Inhibitory effects of FKBP14 on human cervical cancer cells

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Abstract. The FK506-binding protein 14 (FKBP14), which belongs to a subfamily of immunophilins, has been implicated in various biochemical processes. However, its effects on human cervical cancer remain to be elucidated. The present study aimed to determine the exact role of FKBP14 in human cervical cancer cell proliferation, cell cycle progression, apoptosis, invasion and migration. Cell proliferation was measured by Cell Counting Kit-8 assay. Flow cytometry was conducted to determine the effects of FKBP14 on cell cycle progression and apoptosis. Cell invasion and migration were determined by Transwell assay. The results of the present study demonstrated that silencing FKBP14 expression using short hairpin (sh)RNA suppressed proliferation, invasion and migration of HeLa and C-33A cells, and also induced apoptosis and cell cycle arrest. Furthermore, silencing FKBP14 expression decreased the protein expression levels of B-cell lymphoma 2 (Bcl-2), matrix metalloproteinase (MMP)2 and MMP9, and increased the levels of caspase-3 and Bcl-2-associated X protein in FKBP14 shRNA-infected HeLa and C-33A cells. In conclusion, FKBP14 may act as an oncogene through suppressing apoptosis and promoting motility in human cervical carcinogenesis; therefore, it may be considered a potential therapeutic target for the treatment of cervical cancer.

Introduction

Cervical cancer is one of the leading causes of cancer-associated mortality and remains the second most common female malignant disease worldwide (1). Although the majority of cases of cervical cancer are preventable, it currently affects ~500,000 women per year and some patients with high risk factors have an unfavorable prognosis (2). The 5-year survival rate of patients with cervical cancer and lymph node metastasis reduced by 20-30%, compared with patients without lymph node metastasis (3). Therefore, identification of the underlying molecular mechanisms associated with metastasis may contribute to improving the prognosis of cervical cancer.

Cell motility is an essential cellular process associated with cancer cell invasion and metastasis. Cell migration usually occurs in response to a chemoattractant or a growth factor, which is known as chemotaxis (4). In response to a signal, a migrating cell enters the cell motility cycle in an amoeboid-like manner. Previous studies have aimed to improve understanding regarding the molecular mechanisms underlying cell motility, in an attempt to identify novel therapeutic targets that may inhibit tumor invasion and migration (5,6). Gene therapy is a targeted technique that may be used to treat cancer, and various studies have been conducted to investigate the genes that are involved in metastasis of cervical cancer (7,8). However, the highly complex molecular mechanism underlying metastasis remains poorly understood.

FK506-binding protein 14 (FKBP14) belongs to a family of highly conserved proteins known as immunophilins, which bind to the immunosuppressive drug FK506 and often possess peptidyl-prolyl cis-trans isomerase activity (9,10). FKBP family members are present in various organisms and have been implicated in numerous biochemical activities, including protein folding, receptor signaling, protein trafficking and transcription (11,12). Although growing evidence has suggested the important roles of various FKBP family members, knowledge of their specific functions remains unclear. In particular, to the best of our knowledge, there is little information regarding the roles that FKBP family members play in the development and progression of cancer. According to Oncomine (www.oncomine.org/resource/login.html), FKBP5 is overexpressed in brain cancer, prostate cancer, lymphoma, head and neck cancer, and melanoma (13). Conversely, FKBP5 has been reported to be downregulated in pancreatic cancer, colon cancer and testicular cancer (13,14). Furthermore, FKBP38 has been reported to induce the localization of the anti-apoptotic proteins, B-cell lymphoma 2 (Bcl-2) and Bcl-xl, to the mitochondrial membrane and protect cells against apoptosis (15). Previous studies have demonstrated that FKBP14 is overexpressed in ovarian cancer and metastatic prostate cancer (16,17).

