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# Downregulation of FKBP14 by RNA interference inhibits the proliferation, adhesion and invasion of gastric cancer cells

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**Abstract.** FK506 binding protein (FBBP) 14 belongs to the family of FKBPs. Altered expression of FKBPs are observed in several malignancies. The present study aimed to explore the expression and biological function of FKBP14 in gastric cancer. FKBP14 expression levels in 40 gastric cancer samples and matched control samples were evaluated using quantitative polymerase chain reaction. Cell proliferation was evaluated using Cell Counting kit-8 assay. A cell adhesion and a Transwell assay were performed to detect cell adhesion and invasion. Protein expression was determined using western blot analysis. It was found that FKBP14 expression in gastric cancer tissues was elevated compared with normal tissues. Silencing of FKBP14 expression in the gastric cancer MKN-45 and AGS cell lines, which have a higher expression level of FKBP14 compared with four other gastric cancer cell lines, significantly inhibited cellular proliferation, adhesion and invasion. In addition, the protein levels of proliferating cell nuclear antigen, matrix metalloproteinase 2 and the epithelial-mesenchymal-transition (EMT) markers β-catenin, Snail1 and Twist were repressed in gastric cancer cells with FKBP14 silenced. In conclusion, FKBP14 may act as an oncogene by suppressing cellular proliferation, adhesion and invasion and EMT in gastric carcinogenesis. FKBP14 may be a diagnosis marker and potential therapeutic target in gastric cancer.

## Introduction

Gastric cancer is one of the most common types of malignancy and the second leading cause of cancer-associated mortality worldwide (1). Previous improvements to the diagnosis and therapy of gastric cancer have led to a decrease in the rate of mortality (2). However, the therapeutic strategy for gastric cancer is restricted by the deficiency of targeted agents and gastric cancer remains a serious health problem with an overall 5-year survival rate of 20% (3,4). Thus, developing an in-depth understanding of the mechanism underlying gastric cancer is important.

FK506 binding protein (FKBP) 14 is a highly conserved member of the FKBP family. FKBPs contain FKB domains that are known to bind with immunosuppressant drugs, such as FK506 and rapamycin. FKBPs participate in multiple biological processes including kinase activity, receptor signaling, protein folding and stability and trafficking in normal cells. Previously, expression of several members of FKBPs has been identified to be modified in human cancer tissue compared with non-tumorous tissue. For instance, FKBP12 was exhibited to be upregulated in malignant vascular endothelium (5) and childhood astrocytoma (6). FKBP65 expression was increased in early benign lesions compared with normal mucosa (7). FKBP52 has been demonstrated to be overexpressed in breast (8), prostate (9) and hepatocellular carcinoma tissues (10). FKBP38 has also been found to be selectively downregulated in aggressive types of schwannoma tumor cell lines (11). However, the expression profile and biological functions of FKBP14 in tumors remain unknown.

The present study found that FKBP14 was overexpressed in gastric cancer tissues compared with normal tissues. The present study then investigated the function of FKBP14 in gastric cancer cells by RNA interference. Reduced expression of FKBP14 inhibited cellular proliferation, adhesion and invasion. The possible mechanism involved was further investigated. In summary, the present data suggest that FKBP14 is a potential oncogene of gastric cancer and performs an important role in the progression of this disease.

## Materials and methods

*Tissue samples and cell lines.* A total of 40 gastric cancer samples and matched control samples were collected from The People's Hospital of Pudong New Area of Shanghai (Shanghai, China) and stored at -80˚C until RNA extraction. All the specimens were obtained with written informed consent. This study was approved by the Ethics Committee of The People's Hospital of Pudong New Area of Shanghai.

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All cell lines were obtained from the Cell Bank at the Chinese Academy of Sciences (Shanghai, China) and maintained at 37˚C in a 5% humidified $CO_2$ atmosphere. MKN-45, BGC-823, MGC-803, MKN-74, SGC-7901 and AGS were cultured in RPMI-1640, while HEK293T cells were cultured in Dulbecco's modified Eagle's medium. All media (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) were supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.,) and antibiotics.

*RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).* Total RNA of tissue samples was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocol. The RT-qPCR was performed with SYBR Green PCR Master mix (Thermo Fisher Scientific, Inc.) on an ABI 7300 machine (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the following cycling parameters, 10 min at 95˚C, followed by 40 rounds of 15 sec at 95˚C, and 45 sec at 60˚C. GAPDH was used as the internal control. Water was used as a negative control in RT-qPCR. The relative FKBP14 mRNA expression was calculated by the comparative method ($2^{-\Delta\Delta Cq}$) (12). The experiment was performed with triplicate repeats and the experiment was repeated three times. The primers used were as follows: FKBP14 (NM_017946.3) forward, 5'-TGAAGGACAGCACCAATAG-3' and reverse, 5'-GCACATTTACCACCAACTC-3'; and GAPDH (NM_001256799.1) forward, 5'-CACCCACTCCTCCACCTTTG-3' and reverse, 5'-CCACCACCCTGTTGCTGTAG-3'.

*Lentiviral production.* Short hairpin RNA (shRNA) oligos targeting the FKBP14 sequence (5'-GACCACTTTCACTGATTAT-3') and non-silencing sequence (5'-CCTAAGGTTAAGTCGCCCTCG-3') were designed and synthesized by Generay (Shanghai, China). Oligos were annealed and inserted into the PLKO.1-lentiviral vector (Addgene, Inc., Cambridge, MA, USA) and pre-digested with AgeI and EcoRI restriction enzymes, as previously described (13). Lentiviruses were produced by transfection of HEK293T cells according to standard protocol (14). Briefly, HEK293T cells were cotransfected with lentiviral construct and lentiviral packaging vectors by using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Lentiviruses were collected from culture medium 48 h subsequent to transfection.

*Western blotting.* Whole cell lysates were prepared from cultured cells using ice-cold radioimmunoprecipitation assay buffer with protease inhibitor cocktail (Sigma-Aldrich; Merck Millipore, St. Louis, MO, USA). Equal amounts of protein were resolved by 10% or 15% SDS-PAGE and blotted onto polyvinylidene difluoride membranes. The membranes were probed with primary antibodies [FKBP14, (dilution, 1:1,000; #ab105018; Abcam, Cambridge, UK), proliferating cell nuclear antigen (PCNA; dilution, 1:1,000; #ab92552; Abcam), matrix metalloproteinase 2 (MMP2; dilution, 1:1,000; #ab92536; Abcam), Twist (dilution, 1:500; #ab175430; Abcam), Vimentin (dilution, 1:2,000; #ab92547; Abcam), N-cadherin (dilution, 1:1,000; #ab18203; Abcam), Snail (dilution, 1:1,000; #3879s; Cell Signaling Technology Inc., Danvers, MA, USA), β-catenin (dilution, 1:1,000; #8480; Cell Signaling Technology Inc.) and GAPDH (dilution, 1:1,500; #5174; Cell Signaling Technology Inc.)], followed by a corresponding horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse; #A0216; dilution, 1:1,000) and (goat anti-rabbit; #A0208; dilution, 1:1,000); Beyotime Institute of Biotechnology, Shanghai, China). Immunodetection was performed with enhanced chemiluminescence reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in accordance with the manufacturer's protocol. The band intensity was quantified using ImageJ software version 1.6 (National Institutes of Health, Bethesda, MD, USA).

*Cell proliferation assay.* A Cell Counting kit-8 (CCK-8; Signalway Antibody LLC., College Park, MD, USA) assay was performed to monitor cellular proliferation. Briefly, MKN-45 or AGS cells were infected with non-silencing shRNA lentivirus (NC) or FKBP14 shRNA lentivirus (RNAi). At 48 h subsequent to infection, treated and non-treated cells were seeded in 96-well plates (3,000 cells in 100 $\mu$l medium/well). At the indicated time point, cells were treated with 10 $\mu$l CCK-8 solution per well at 37˚C for 1 h. The absorbance was determined at 450 nm with a microplate reader (Bio-Rad Laboratories, Inc.).

*Cell adhesion assay.* The cell adhesion assay was performed in fibronectin-coated 12-well plates. MKN-45 or AGS cells infected with non-silencing shRNA lentivirus or FKBP14 shRNA lentivirus were plated onto plates at a density of $10^5$ cells per well. Cells were allowed to adhere to plates for 1 h at 37˚C. The non-adherent cells were removed by PBS. The remaining cells were fixed with 10% formalin, stained with giemsa solution, and counted in 5 random fields using an IX71 Olympus Inverted microscope (Olympus Corporation, Tokyo, Japan).

