared with normal endometrial epithelial cells. Cell proliferation was gradually enhanced, and apoptosis was reduced following differentiation declination. Si-FRα and/or PD98059 treatment clearly weakened ERK, c-Fos, and c-Jun phosphorylation, downregulated survivin expression, suppressed cell proliferation, and elevated cell apoptosis. FRα is abnormally overexpressed in endometrial carcinoma tissue. Elevated FRα can promote cancer cell proliferation and suppress apoptosis by activating the ERK signaling pathway to upregulate survivin expression.

Keywords: Folate receptor, endometrial carcinoma, ERK, survivin, proliferation, apoptosis

Introduction

Endometrial carcinoma (EC) is a type of epithelial endometrial malignant tumor that occurs in the endometrium. It has various histologic types, of which endometrial adenocarcinoma (EA) is the most common [1]. The incidence of EC is only second to cervical cancer, accounting for 20%–30% of female reproductive system tumors [2]. Folate receptor alpha (FRα) is a kind of glycoprotein that couples with phosphatidyl inositol on the cell membrane. It expresses low in normal tissues, but it is highly overexpressed in a variety of tumors, such as lung cancer [3], ovarian cancer [4], and breast cancer [5], etc. It was reported that about 90% of EC presented FRα overexpression [6]. However, the relationship between FRα overexpression and the risk of EC is still unclear. Extracellular signal regulated kinase (ERK) is the classic family member of the mitogen activated protein kinase (MAPK) [7]. It is activated by a growth factor or other mitosis stimulation, with the effect of promoting cell growth and proliferation [8], and anti-apoptosis [9]. Ras/Raf/MEK/ERK is the main ERK pathway, so it is called the ERK pathway [10]. After the extracellular signal molecules bind with the corresponding receptors, ERK can be phosphorylated by an upstream intracellular protein cascade reaction, such as c-Src, Ras, Raf, and MEK [11]. ERK is the downstream core element of the Ras MAPK. Phosphorylated ERK
FRα regulates ERK to facilitate cancer proliferation

can promote target protein phosphorylation or regulate other protein kinase activity. More importantly, it can enter the nucleus to phosphorylate a series of nuclear transcription factors, such as c-Fos and c-Jun, affecting multiple biological processes, including cell growth, differentiation, and apoptosis [12]. It was found that the folate receptor liposome coupling compound may phosphorylate ERK in cancer cells [13]. A recent study discovered that the FRα that mediates folic acid can activate the ERK signaling pathway through non-receptor tyrosine kinase (c-Src) [14]. Its roles in FRα abnormal expression and the ERK signaling pathway in EC are still controversial.

Materials and methods

Main reagents and materials

The well-differentiated EC cell line Ishikawa, which moderates the differentiated EC cell line JEC, and the normal endometrial epithelial cell line ESC were bought from the Shanghai Cell Bank of Chinese Academy of Sciences. DMEM, RPMI 1640, fetal bovine serum, and penicillin-streptomycin were obtained from Gibco. Lipofectamine 2000 was purchased from Invitrogen. The GoScript™ Reverse Transcription System Kit was bought from Promega. The SYBR Green Real-Time PCR Master Mixes was purchased from Life Technologies. The siRNA interference sequence and the PCR primers were designed and synthetized by Genepharma. The rabbit anti human p-ERK1/2 (Thr202/Tyr204) antibody and the goat anti human FRα antibody were bought from Santa Cruz. The rabbit anti human p-c-Fos, p-c-Jun, and survivin antibodies were from Abcam. The FITC labeled Ki-67 antibody was from eBioscience, and the BCA Protein Assay Kit was from Shanghai Yiyan Biotechnology. The Annexin V/PI double staining apoptosis kit and the Caspase-3 activity detection kit were from Beyotime. The ERK1/2 inhibitor PD98059 was purchased from Med-Chemexpress.

Clinical information

A total of 56 cases of EC patients in the First Affiliated Hospital of Bengbu Medical College between March 2014 and September 2015 were enrolled, including 16 cases in stage I, 22 cases in stage II, and 18 cases in stage III. The mean age was 55.6 ± 6.1 (49-68) years old. EC tissue was collected after each hysterectomy. Other normal endometrium tissues from each hysteroscopy were collected as a control. All specimens were stored in liquid nitrogen within 10 min, and then stored at -80°C in a freezer.

