Abstract. Non-small cell lung cancer (NSCLC) as the most frequently diagnosed lethal cancer remains the major cause of overall cancer-related death worldwide. Testes-specific protease 50 (TSP50) has been proved as a critical biomarker in various cancers, and we previously reported that TSP50 protein expression is overexpressed in clinical resected NSCLC tumor tissues and related to poor prognosis in NSCLC patients. Hence, the present study was designed to further investigate the potential oncogenesis mechanism of TSP50 in NSCLC cells. Real-time quantitative PCR, immunohistochemical assay and western blot analysis were used to analyze the TSP50 mRNA and protein expression in 20 NSCLC cases, and TSP50 expression was observed to have high levels in the NSCLC specimens and paired metastatic lymph node tissues when compared to the levels in corresponding normal lung tissues and normal lymph nodes. In the experiments in NSCLC cell lines, lentiviral short hairpin RNA (shRNA) delivery system was applied to knock down TSP50 in 95D cells, and the following investigations revealed that downregulation of TSP50 expression markedly reduced cell proliferation, colony formation and migration ability in vitro. Furthermore, the inhibition of TSP50 induced G0/G1-phase arrest and decreased expression levels of cell cycle relative markers CDK4, CDK6, and CyclinD1 and increased expression of p21 and p53 in 95D cells. In conclusion, this study indicates that TSP50 plays a significant role in NSCLC cell proliferation and may act as a novel oncogene in the development and progression of NSCLC, offering a potential cancer therapeutic target for the treatment of NSCLC.

Introduction

Non-small cell lung cancer (NSCLC), accounting for about 80-85% of lung cancer, is currently the leading cause of cancer-related mortality in the world (1). Despite growing advance in standard diagnosis and treatment, the expected 5-year survival is only approximately 15% (2). Patients with early-stage NSCLC could often die from relapse and distant metastasis after curative surgical resection (3), and patients with advanced-stage NSCLC received palliative resection or combined with alleviative chemotherapy and radiotherapy frequently fail to approach satisfactory effect (4). In recent years, targeted cancer therapies, as a new class of complementary therapeutical method that specifically target certain molecular pathways leading to cancer phenotypes, demonstrated its advantage in personalized medical treatment in advanced-stage NSCLC (5), while indication of limitations and growing drug resistance of tyrosine kinase inhibitor (TKI) therapy have become critical issues (6). Consequently, better understanding of the molecular mechanisms and identification of tumor related biomarkers are necessary for the development of new effective diagnostic or therapeutic targets for NSCLC.

Testes-specific protease 50 (TSP50), also known as PRSS50 or CT20, encoding a protein homologous to many serine proteases, was isolated from a human testes cDNA library by using a genomic DNA probe (7). Although TSP50 shared two critical catalytic triads, histidine and aspartic acid with traditional serine proteases, the most critical catalytic serine site was substituted with threonine, hence it could be classified as a novel protease (8). Normally, northern blot analysis of multiple human tissue RNA expression panels showed that TSP50 is specifically and strongly expressed in the testes which suggest it might be involved in the human reproductive pathway (9). Reverse transcription-PCR analysis of 18 paired breast cancer tissues first found that the TSP50 gene was differentially expressed in 28% of the cancer samples (7), and Shan et al confirmed that TSP50 gene activation and overexpression in breast tumors took place in malignant mammary epithelial cells (9). To elucidate the regulatory mechanisms related to differential expression of TSP50 gene in breast cancer, Xu et al further demonstrated a putative p53-binding site and several Sp1-binding sites in the TSP50 promoter and p53 play a critical role in negatively regulating the TSP50 gene,
and breast cancer cells containing mutated p53 hence could produce high or low levels of TSP50 transcripts (10); while overexpression of Sp1 and C/EBPβ transcriptional factors upregulated the activities of the TSP50 promoter in human breast cancer and bFGF mediates TSP50 downregulation by ERK activation, leading to the phosphorylation of Sp1 in this process, which was consistent with the previous research (11,12). Recent results suggested that downregulation of TSP50 induces apoptosis, reduces cell proliferation and colony formation in p19 cells (13). Moreover, knockdown of matrix metalloproteinase-9 (MMP9) resulted in inhibition of cell migration and invasion in vitro, and TSP50 overexpression enhanced expression and secretion of MMP9, hence TSP50 activation of MMP9 is a novel signaling mechanism underlying human breast cancer invasion and metastasis (14).

