Tumori, 96: 289-295, 2010

Knockdown of TSPAN1 by RNA silencing and antisense technique inhibits proliferation and infiltration of human skin squamous carcinoma cells

Li Chen[1], Yuanyuan Zhu[2], Hui Li[1], Guei-lan Wang[1], Yuan-yuan Wu[1], Yu-xiang Lu[2], Jin Qin[1], Jinsheng Tuo[3], Jian-li Wang[4], and Jianwei Zhu[5]

[1]Department of Pathological Anatomy, Nantong University, Nantong; [2]Biotechnologies (Nantong) Co, Ltd, Nantong, Jiangsu Province; [3]Department of Ophthamology, [4]Department of Dermatology, and [5]Department of General Surgery, Affiliated Hospital, Nantong University, Nantong, China

ABSTRACT

Aim. To explore the function of TSPAN1 in squamous cell skin carcinoma by means of TSPAN1-specific siRNA and antisense oligonucleotide techniques.

Methods. pU6H1-GFP-siRNA TSPAN1 and pcDNA3.1 antisense TSPAN1 were constructed and transfected into squamous cell skin carcinoma cell line A431 cells to knock down TSPAN1 gene expression. The levels of TSPAN1 mRNA and protein expression were detected by semiquantitive RT-PCR and Western blot, respectively. The proliferation rates of A431 cells were determined by MTT assay and flow cytometry. Lastly, the migration and infiltration of A431 cells were determined by the Transwell migration assay.

Results. Transfection with either pU6H1-GFP-siRNA TSPAN1 or pcDNA3.1 antisense TSPAN1 led to an obvious reduction of expression levels of TSPAN1 mRNA and protein in A431 cells, respectively. The proliferation, migration and infiltration of A431 cancer cells were significantly inhibited at 48 hours after transfection of plasmids harboring TSPAN1 siRNA and antisense RNA.

Conclusion. The TSPAN1 gene might play a role in the proliferation of squamous cell carcinoma of the skin and be associated with cancer cell motility, implying a function of the gene in the development of skin cancer. **Free full text available at www.tumorionline.it**

Key words: TSPAN1, RNA interference, antisense oligonucleotide, skin squamous cell carcinoma.

Acknowledgments: This research was supported by the University High-New-Tech Development Fund of Jiangsu Province (No. JHO2-118), the Science Foundation of Nantong City, Jiangsu Province, China (No. K2006020), the Natural and Scientific Foundation of Jiangsu Province (BK 2006058), and the National Natural and Science Foundation (30771126).

Correspondence to: Jianwei Zhu, Department of General Surgery, Affiliated Hospital, Nantong University, Nantong, China.
Tel +86-513-81161221;
e-mail usazhujianwei@yahoo.com.cn
or Jian-li Wang, Department of Dermatology, Affiliated Hospital, Nantong University, 20 Xi Si Road, Nantong 226001, Jiangsu, China

Received May 19, 2009;
accepted September 8, 2009.

Introduction

CTSPAN1 is a member of the guanine nucleotide exchange factor (GEF) family and homologous to the tetraspanin/TM4SF family (GenBank Accession No. AF065388). The TSPAN1 gene is located at chromosome 1p34.1 and encodes a protein of 241 amino acids[1,2]. While studies have reported that overexpression of TSPAN1 proteins was associated with the development of tumors including liver cancer[3], gastric carcinoma[4], prostate cancer[5] and cervix cancer[6], the function of the TSPAN1 gene in squamous cell carcinoma of the skin has yet to be understood. Indeed, we found TSPAN1 to be overexpressed also in squamous cell skin cancer tissues (Data published in Chinese).

The skin is the largest organ in the human body. Basal cell carcinoma and squamous cell carcinoma are common skin tumors, with squamous cell carcinoma being the most frequent of the two[7]. Prognosis is dependent on tumor size, predisposing conditions, depth of invasion, immunosuppressed states, and neural or vascular invasion[7,8]. It is therefore important to know the mechanism of proliferation and infiltration of skin squamous cancer cells. A large number of genes have been found to

be involved in the different steps of carcinogenesis and tumor development[9]. In our previous studies, we found that the TSPAN1 gene was associated with the development of liver, gastric and colorectal cancer, suggesting a potential link of the gene to proliferation and metastasis. In the present study we examined the role of TSPAN1 in the skin squamous cancer cell line A431 by construction and cell transfection of vectors harboring TSPAN1-specific siRNA and antisense TSPAN1 oligonucleotide, which led to knockdown of TSPAN1 expression. We found that knockdown of TSPAN1 expression by TSPAN1 siRNA and antisense RNA could inhibit the proliferation and infiltration of human skin squamous carcinoma cells. This study demonstrated for the first time that the TSPAN1 gene plays an important role in the development of squamous cell carcinoma of the skin.