*Cell invasion assay.* Invasive ability of cells into Matrigel was determined using 24-well Matrigel Invasion Chambers (8 $\mu$m pore size with polycarbonate membrane; BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol. The Matrigel invasion chambers were rehydrated with serum-free RPMI-1640 (500 $\mu$l/well) at 37˚C for 2 h. MKN-45 or AGS cells infected with non-silencing shRNA lentivirus or FKBP14 shRNA lentivirus were suspended in serum-free medium and plated onto the upper membrane at a density of $5x10^4$ cells/well. Subsequently, the lower compartment was filled with 20% FBS-containing medium. After 24 h, cells on the upper surface of membrane were removed with a cotton swab. The invasive cells were fixed, stained with 0.5% crystal violet staining solution, and then counted in 5 random fields using an IX71 Olympus Inverted microscope.

*Statistical analysis.* Data were presented as the mean ± standard deviation of 3 independent experiments and compared by Student's *t*-test. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*FKBP14 mRNA expression is significantly increased in gastric cancer tissues.* The present study explored FKBP14 expression in 40 pairs of gastric cancer tissues utilizing qPCR. The level

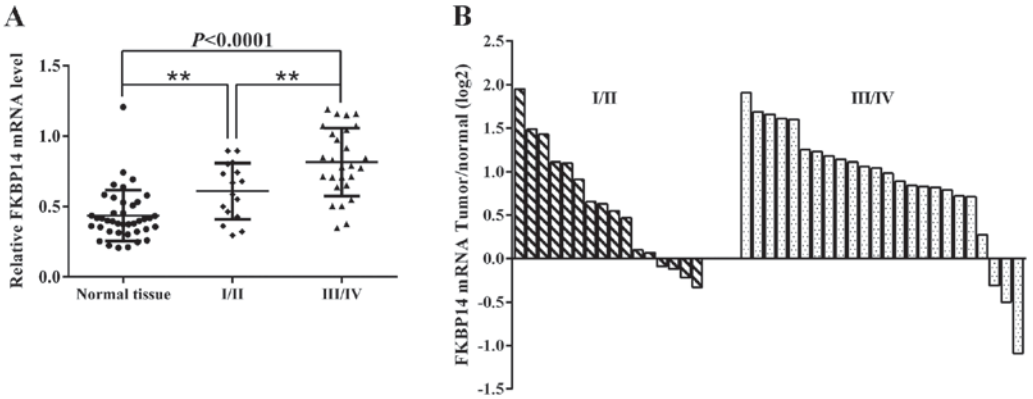


Figure 1. Analysis of FKBP14 expression in human gastric cancer. (A) FKBP14 mRNA levels in 40 tumor tissues (Stage I/II, 16 cases and Stage III/IV, 24 cases) and matched normal tissues by quantitative polymerase chain reaction. FKBP14 mRNA was normalized with GAPDH expression. **P<0.01. (B) The log2 ratio of FKBP14 expression in tumor tissues and normal tissues was calculated. The striped bars refer to tissues classified as Stage I/II and the dotted bars refer to tissues classified as Stage III/IV. FKBP, FK506 binding protein.

of FKBP14 expression was significantly increased in gastric cancer tissues compared with the normal tissues (Fig. 1A; Stage I/II, P=0.0028; Stage III/IV, P<0.0001). In addition, FKBP14 expression was higher in tumors of Stage III/IV (n=24) than Stage I/II (n=16; P=0.0063). FKBP14 expression was then expressed as the log2 ratio of tumor samples over normal samples. Positive log2 indicated an increased expression of FKBP14 in tumor samples and a sample with negative log2 suggested a decreased expression of FKBP14 in tumor samples as compared with the matched normal samples. As presented in Fig. 1B, FKBP14 expression was higher in 33 gastric cancer tissues compared with in their pair-matched adjacent normal tissues (Stage I/II, 12 cases, P=0.0019; Stage III/IV, 21 cases, P<0.0001).