In addition, TSP50 was also found to be abnormally activated and expressed in laryngocarcinoma and TSP50 knockdown inhibited Hep2 cell proliferation and induced apoptosis in vitro in a NF-κB-mediated pathway (15,16). TSP50 also depends on its threonine protease activity and its interactions with TNF-α-induced NF-κB playing an important role in human cervical tumorigenesis (17). Recently, we have reported that TSP50 protein expression is elevated in resected NSCLC tumor tissues as compared with paired non-tumorous tissues using immunohistochemical assay, and found that high levels of TSP50 is related to poor-differentiated tumor, late tumor status and late TNM stage, as well as a reduced disease-free survival and overall survival rate in all NSCLC patients (18). However, the specific molecular mechanisms of TSP50 involved in the development and progression of lung cancer remain largely unknown.

To further investigate the TSP50 expression and its significant role in NSCLC as well as confirm the previous observations, cancer tissue samples with corresponding metastatic lymph nodes from previous NSCLC samples and lung cancer cell lines were detected by immunohistochemical assay, real-time quantitative PCR and western blot analysis. Moreover, we used specific short hairpin RNA (shRNA) to downregulate TSP50 expression in NSCLC cell lines 95D in vitro, and then investigated the proliferation, colony formation, wound healing capacity and cell cycle progression of the cells.

**Materials and methods**

**Immunohistochemistry.** Twenty cases of NSCLC tissues with metastatic lymph nodes and their corresponding adjacent normal tissues were specially selected from 20 patients who underwent surgical resection at the Department of Thoracic Surgery, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, between from January 2015 to June 2015. Surgically-removed tumors and matched normal tissues were partially immediately frozen in liquid nitrogen and kept at -80˚C until the extraction of the RNA and protein, and partially fixed in neutral-buffered formaldehyde for IHC. These formalin-fixed and paraffin-embedded tissue blocks were cut into 3-4 μm sections and routinely deparaffinized, dehydrated and heated in antigen retrieval with 0.01 mol/l citrate buffer solution (pH 6.0). Then endogenous peroxidase was blocked in 3% H2O2 and non-specific antigen was blocked with 10% normal goat serum before they were incubated with a rabbit polyclonal antibody against TSP50 (1:150; 12574-1-AP, Proteintech) at 4˚C in a humidified chamber overnight. After being washed with PBS (pH 7.4), the slides were hybridized with goat anti-rabbit second antibody conjugated with streptavidin-biotin peroxidase for 20 min and visualized with 3,3’-diaminobenzidine (DAB), then followed by counterstaining with Mayer's hematoxylin and dehydrated. Each slide was evaluated by two experienced pathologists. The percentage of positive cells in the fields was examined and graded as score 0 (negative), score 1 (0-10%), score 2 (10-50%), score 3 (51-75%) and score 4 (75-100%). The staining intensity of the immunohistochemical staining was graded as follows: 0 (no staining), 1 (weak yellow), 2 (yellow), or 3 (brown). The final score was the sum of the positive cell rate and the intensity of the immunohistochemical staining as 0, 1-3, 4-5, or 6-7, which represent negative (-), weakly positive (+), moderately positive (++), strongly positive (+++) and very strongly positive (+++), respectively. This study was approved by the Ethics Committee of Shanghai Jiao Tong University School of Medicine and written informed consent was obtained from each patient.

**Cell lines and cell culture.** Human lung cancer cell lines A549, 95D, Calu-1, H1299, H460, SPC-A-1 and human embryonic kidney cell line HEK293T were all purchased from the Cell Bank of Chinese Academy of Science (Shanghai, China) and maintained in a humidified atmosphere at 37˚C in 5% CO2. 95D, H1299, H460 and SPC-A-1 cells were cultured in RPMI-1640 medium (Hyclone), A549 and 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM; Hyclone), and Calu-1 were cultured in McCoy's 5A medium (Sigma, St. Louis, MO, USA). All culture media were supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone), L-glutamine (2 mmol/l) (Life Technologies), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Life Technologies).

