SPANDIDOS PUBLICATIONS

ONCOLOGY REPORTS 27: 1353-1359, 2012

The TPX2 gene is a promising diagnostic and therapeutic target for cervical cancer

HAIPING CHANG, JINGZHI WANG, YUAN TIAN, JIE XU, XIAOJUAN GOU and JIANXIN CHENG

Department of Obstetrics and Gynecology, The Fourth Hospital of Hebei Medical University, Shijiazhuang, Hebei 050011, P.R. China

Received November 2, 2011; Accepted January 12, 2012

DOI: 10.3892/or.2012.1668

Abstract. The target protein for Xklp2 (TPX2), a microtubule-associated protein, can be used to evaluate more precisely the proliferative behavior of tumor cells. The abnormal expression of TPX2 in various types of malignant tumors has been reported, but less is known for cervical cancer. We studied the relationship between TPX2 expression and the biological behavior of cervical cancer. Immunohistochemistry and RT-PCR were used to detect the expression of TPX2 in cervical cancer tissues. The inhibitory effect of TPX2-siRNA on the growth of HeLa human cervical carcinoma cells was studied *in vitro*. TPX2 expression was found to be significantly higher in cervical carcinoma compared to normal cervical tissues and CIN. The expression of TPX2 in cervical cancer was correlated with histological grading, FIGO staging and lymph node metastasis. TPX2 RNAi in HeLa cervical cancer cells caused S-phase cell cycle arrest, induced apoptosis and inhibited cell proliferation and invasion. In conclusion, TPX2 shows potential to be used as a new marker for cervical cancer diagnosis and therapy.

Introduction

TPX2 (targeting protein for Xenopus kinesin-like protein 2), also known as REPP86 (restrictedly expressed proliferation-associated protein), is a microtubule-associated protein, which plays an important role in the formation of mitotic spindle. Its expression is controlled strictly by cell cycle, which appears between G1-S stage and vanishes after the completion of cytokinesis (1). Therefore, TPX2 can evaluate more precisely the proliferative behavior of tumor cells. In recent years, it has been discovered that TPX2 was overexpressed in certain malignant tumors, which resulted in abnormal centrosome amplification, aneuploidy formation and malignant transformation of cells, promoting proliferation, involved in cell cycle and apoptosis regulation, associated with tumor differentiation and metastasis and recurrence. Blocking its expression could inhibit tumor cell growth and might become a candidate target for tumor treatment (2-5). The overexpression of TPX2 has been found in saliva gland cancer (3), lung squamous cell carcinoma (4) and breast cancer (5), however, little information exists on its expression in cervical cancer until now. Cervical cancer is one of common gynecologic malignancies. Its occurrence, development and outcome are correlated with many factors, whether TPX2 is involved has not been reported yet. In this study, we analyzed the expression of TPX2 in cervical cancer and the correlation between its expression with clinical stages, tumor differentiation and lymph node metastasis. In order to provide new experimental bases for the diagnosis and treatment of cervical cancer, we studied the inhibitory effect of TPX2-siRNA on growth of human cervical carcinoma HeLa cells *in vitro*.

Materials and methods

Specimen preparation. Complete data of cervical cancer resection specimens of 62 patients were collected at The Fourth Hospital of Hebei Medical University from October 2009 to October 2010, and all the specimens identified by pathologist were collected before radiotherapy and chemotherapy. All the fresh cervical tissues were put into liquid nitrogen immediately after taking the specimens and stored at -80˚C. The cervical cancer patients' age was between 30-72 (average 49.5±3.10) years. According to the clinical FIGO stage by International Federation of Obstetrics and Gynecology, there were 34 cases of stage I and 28 cases of stage II. Histological grade: I in 26 cases, II in 17 cases, III in 19 cases. Lymph node metastasis group had 9 cases, non-lymph node metastasis group had 53 cases. Thirty cases in cervical intraepithelial neoplasia (CIN) groups, and the paraffin blocks were taken from the Department of Pathology Archive, the patients' age was between 34-62 (mean 49±3.88) years. The normal control group had 15 specimens obtained from the tissues of total hysterectomy due to myoma of the uterus, the patients' age range was 34-65 (mean 49±6.37) years. All the tissue samples were obtained with informed consent and the experi-

