RNAi-Mediated Downregulation of FKBP14 Suppresses the Growth of Human Ovarian Cancer Cells

Meng Lu, Yi Miao, Lan Qi, Mingzhu Bai, Jiarong Zhang, and Youji Feng

Department of Obstetrics and Gynecology, Shanghai General Hospital of Nanjing Medical University, Shanghai, China

FKBP14 belongs to the family of FK506-binding proteins (FKBPs). Altered expression of FKBPs has been reported in several malignancies. This study aimed to reveal the expression profile of FKBP14 in ovarian cancer and evaluate whether FKBP14 is a molecular target for cancer therapy. We found that the FKBP14 mRNA level was significantly higher in ovarian cancer tissues than in normal tissues. FKBP14 expression was then knocked down in two ovarian cancer cell lines, SKOV3 and HO8910 cells, by a lentiviral short hairpin RNA (shRNA) delivery system. Reduced expression of FKBP14 markedly impaired the proliferative ability of ovarian cancer cells. Additionally, ovarian cancer cells infected with FKBP14 shRNA lentivirus tended to arrest in the G0/G1 phase and undergo apoptosis. Moreover, knockdown of FKBP14 induced cell apoptosis via increasing the ratio of Bax to Bcl-2. These results indicated that FKBP14 might be a diagnostic marker for ovarian cancer and could be a potential molecular target for the therapy of ovarian cancer.

Key words: FKBP14; Ovarian cancer; Cell proliferation; Cell cycle; Cell apoptosis

INTRODUCTION

Ovarian cancer is the most lethal form of gynecologic malignancy and is characterized by a high rate of mortality among gynecologic oncology patients (1). Ovarian cancer is frequently diagnosed in an advanced stage, contributing to a relatively high mortality rate and poor survival. The 5-year survival rate is only 30% (2). The poor clinical outcome of ovarian cancer patients emphasizes the requirement of developing better methods of diagnosis, treatment, and prevention of this disease based on an in-depth understanding of molecular pathogenesis and progression of ovarian cancer (3).

FKBP14 belongs to FK506-binding proteins (FKBPs), which are known to be intracellular receptors for immunosuppressive drugs FK506 and rapamycin. The mutations of FKBP14 cause a variant of Ehlers–Danlos syndrome (4,5), which is a disorder of connective tissue characterized by cutaneous hyperextensibility, joint hypermobility, progressive kyphoscoliosis, and hearing impairment. A recent study reported that FKBP14 regulated presenilin protein levels and Notch signaling during the development of Drosophila (6). FKBPs often exhibit peptidyl-prolyl cis–trans isomerase (PPlase) activity (7,8). Recent reports have revealed the roles of FKBPs on gene expression, DNA repair, and DNA replication. FKBP12 is shown to be a physiologic regulator of cell cycle (9) and intracellular calcium homeostasis (10). FKBP38 is suggested as a negative regulator of cell apoptosis (11). Although few investigations have been carried out on the expression profile and biological functions of FKBP14 in human tumors, several members of FKBPs are reported to be involved in the development and progression of cancer. For instance, FKBP11 (12) and FKBP52 (13) were found to be overexpressed in hepatocellular carcinoma. FKBP5 was overexpressed in prostate cancer (14), melanoma (15), and glioma (16). FKBP65 expression was decreased in high-grade ovarian serous carcinoma (17).

In the present study, we determined that FKBP14 was overexpressed in ovarian cancer tissues when compared with matched normal tissues. The effects of FKBP14 knockdown on the proliferation, cell cycle progression, and apoptosis of ovarian cancer cells were then evaluated. We also tried to investigate the possible mechanism that was involved. Our study suggested that FKBP14 is a possible oncogene in ovarian cancer and may serve as an effective therapeutic target for this disease.

MATERIALS AND METHODS

Tissue Samples and Cell Lines

Forty pairs of ovarian cancer tissues and noncancerous tissues were collected from the Department of Obstetrics
and Gynecology, Shanghai General Hospital of Nanjing Medical University (Shanghai, China) and stored at −80°C until RNA extraction. Written informed consent was obtained from all patients. This study was approved by the Ethics Committee of Shanghai General Hospital of Nanjing Medical University.