**Lentivirus vector construction and infection.** The lentiviral vector (PLKO.1, Addgene) was used to construct short hairpin RNA (shRNA) specific for TSP50. The TSP50 cDNA sequence was from GenBank with accession number NM_013270.4, the shRNA specific against TSP50 (sh-TSP50) was 5'-CAGTTCTGCATAGGCTAACTGTCGAGCCATTGGCTCA GCAGAACTTTTTT-3', and a non-specific scramble shRNA sequence (sh-Mock) was 5'-GCCGAGGTGTGAAAGAA TATCTCGAGATATCTTCAAACTCTCGCTTTTTT-3'. After being sequenced for correct ligation, these recombinant vectors were transfected into HEK293T cells with lentiviral packaging vectors with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's procedure. The 95D cells were grown in 6-well plates at a density of 5x104 cells per well 24 h before the infection, then the constructed lentiviruses containing TSP50 shRNA and non-silencing shRNA (sh-Mock) were individually transfected into the 95D cells at a multiplicity of infection (MOI) of 6 with polybrene (5 μg/ml) (Sigma). The efficiency of lentiviral infection was primarily determined by intensity of green florescence protein (GFP) after 96 h.

**RNA extraction and quantitative RT-PCR.** Quantitative real-time PCR (qRT-PCR) was used to observe TSP50 expression in 20 pairs of NSCLC cancer and matched metastatic lymph...
node specimens (kept in liquid nitrogen) and knockdown efficiency in 95D cells. Total RNA of specimens and cells harvested and washed with PBS was extracted with TRIzol reagent (Invitrogen). Samples with 260/280 values of 1.8-2.0 were considered for further research. cDNA was then reverse transcribed with oligodT using M-MLV reverse transcriptase (Thermo Fisher Scientific, Rockford, IL, USA). Quantitative PCR was performed with Maxima SYBR Green qPCR Master Mixes (Thermo Fisher Scientific) in an ABI 7300 system (Applied Biosystems, Foster City, CA, USA), and approx. 20 µl of qRT-PCR mixtures containing were 2X SYBR Premix Ex Taq 10 µl, forward primers 0.5 µl (2.5 µM), reverse primers 0.5 µl (2.5 µM), cDNA 5 µl, and ddH₂O 4.5 µl. All 40 PCR cycles were run using the following cycling parameters, predegeneration for 1 min at 95˚C, denaturation for 5 sec at 95˚C, and extension for 30 sec at 60˚C. The primer sequences for PCR amplification of the TSP50 were F: 5'-GCTCCTGTGGCTTTTCCTAC-3' and R: 5'-CGGAGGCGGTCTGCGTCAT-3'. The primer sequences for β-actin were F: 5'-GTGGACATCCGCAAAAGC-3' and R: 5'-AAAGGGTGTAACGCAACTA-3'. The fold change of TSP50 mRNA expression was calculated by the 2-∆∆Ct method, using β-actin mRNA expression level for normalization. All experiments were repeated in triplicate.

Western blot analysis. The liquid nitrogen-conserved NSCLC tissues were homogenized and lysed in the RIPA buffer on ice. Protein concentrations were determined by the Bradford method using bovine serum albumin as the standard. The 95D cells were collected 7 days after lentivirus infection and washed with ice-cold PBS 2 times then harvested in RIPA lysis buffer (150 mM Tris-HCl, 50 mM NaCl, 1% NP-40, 0.1% Tween-20) with freshly added protease inhibitor cocktail (Sigma). Equal amount of protein (30 µg) were mixed with 5X loading buffer, boiled for 5 min, and loaded into a 10% sodium dodecyl sulfate-polyacrylamide (SDS) gel electrophoresis and transferred onto a polyvinylidene fluoride (PVDF, Millipore, Bedford, MA, USA) membrane by electrophobting for 90 min. Non-specific sites on membranes were then blocked with 5% skim-milk for 1 h then incubated with primary antibodies anti-TSP50, anti-p21 and anti-CDK6 (Abcam, Cambridge, MA, USA); anti-CDK4, anti-CyclinD1, anti-GAPDH and p53 (Cell Signaling Technology, Danvers, MA, USA) overnight at 4˚C, and then probed with goat anti-rabbit HRP-linked secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactive bands were identified using the enhanced chemiluminescence (ECL) system (Bio-Rad Laboratories, Richmond, CA, USA) following the manufacturer's instructions. GAPDH was used as a loading control.