The present study aimed to determine the precise role of FKBP14 in human cervical cancer cell proliferation, cell cycle progression, apoptosis, invasion and migration. The present study indicated that FKBP14 is overexpressed in human cervical cancer cell lines. Notably, FKBP14 knockdown markedly inhibited HeLa and C-33A cell proliferation, invasion and migration, and induced cell cycle arrest and apoptosis. Furthermore, FKBP14 is associated with cell apoptosis, invasion...
and migration via the regulation of caspase-3, Bcl-2-associated X protein (Bax)/Bcl-2, matrix metalloproteinase (MMP)2 and MMP9 expression. These findings support a potential role for FKBP14 as a tumor promoter, and suggest that it may have efficacy as a target for the treatment of human cervical cancer.

Materials and methods

Cell culture. The following human cervical cancer cell lines: HeLa, C4-1, C-33A, SiHa and CaSk i in addition to normal cervical epithelial cells (CRL2614) were obtained from the Institute of Biochemistry and Cell Biology (Shanghai, China). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen; Thermo Fisher Scientific, Inc.). 100 U/ml penicillin and 100 µg/ml streptomycin, and were incubated in a humidified atmosphere containing 5% CO2 at 37°C.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from cervical cancer cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and was detected by 1% agarose gel electrophoresis to determine quantity and quality. As the template for PCR reactions, cDNA was synthesized from 1 mg RNA using AMV reverse transcriptase (Fermentas; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. RT-qPCR was conducted using SYBR-Green® 10X SuperMix (Takara Bio, Inc., Otsu, Japan) in a 25 µl total volume using a Roche Light Cycler® 480 II system (Roche Diagnostics, Basel, Switzerland). The PCR cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 45 sec, and a final extension step of 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec and 60°C for 15 sec. Primer pairs for human genes were designed using Primer Express software (version 3.0.1; Applied Biosystems; Thermo Fisher Scientific, Inc.) as follows: FKBP14 forward, 5’-TGA AGG CAC UUU GCA UGA CAU UA-3’; Sangon Biotech Co., Ltd.) was cloned into the pLVX-AcGFP-C1 lentiviral vector, and was used as the internal control. All PCR reactions were performed in triplicate and the relative expression levels of FKBP14 were calculated normalized to the mRNA expression levels of GAPDH using the ΔΔCq method (18).

RNA interference (RNAi) and construction of stable cell lines. A short hairpin (sh)RNA (shRNA, 5’-GACCACCUU CACUGAUUU A-3’; Sangon Biotech Co., Ltd., Shanghai, China) targeting human FKBP14 mRNA was cloned into the pLVX-AcGFP-C1 lentiviral vector (Sangon Biotech Co., Ltd.) using EcoRI and BamHI. Scramble shRNA (5’-CUUGGAGAA GCUUGACAUU A-3’; Sangon Biotech Co., Ltd.) was cloned into the pLVX-AcGFP-C1 lentiviral vector, and was used as a negative control (shNC). The constructs were subsequently transfected into human embryonic kidney-293T cells (Shanghai Institute of Biochemistry and Cell Biology, Shanghai, China) with pLVX-AcGFP-C1 lentiviral packaging vectors using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Transfected cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, and were incubated in a humidified atmosphere containing 5% CO2 at 37°C. Viruses were collected by centrifugation at 1,000 x g for 5 min at 37°C 48 h post-transfection, and were used to infect HeLa and C-33A cells at a multiplicity of infection of 20 in the presence of 8 mg/ml polybrene (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Subsequent experiments were performed 48 h post-infection.

Cell proliferation assay. The effects of FKBP14 shRNA on the proliferation of HeLa and C-33A cells were determined by Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Rockville, MD, USA) assay. Briefly, 5x104 cells were seeded into 96-well culture plates at 100 µl/well. Subsequently, cell proliferation was evaluated using the CCK-8 assay according to the manufacturer’s protocol. The absorbance of each supernatant was measured at a wavelength of 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.).