A2780, OVCAR3, SKOV3, 3AO, HO8910, and HEK293T were obtained from the Cell Bank at the Chinese Academy of Sciences and maintained at 37°C in 5% CO₂. A2780, OVCAR3, 3AO, and HO8910 were cultured in RPMI-1640, while SKOV3 and HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM). All media (Gibco, Carlsbad, CA, USA) were supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine.

RNA Extraction and qRT-PCR
Total RNA was extracted from tissues and cell lines with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and RNA concentrations were determined by measuring absorbance at 260 nm. The quantitative real-time PCR was performed in triplicate on an ABI 7300 machine (Applied Biosystems, Foster City, CA, USA) using SYBR Green PCR Master Mix (TOYOBO, Osaka, Japan). GAPDH was amplified as the internal control. The primers were as follows: FKBP14 (forward: 5'-TGAAGGACAGCACCAATAG-3'; reverse: 5'-GCACATTTACCACCAACTC-3'); GAPDH (forward: 5'-CACCCACCCTGTTGCTGTAG-3'; reverse: 5'-CCACCACCGTGTTCGTTAG-3').

Immunohistochemistry
For immunohistochemistry, tissue array sections containing 75 ovarian cases (Outdo Biotech, Shanghai, China) were deparaffinized in xylene, rehydrated in graded alcohol, and probed with FKBP14 primary antibody (Abcam, Cambridge, MA, USA) at 4°C overnight. The sections were then incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. The sections were developed with diaminobenzidine tetrahydrochloride, then counterstained with hematoxylin. The intensity of immunoreactivity was graded according to the extent of positivity as follows: FKBP14 low expression: <25% of the tumor cells showed positive stain; FKBP14 high expression: >25% of tumor cells showed positive stain.

Lentiviral Vector Production
Small interfering RNA (siRNA) targeting FKBP14 sequence (GACCACCTTCACGTATTAT) and non-silencing sequence (CCTAGGTTAAGTGCCCTTCG) were transformed into short hairpin RNA (shRNA) and were cloned into PLKO.1-lentiviral vector (Addgene, Cambridge, MA, USA).

The constructs and lentiviral packaging vectors were then transfected into HEK293T cells via Lipofectamine 2000 (Invitrogen). After being incubated for 48 h, lentivirus was collected from culture medium to infect target cells.

Western Blotting
Total protein was isolated from cultured cells using ice-cold RIPA buffer in the presence of a protease inhibitor cocktail (Pierce, Rockford, IL, USA). Protein concentration was determined using a BCA Protein Assay Kit (Pierce). Equal amounts of protein were electrophoresed in SDS-PAGE gels and transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). After being blocked with 5% nonfat milk, the membranes were incubated with primary antibodies and then corresponding horseradish peroxidase-conjugated secondary antibody (Beyotime, Shanghai, China). Signals were detected with enhanced chemiluminescence reagent (Millipore). Antibodies against FKBP14, PCNA, and cleaved caspase 3 were purchased from Abcam, anti-Bcl-2 and anti-Bax were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-GAPDH (a loading control) was from Cell Signaling Technology (Danvers, MA, USA).

Cell Proliferation Assay
Cell proliferation was monitored with Cell Counting Kit-8 (CCK-8; Beyotime) according to the manufacturer’s instructions. Briefly, cells infected with FKBP14 shRNA lentivirus (RNAi) or nonsilencing shRNA lentivirus (NC), along with nontreated cells (WT), were seeded onto 96-well plates (2,000 cells/well). After 0, 24, 48, and 72 h, 10 μl of CCK-8 solution was added to each well and incubated at 37°C for 1 h. The absorbance at 450 nm was measured in a microplate reader (Epoch, BioTek, Luzern, Switzerland).

Cell Cycle Analysis
Cells cultured in six-well plates were treated with FKBP14 shRNA or NC. After 48 h, cells were harvested and fixed overnight at 4°C in ice-cold 70% ethanol. The cells were then rehydrated and stained with propidium iodide (PI) staining buffer (Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 30 min. Cells were then analyzed using flow cytometry (BD Biosciences, San Jose, CA, USA).