*Knockdown of FKBP14 in gastric cancer cells*. The fact that FKBP14 is frequently upregulated in gastric cancer tissues prompted the present study to investigate the role of FKBP14 in gastric cancer cells by knocking down its expression. Firstly, the FKBP14 protein level (Fig. 2A) in 6 gastric cancer cell lines was examined. The MKN-45 and AGS cell lines exhibited higher FKBP14 protein expression levels, which were selected for the following RNAi-mediated knockdown assays. The present study then produced FKBP14 shRNA lentivirus (RNAi) and non-silencing shRNA lentivirus (NC) to infect MKN-45 and AGS cells (Fig. 2B). FKBP14 shRNA lentivirus could effectively suppress the expression of FKBP14 with a knockdown ratio of 70% in the two cell lines (MKN-45, P=0.0024; AGS, P=0.0011), whereas no evident difference was observed between NC and non-treated cells (WT).

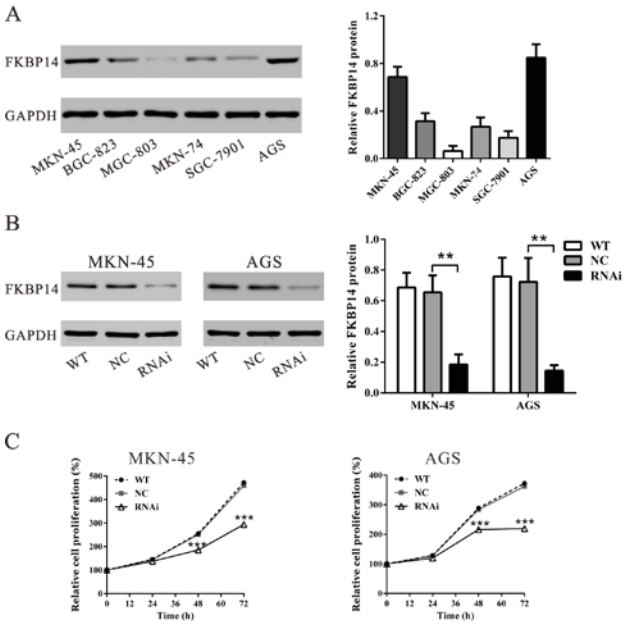


Figure 2. FKBP14 expression in cell lines and its knockdown inhibits cancer cell proliferation. (A) FKBP14 protein levels in 6 gastric cancer cell lines were analyzed using Western blot analysis. The band intensity was quantified and FKBP14 protein level was normalized using GAPDH expression. (B) Western blot analysis of FKBP14 expression in MKN-45 and AGS cell lines infected with indicated lentivirus. (C) Effects of FKBP14 knockdown on proliferation was evaluated by Cell Counting Kit-8 assays. Results of cell proliferation were normalized to the initial cell numbers (100%). **P<0.01, ***P<0.001 compared with NC. FKBP, FK506 binding protein; WT, non-treated cells; NC, cells infected with non-silencing shRNA lentivirus; RNAi, cells infected with FKBP14 shRNA lentivirus.

*Downregulation of FKBP14 expression inhibited the proliferation of gastric cancer cells*. Following 48 h of lentiviral infection, cell growth was detected by CCK-8 assays at 24 , 48 and 72 h (Fig. 2C). Results of cell growth were normalized to the initial cell numbers (100%). The present study found that FKBP14 shRNA lentivirus (RNAi)-infected cells experienced a significant inhibition of cell viability compared with the control groups (WT and NC) at 48 and 72 h (MKN-45; 48 h, P=0.00049; 72 h, P=0.00013; AGS; 48 h, P=0.00048; 72 h, P=0.00017).

*Knockdown of FKBP14 inhibited the adherent and invasive ability of gastric cancer cells*. Metastasis is an important characteristic of gastric cancer. Cellular adhesion and invasion are essential for tumor metastasis (15). The present study then evaluated the effects of FKBP14 on the cell adherent and invasive ability of MKN-45 and AGS cells by cell adhesion assay (Fig. 3A) and Matrigel assay (Fig. 3B).