Flow cytometry. For cell cycle analysis, FKBP14 shRNA-infected HeLa and C-33A cells were seeded into 12-well plates at a density of 3x105 cells/well, and the percentage of cells in the various phases of the cell cycle were evaluated by determining the DNA content after propidium iodide (PI) staining. Briefly, cells were washed with PBS, trypsinized and centrifuged at 400 x g for 5 min at 4°C. Pellets were fixed overnight in 70% cold ethanol. Following fixation, cells were washed twice with PBS and incubated in PBS containing RNase (1 mg/ml) for 10 min at room temperature. Finally, samples were stained with PI (1 mg/ml) for 30 min at 4°C. Data acquisition was performed by flow cytometry (BD Accuri C6; software version 1.0.264.21; BD Biosciences, Franklin Lakes, NJ, USA). To analyze apoptosis, an Annexin V-fluorescein isothiocyanate (FITC)/PI double stain assay (BioVision, Inc., Milpitas, CA, USA) was conducted according to the manufacturer's protocol. Floating and trypsinized adherent cells (3x105 cells/well; 12-well plates) were collected, resuspended in 200 µl binding buffer containing 195 µl Annexin V-FITC and 5 µl PI, and were incubated for 10 min in the dark at room temperature prior to flow cytometric analysis (BD Accuri C6, software version 1.0.264.21; BD Biosciences).

Transwell assay. A total of 5x104 cells infected with FKBP14 shRNA were plated into the upper wells of a Transwell chamber (Greiner Bio-One GmbH, Frickenhausen, Germany), which was precoated with Matrigel (BD Biosciences) for the invasion assay and was uncoated for the migration assay. In both assays, the lower wells were filled with 600 µl DMEM containing 10% FBS. After 48 h at 37°C, cells in the upper wells were removed using a cotton bud. The cells attached to the lower surface were washed with PBS, fixed in 4% paraformaldehyde for 10 min at room temperature and stained with 0.5% crystal violet for 30 min at room temperature. Images of the cells were captured and cell numbers were counted under a light microscope (magnification, x200; Olympus Corporation, Tokyo, Japan).

Western blot analysis. Human cervical cancer cell lines were harvested and washed twice with PBS, after which the cells were lysed in ice-cold radioimmunoprecipitation assay buffer
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Beyotime Institute of Biotechnology (Shanghai, China) and were incubated on ice for 30 min. The protein concentration was measured using a bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.), and absorbance was measured using a microplate reader (SM600 Labsystem; Shanghai Utrao Medical Instrument Co., Ltd., Shanghai, China). The cell lysates were centrifuged at 13,000 x g for 10 min at 4˚C and the supernatant (20-30 µg protein) was separated by 10% SDS-PAGE. Proteins were then electrophoretically transferred onto a polyvinylidene fluoride membrane (EMD Millipore, Shanghai, China). The blots were blocked with 5% skim milk, followed by incubation with specific antibodies against FKBP14 (cat no. ab105018; 1:1,000), cleaved caspase-3 (cat no. ab32351; 1:5,000), Bax (cat no. ab53154; 1:1,000), Bel-2 (cat no. ab59348; 1:1,000), MMP2 (cat no. ab37150; 1:500) and MMP9 (cat no. ab38898; 1:1,000) (all from Abcam, Cambridge, MA, USA), and GAPDH (cat no. 5174; 1:1,500; Cell Signaling Technology, Inc., Danvers, MA, USA), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit/anti-mouse secondary antibodies (cat nos. A0208 and A0216; dilution 1:1,000; Beyotime Institute of Biotechnology) for 1 h at 37˚C, prior to being washed three times with Tris-buffered saline containing 20% Tween-20 (Amresco, Solon, OH, USA). The blots were visualized using enhanced chemiluminescence (EMD Millipore, Billerica, MA, USA) and signals were quantified by densitometry (Quantity One software; version 4.62; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. All values are presented as the mean ± standard deviation. All experiments were performed in triplicate. Data were analyzed by unpaired, two-tailed Student's t-test and one-way analysis of variance with Tukey's post hoc test, using Graphpad Prism 6.0 software (GraphPad, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