This study was pre-approved by the ethical committee of the First Affiliated Hospital of Bengbu Medical College. All subjects signed the consent forms before being recruited in this study.

Si-FRα transfection and grouping

The Ishikawa and ESC cells were maintained in RPMI 1640 medium supplement with 10% FBS and 1% penicillin-streptomycin. The JEC and KLE cells were maintained in a DMEM medium supplement with 10% FBS and 1% penicillin-streptomycin. The cells were used for experiments after the fusion rate reached 70-80%. The SiRNA interference sequences were as follows. Si-FRα, forward, 5'-GGACUGAGCUUCUAUGUTT-3', reverse, 5'-ACAUCUUGAGCUACGUC-3'; si-NC, forward, 5'-UUCUCCGAACGUCT3'; reverse, 5'-ACGUGACGUUUGAATT-3'. SiRNA and lipofectamine 2000 were diluted by Opti-MEM at RT for 5 min, respectively. Then they were mixed at RT for 20 min and added to the cells for 6 h. After changing the medium, the cells were further incubated for 48 h for the following experiments. The KLE cells were cultured in vitro and divided into five groups, including the control group, the si-NC group, the si-FRα group, the PD98059 (ERK1/2 inhibitor) group, and the si-FRα + PD98059 group.

qRT-PCR

Total RNA was extracted from tissue or cells by Trizol. Specially, a 50-100 mg sample was rinsed in liquid nitrogen and added to 1 ml Trizol at RT for 5 min. A total of 5~10×10^7 cells were collected as added to 1 ml Trizol at RT for 5 min. After chloroform extraction and isopropanol sediment, the RNA was washed by 75% ethanol and dissolved in RNAse free H2O. Then the RNA was reverse transcribed to cDNA with the system containing 2 μg total RNA, 4 μL reaction buffer (5×), 1 μL dNTP, 1 μL oligo dT + random primer mix, 1 μL reverse transcriptase, 1 μL ribonuclease inhibitor, and ddH2O. The reaction was performed at 25°C for 5 min,
FRα regulates ERK to facilitate cancer proliferation

37°C for 60 min, and 70°C 15 min. The cDNA was used as a template to perform PCR amplification. The primer sequences were as follows. FRαP_f: 5’-GCTCAGCGGATGACAACACA-3’, FRαP_r: 5’-CCTGGCCCATGCAATCCTT-3’; SurvivinP_f: 5’-AGGACCACCGCATCTCATAC-3’, SurvivinP_r: 5’-AAGTCTGGCTCGTTCTCAGTG-3’; β-actinP_f: 5’-GAACCCTAAGGCCAAC-3’, β-actinP_r: 5’-TGTCACGCACGATTTCC-3’. The reaction system was constructed by 2×SYBR for 12.5 μL, 2 μm/L primers for 0.5 μL, cDNA for 1 μL, reaction buffer (5×) for 5 μL, DNA polymerase for 0.25 μL, and ddH₂O. The PCR reaction consisted of 40 cycles at 95°C for 15 s, 60°C for 30 s, and 74°C for 30 s. Each sample was repeated three times. The comparative Ct method (2^(-ΔΔCT)) was adopted to calculate the results.

**Western blot**

The cells were washed by PBS and treated using a SDS lysate. The protein concentration was determined by the BCA method. The protein was diluted using a loading buffer and boiled at 100°C for 5 min. A total of 50 μg protein was separated by 10% SDS-PAGE for 3 h and transferred to a PVDF membrane. After being blocked with 5% skim milk at RT for 1 h, the membrane was incubated in a primary antibody at 4°C overnight. After being washed with PBST, the membrane was further incubated in a HRP labeled secondary antibody at RT for 1 h. Finally, the membrane was developed using the ECL method. The result was analyzed on Quantity One.

**Flow cytometry detection of cell apoptosis**

The cells were digested by an enzyme and resuspended in 195 μl of annexin V-FITC binding buffer. Next, the cells were incubated in 5 μl annexin V-FITC and 10 μl of PI at RT in the dark for 10-20 min. Lastly, the cells were tested using flow cytometry.