Cell Apoptosis Analysis
Cells cultured in six-well plates were treated with FKBP14 shRNA or NC. After 48 h, cells were harvested and incubated with Annexin-V-fluorescein isothiocyanate (FITC)/PI apoptosis kit (KeyGen, Nanjing, China) for 15 min in the dark and analyzed within 1 h.

Statistical Analysis
Results of experiments are expressed as mean±SD. Statistical significance was determined using the Student’s
RESULTS

FKBP14 Expression in Ovarian Cancer Tissues and Cell Lines

To investigate the expression pattern of FKBP14 in ovarian cancer, we assessed the FKBP14 mRNA levels in 40 pairs of ovarian cancer tissues and noncancerous tissues. Statistical analysis with Student’s t-test demonstrated that FKBP14 expression was significantly elevated in ovarian cancer tissues compared with that in noncancerous tissues ($p<0.0001$) (Fig. 1A). For each cancer sample, the log2 tumor/normal ratio was calculated. As shown in Figure 1A, 34 cases of cancer tissues showed positive log2 (Tumor/Normal), indicating the elevated expression of FKBP14 in these tumor tissues, while another six cases displayed negative log2, indicating the decreased expression of FKBP14 in these tumor tissues.

We then performed immunohistochemistry tissue array analyses of FKBP14 expression using samples from 75 patients with ovarian cancer. Higher level of FKBP14 expression was observed in 51 ovarian cancer tissues

Figure 1. FKBP14 expression in ovarian cancer tissues and cell lines. (A) The expression level of FKBP14 in ovarian cancer tissues ($n=40$) was examined by qRT-PCR. (B) Expression of FKBP14 was higher in 34 ovarian tissues than in their pair-matched adjacent normal tissues. Each bar represents the log2 ratio of FKBP14 expression in each cancer sample and normal control. Positive log2 is represented in gray, and negative log2 is in white. (C) The expression level of FKBP14 by immunohistochemistry staining in 75 ovarian cancer tissues. mRNA (D) and protein (E) levels of FKBP14 were evaluated in five ovarian cancer cells. GAPDH was used as a loading control.
Table 1. Correlations Between FKBP14 Expression and Clinicopathologic Features in Patients With Ovarian Cancer

| Clinicopathologic Features | Total | Low (n=24) | High (n=51) | p Value |
|----------------------------|-------|------------|-------------|---------|
| Age (years)                |       |            |             | 0.6061  |
| <60                        | 49    | 17         | 32          |         |
| ≥60                        | 26    | 7          | 19          |         |
| Histologic type            |       |            |             | 0.4604  |
| Serous                     | 12    |            | 28          |         |
| Endometrioid               | 5     |            | 3           |         |
| Mucinous                   | 3     |            | 6           |         |
| Clear cell                 | 2     |            | 5           |         |
| Mixed                      | 2     |            | 2           |         |
| Tumor grade                |       |            |             | 0.0271* |
| I–II                       | 42    | 18         | 24          |         |
| III                        | 33    | 6          | 27          |         |
| Tumor size                 |       |            |             | 0.0002***|
| <5 cm                      | 24    | 15         | 9           |         |
| ≥5 cm                      | 51    | 9          | 42          |         |

*p<0.05; ***p<0.0001.

Figure 2. Knockdown of FKBP14 inhibited the proliferation of ovarian cancer cells. (A, B) Identification of FKBP14 knockdown efficiency using shRNA lentivirus by Western blotting in SKOV3 and HO8910 cells. (C, D). Treated or nontreated cells seeded onto 96-well plates, and cell viability was determined at indicated time points by CCK-8 assay. Assays were performed in triplicate. WT, nontreated cells; NC, nonsilencing shRNA lentivirus; RNAi, FKBP14 shRNA lentivirus. ***p<0.001 compared with NC.
Chi-square test indicated that FKBP14 expression was closely related with tumor size and tumor grade. There was no correlation between FKBP14 expression level and age and histologic type (Table 1).

We also examined the FKBP14 mRNA (Fig. 1D) and protein level (Fig. 1E) of five ovarian cancer cell lines. Two cell lines, SKOV3 and HO8910, showed higher FKBP14 mRNA and protein expression.