The adherent ability of tumor cells to the extracellular matrix is a prerequisite for cell invasion into the basement membrane. The present study observed that the number of

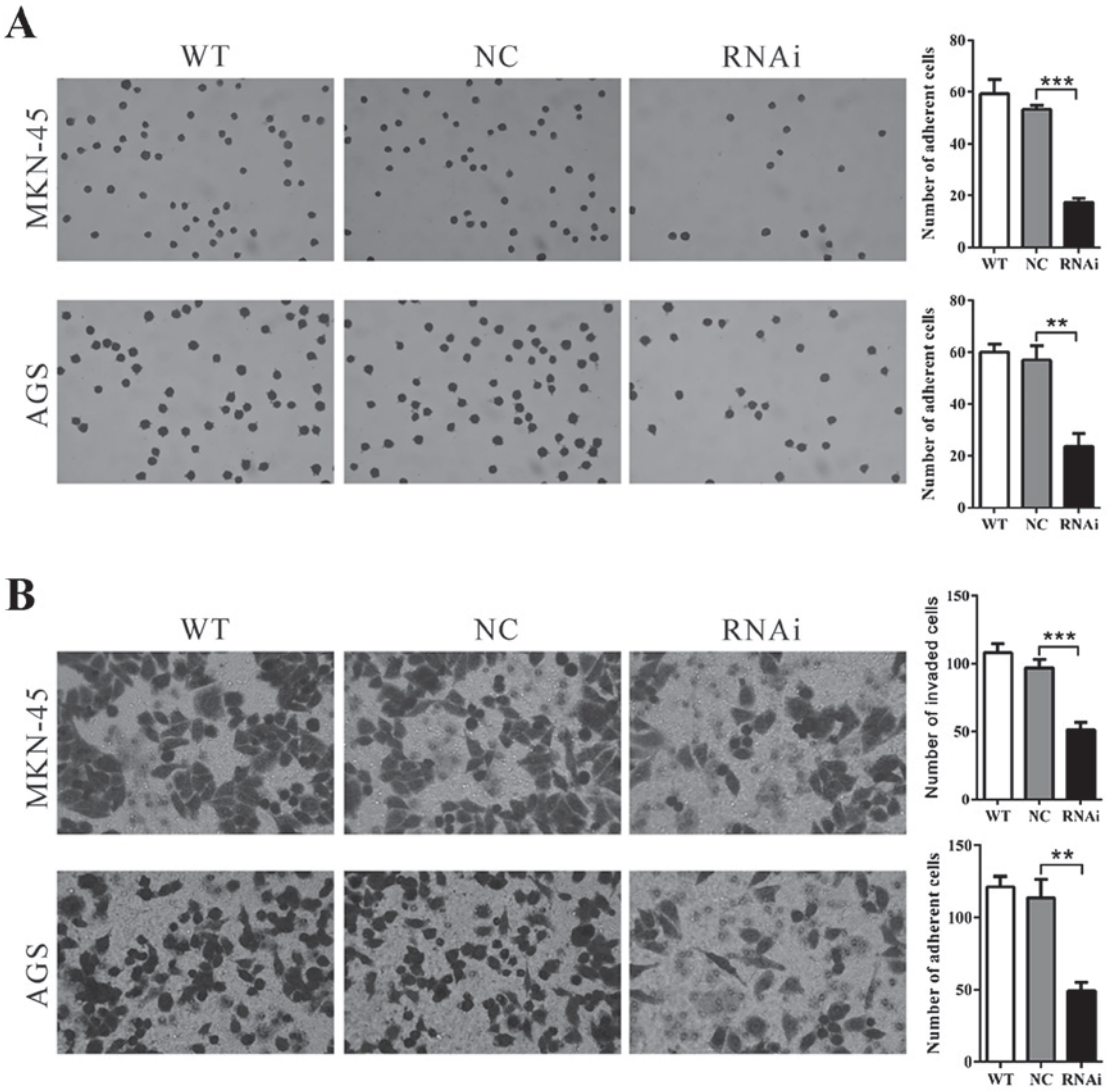


Figure 3. Effect of FKBP14 knockdown on gastric cancer cell adhesion and invasion *in vitro*. (A) Cell adhesion assays were carried out on 3 groups of cells in 12-well plates. The adherent cells were counted in 5 random fields (magnification, x200). (B) Cells of 3 groups were harvested and resuspended in serum-free culture medium and allowed to migrate toward the Matrigel-coated chamber for 24 h. Invading cells were stained with 0.5% crystal violet and counted in 5 random fields (magnification, x200). Each experiment was repeated thrice independently. $^{**}$P<0.01, $^{***}$P<0.001 compared with NC. FKBP, FK506 binding protein; WT, non-treated cells; NC, cells infected with non-silencing shRNA lentivirus; RNAi, cells infected with FKBP14 shRNA lentivirus.

cells that adhered to fibronectin was significantly decreased by FKBP14 knockdown (Fig. 3A; MKN-45, P=0.0008; AGS, P=0.0015).