Materials and methods

Construction of vectors

The siRNA targeting TSPAN1 (19 nt) was selected within the 50th-100th nucleotide from the start codon AUG. The specificity of siRNA was checked by a BLAST search (www.ncbi.nlm.nih.gov/Blast.cgi), which showed that only the TSPAN1 gene was targeted. The selected siRNAs contained a low G ratio and consecutive G section[10]. siRNA was synthesized and inserted into the pU6H1-GFP vector (Biomics Biotechnologies, Nantong, China). In addition, the whole sequence of TSPAN1 cDNA was amplified by PCR, purified and digested by EcoR1 and BamH1, respectively, and inserted into the pcDNA3.1 vector (Qiagen, USA) to construct a recombinant pcDNA3.1 plasmid encoding antisense TSPAN1 (Biomics Biotechnologies). Plasmids including pU6H1-GFP-siRNA off-target with random dsRNA (21 nt) and pcDNA3.1 plasmid encoding sense TSPAN1 were prepared with the same procedure. All recombinant plasmids were verified by sequencing. The information of all recombinant plasmids is listed in Table 1.

Cell culture and DNA transfection

The human skin squamous cell carcinoma cell line A431 (ATCC) was cultured with Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Invitrogen) in a 37 °C 5%CO_2 incubator. A431 cells in the logarithmic growth phase were seeded in 6-well plates (Nunc, Rochester, NY, USA) at a density of 1.5×10^5 cells per well the day before transfection. The cells were transfected with Lipofectamine^{2000} (Invitrogen, USA) according to the protocol provided by the manufacturer. Cells were harvested 48 hours after transfection. The efficiency was evaluated by measurement of GFP fluorescence using fluorescence microscopy (Olympus, BX51, Japan) at ×400 magnification. The culture supernatant was collected for further analysis.

To assess the transfection efficiency, 48 hours after transfection the transfected cells were washed with phosphate-buffered saline (PBS) and observed without fixation using a fluorescence microscope. The expression of GFP in A431 cells was counted in 10 randomly selected fields. The transfection efficiency was evaluated by calculated the percentage of GFP-expressing cells of the total cells (Figure 1).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total cell RNA was isolated using the PicoPure™ RNA isolation kit (Arcturus Bioscience, Mountain View, CA, USA) according to the manufacturer's instructions. RT-PCR was performed using a RiboAmp® Hs RNA amplification kit (Arcturus Bioscience) according to the manufacturer's instructions. Glycoraldelyde-3-phosphate dehydrogenase (GAPDH) was amplified as an internal control. Primers (20 pmol/μL) were added to the reaction buffer with a total volume of 25 μL together with 2 μL template cDNA, 5 μL dNTP0 and 0.5 μL Taq enzyme. PCR was performed in the following conditions: 30 seconds at 94 °C, 30 seconds at 62 °C, and 40 seconds at 72 °C for 40 cycles. PCR products were electrophoresed in

Table 1 - The insert sequences of TSPAN1 in recombinant vectors

Name	Sequence	Length (bp)
pU6H1-GFP-siRNA TSPAN1		
TSPAN1-S	5′-TGTGGTCTTTGCTCTTGGTTTCC-3′	19
TSPAN1-AS	3′-ACACCAGAAACGAGAACCAAAGG-5′	
pU6H1-GFP-siRNA off-target		
random sequence-S	5′-GAGTGATTGGAGGTTGGGGAC-3′	21
random sequence-AS	3′-CTCACTAACCTCCAACCCCCTG-5′	
pcDNA3.1 antisenseTSPAN1		
TSPAN1-S	5′-CG GAATTC TCCCTCTTTCAGAACTCACT-3′	806
TSPAN1-AS	5′-AA GGATCC GCAGAGGCAGAAGTGGAC-3′	
pcDNA3.1 sense TSPAN1		
TSPAN1-S	5′-CC GGATCC TCCCTCTTTCAGAACTCACT-3′	806
TSPAN1-AS	5′-CG GAATTC GCAGAGGCAGAACTGGAC-3′	