Cell viability assay. Transfected cell proliferation was determined using an MTT assay. After lentivirus infection, 95D cells were placed with 100 µl at a density of 2x10³ cells/well in 96-well microtitre plates. During the following 5 days, the absorbance data of one specified plate was analyzed each day, and the cells in the rest of the plates were cultured continuously. A total of 20 µl MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (5.0 mg/ml) was added into each well and incubated with cells at 37˚C for 4 h, then the reaction was terminated by adding 100 µl acidic isopropanol (5% isopropanol, 10% SDS, and 0.01 mol/l HCl). Fast colorimetric assay for cellular growth was measured the next day by a microplate reader (Bio-Rad Laboratories) in absorbance at wavelength 595 nm. The results were plotted as the mean ± standard deviations (SD) of five determinations.

Colony formation assay. Three kinds of 95D cells were plated onto 6-well plates with 2 ml at a density of 500 cells per well for 8 days with the culture medium changed every 3 days. When most of the colonies were >50 cells, adherent cells were gently washed with ddH₂O 2 times and fixed in 800 µl fresh prepared 4% paraformaldehyde at room temperature for 15 min. The

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Table I. Association of TSP50 expression between NSCLC and non-tumor tissues.

<table>
<thead>
<tr>
<th>Type of tissue</th>
<th>No. of cases</th>
<th>Negative (-)</th>
<th>Medium (+)</th>
<th>Positive (+++)</th>
<th>Strong Positive (+++)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSCLC tissues</td>
<td>20</td>
<td>4</td>
<td>5</td>
<td>9</td>
<td>2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Non-tumor tissues</td>
<td>20</td>
<td>18</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Table II. Association of TSP50 expression between metastatic and non-metastatic lymph node status.

<table>
<thead>
<tr>
<th>Lymph node tissue</th>
<th>No. of cases</th>
<th>Negative (-)</th>
<th>Medium (+)</th>
<th>Positive (+++)</th>
<th>Strong Positive (+++)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metastatic lymph node</td>
<td>20</td>
<td>9</td>
<td>5</td>
<td>6</td>
<td>0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Non-metastatic lymph node</td>
<td>20</td>
<td>11</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
cells were then stained with Giemsa (Sigma-Aldrich) for 10 min and washed with ddH2O 3 times. Colonies with >50 cells were photographed with fluorescence microscope. Three independent experiments were repeated.

**Wound healing assay.** To measure the invasion ability of cells, each group of cells were grown in 96-well microtitre plates incubated with RPMI-1640/fetal bovine serum (10%) in a volume of 100 µl per well overnight. When primary cultures became confluent and a wound was created with a sterile pipette tip. After being washed with PBS 2 times, 2% RPMI-1640/fetal bovine serum was added into plates and each group of cells were fixed and observed by microscopy at the time point of 24 h. The experiment was independently performed for three times.

**Cell cycle analysis.** The role of TSP50 silencing on 95D cell cycle distribution was evaluated by flow cytometry with propidium iodide (PI, Sigma) staining. Briefly, 95D cells were seeded in 6-cm dishes with 5 ml volume at a density of 1x10⁶ cells/well after infected with indicated virus for 6 days, then cells were harvested with trypsinization after 48 h, washed with ice-cold PBS, and fixed in 75% ice-cold ethanol overnight at 4°C. After the ethanol was removed by centrifugation, cells were washed with PBS two times and incubated with PI (50 µg/ml, Sigma) solution containing 100 µg/ml of ribonuclease A (Sigma) at room temperature for 30 min. DNA content of all stained cells was then analyzed on flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Experiments were performed in triplicate.