To investigate the role of FKBP14 in ovarian cancer, shRNA targeting FKBP14 or nonsilencing sequences were cloned into a lentiviral vector. FKBP14 shRNA lentivirus or nonsilencing shRNA lentivirus was produced and infected into two ovarian cancer cell lines with higher FKBP14 expression (SKOV3 and HO8910). As shown in Figure 2A and B, FKBP14 protein level was reduced.

Figure 3. FKBP14 silencing induced a G0/G1 arrest and cell apoptosis in ovarian cancer cells. (A) FACS histograms and cell cycle distribution analysis of SKOV3 and HO8910 cells by PI staining at 48 h after lentivirus infection. (B) Cell apoptosis analysis by Annexin-V-FITC/PI staining at 48 h after lentivirus infection. WT, nontreated cells; NC, nonsilencing shRNA lentivirus; RNAi, FKBP14 shRNA lentivirus. **p<0.01, ***p<0.001 compared with NC.
by about 90% in both cell lines infected with FKBP14 shRNA lentivirus (RNAi), compared with cells infected with nonsilencing shRNA lentivirus (NC). There was no difference between NC and WT cells.

FKBP14 Is Important for Ovarian Cancer Cell Growth

To further assess the role of FKBP14 in regulating ovarian cancer cell proliferation, CCK-8 assays were performed on both SKOV3 and HO8910 cells following lentivirus infection for 48 h (Fig. 2C, D). No statistically significant differences in cell proliferation were observed between NC and WT cells, indicating that the lentiviral system had no cytotoxic effect on cells, whereas the proliferation was markedly inhibited by FKBP14 knockdown at 48 and 72 h in both cell lines (p<0.001, compared with NC). These findings indicated that knockdown of FKBP14 greatly suppressed cell proliferative ability.

Knockdown of FKBP14 Induced G0/G1, Arrest and Apoptosis of Ovarian Cancer Cells

Considering that silencing of FKBP14 significantly slowed cell proliferation, we further performed cell cycle and apoptosis analyses to reveal the mechanism of the inhibitory effect of FKBP14 shRNA lentivirus on cell proliferation. As shown in Figure 3A, FKBP14 shRNA lentivirus infection significantly increased the percentage of cells in the G0/G1 phase (p<0.001) and decrease the percentage of cells in the S phase (p<0.01), compared with the NC cells. As shown in Figure 3B, FKBP14 knockdown resulted in an approximately ninefold increase in the apoptotic ratio, compared with the NC cells (p<0.001). No obvious difference in cell cycle distribution or cell apoptotic ratio was found between NC and WT cells. Our results revealed that FKBP14 shRNA inhibited ovarian cell proliferation via inducing G0/G1 cell cycle arrest and

![Figure 4](imageurl)  
**Figure 4.** Effect of FKBP14 shRNA on the protein expressions of PCNA, cleaved caspase 3, Bcl-2, and Bax was evaluated by Western blotting. Representative Western blotting (upper panel) and quantitative analysis based on three independent experiments (lower panel) are shown. WT, nontreated cells; NC, nonsilencing shRNA lentivirus; RNAi, FKBP14 shRNA lentivirus. **p<0.01, ***p<0.001 compared with NC.
cell apoptosis, indicating that FKBP14 may promote the growth of ovarian cancer cells.

FKBP14 Affected the Expression of Cell Proliferation and Apoptosis-Related Protein

The expression levels of proliferation and apoptosis-regulating proteins (cleaved caspase 3, Bcl-2, and Bax) were then detected by Western blotting (Fig. 4). FKBP14 knockdown resulted in a notable decrease in a marker of cell proliferation (PCNA) and antiapoptosis protein (Bcl-2), and a significant increase of apoptosis marker (cleaved caspase 3) and apoptosis-promoting protein (Bax). The ratio of Bax/Bcl-2 was significantly increased when FKBP14 expression was suppressed. These results indicated the growth-promoting and antiapoptosis role of FKBP14 in ovarian cancer cells.

DISCUSSION

Although altered expression of FKBPs have been involved in various human cancers (12–17), the expression profile and functional implication of FKBP14 in cancers has been poorly investigated. Here we found a marked elevation of FKBP14 in ovarian cancer tissues. Further in vitro experiments demonstrated that reduced expression of FKBP14 in two ovarian cancer cell lines inhibited cell growth via inducing G1/G0 arrest and cell apoptosis.