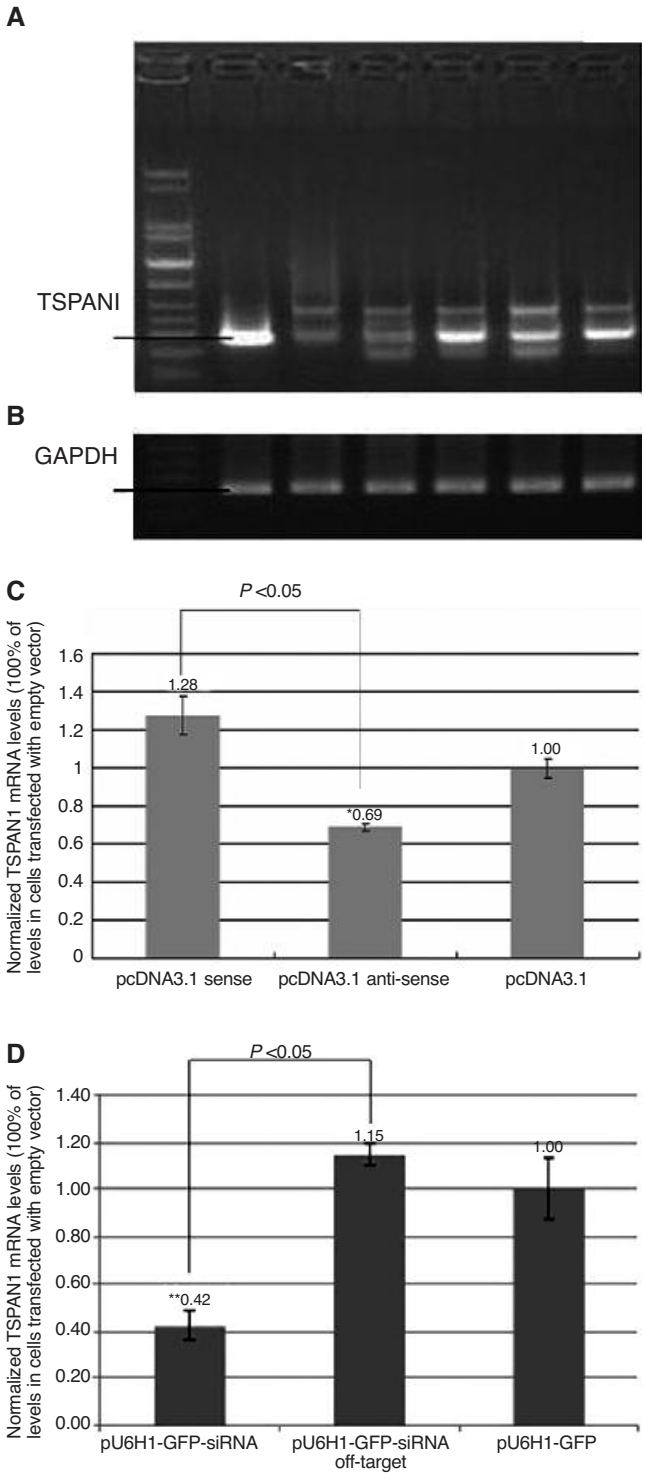

Figure 1 - RT-PCR assay of TSPAN1 mRNA levels in TSPAN1 siRNA and antisense RNA treated cells. A) RT-PCR electrophoresis pattern of TSPAN1 mRNA in A431 cell line 48 hours after transfection. Lane M, 1 kb DNA marker (Tiangen Biotech, Beijing, China); Lane A, pcDNA3.1 sense TSPAN1; Lane B, pcDNA3.1 antisense TSPAN1; Lane C, pU6H1-GFP-siRNA TSPAN1; Lane D, pU6H1-GFP-siRNA off-target; Lane E, pcDNA3.1; Lane F, pU6H1-GFP. B) RT-PCR electrophoresis pattern of GAPDH as control. C) and D) Densitometric quantitation of TSPAN1 mRNA bands normalized to the signal intensity of GAPDH control after TSPAN antisense RNA and siRNA treatments.

1% agarose gel (electric voltage 220 V, 30 minutes). TSPAN1 and GAPDH bands were visualized under ultraviolet light and were analyzed densitometrically using a gel imaging system (Gel-Pro Analyzer 4.0 software). The primer sequences and expected product sizes for TSPAN1 and GAPDH are listed in Table 2.