**Statistical analysis.** Results were compared using the Student's t-test and considered statistically significant at a P-value of <0.05. Data were expressed as mean ± SD and all experiments were carried out independently at least three times.
Results

High levels of TSP50 expression in NSCLC and metastatic lymph nodes. Overall results of the TSP50 protein in NSCLC and metastatic lymph node tissues is summarized in Tables I and II, positive staining of TSP50 was detected in the cytoplasm of adenocarcinoma and squamous cell carcinoma tissues, significant correlation was observed both in between NSCLC and non-cancer tissues, and between the metastatic lymph node and non-metastatic lymph node subgroups, respectively (Fig. 1). In addition, the RT-qPCR data confirmed increased levels of TSP50 mRNA expression in the 20 NSCLC specimens compared with the adjacent non-tumor tissues (Fig. 2A). Consistent with the mRNA level, western
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blot analysis also showed that 13 (65%) NSCLC samples had significantly higher TSP50 protein levels than that of the adjacent non-tumor tissues (Fig. 2B).

**Figure 5.** The role of TSP50 knockdown on cell proliferation, colony formation and migration ability in 95D cells. (A) Effect of TSP50 silencing on cell proliferation. Methylthiazol tetrazolium (MTT) assay showed that the growth curve was significantly inhibited in 95D cells after TSP50 knockdown. (B) Effect of TSP50 knockdown on the colony formation. Representative photomicrographs of Giemsa-stained colonies of 95D cells growing in culture plates for 8 days after infection. Obvious decrease was observed in the total number of colonies formed in 95D cells transfected with sh-TSP50 against control groups. (C) Colonies consisting of more than 50 cells in each well were counted. Comparing with the number of colonies in control groups, the number of colonies in the sh-TSP50 group was significantly reduced. Data are expressed as mean ± SD of three independent experiments. **P<0.01.

Knockdown of TSP50 with shRNA lentiviruses systems in 95D cells. TSP50 in six NSCLC cell lines including A549, H460, Calu-1, H1299, SPC-A-1 and 95D lung cancer cells was evaluated by real-time PCR and western blot analysis. As shown in Fig. 3A and B, 95D cells showed the highest transcription level of TSP50 and indicated highest levels of TSP50 protein expression, thus they were chosen to further investigate the potential biological function of TSP50 in NSCLC.

shRNA against TSP50 (shTSP50) and non-specific scramble shRNA sequence (sh-Mock) were cloned into a lentiviral vector expressing GFP which were further infected into 95D cells, respectively, and GFP expression was generated and examined with fluorescence microscopy to assess the lentiviral infection efficiency. The infection efficiency of lentivirus was >90% after 96 h of infection (Fig. 4A). In addition, the knockdown efficiency was further confirmed by qRT-PCR and western blot analysis. As shown in Fig. 4B, the qRT-PCR assay revealed that the TSP50 mRNA level was reduced by 80% in the sh-TSP50 group in comparison with that in the sh-Mock and Control group. The TSP50 protein expression was also markedly downregulated in the shTSP50 group in contrast with the other two groups (Fig. 4C). Therefore, the recombinant RNA interference lentivirus system was able to efficiently knock down TSP50 expression in 95D cells.

Silencing of TSP50 reduces cell proliferation and colony formation. To further analyze the biological function of TSP50 in the development and progression of NSCLC, we performed MTT and colony formation after infecting 95D cells with shRNA and sh-Mock controls. As shown in Fig. 5A, MTT cell proliferation assay indicates that the growth curves of sh-TSP50 group was significantly inhibited and the proliferation rate of 95D cells was reduced by 48.6 and 53.3% on the 4th and 5th day, respectively, compared to that of control groups. However, there was no difference between sh-Mock infected and non-infected 95D cells. This finding indicates that the knockdown of TSP50 markedly decreased the cell proliferative ability in NSCLC cancer cells.