**Ki-67 detection**

The cells were digested by an enzyme and centrifuged at 1000 rpm for 5 min. After being fixed by 1 ml 4% paraformaldehyde at RT for 30 min, the cells were further incubated in PBS containing 0.1% Triton X-100 and 0.5% BSA at RT for 30 min. Next, the cells were resuspended in 100 μl PBS containing 0.5% BSA and an FITC labeled Ki-67 antibody was added at 4°C in the dark for 40 min. Finally, the cells were
FRα regulates ERK to facilitate cancer proliferation

resuspended in a 500 μl incubation buffer and tested using flow cytometry.

Caspase-3 activity detection

pNA (10 mM) was diluted to 0, 10, 20, 50, 100, and 200 μM. The standard substance was tested on a microplate reader at 405 nm to draw the standard curve. The cells were digested by enzymes and centrifuged at 800 g and 4°C for 5 min. A total of 2×10⁶ cells were cracked at 4°C for 20 min and centrifuged at 18000 g for 15 min. Next, the supernatant was moved to a pre-cooled Ep tube to test the caspase-3 activity. 10 μl Ac-DEVD-pNA (2 mM) was added to the supernatant, and we incubated the solution at 37°C for 2 h. At last, the plate was read at 405 nm.

Statistical analysis

SPSS 18.0 was used for the statistical analysis. The measurement data were depicted as the mean ± standard deviation and compared using a t test. P < 0.05 was considered statistically significant.

Results

FRα overexpressed in EC tissue

The qRT-PCR revealed that, compared with normal endometrial tissue, FRα mRNA (Figure 1A) and protein (Figure 1C) expression was obviously increased in EC tissue with pathological grading dependence. Similar to the elevation trend of FRα, the p-ERK1/2 protein level was

Figure 2. FRα expression in EC cells with different differentiation degrees. A. qRT-PCR detection of mRNA expression. B. Western Blot detection of protein expression. C. Spectrophotometry detection of caspase-3 activity. D. Flow cytometry detection of Ki-67 protein expression. E. Flow cytometry detection of cell apoptosis.
FRα regulates ERK to facilitate cancer proliferation

The Survivin mRNA (Figure 1B) and protein (Figure 1C) levels were also significantly higher in the EC tissue compared with the normal endometrial tissue and presented an elevation trend following the pathological grading.

FRα expression in EC cells with different differentiation degrees

We tested the related molecules expression in the EC cells with different differentiation degrees. The results demonstrated that EC cells presented a higher FRα expression with an increasing trend following the declination of cell differentiation (Figure 2A and 2B). The ERK protein phosphorylation also showed a similar trend (Figure 2B). The survivin levels in the various cell lines were in accordance with FRα, as they were enhanced following the differentiation reduction (Figure 2A and 2B). The caspase-3 activity in the normal endometrial cells and in the well-differentiated EC cells was relatively higher, but it was lower in the moderate and poorly differentiated EC cells (Figure 2C). The cell proliferation was gradually enhanced following the differentiation reduction, as the Ki-67 elevation (Figure 2D) and cell apoptosis declined (Figure 2E).

Silencing FRα affected ERK activity, promoted KLE cell apoptosis, and suppressed cell proliferation

This study applied KLE cells as a tool to investigate the FRα impact on cell proliferation and apoptosis, as there was a high FRα level and...
FRα regulates ERK to facilitate cancer proliferation