Western blot analysis

A431 cells were harvested 48 hours after transfection. Cells were lysated with buffer containing 0.1 mol/L Tris-HCl (pH6.8), 4% SDS, 20% glycerine, 0.1% BPB, and 5% β-mercaptoethanol. The complex was heated in boiling water for 5 minutes. Proteins were separated by 10% polyacrylamide gel electrophoresis and then transferred onto polyvinylidene fluoride (PVDE) membranes (Millipore, USA) at 350 mA for 2 hours, which were later soaked for 2 hours on a blocking solution (Tris-buffered saline containing 5% non-fat dry milk and 0.01% vol/vol Tween-20), and incubated for 1 hour at room temperature in the presence of anti-TSPAN1 rabbit polyclonal antibody[4,5], or anti-β-actin monoclonal antibody (Sigma, USA) used as internal control, then incubated at 47 overnight. After incubation, the membrane was washed 3 times, and peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (ICN Laboratories, Irvine, CA, USA; diluted 1:10,000) were added and incubated for an additional hour. The reaction was visualized by the ECL chemiluminescence detection system (Pierce, USA) on radiographic films (Kodak, USA). The molecular weights of TSPAN1 and β-actin were 38 kDa and 41 kDa, respectively. The results were analyzed using Gel-Pro Analyzer 4.0 software.

MTT assay

To examine the effect of TSPAN1 siRNA on the proliferation of A431 cells, 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl-2H tetrazolium bromide (MTT) assay was carried out 0, 24, 48, and 72 hours after transfection. Briefly, 3×10^4 cells were seeded into 96-well plates the day before transfection. The cells were cultured in a 5% CO_2 incubator at 37 °C with 150 µL DMEM containing 20 µL MTT (5 g/L) per well. After incubation for 4 hours the medium was discarded, and 150 mL of dimethyl sulfoxide (DMSO) was added to each well to dissolve formazan crystals. The optical density was recorded with an automated plate reader at 570 nm.

Flow cytometry analysis

1.5×10^5 cells were seeded into 6-well plates the day before transfection. Cell suspensions were collected and centrifuged at 1000 rpm for 5 minutes. The pellets were washed twice with ice-cold PBS and fixed in 70% ethanol at 4 °C overnight. After washing twice with PBS, cells were incubated in PBS with 5 mg/mL propidium iodide (PI) staining of nuclei and 50 mg/mL RNase A for

Table 2 - Primer sequences and expected product sizes for TSPAN1 and GAPDH genes in reverse transcription-polymerase chain reaction (RT-PCR)

Name	Primer sequence	Amplifying length (bp)
TSPAN1-S	5′- CCAATAAGCTTATGCAGTGCTTCAGCTTCATTAAGA-3′	300
TSPAN1-AS	5′- CCAATGAATTCTTGTAGATTGCAGTACAGATACATG-3′	
GAPDH-S	5′-TGATGACATCAAGAAGGTGGTGAAG-3′	240
GAPDH-AS	5′-TCCTTGGAGGCCATGTGGGCCAT-3′	

30 minutes at 4 °C. The red fluorescence of DNA-bound PI in cells was measured at 488 nmol/L with a FACSCalibur flow cytometer (Becton Dickinson, USA). The results were analyzed using the CellQuest software. The percentages of cells in each cycle were measured for each sample.

Transwell migration assay

1.5×10^5 cells were seeded into 24-well plates the day before transfection. Cells were harvested 24 hours after transfection and seeded into 24 transwell inserts (the upper chamber) in DMEM without serum. The lower chamber was supplemented with 500 µL DMEM medium with 10% FCS. The bottom membrane of the upper chamber was an 8-mm-pore polycarbonate membrane (Costar, Corning, NY, USA) coated with 50 µL of 0.5 mg/mL Matrigel. The cells on the upper surface were carefully removed with a cotton swab 36 hours and 48 hours after transfection. Migration was quantified by counting the cells remaining on the surface at the other side of the bottom membrane. Cell numbers were counted in 5 random fields under the microscope.

Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean ± standard error. All data were analyzed with the SPSS v. 13.0 statistical software. The 1-way ANOVA and chi-square tests were used for data analysis. P values <0.05 were considered statistically significant.

Results

TSPAN1 expression in A431 cells was inhibited by TSPAN1 siRNA and antisense RNA

RT-PCR was performed to determine whether transfection with pU6H1-GFP-siRNA TSPAN1 and pcDNA3.1 antisense TSPAN1 would result in reduction of TSPAN1 mRNA in A431 cells. Transfection of A431 cells with either pU6H1-GFP-TSPAN1 siRNA or pcDNA3.1 antisense TSPAN1 resulted in 58% and 31% reduction of TSPAN1 mRNA levels, respectively, when compared with control cells transfected with either pU6H1-GFP or pcDNA3.1 (P <0.05) (Figure 1). There were significant differences in TSPAN1 mRNA levels between pU6H1-GFP-siRNA TSPAN1 and pcDNA3.1 antisense TSPAN1 transfected cells (P <0.05), and no inhibition of TSPAN1 expression was found in cells transfected with either pU6H1-GFP-siRNA off-target or pcDNA3.1 sense TSPAN1 (Figure 1).