Correspondence to: Professor Jianxin Cheng, Department of Obstetrics and Gynecology, Eastern Hospital of The Fourth Hospital of Hebei Medical University, 169 Tianshan Street, High-Tech Development Zone, Shijiazhuang, Hebei 050035, P.R. China
E-mail: jianxin_cheng@263.net

Key words: cervical cancer, targeting protein for Xenopus kinesin-like protein 2, siRNA, diagnosis, treatment

mental procedures in this study were reviewed and approved by the Ethics Committee of The Fourth Hospital of Hebei Medical University.

RT-PCR analysis. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturers' instructions, and the purity and concentration of RNA were determined. Total RNA (1-2 μg) was reverse transcribed according to reverse transcription kit manual (Fermentas Company). Primers were based on sequences reported on Genebank (NM-012122). TPX2 sense sequence was 5'-AACA ATCCATTCCGTCAA-3' and TPX2 anti-sense sequence 5'-TGCAGGTGGCATACAAGG-3'. The expected product size of TPX2 cDNA was 332 bp. GAPDH sense sequence was 5'-ACCTGACCTGCCGTCTAGAA-3' and GAPDH anti-sense sequence 5'-TCCACCACCCTGTTGCTGTA-3'. The expected product size of GAPDH cDNA was 247 bp. PCR amplification was performed in 25 μl reaction volumes containing 0.2 μM dNTPs, 20 pmol of each oligonucleotide primer, and 0.2U Tag polymerase in PCR buffer. cDNA was amplified on a PCR thermal controller with an initial denaturation at 95˚C for 3 min, and then 30 cycles of denaturation for 30 sec at 95˚C, annealing for 30 sec at 56˚C, and extension for 40 sec at 72˚C. A final elongation step was carried out at 72˚C for 6 min. The amount of starting cDNA was adjusted using GAPDH intensity.

Immunohistochemical detection and result evaluation. The immunohistochemical SP method was used. Experimental operation was performed according to the SP kit manual (Beijing 4A Biotech Co., Ltd.). The sections were conventionally dewaxed, then repaired in pressure cooker at 130˚C for 13 min, adding 3% H_2O_2 for 15 min to eliminate endogenous peroxidase, next normal goat serum was used at 37˚C for 30 min, adding TPX2 antibody (1:75), (Bioward company) at 4˚C overnight, 37˚C for 30 min, adding the second antibody, incubated at 37˚C for 30 min, PBS was used to wash for 5 min 3 times, adding the third antibody, incubated at 37˚C for 30 min, then DAB and haematoxylin dyes were used, and the sections were sealed. Comprehensive assessment was in accordance with stain-positive cells and dye intensity. The positive expression of TPX2 was located in the nucleus. The 5 high power fields were randomly selected, counting 100 cells in every field of vision and counting the percentage of positive cells. No staining was 0 point; weak staining (light yellow) 1 point; moderate staining (yellow-brown) 2 points; strong staining (brown) 3 points. The positive rate of cells between 0-5% was 1 point, 5-25% 2 points, 25-50% 3 points, >50% 3 points. The two items mentioned above were added together as follows: 0 point was negative (-), 1-2 (+), 3-4 (++), 5-6 (+++).

Cell culture. Human cervical carcinoma HeLa cell line was obtained from The Fourth Hospital Research Center of Hebei Medical University. Cells were grown in RMPI-1640 (Gibco™) medium supplemented with 10% fetal bovine serum, penicillin G (100 U/ml), and streptomycin (100 μg/ml) termed complete medium. Cells were maintained in monolayer culture at 37˚C in humidified air with 5% CO_2.