To further investigate and confirm the effect of TSP50 silencing on the proliferation of 95D cells, the colony formation assay was performed. As depicted in Fig. 5B, 95D cells infected with sh-TSP50 formed smaller colony size and lower colony density compared with controls, and the number of colonies was correspondingly decreased by 61.2 and 62.6% after shTSP50 infection, respectively (Fig. 5C, P<0.01). The results further demonstrated that silencing of TSP50 could significantly suppress the colony-forming ability of NSCLC cells. Collectively, these data suggest that TSP50 may act as a positive regulator of cell growth and plays a critical role in NSCLC carcinogenesis.

Suppression of 95D cell migration in vitro by TSP50 knockdown. We further evaluated the effect of TSP50 on the migratory ability of the 95D cells by wounded healing assay after lentivirus infection with sh-TSP50 and sh-Mock controls. We observed that migratory ability of 95D cells transfected with sh-TSP50 against control groups was much weaker than those in sh-Mock group at the 48 h point after infection (P<0.05) (Fig. 6). Therefore, these results preliminarily indicate that TSP50 may play a key role in the migration of 95D cells.

Suppression of TSP50 induces G0/G1 phase arrest of 95D cells. In view of the findings that knockdown of TSP50 in 95D cells markedly inhibits cell proliferation and decrease cell colony formation ability, we further performed cell cycle analysis to investigate the mechanism underlying the inhibition of 95D cell growth by flow cytometry analysis after PI staining. As shown in Fig. 7A and B, we observed that migratory ability of 95D cells transfected with sh-TSP50 against control groups was much weaker than those in sh-Mock group at the 48 h point after infection (P<0.05) (Fig. 6). Therefore, these results preliminarily indicate that TSP50 may play a key role in the migration of 95D cells.
percentages in the sh-Mock infected 95D cells; however, no significant difference in percentage of cells was observed between the groups of sh-Mock and Control during G0/G1, S, and G2/M phases. Further, we detected the protein levels of G0/G1-associated genes including CyclinD1, CDK4, CDK6, p21 and p53 by western blot analysis. As shown in Fig. 7C, knockdown of TSP50 resulted in decrease in CDK4, CDK6 and cyclinD1 expression, and increase in p21 and p53 gene expression. Consequently, our results suggested that TSP50 silencing was able to inhibit the growth of NSCLC cells via inducing G0/G1 cell cycle arrest.

Discussion

NSCLC as the predominant form of lung cancer accounting for the majority of lung cancer associated deaths worldwide (1). There has been an unceasing and positive effort to improve prognosis of NSCLC, and novel treatment strategies have arisen from the research of the molecular and cellular biology of NSCLC. Here, we investigated the functional role of TSP50 gene in NSCLC and its potential application as a therapeutic target. Firstly, high levels of TSP50 expression in NSCLC tissues was detected at both the RNA and protein levels and in corresponding metastatic lymph nodes, these results were in accordance with a previous study and suggest that TSP50 may be related to both tumorigenesis and development of NSCLC, hence it could be a potential therapeutic target (19,20).

TSP50 as a testis-specific gene re-expressed in different types of malignant somatic cells indicate the TSP50 gene may be an oncogene. Thus, to further investigate the functional significance of TSP50 and evaluate its application prospect of targeted gene therapy in NSCLC cells, we conducted an in vitro experiment using lentivirus shRNA system that steadily and effectively knocked down the expression of TSP50, and observed the subsequent effect of TSP50 silencing on progression in 95D cells. To our knowledge, this is the first study to report TSP50 expression in NSCLC cells and investigate the role in functional phenotype of NSCLC cells. qRT-PCR and western blot analysis showed targeting gene was inhibited effectively, hence ensuring the credibility of the following assays. As expected, knockdown of TSP50 significantly reduced cell proliferation, colony formation, migration ability and arrested cell cycle in 95D cell lines. These results were coincident with recent reports that downregulation of TSP50 expression inhibited the growth of breast cancer and laryngocarcinoma cells (14,16).