In addition, we detected TSPAN1 protein expression in transfected cells by Western blot analysis. As shown in Figure 2A and B, significant inhibition of TSPAN1 protein expression was found in A431 cells transfected with either pcDNA3.1 antisense TSPAN1 or pU6H1-GFP-siRNA TSPAN1 (Figure 2C and 2D) in comparison with cells transfected with either pcDNA3.1 or pU6H1-GFP (P <0.01). However, there were no significant differences in the inhibition effect between cells transfected with pU6H1-GFP-siRNA TSPAN1, pcDNA3.1 sense TSPAN1, or pcDNA3.1 antisense TSPAN1. Furthermore, we analyzed the relationship between mRNA expression and protein expression of TSPAN1 and found a close correlation between the two.

Proliferation of A431 cells was inhibited by TSPAN1 siRNA and TSPAN1 antisense RNA

To evaluate the proliferation of transfected A431 cells, cell growth curves were obtained by the MTT assay (Figure 3A). There was a significant reduction of cell proliferation after transfection with either pcDNA3.1 antisense RNA of TSPAN1 (55%) (Figure 3B) or pU6H1-GFP-siRNA TSPAN1 (50%) (Figure 4C), in comparison with cells transfected with either pcDNA3.1 or pU6H1-GFP (P <0.01). A higher inhibition rate was found at 72 hours after transfection compared to 48 hours, showing that the effect was time dependent. There was no significant difference in cell proliferation rates between pU6H1-GFP-siRNA TSPAN1 and pcDNA3.1 antisense TSPAN1 transfected cells.

Flow cytometry was performed to determine the cell cycle distribution by PI staining of nuclei. Cytometry analysis of transfected A431 cells displayed different changes in the percentage of cells in G_2 and S phase (Table 3). G_2 and S phase cells were apparently reduced after transfection with either pcDNA3.1 antisense TSPAN1 or pU6H1-GFP-siRNA TSPAN1. These results suggested that downregulation of TSPAN1 levels in A431 cells by transfection with either pcDNA3.1 antisense TSPAN1 or pU6H1-GFP-siRNA TSPAN1 could reduce the percentage of cells in the proliferation phases (G_2 and S).

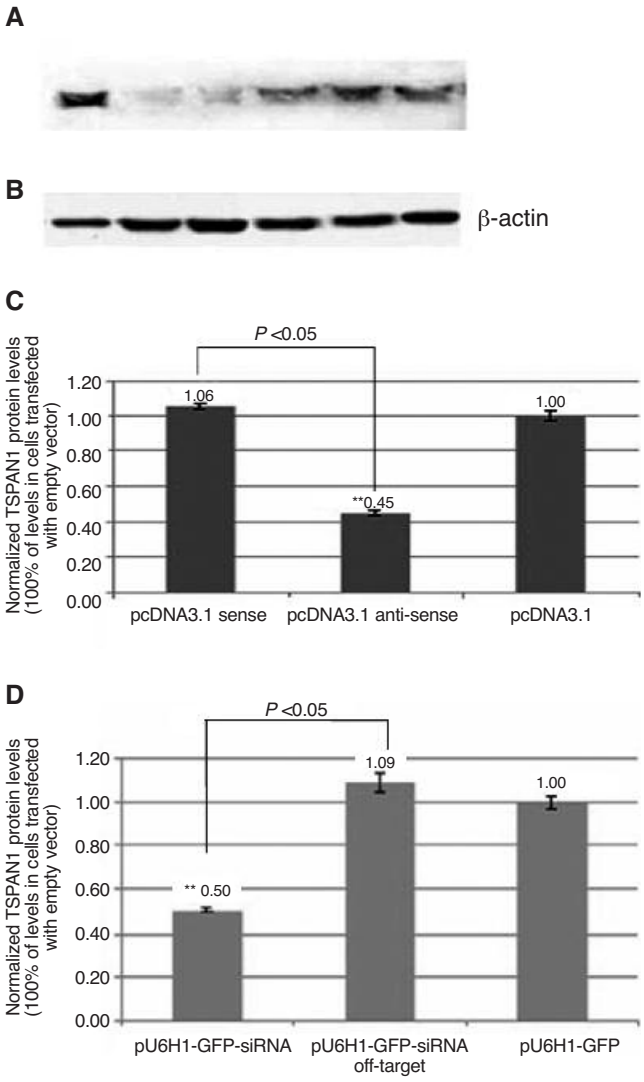

Figure 2 - Western blot assay of TSPAN1 protein levels in TSPAN1 siRNA and antisense RNA treated cells. A) Western blot analysis of TSPAN1 protein in A431 cells 48 hours after transfection. Lane A, pcDNA3.1 sense TSPAN1 treatment; Lane B, pcDNA3.1 antisense TSPAN1 treatment; Lane C, pU6H1-GFP-siRNA TSPAN1 treatment; Lane D, pU6H1-GFP-siRNA off-target treatment; Lane E, pcDNA3.1; Lane F, pU6H1-GFP. B) Western blot analysis of β-actin as control. C) and D) Densitometric analysis of samples normalized to β-actin bands.