RNAi and transfection. Human TPX2 siRNA and negative control siRNA were purchased from GenePharma RNAi Company (Shanghai, China). Non-transfected cells were used as blank control group. The sequences of TPX2-siRNA (GenBank accession no. NM-012122) duplex are: sense strand, 5'-GCUCG AGAAAUUGCAACAATT-3; antisense strand, 5'-UUGUUGC AAUUUCUCGAGCTT-3'. The sequences of negative control siRNA duplex are: sense strand, 5'-UUCUCCGAACGUGUC ACGUTT-3'; antisense strand, 5'-ACGUGACACGUUCG GAGAATT-3', which bear no homology with relevant human genes. Both siRNAs were chemically modified by 2' Ome. HeLa cells were seeded in 6-well culture plates at a density of 10^5 cells. After incubation overnight, cells were transfected with TPX2-siRNA (2 μg/well) or the negative siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Western blot analysis. After transfection for 48 h, total protein was extracted in RIPA buffer (Beyotime, Shanghai, China) with protease inhibitors (PMSF). Protein concentrations were measured using the BCA-100 Protein Quantitative Analysis kit (Shenergy Biocolor, Shanghai, China). Equal amounts of total proteins were separated in 10% sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in 5% nonfat dry milk in Tris-buffered saline Tween-20 (TBST) for 2 h at room temperature and incubated with rabbit anti-TPX2 antibodies (1:500, Bioworld Technology, Inc., USA) overnight at 4˚C. After three washes with TBST, membranes were incubated with anti-rabbits IgG-horseradish-peroxidase for 1 h at room temperature. Enhanced chemiluminescence detection reagent was used, and the protein bands were visualized after exposure to X-ray film. Anti-β-actin expression was used as the housekeeping gene control. Image analyses were performed with Quantity One Software.

MTT assay. The effect of TPX2-siRNA on the viability of cells was determined by the MTT assay. Briefly, HeLa cells were plated at $1x10^4$ cells per well in 96-well microtitre plates. After incubation for 24, 48, 72, 96 h, cell viability was determined. MTT (10 μl) with 5 mg/ml concentration was added to each well and cultured at 37˚C for 4 h. Then, the medium was discarded and 100 μl DMSO was added to each well and shaken carefully for 10 min. The absorbance of each well was recorded with a microplate reader (model 550, Bio-Rad, Richmond, CA) at a 570-nm wavelength.

Cell cycle analysis. For flow cytometric cell cycle analysis, at 48 h after transfection, $2x10^6$ HeLa cells of each group were harvested by trypsinization, washed twice with PBS, and fixed with cold 70% ethanol at 4˚C and stored at -20˚C until staining. The cells were then washed once with PBS, digested by 200 μl RNase (1 mg/ml) at 37˚C for 30 min, and stained with 800 μl propidium iodide (50 μg/ml) at room temperature for 30 min. Cell cycle was analyzed by flow cytometry.

Cell apoptosis analysis. Apoptotic cells in early and late stages were detected using an annexin V-FITC Apoptosis Detection kit from BioVision (Mountain View, CA, USA). In brief, the cells were transfected with siRNA. At 48 h after transfection,

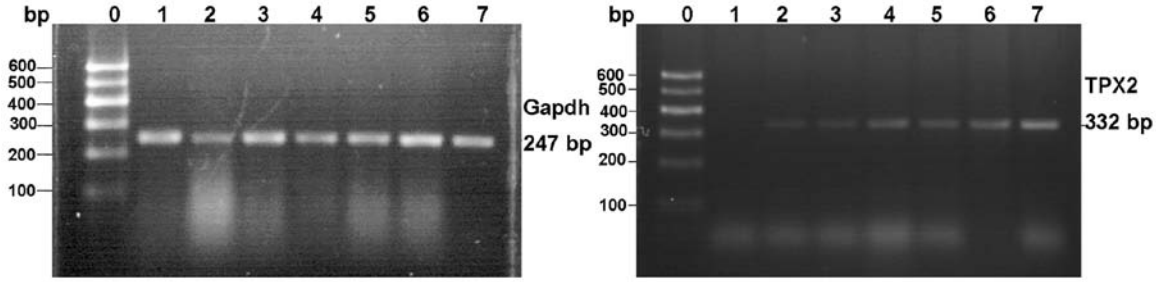

Figure 1. The expression of TPX2 mRNA in normal cervical tissue and cervical squamous cell carcinoma. 0: marker. From the bottom: 100, 200, 300, 400, 500, and 600 bp. Lane 1, normal cervical tissues. Lane 2-7, cervical squamous cell carcinoma. The expected product size of TPX2 cDNA was 332 bp. GAPDH mRNA was used as control for internal mRNA loading. The expected product size of GAPDH cDNA was 247 bp.