Discussion

Folic acid plays a critical role in cell metabolism, DNA synthesis and repair, and the rapid proliferation of cancer cells [15]. Cancer cells in rapid division need a large amount of folic acid because of DNA synthesis. The folate receptor is a kind of glycoprotein coupled with phosphatidylinositol on the membrane, including three subtypes: FRα, FRβ, and FRγ [16]. Although all folate receptor subtypes can bind with folic acid, only FRα combining with folic acid is actually combined, and only the combination of FRα and folic acid is physiological. Moreover, among all subtypes, FRα has the strongest affinity with folic acid and is also the most closely related to human disease [17]. In recent years, FRα has become a hot spot in cancer research because of its efficient combining ability with folic acid and intracellular transferring. FRα expression in normal tissue is extremely low, but its abnormal elevation can be found in a variety of cancer tissues, such as breast cancer [3], lung cancer [18], and pituitary adenoma [19]. Targeted biological therapy focusing on folic acid receptors showed a good clinical effect and prospects, providing new hope for patients [20]. FRα elevation occurs in about 90% of EC patients, but its relationship with the pathogenesis of EC is still unclear. Our results showed that FRα mRNA and protein had a low expression in normal endometrium tissue, but they were abnormally increased in EC tissues with pathological grading dependence. It is suggested that an abnormal overexpression of FRα may be involved in the pathogenesis of EC. Ras/Raf/MEK/ERK is the main component of the ERK pathway. After the extracellular signal molecules bind with the corresponding receptors, ERK can be phosphorylated as the core component of the Ras MAPK, regulating a series of nuclear transcription factors’ phosphorylation and transcriptional activity, such as c-Fos and c-Jun. c-Fos and c-Jun can form transcription factor AP-1 in the form of a heterodimer to bind with DNA and regulate target gene expression at the transcriptional level, so as to participate in the biological process regulation, including cell growth, differentiation, and apoptosis [12]. It was also found that folate receptor liposome coupling compound may phosphorylate ERK in cancer cells [13]. A recent study discovered that FRα mediating folic acid can activate the ERK signaling pathway through c-Src [14]. This study confirmed that, as with an increasing trend of FRα, the ERK phosphorylation level in the different pathological grades of EC tissues were obviously higher than in normal endometrium tissues with pathological grading dependence, indicating that the activation of the ERK signaling pathway may be involved in the pathogenesis of EC, and FRα elevation may play a role in ERK signaling pathway activation. Survivin, a member of the inhibitor of apoptosis family of proteins (IAPs), has been found to be the strongest apoptosis inhibiting factor so far. It plays a role in antiapoptosis by inhibiting caspase-3 catalytic activity. It is involved in regulating cell proliferation and apoptosis, and its elevation is related to the occurrence of a variety of tumors [21-23]. It has been shown that survivin expression is regulated by the ERK signaling pathway, and inhibiting the activity of ERK signaling pathway can obviously reduce survivin levels [24]. Our study investigated the relationship of the ERK signaling pathway and survivin expression with EC patients. The results demonstrated that survivin expression in EC tissue was significantly higher than of its expression in normal endometrium tissues with pathological grading dependence. Takai, et al. [25] revealed that survivin levels were markedly elevated in EC tissues and closely related to...
FRα regulates ERK to facilitate cancer proliferation

clinical stage, pathological grading, and prognosis, which is consistent with our results. FRα and survivin expression in normal endometrial epithelial cells is low, and ERK activity is the weakest. FRα and survivin expression, together with ERK activity, were higher following EC cell line differentiation reduction. Furthermore, our results discussed whether FRα abnormal elevation may regulate cell proliferation and apoptosis by affecting ERK activity and survivin expression. After silencing FRα expression and (or) restraining ERK1/2 phosphorylation, c-Fos and c-Jun phosphorylation levels decreased, and survivin was significantly reduced, cell proliferation was obviously weakened, caspase-3 activity markedly was enhanced, and apoptotic cells increased. Their combination showed the strongest effect. The results pointed to the apparent role of FRα elevation in enhancing ERK signaling pathway activity, upregulating survivin expression, promoting EC cell proliferation, and inhibiting apoptosis. Pallares, et al. demonstrated that an increase in survivin in EC patients was associated with STAT-3 and PI3K/AKT activation. This study illustrated from another angle that FRα may affect ERK activity to regulate survivin expression in EC.

Conclusion

FRα is abnormally overexpressed in EC tissue. FRα may promote EC cell proliferation and suppress apoptosis by activating the ERK signaling pathway and enhancing c-Fos and c-Jun transcription activity to upregulate survivin expression.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jing Zhang, Department of Gynecologic Oncology, The First Affiliated Hospital of Bengbu Medical College, No. 287, Changhai Road, Bengbu 233004, Anhui Province, P. R. China. Tel: +86-0552-3086191; Fax: +86-0552-3086191; E-mail: jishi038182407@yeah.net

References

[13] Chaudhury A, Tan BJ, Das S, Chiu GN. Increased ERK activation and cellular drug accumulation in the enhanced cytotoxicity of folate receptor-