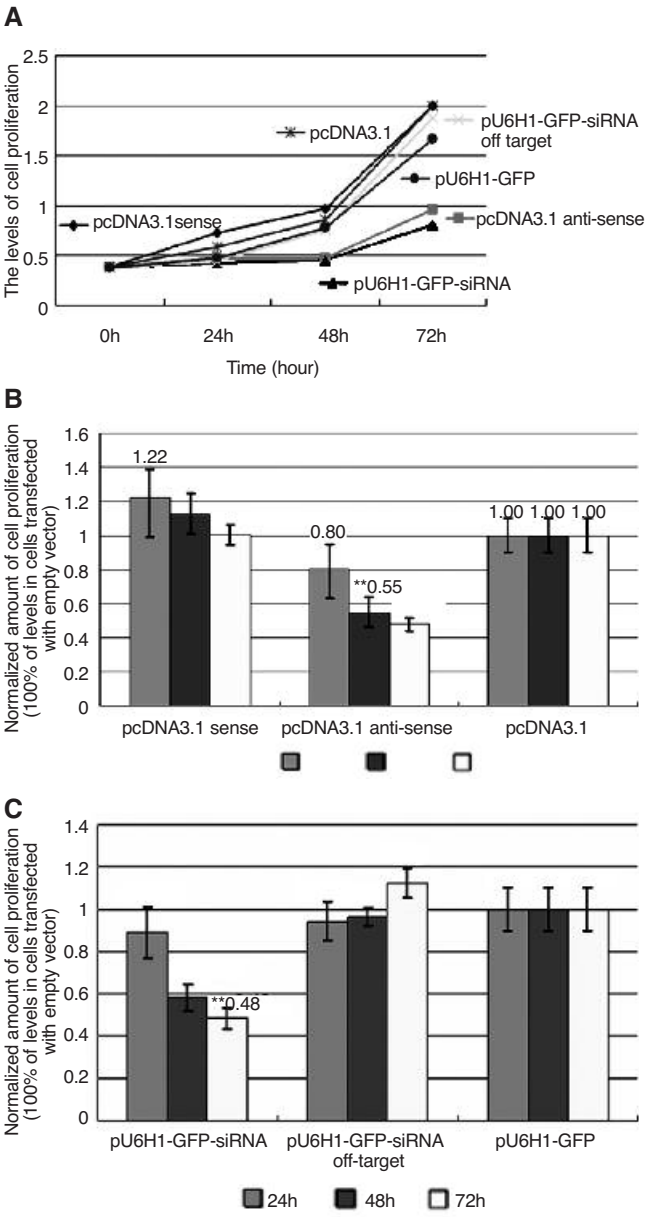

Figure 3 - Cancer cell proliferation was inhibited by TSPAN1 siRNA and antisense RNA treatment.
Transfection of each vector was performed 24 hours after cell culture as described in Materials and Methods. Cell proliferation was analyzed by MTT assay. A) Significant difference of proliferation levels presented 72 hours after cell transfection as follows: Line A, pcDNA3.1 sense TSPAN1; Line B) pcDNA3.1 antisense TSPAN1; Line C, pU6H1-GFP-siRNA TSPAN1; Line D, pU6H1-GFP-siRNA off-target; Line E, pcDNA3.1; Line F, pU6H1-GFP. B) and C) Proliferation levels of A431 cells.

Infiltration of A431 squamous carcinoma cells was inhibited by knockdown of TSPAN1 expression

A transwell chamber was used to observe the migration of cancer cells and evaluate the change of infiltration ability of cancer cells after knockdown of TSPAN1 expression in cells transfected with TSPAN1 siRNA and antisense RNA. The amount of cells located on the outside of the bottom membrane of the transwell chamber were counted 48 hours after transfection with each vector (Figure 4A). Transfection with either pcDNA3.1 antisense TSPAN1 or pU6H1-GFP-siRNA TSPAN1 reduced infiltration of cancer cells by 33% (Figure 4B) and 39% (Figure 4C), respectively, in comparison with the empty-vector controls pcDNA3.1 and pU6H1-GFP (P <0.01).