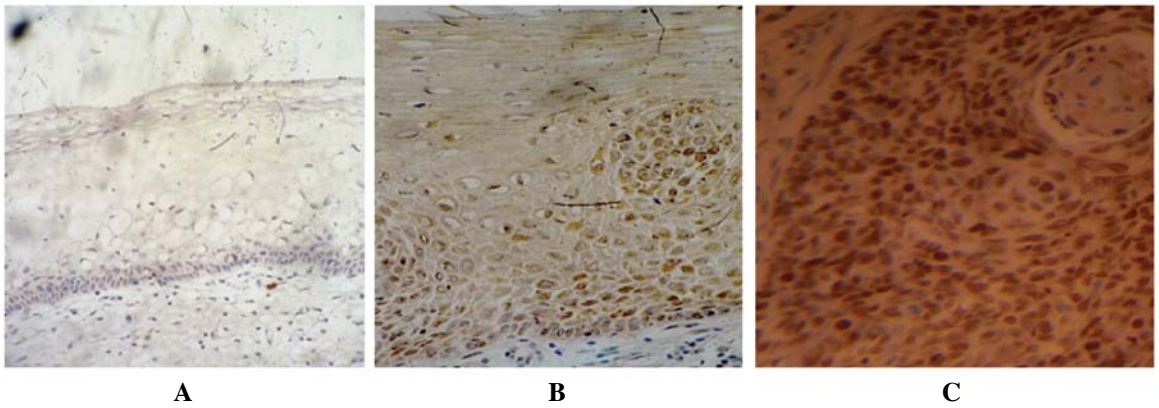

Figure 2. Evaluation of TPX2 expression in cervical squamous epithelial tissues. (A) No epithelial cell staining in normal cervical squamous epithelium, (original magnification, x400). (B) Moderate epithelial cell staining in cervical intraepithelial neoplasia (original magnification, x400). (C) Strong epithelial cell staining in cervical squamous cell carcinoma (original magnification, x400).

culture media and cells were collected and centrifuged. After washing, cells were resuspended in 490 μl annexin V binding buffer, followed by the addition of 5 μl annexin V-FITC and 5 μl propidium iodide. The samples were incubated in the dark for 5 min at room temperature and analyzed using flow cytometry.

Cell invasion assay. The cell invasion assay was carried out using modified Boyden chambers consisting of transwell-precoated matrigel membrane filter inserts with 8-μm pores in 24-well tissue culture plates (BD Biosciences, San Diego, CA, USA). After transfection for 48 h, The cells TPX2 siRNA transfected or control transfected cells were then seeded at $5x10^4$ cells/well in the upper transwell chambers of inserts precoated with matrigel in serum and growth factor-free medium. The bottoms of the chambers were filled with 500 μl medium containing 10% FBS. The culture plate were incubated overnight at 37˚C in a humidified atmosphere containing 5% CO_2. Remaning cells on the upper surface were mechanically removed. Membranes were then washed, fixed, and stained by hexamethylpararosaniline. The number of cells that migrated to the lower surface of the filters was determined by counting stained cells under a light microscope in three random fields.

Statistical analysis. SPSS13.0 statistical software was used for statistical analyses. Statistical data are presented as mean ± standard deviation. Student test was used to compare two samples, and the single-factor analysis of variance (One-way ANOVA) was used to compare multiple samples. Kruskal-Wallis test for group comparisons, as well as the Mann-Whitney U test for nonparametric independent two-group comparisons were performed. A P-value of <0.05 was considered as statistically significant.

Results

Expression of TPX2 in the cervical tissue detected by RT-PCR. In the normal cervical tissue, TPX2 mRNA expression was almost absent, relative expression quantity was 0.08±0.03, but it was highly expressed in cervical cancer, the relative expression quantity was 0.441±0.011, significant difference ($t=19.568$, $P<0.01$) (Fig. 1).