Matrigel assay revealed a significant decrease in the number of cells that penetrated through the Matrigel-coated membrane with FKBP14 knockdown, suggesting impaired invasion ability of MKN-45 and AGS cells (Fig. 3B; MKN-45, P=0.0006; AGS, P=0.0015). These data suggested that FKBP14 promoted the invasion of gastric cancer cells.

*Effect of FKBP14 knockdown on the expression of a proliferation marker and invasion-related proteins in gastric cancer cells*. The present study investigated the proliferation marker (PCNA) and invasion-related protein (MMP2, β-catenin, Snail and Twist) expression levels in infected and uninfected cells by western blotting (Fig. 4). Knockdown of FKBP14 markedly reduced the expression of PCNA (MKN-45, P=0.0009; AGS, P=0.0031), MMP2 (MKN-45, P=0.0005; AGS, P=0.0008) and epithelial-mesenchymal-transition (EMT) marker [β-catenin (MKN-45, P=0.0001; AGS, P=0.0148), Snail1 (MKN-45, P=0.0001; AGS, P=0.0031), Twist (MKN-45, P=0.0005; AGS, P=0.0191), Vimentin (MKN-45, P=0.0062; AGS, P=0.0063) and N-cadherin (MKN-45, P=0.0017; AGS, P=0.0096)].

## Discussion

FKBP14 is an important regulator for the development of *Drosophila* (16) and its mutation is associated with a variant of Ehlers-Danlos syndrome (17,18). FKBP14 belongs to the FKBP family, which perform important biological functions in the cell. Several members of the FKBP family are established to contribute to carcinogenesis, including FKBP12 (5,6), FKBP65 (7) and FKBP52 (8-10), while other FKBPs are downregulated in human cancer, including FKBP38 (11). However, the functions of FKBP14 in human cancer remain to be elucidated. The present study highlights the significance of FKBP14 as an oncogene in gastric cancer.