Discussion

TSPAN1 is a newly reported tumor-related gene that is closely associated with the development and progno-

Table 3 - The percentage of A431 cells in different phases of the cell cycle

Treatment	Cell cycle phase		
	G1/G1 (%)	G2/M (%)	S (%)
pcDNA3.1 sense TSPAN1	51.89	15.03	33.08
pcDNA3.1 antisense TSPAN1	63.95	12.48	23.57
pU6H1-GFP-siRNA TSPAN1	68.63	10.24	21.13
pU6H1-GFP-siRNA off-target	51.86	15.69	32.55
pcDNA3.1	53.16	12.61	34.23
pU6H1-GFP	52.13	14.59	33.28

sis of several malignant tumors[4-7]. We and others have previously reported that TSPAN1 was expressed in almost all normal human tissues and upregulated or overexpressed in some human tumors including liver cancer, gastrointestinal cancer, prostate carcinoma, uterine cervix carcinoma, and squamous cell skin cancer[4-7]. So far there has been no report about the function of this gene in skin carcinoma. In the present study, we designed and constructed siRNA in addition to antisense RNA of TSPAN1, and transfected it into the human skin squamous cell carcinoma line A431. We examined for the first time the role of TSPAN1 in A431 skin cancer cells.

We designed 19-nt siRNA to avoid activating nonspecific interference reactions in cells. The pU6H1-GFP expression vector with a polyclonal site between initiation sequence AAAAA of H1 and termination sequence TTTTT of U6 promoter can improve the efficiency and specificity of silencing[11]. A test for the specific inhibition ability of the pU6H1-GFP-siRNA TSPAN1 plasmid was performed through transfection of pU6H1-GFP-siRNA off-target with a random siRNA sequence. It finally revealed the specificity of the expression inhibition of TSPAN1 in A431 cells. The effect was sequence specific. No inhibition of TSPAN1 expression was found after transfection with pU6H1-GFP-siRNA off-target in A431 cells. After transfection of pU6H1-GFP-siRNA TSPAN1 and pcDNA3.1 antisense TSPAN1 into cancer cells, the levels of TSPAN1 mRNA and protein in the cells displayed a significant reduction by RT-PCR and Western blot assay. This demonstrated that TSPAN1 siRNA could effectively inhibit TSPAN1 levels in A431 cells.

The proliferation and infiltration of skin squamous cell carcinoma is the key to malignant progression. In this study the proliferation ability of the A431 cells was detected by MTT assay and flow cytometry. Knockdown of TSPAN1 expression could apparently inhibit cell proliferation of A431 cells without an increase in apoptosis. Moreover, cancer cell motility was examined by transwell migration assay. The results revealed that the extent of migration and infiltration was dramatically reduced after cell transfection with pU6H1-GFP-siRNA