Detection of TPX2 protein in different tissues of the cervix by immunohistochemistry. The expression of TPX2 protein was localized in the cell nucleus. The expression of TPX2 protein in CIN and cervical carcinoma tissues gradually increased, however, no expression in normal cervix tissue was found. There were significant differences among the three groups ($P<0.01$) (Fig. 2 and Table I).

The relationship between the expression of TPX2 protein in cervical squamous carcinoma cells with the clinical and

Table I. TPX2 protein expression in cervical squamous epithelial tissues.

Group	Cases	TPX2			
		-	+	++	+++
Normal cervical squamous epithelia	15	15[a]	0[a]	0[a]	0[a]
Cervical intraepithelial neoplasia	30	10[b]	11[b]	7[b]	2[b]
Cervical squamous carcinoma	62	5[c]	11[c]	27[c]	19[c]

[a]Z=-4.012, P<0.01, compared with cervical intraepithelial neoplasia. [b]Z=-4.151, P<0.05, compared with cervical squamous carcinoma. [c]Z=-5.728, P<0.01, compared with normal cervical epithelia.

Table II. Correlation between TPX2 expression and the clinicopathological factors of cervical squamous cell carcinoma.

Item	Cases	TPX2			
		-	+	++	+++
Age (years)					
≤40	26	2[d]	5[d]	11[d]	8[d]
>40	36	3	6	16	11
FIGO stage					
I	34	3[a]	7[a]	19[a]	5[a]
II	28	2	4	8	14
Lymph node metastasis					
Negative	53	5[b]	10[b]	25[b]	13[b]
Positive	9	0	1	2	6
Pathologic grade					
I	26	3[c]	6[c]	13[c]	4[c]
II+III	36	2	5	14	15

[a]Z=-2.258, P<0.05, compared with FIGO stage II. [b]Z=-2.244, P<0.05, compared with lymph node positive group. [c]Z=-2.156, P<0.05, compared with pathologic grade II+III. [d]Z=-0.061, P>0.05, compared with age (years) >40.

pathological parameters. The expression intensity of TPX2 had no correlation with patients' age (P>0.05). The expression intensity of TPX2 in clinical stage II was significantly stronger than that in stage I (P<0.05). The expression levels of TPX2 in lymph node metastasis group were significantly higher than those in non-lymph node metastasis group (P<0.05). Pathological grade II and III were significantly higher than those in grade I (P<0.05) (Table II).

TPX2-siRNA reduces TPX2 mRNA and protein levels in HeLa cells. The ability of siRNA to reduce TPX2 mRNA and protein expression was analyzed using RT-PCR and western blot analysis, respectively. Expression of TPX2 mRNA in HeLa cells transfected with TPX2-siRNA was reduced (82.5±0.43%) compared with blank control groups (P<0.01) (Fig. 3A). TPX2 protein levels were lower (63.4±1.05%) in HeLa cells transfected with TPX2-siRNA compared with blank control groups (P<0.05), whereas negative control groups did not affect TPX2 protein levels (Fig. 3B).

MTT essay. Cells in three groups were harvested at 24, 48, 72, and 96 h after transfection. The proliferation rate of HeLa cells was significantly lower in the TPX2-siRNA group than that in the blank control group and negative siRNA control group (both, P<0.01). There was no statistical difference between blank control groups and negative control groups (both, P>0.05) (Fig. 4). These results suggest that down-regulation of TPX2 significantly inhibits the proliferation of HeLa cells.

Downregulation of TPX2 affects cell cycle distribution in HeLa cells. The effect of TPX2-siRNA on cell cycle was evaluated by flow cytometry assay (Fig. 5). Three groups of cells were collected for cell cycle analysis at 48 h after transfection. The percentages of S-phase and G2-phase cells in TPX2 siRNA transfected group was higher than that in blank control group and negative siRNA control group (both, P<0.01). Therefore, TPX2 silencing may arrest the cell cycle at the S phase and G2 phase in HeLa cells.