Additionally, TSP50 as a testis-specific gene overexpressed in various malignant cells was considered as an oncogenic cancer/testis (CT) antigen family member, also including SSX-2, MAGE-A1, NY-ESO-1 and others (21). Importantly,
many CT antigens have been shown to support cancer cell growth (22). Recent observations indicated that knockdown of the CT antigen SSX2 in melanoma cells significantly decrease cellular proliferation ability (23), GAGE7 played a preventing role in response to different types of apoptotic stimuli (24,25), and silencing of PAGE4 could attenuate tumor growth by inducing cell death in vivo (26). Consequently, our data indicate similar features of TSP50 with other CT antigens from another angle, further demonstrating TSP50 plays a crucial role in tumorigenesis of NSCLC. In addition, migration ability is the essential processes towards metastasis of cancer, combined with the high levels of TSP50 expression in the paired metastatic lymph nodes, we believe that the effects of TSP50 may be closely related with the metastasis process of NSCLC.

Noteworthy, our data first revealed that TSP50 silencing had an inhibitory effect on cell cycle progression of 95D cell lines via G0/G1 phase, which has not been reported in other cancer cell lines. To clarify the possible molecular mechanism involved in 95D cell proliferation, we detected modifications of some crucial checkpoint and regulatory molecules related to G0/G1 to S phase in 95D cells with TSP50 knockdown. Previous studies have confirmed cell cycle progression is positively regulated by a series of Cyclins and CDKs (27). CyclinD1 is regarded as one of the significant oncogenic drivers in cancer cells and is the most distinctive G1-phase cyclin (28), and CDK4/6 along with its activating cyclin partner D1 has a critical role in cell cycle control (29).

CyclinD1 binding to CDK4/6 forms the active complex of CyclinD1-CDK4/6, which could further assemble cyclin and CDK subunits and activate specific gene expression required for G0/G1 to S phase progression (30,31). However, the CDK inhibitor family members including p21, p16 and p27 have a negative regulatory role in this process (32,33). For instance, p21 could inactivate and degrade the CyclinD1-CDK4/6 compound and bind and inhibit the proliferating cell nuclear antigen to arrest the G0/G1 to S phase transition (34). Besides, p53 known as a ‘superstar’ marker could be activated by DNA damage checkpoint kinases to simultaneously control the G1/S and G2/M cell cycle checkpoints, with the p53 binding sites in p21 promoter, p21 gene could be activated and regulated by p53 gene at the transcriptional level (35,36). In our research, we found that knockdown of TSP50 reduced the levels of CDK4, CDK6 and cyclinD1 expression, and increased the level of p21 and p53 expression in 95D cells, which indicated that TSP50 knockdown induced G0/G1 phase arrest partly via suppression of these related cell cycle regulators. Besides, the trend of p53 change in sh-TSP50 and sh-Mock 95D cells according to a
previous study showing TSP50 is a downstream protein of p53 for its promoter containing a putative p53-binding site and it was negatively regulated by the p53 transgene in diverse types of cell lines (10).

CT antigens are immunogenic proteins expressed in normal testis and in different types of tumors. Because of their immunogenicity and testis-restricted normal tissue expression, CT antigens are prioritized targets for antigen-specific cancer immunotherapy and the identification of novel CT antigens is a prerequisite for the development of cancer vaccines (37,38). Moreover, Kim et al demonstrated CT antigens are potential candidates in lung cancer patients for polyclonal immunotherapy (21). Since our data have proved that TSP50 can be used as a prognostic or predictive biomarker in NSCLC, further immunotherapy of TSP50 in NSCLC as a supplement to conventional treatment will be conducted.

In summary, this is the first study discussing the possible role of TSP50 in NSCLC cell growth and migration ability, which provide further specific molecular mechanisms to our previous study indicating the considerable potential of TSP50 to be a new therapeutic target for the treatment of NSCLC.

Acknowledgements

This study was supported by grants from The National Natural Science Foundation of China (General Program) (81372521). We are grateful to all the Staff of the Experimental Center of Shanghai General Hospital for their sincere help and technical support.

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