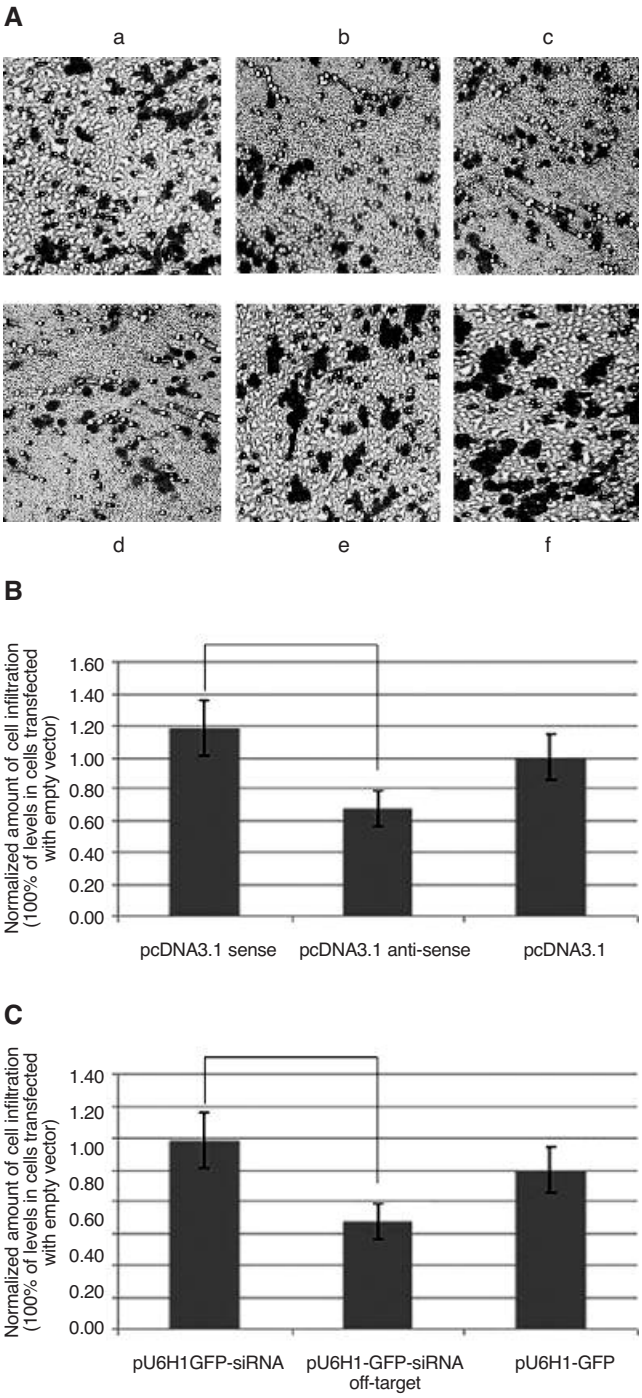

Figure 4 - Cancer cell infiltration was inhibited by TSPAN1 siRNA and antisense RNA treatment. A) The amount of cancer cell infiltration in Boyden chamber 48 hours after transfection of each vector as follows: a) pcDNA3.1 sense TSPAN1; b) pcDNA3.1 antisense TSPAN1; c) pcDNA3.1; d) pU6H1-GFP-siRNA TSPAN1; e) pU6H1-GFP-siRNA off-target; f) pU6H1-GFP. B) and C) Amount of A431 cell infiltration.

TSPAN1 and pcDNA3.1 antisense TSPAN1. These results suggested that expression of the TSPAN1 gene might play a role in the proliferation, migration and infiltration of skin squamous cell carcinoma.

In summary, siRNA and antisense oligonucleotide techniques could be used *in vitro* to reduce the levels of TSPAN1 mRNA and protein product and inhibit the function of the TSPAN1 gene in A431 squamous cell skin carcinoma cells. The expression of TSPAN1 in A431 cells was related to biological behaviors of the cells including proliferation, migration, and infiltration. *In vivo* experiments should be performed next to further examine whether malignant development would be postponed after TSPAN1 expression was inhibited in the cells.

References

1. Iwai K, Ishii M, Ohshima S, Miyatake K, Saeki Y: Expression and function of transmembrane-4 superfamily (Tetraspanin) proteins in osteoclasts: reciprocal roles of Tspan-5 and NET-6 during osteoclastogenesis. Allergol Int, 56: 457- 463, 2007.
2. Serru V, Dessen P, Boucheix C, Rubinstein E: Sequence and expression of seven new tetraspans. Biochim Biophs Acta, 1478: 159-164, 2000.
3. Chen L, Wang Z, Zhan X, Zhu J: Association of TSPAN1 gene expression with human hepatocellular carcinoma. Int J Surg Pathol, 15: 346-353, 2007.
4. Chen L, Li X, Wang G: Clinicopathological significance of overexpression of TSPAN1, Ki67 and CD34 in gastric carcinoma. Tumori, 94: 529-536, 2008.
5. Xu J, Stolk JA, Zhang X: Identification of differentially expressed genes in human prostate cancer using subtraction and microarray. Cancer Res, 60: 1677-1682, 2000.
6. Wollscheid V, Kühne-Heid R, Stein I, Jansen L, Köllner S, Schneider A, Dürst M: Identification of a new proliferation-associated protein NET-1/C4.8 characteristic for a subset of high-grade cervical intraepithelial neoplasia and cervical carcinomas. Int J Cancer, 99: 771-775, 2002.
7. Smoller BR: Squamous cell carcinoma: from precursor lesions to high-risk variants. Mod Pathol, 19: 88-92, 2006.
8. Evans HL, Smith JL: Spindle cell squamous cell carcinoma and sarcoma-like tumors of the skin: a comparative study of 38 cases. Cancer, 45: 2687-2697, 1980.
9. Hafner C, Becker B, Landthaler M, Vogt T: Expression profile of Eph receptors and ephrin ligands in human skin and downregulation of EphA1 in nonmelanoma skin cancer. Mod Pathol, 19: 1369-1377, 2006.
10. Juni A, Ueda R, Saigo K: Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. Nucl Acids Res, 32: 936-948, 2004.
11. Jian R, Peng T, Deng S, Jiang J, Hu F, An J, Cheng X: A simple strategy for generation of gene knockdown constructs with convergent H1 and U6 promoter. Eur J Cell Biol, 85: 433-440, 2006.