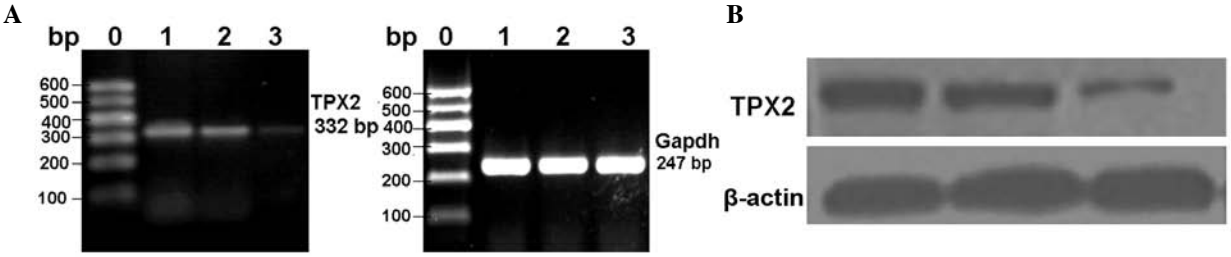

Figure 3. (A) The result of RT-PCR analysis after transfection with TPX2-siRNA. 0, marker. From the bottom: 100, 200, 300, 400, 500, and 600 bp. Lane 1, blank control group. Lane 2, negative siRNA control group. Lane 3, TPX2-siRNA group. GAPDH mRNA was presented as controls for internal mRNA loading. (B) The result of western blot analysis after transfection with TPX2-siRNA. Lane 1, blank control group. Lane 2, negative siRNA control group. Lane 3, TPX2-siRNA group. β-actin protein was presented as controls for internal protein loading.

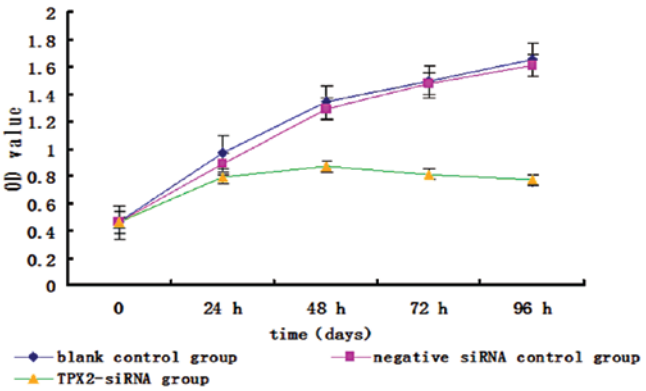

Figure 4. The growth capacity of HeLa cells lines was detected by MTT.

TPX2 silencing induces apoptosis in HeLa cells. Three groups of cells were harvested at 48 h after transfection and the apoptosis rate was analyzed using flow cytometry (Fig. 6). The apoptosis rate of TPX2-siRNA groups (13.183±1.170%) was significantly higher than that in blank control groups (2.707±0.573%) ($t=13.927$, $P<0.01$) and negative control groups (3.447±0.7252%) ($t=12.252$, $P<0.01$). There was no statistical difference between blank control groups and negative control groups ($t=1.388$, $P>0.05$). These results indicate that TPX2-siRNA induces cell apoptosis in HeLa cells.

Invasion assay. The invasive potential of HeLa cells was determined by using a Matrigel invasion assay (Fig. 7). Cells transfected with TPX2-siRNA showed decreased migration (13.33±1.53) through the Matrigel compared with the blank control group (54.33±2.52) and negative siRNA control cells (53.00±4.00) ($P<0.05$). In addition, no difference between the blank control group and negative siRNA control cells was observed ($P>0.05$). These results suggest that downregulation of TPX2 significantly inhibits the invasive capacity of HeLa cells.

Discussion

TPX2 is a microtubule-associated protein downstream of Ran-GTP that plays a central role in mitotic spindle formation and therefore proper segregation of chromosomes during cell division (6). Throughout interphase TPX2 is sequestered in a cell's nucleus by interaction with the nuclear pore proteins importin α/β, but is released at the early stages of mitosis in a RanGTP-dependent manner (7). During mitosis, TPX2 is able to interact with downstream partners, which includes the Aurora A kinase resulting in the localization of Aurora A to the microtubules of the mitotic spindle (1). Furthermore, TPX2 activates the kinase activity of Aurora A by locking it in an active conformation (8). Therefore, TPX2 exerts two levels of regulation on Aurora A kinase signaling (localization and