FKBP14 expression in human cervical cancer cell lines. The present study initially examined the expression levels of FKBP14 in five human cervical cancer cell lines: HeLa,
C4-1, C-33A, SiHa and CaSki, by RT-qPCR and western blot analysis, using GAPDH as a reference. The protein expression of FKBP14 in normal cervical epithelial cells (CRL2614) was additionally detected by western blot analysis. The results demonstrated that the mRNA and protein expression levels of FKBP14 were significantly higher in HeLa and C-33A cells compared with the three other cervical cancer cell lines (Fig. 1A and B). Notably, the protein expression of FKBP14 was significantly increased in HeLa and C-33A cells compared with CRL2614 cells (Fig. 1C). Therefore, the FKBP14-specific shRNA was used to infect HeLa and C-33A cells. The shNC-infected HeLa and C-33A cells were used as negative controls, and uninfected HeLa and C-33A cells were used as controls. As presented in Fig. 1D and E, the protein expression levels of FKBP14 were successfully reduced to ~92.6 and 60.3% of the levels in the control HeLa and C-33A cells 48 h post-infection, respectively. In addition, the expression of FKBP14 in the shNC-infected cells was the same as in control HeLa and C-33A cells.

**FKBP14 knockdown inhibits cell proliferation and induces cell cycle arrest.** The CCK-8 assay revealed that FKBP14 knockdown significantly inhibited cell proliferation in a time-dependent manner in FKBP14 shRNA-infected HeLa and C-33A cells. Cell proliferation was decreased by 9.8, 30.9 and 45.0% at 24, 48 and 72 h, respectively, compared with the control HeLa group (Fig. 2A). Cell proliferation was decreased by 9.5, 28.6 and 40.7% at 24, 48 and 72 h, respectively, compared with the control C-33A group (Fig. 2B).

Subsequently, the potential inhibitory effects of FKBP14 knockdown on cell cycle progression were investigated. As presented in Fig. 2C-F, suppression of FKBP14 resulted in
an increased number of cells in G1 phase (HeLa, mean=58.9; C-33A, mean=69.7) compared with the corresponding controls (HeLa, mean=41.9; C-33A, mean=51.05; P<0.001). There was a concomitant reduction in the number of cells in S and G2/M phases in HeLa and C-33A cells infected with FKBP14 shRNA. These data suggested that knockdown of FKBP14 induces cell cycle arrest at G1 phase in human cervical cancer cell lines, which may be associated with the inhibition of cell proliferation.

**FKBP14 knockdown induces cell apoptosis.** To assess the effects of FKBP14 on cell apoptosis, Annexin V/PI staining was performed. The mean apoptotic rate was significantly increased to 23.5 and 24.7% in FKBP14 shRNA-infected HeLa and C-33A cells, respectively, compared with the control groups (HeLa, mean=1.6%; C-33A, mean=3.7%, P<0.001; Fig. 3). These results suggested that FKBP14 may serve an anti-apoptotic role in human cervical cancer cell lines, as indicated by FKBP14 knockdown-induced inhibition of cell proliferation and induction of cell cycle arrest.

**FKBP14 knockdown inhibits cell invasion and migration.** The effects of FKBP14 knockdown on cervical cancer cell invasion and migration were determined by Transwell assay. As presented in Fig. 4, FKBP14 shRNA markedly suppressed the invasion of HeLa and C-33A cells by 46.1 and 56.1%, respectively, compared with the control groups. The ability of cells to migrate across uncoated chambers was also measured. The number of migratory FKBP14 shRNA-infected HeLa and C-33A cells was significantly decreased by 41.7 and 50.1% compared with the HeLa and C-33A control groups (Fig. 5).

**FKBP14 knockdown regulates the expression of apoptosis-, invasion- and migration-associated proteins.** The present study examined the expression levels of proteins associated with cell apoptosis, invasion and migration by western blot analysis. The expression levels of apoptosis-associated proteins, including caspase-3 and Bax, were significantly increased by 22.2 and 43.4%, respectively, whereas Bcl-2 was significantly decreased by 92.7%, in FKBP14 shRNA-infected HeLa cells compared with the control HeLa cells (Fig. 6A). In addition, the expression levels of proteins involved in cell invasion and migration, MMP2 and MMP9, were significantly decreased by 73.2 and 86.9%, respectively, in FKBP14 shRNA-infected HeLa cells compared with the control HeLa cells (Fig. 6A). In FKBP14 shRNA-infected C-33A cells, the protein expression levels of caspase-3 and Bax were increased by 68.3 and 85.1%, whereas the protein expression levels of Bcl-2, MMP2 and MMP9 were decreased by 40.5, 43.2 and 57.1%, respectively, in FKBP14 shRNA-infected cells compared with the control C-33A cells (Fig. 6B). These findings suggested that the effects of FKBP14 on cervical cancer cell apoptosis, invasion and migration may be associated with regulation of the protein expression levels of caspase-3, Bax, Bcl-2, MMP2 and MMP9.