First, FKBP14 was identified as a useful diagnostic biomarker for ovarian cancer (Fig. 1A–C). FKBP14 expression was associated with tumor size and tumor grade (Table 1). We then presumed that FKBP14 may act as an oncogenic factor in ovarian cancer development. We used a lentiviral shRNA delivery system to effectively suppress the expression of FKBP14 protein in two ovarian cell lines with a higher expression of FKBP14 (Fig. 2A, B). Silencing of FKBP14 expression in both ovarian cell lines significantly decreased cancer cell proliferation (Fig. 2), which was further confirmed by detection of PCNA protein expression with Western blotting (Fig. 4). Other members of FKBPs have been involved in the regulation of cell cycle and apoptosis. Cells from FKBP12-deficient mice manifest cell cycle arrest in G1 phase (9). Overexpression of FKBP38 blocks apoptosis, whereas functional inhibition of this protein promoted cell apoptosis (11). In order to discover the mechanism by which FKBP14 affects ovarian cancer growth, we then applied cell cycle and cell apoptosis analysis by flow cytometry (Fig. 3A). Our data revealed that FKBP14 shRNA had an inhibitory effect on ovarian cancer cell growth via inducing G1/G0 arrest and cell apoptosis.

Cleaved caspase 3 is a well-known marker for cell apoptosis (18). Bcl-2 family proteins exert inhibitory (e.g., Bcl-2) or promotional (e.g., Bax) effects on cell apoptosis. The ratio of Bax to Bcl-2 is a useful index to evaluate cell apoptosis (19). We found that reduced expression of FKBP14 increased the expression of cleaved caspase 3 and the ratio of Bax to Bcl-2 (Fig. 4), which suggested that FKBP14 exerted an inhibitory effect on cell apoptosis via regulating the ratio of Bax/Bcl-2.

The present study proved, for the first time, that FKBP14 expression was elevated in ovarian cancer tissues when compared with matched normal tissues. Lentiviral shRNA mediated knockdown of FKBP14 and suppressed the growth of ovarian cancer cells via arresting the cell cycle in the G1/S, G2/M phases and stimulating cell apoptosis. Moreover, cell apoptosis induced by FKBP14 RNAi was mediated by enhancing the ratio of Bax/Bcl-2. Our data indicated that FKBP14 may be an oncogene during the development of ovarian cancer. FKBP14 has considerable potential as a therapeutic target for ovarian cancer treatment.