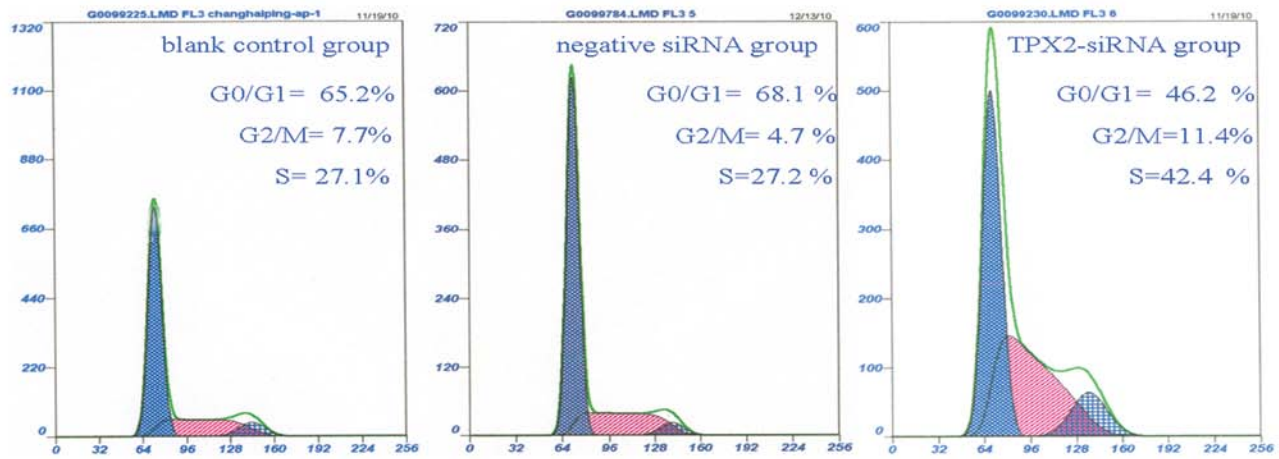

Figure 5. Cell cycle distrbution of the HeLa cell line was detected by FCM.

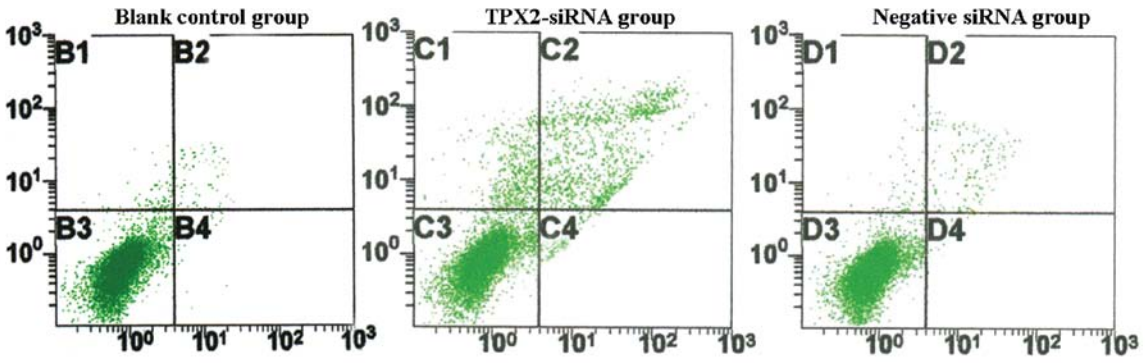

Figure 6. The apoptosis rate of HeLa cells was detected by using flow cytometry.