**Discussion**

Cervical cancer is the most prevalent malignancy of the female reproductive system. Although great progress has been achieved, current therapies for the treatment of patients with advanced cervical cancer are still unfavorable, due to uncontrolled local cancer, recurrence and/or distant metastasis (19). Members of the FKBP family have been implicated in various processes, particularly in cancer, including cell...
cycle progression, and survival and apoptotic signaling pathways (20). Since dysregulation of FKBP expression has been observed in cancer tissues, it has been hypothesized that FKBP members serve an important role in tumorigenesis and the response to chemotherapy and radiotherapy; in addition, it has been suggested that FKBP members may act as oncogenes or tumor suppressors depending on the tissue type. A previous study reported that FKBP14 is frequently upregulated in various human malignancies (21). In the present study, the mRNA and protein expression levels of FKBP14 were higher in HeLa and C-33A cells compared with in three other human cervical cancer cell lines.

To investigate the roles of FKBP14 in cervical cancer cells, RNAi was conducted. The results of the present study demonstrated that FKBP14 knockdown significantly inhibited cell proliferation, and induced cell cycle arrest and apoptosis, in HeLa and C-33A cells. Other members of the FKBP family have also been reported to be involved in the regulation of cell cycle progression and apoptosis. For example, cells from FKBP12-deficient mice exhibited cell cycle arrest in G1 phase (22). Furthermore, overexpression of FKBP38 resulted in the suppression of apoptosis, whereas functional inhibition of this protein promoted cell apoptosis (23). Cleaved caspase-3 is a well-known marker for cell apoptosis (24). The Bcl-2 family proteins exert anti-apoptotic (e.g. Bcl-2) or proapoptotic (e.g. Bax) effects, and the ratio of Bax/Bcl-2 is a useful index to evaluate cell apoptosis (25). The present study revealed that knockdown of FKBP14 increased the expression of cleaved caspase-3 and the ratio of Bax/Bcl-2, thus suggesting that FKBP14 may exert an inhibitory effect on cell apoptosis by regulating caspase-3 expression and the ratio of Bax/Bcl-2.

Invasion and metastasis are important malignancy-associated biological behaviors, which are the main causes of mortality in patients with advanced cervical cancer (26).
Active cell migration is a critical factor in the invasion and metastasis of cancer, and highly invasive cancer cells usually exhibit more active cell migration. In the present study, the effects of FKBP14 shRNA infection on the abilities of HeLa and C-33A cells to invade and migrate were determined by Transwell assay. The results indicated that the number of invasive and migratory HeLa and C-33A cells was significantly decreased following FKBP14 knockdown. Degradation and destruction of the extracellular matrix and basement membrane are crucial steps in the invasion and metastasis of cancer, in which MMPs serve an important role (27,28). Two of these enzymes, MMP2 and MMP9, are associated with tumor invasion and metastasis. Overexpression of MMP2 and MMP9 has been detected in pre-cancer and cancerous lesions of the cervix (29). Previous studies have reported that MMP9 is involved in cancer angiogenesis via vascular endothelial growth factor regulation, and serves a central role in the cleavage of certain cytokine receptors on tumor-infiltrating lymphocytes derived from human cervical cancer (30,31). The results of the present study indicated that the expression levels of MMP2 and MMP9 were decreased in FKBP14 shRNA-infected HeLa and C-33A cells. These findings indicated that FKBP14 promotes invasion and migration by modulating the expression of MMP2 and MMP9.

In conclusion, further studies regarding the association of FKBP14 with apoptosis, invasion and migration of human cervical cancer are required to determine the exact mechanism underlying the oncogenic effects of FKBP14.

References