REFERENCES

1. Kim, A.; Ueda, Y.; Naka, T.; Enomoto, T. Therapeutic strategies in epithelial ovarian cancer. J. Exp. Clin. Cancer Res. 31:14; 2012.
2. Rustin, G.; van der Burg, M.; Griffin, C.; Qian, W.; Swart, A. M. Early versus delayed treatment of relapsed ovarian cancer. Lancet 377:380–381; 2011.
3. Canevari, S.; Gariboldi, M.; Reid, J. F.; Bongarzone, I.; Pierotti, M. A. Molecular predictors of response and outcome in ovarian cancer. Crit. Rev. Oncol. Hematol. 60:19–37; 2006.
4. Baumann, M.; Giunta, C.; Krabichler, B.; Ruschendorf, F.; Zoppo, N.; Colombi, M.; Bittner, R. E.; Quijano-Roy, S.; Muntoni, F.; Cirak, S.; Schreiber, G.; Zou, Y.; Hu, Y.; Romero, N. B.; Carlier, R. Y.; Amberger, A.; Deutschmann, A.; Straub, V.; Rohrbach, M.; Steinmann, B.; Rostasy, K.; Karall, D.; Bonnemann, C. G.; Zschocke, J.; Fauth, C. Mutations in FKBP14 cause a variant of Ehlers-Danlos syndrome with progressive kyphoscoliosis, myopathy, and hearing loss. Am. J. Hum. Genet. 90:201–216; 2012.
5. Murray, M. L.; Yang, M.; Fauth, C.; Byers, P. H. FKBP14-related Ehlers-Danlos syndrome: Expansion of the phenotype to include vascular complications. Am. J. Med. Genet. A 164a:1750–1755; 2014.
6. van de Hoef, D. L.; Couven, J. M.; Bouilanne, G. L. FKBP14 is an essential gene that regulates presenilin protein levels and Notch signaling in Drosohphila. Development 140:810–819; 2013.
7. Barik, S. Immunophilins: For the love of proteins. Cell Mol. Life Sci. 63:2889–2900; 2006.
8. Kang, C. B.; Hong, Y.; Dhe-Paganon, S.; Yoon, H. S. FKBP14-related epithelial cell family proteins: Immunophilins with versatile biological functions. Neurosignals 16:318–325; 2008.
9. Aghdasi, B.; Ye, K.; Resnick, A.; Huang, A.; Ha, H. C.; Guo, X.; Dawson, T. M.; Dawson, V. L.; Snyder, S. H. FKBP12, the 12-kDa FK506-binding protein, is a physiologic regulator of the cell cycle. Proc. Natl. Acad. Sci. USA 98:2425–2430; 2001.
10. Sood, S.; Chelu, M. G.; van Oort, R. J.; Skapura, D.; Santonastasi, M.; Dobrev, D.; Wehrens, C. H. Intracellular calcium leak due to FKBP12.6 deficiency in mice facilitates the inducibility of atrial fibrillation. Heart Rhythm 5:1047–1054; 2008.
11. Shirane, M.; Nakayama, K. I. Inherent calcineurin inhibitor FKBP38 targets Bcl-2 to mitochondria and inhibits apoptosis. Nat. Cell Biol. 5:28–37; 2003.
12. Lin, I.-Y.; Yen, C.-H.; Liao, Y.-J.; Lin, S.-E.; Ma, H.-P.; Chan, Y.-J.; Chen, Y.-M. A. Identification of FKBP11 as a biomarker for hepatocellular carcinoma. Anticancer Res. 33:2763–2769; 2013.
13. Liu, Y.; Li, C.; Xing, Z.; Yuan, X.; Wu, Y.; Xu, M.; Tu, K.; Li, Q.; Wu, C.; Zhao, M.; Zeng, R. Proteomic mining in the dysplastic liver of WHV/c-myc mice—Insights and indicators for early hepatocarcinogenesis. FEBS J. 277:4039–4053; 2010.
14. Velasco, A. M.; Gillis, K. A.; Li, Y.; Brown, E. L.; Sadler, T. M.; Achilleos, M.; Greenberger, L. M.; Frost, P.; Bai, W.; Zhang, Y. Identification and validation of novel androgen-regulated genes in prostate cancer. Endocrinology 145:5913–5924; 2004.
15. Romano, S.; D’Angelillo, A.; Pacelli, R.; Staibano, S.; De Luna, E.; Bisogni, R.; Eskelinen, E. L.; Mascolo, M.; Cali, G.; Arra, C.; Romano, M. F. Role of FK506-binding protein 51 in the control of apoptosis of irradiated melanoma cells. Cell Death Differ. 17:145–157; 2010.
16. Jiang, W.; Cazacu, S.; Xiang, C.; Zenklusen, J. C.; Fine, H. A.; Berens, M.; Armstrong, B.; Brodie, C.; Mikkelsen, T. FK506 binding protein mediates glioma cell growth and sensitivity to rapamycin treatment by regulating NF-kappaB signaling pathway. Neoplasia 10:235–243; 2008.
17. Quinn, M. C.; Wojnarowicz, P. M.; Pickett, A.; Provencher, D. M.; Mes-Masson, A.-M.; Davis, E. C.; Tonin, P. N. FKBP10/FKBP65 expression in high-grade ovarian serous carcinoma and its association with patient outcome. Int. J. Oncol. 42:912–920; 2013.
18. Porter, A. G.; Jänicke, R. U. Emerging roles of caspase-3 in apoptosis. Cell Death Differ. 6:99–104; 1999.
19. Ola, M. S.; Nawaz, M.; Ahsan, H. Role of Bcl-2 family proteins and caspases in the regulation of apoptosis. Mol. Cell. Biochem. 351:41–58; 2011.