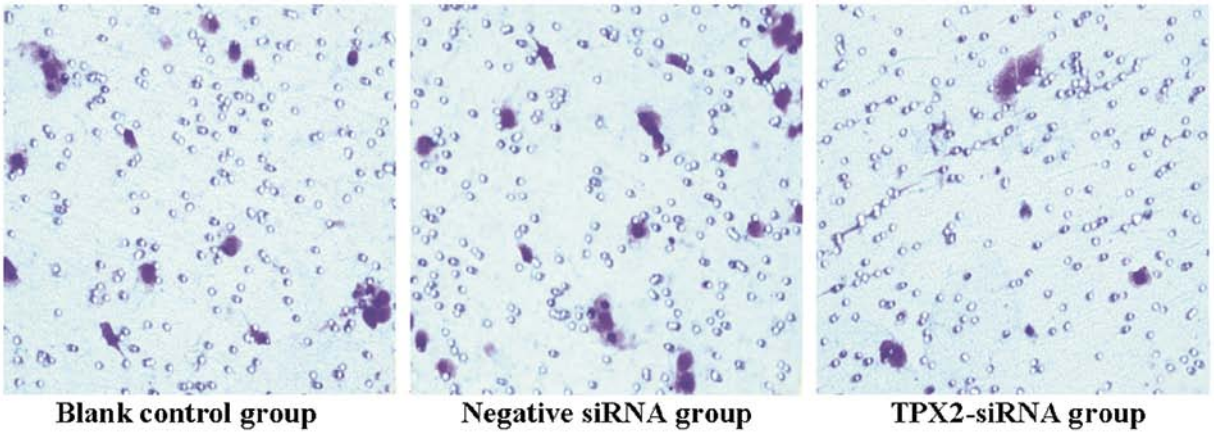

Figure 7. The invasive capacity of HeLa cells was detected by Matrigel invasion assay.

enzymatic activity). TPX2 is identified as a candidate oncogene amplified from chromosome 20q11.2, which appears in the cell cycle of G1-S phase and disappears after the completion of cytokinesis (9). Its expression has been associated with highly proliferative tissues. TPX2 can promote tumor cell proliferation, and is involved in cell cycle and apoptosis regulation (2). The suppression of TPX2 expression by RNA intervention technique can cause the defects in microtubule organization during mitosis, as a result the formation of microtubule asters of spindle can not be formed (6). TPX2 was found to be upregulated in squamous cell carcinoma of the lung with the expression correlating to tumor grade, stage and nodal status (4). The high-expression of TPX2 has been demonstrated in other cancers such as ovarian cancer (10) breast cancer (5) pancreatic cancer (11), meningioma (12), astrocytoma (13), mantle cell lymphoma (14), hepatocellular carcinoma (15), oral squamous cellcarcinomas (16), and giant-cell tumor of the bone (17). The high-expression of TPX2 has predictive value for the biological behavior of the disease, and can be used as a prognostic indicator.

In the present study, we showed that TPX2 is expressed at high levels in cervical cancer and that in some cases amplification of the TPX2 locus might be responsible for the increased expression. Immunohistochemical staining of the cervical tissue showed that TPX2 was highly and extensively expressed in cervical squamous cancer tissues. It was weakly expressed in CIN tissues, not expressed in normal cervical tissues. The data suggest that TPX2, as a proliferation stimulator, facilitates the stepwise tumorigenesis occurring in cervical squamous epithelia and that TPX2 could be a candidate early biomarker for malignant transformation of cervical squamous epithelial tissue.

TPX2 was found to be upregulated in squamous cell carcinoma of the cervix with the expression correlating to tumor grade, stage and nodal status. Therefore, TPX2 may enhance the metastasis capability of the tumor cells, and the TPX2 could be considered a risk factor for metastasis of cervical cancer. We transfected TPX2-siRNA into HeLa cells to study the effect of TPX2 downregulation on cell proliferation, cell cycle, apoptosis and invasiveness. We found that downregulation of TPX2 using TPX2-siRNA inhibited proliferation and invasiveness of cervical carcinoma HeLa cells. Inhibiting the expression of TPX2 by RNAi increased the percentage of S-phase and G2-phase cells and cell apoptosis. We observed that cell apoptosis and cell cycle arrest may contribute to the decreased cell proliferation and invasiveness. Previously it has been found that TPX2 plays an important role in the action mechanism of paclitaxel in treatment of cancers (18). Other have also obtained satisfactory effects in treating mouse pancreatic cancer through targeted inhibition of TPX2 by RNA intervention (11). Thus, we believe that TPX2 might become

a new potential marker for cervical cancer diagnostics and therapy.

Acknowledgements

We thank the staff of Department of Obstetrics and Gynecology, The Fourth Hospital of Hebei Medical University, for assistance in obtaining clinical samples.

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