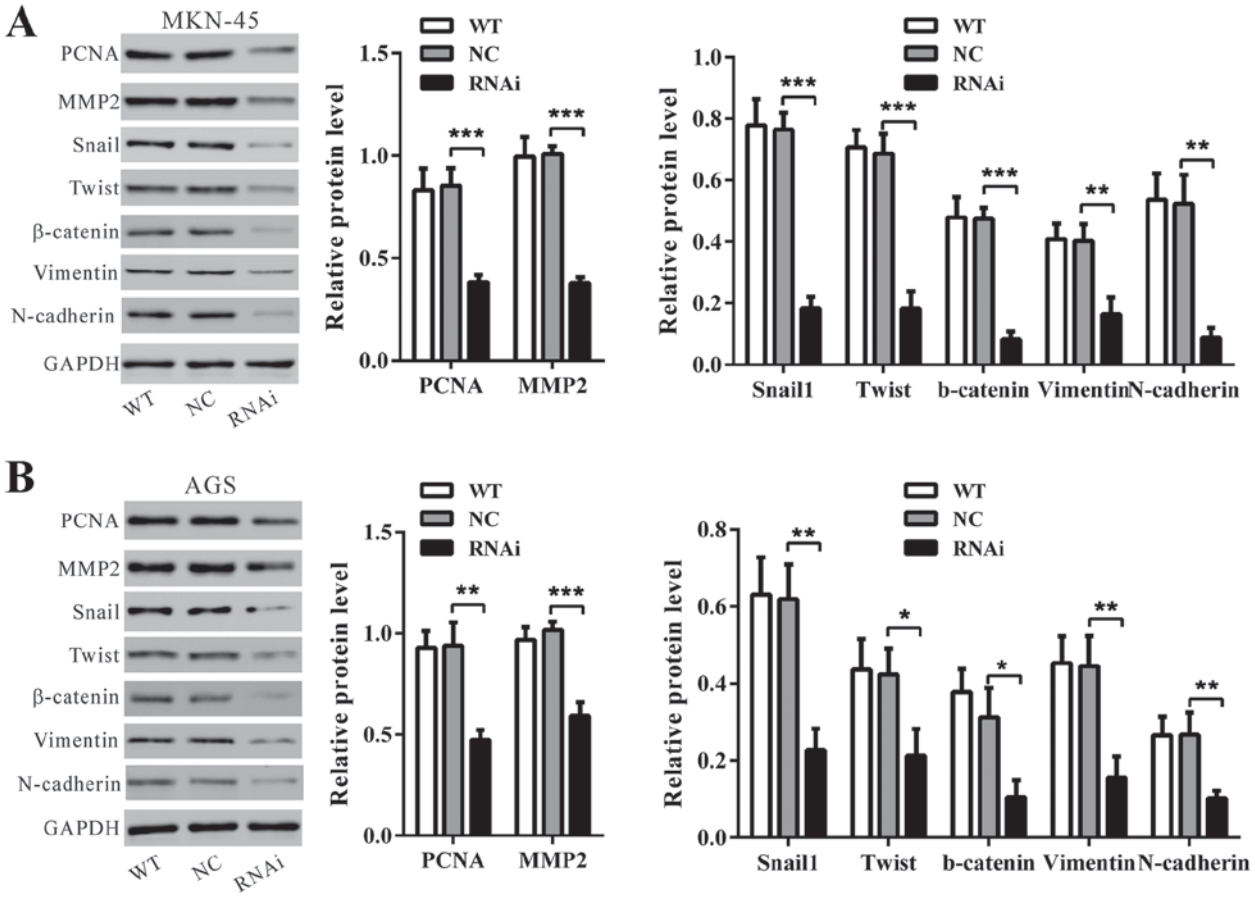


Figure 4. Effect of FKBP14 shRNA on the protein expressions of PCNA, MMP2, Snail1, Twist, β-catenin, Vimentin and N-cadherin was evaluated by western blotting. Representative western blotting and quantitative analysis of (A) MKN-45 and (B) AGS cell lines based on 3 independent experiments. $^{*}$P<0.05, $^{**}$P<0.01, $^{***}$P<0.001 compared with NC. FKBP, FK506 binding protein; shRNA, small hairpin RNA; PCNA, proliferating cell nuclear antigen; MMP2, matrix metalloproteinase 2; WT, non-treated cells; NC, non-silencing shRNA lentivirus; RNAi, FKBP14 shRNA lentivirus.

The present study detected FKBP14 mRNA expression in gastric cancer and normal tissues (Fig. 1). The results demonstrated that FKBP14 mRNA was significantly elevated in gastric cancer tissues and may serve as a useful diagnostic marker for gastric cancer. In order to investigate the functions of FKBP14 on gastric cancer, the expression of FKBP14 was knocked down in 2 gastric cancer cells with high expression of FKBP14 by lentiviral shRNA. Uncontrolled cell proliferation is the characteristic feature of cancer. In the present study, FKBP14 shRNA exerted its inhibitory effects on the proliferation of gastric cancer cells manifested by CCK-8 assay (Fig. 2) and detection of PCNA expression (Fig. 4). These data demonstrate the association of FKBP14 with gastric carcinogenesis.

High incidence of metastasis remains the main cause of mortality in patients with gastric cancer (19). Cell invasion is an important feature associated with tumor metastasis. Cancer cells that enter the circulation are capable of establishing new tumors in the body using cell adhesion mechanisms (20,21). Data in the present study demonstrated that silencing of FKBP14 significantly suppressed the adhesion and invasion of gastric cancer cells (Fig. 3). These data demonstrated the association of FKBP14 with gastric cancer metastasis. EMT process is involved in cancer invasion, metastasis and therapeutic resistance (22-24). FKBP14 shRNA treatment decreased the expression of the 3 known inducers of EMT, β-catenin, Snail1 and Twist (25) and the EMT markers Vimentin and N-cadherin (26). The present data suggested that FKBP14 shRNA may decrease gastric cancer cell invasion through inhibiting EMT.

In summary, the present study has identified that FKBP14 expression was substantially increased in gastric cancer tissues compared with normal tissues. In addition, the present study has demonstrated that the RNAi mediated targeting of FKBP14 suppressed the proliferation, adhesion and invasion of gastric cancer cells. Furthermore, FKBP14 shRNA may decrease gastric cancer cell invasion through inhibiting EMT. The present data indicated that FKBP14 may serve as an oncogene in the development and metastasis of gastric cancer. Therefore, FKBP14 may be a therapeutic target for the treatment of gastric cancer.

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