Abstract. Pancreatic cancer (PCC) is one of the most difficult cancers to treat and the 10th leading cause of cancer-related death in worldwide. Studies have demonstrated that the tetraspanin 1 (Tspan1) is overexpressed in various cancers and may be a potential therapeutic strategy for the treatment of different cancers. However, the possible role of Tspan1 in PCC is still unknown. In the present study, our data revealed that the increased Tspan1 in PCC tissues was associated with the clinicopathological features and survival rate of PCC patient. We also investigated the effects of Tspan1 gene knockdown on the biological behavior of human PCC. The expression of Tspan1 (detected by immunohistochemistry, qRT-PCR and western blot analysis) derived from human PCC tissues and cell lines (AsPC-1 and PANC-1), were significantly elevated compared with those of the control (P<0.05). Transfection with siRNA-targeting Tspan1 significantly decreased proliferation, increased the apoptosis and reduced migration and invasion of AsPC-1 and PANC-1 cells. The present study demonstrated that Tspan1 plays an important role in PCC carcinogenic progression, including migration and invasion. The siRNA targeting of Tspan1 may be a potential therapeutic strategy for the treatment of PCC.

Introduction

Pancreatic cancer (PCC) represents one of the leading causes of cancer-related mortality in industrialized countries (1). Despite surgical resection, radiation, and chemotherapy, >94% of patients with PCC do not survive beyond 5 years. The poor prognosis of this disease is mainly due to its early systemic metastasis (1-3).

It is well established that the tetraspanin family of four-pass transmembrane proteins has been implicated in fundamental biological processes, including cell adhesion, migration, and proliferation (4). Tetraspanins interact with various transmembrane proteins, establishing a network of large multimolecular complexes that allows specific lateral secondary interactions (5). In animals, the tetraspanins are a large superfamily of membrane proteins that play important roles in organizing various cell-cell and matrix-cell interactions and signal pathways based on such interactions (6,7).

Tetraspanins are a heterogeneous group of 4-transmembrane proteins that segregate into so-called tetraspanin-enriched microdomains (TEMs) along with other cell surface proteins such as integrins. TEMs of various types are reportedly involved in the regulation of cell growth, migration and invasion of several tumor cell types, both as suppressors or supporting structures (8). Tetraspanin 1 (Tspan1), a member of the transmembrane 4 superfamily of tetraspanins, is overexpressed in high-grade cervical intraepithelial neoplasia (CIN) and terminal carcinomas (8), lung cancer (9), colon cancer (10), breast cancer (11), as well as squamous cell carcinoma (12). However, the precise function of Tspan1 in the context of PCC is not known.

In the present study, quantitative RT-PCR (qRT-PCR) and western blot analysis were employed to explore the expressions of Tspan1 in human PCC tissues, adjacent normal tissues and human AsPC-1 and PANC-1 cell lines. Immunohistochemistry (IHC) was also used to detect the subcellular locations of Tspan1 in human PCC tissues. The corrections between Tspan1 expression in PCC and clinicopathological features were analyzed. Then, virus carrying a small interference RNA (siRNA) targeting the human Tspan1 gene was constructed and transfected into PCC cells. After transfection, Tspan1 expression was detected by qRT-PCR and western blot analysis. The cell proliferation and apoptosis fractions were also evaluated by MTT assay and flow cytometry (FCM). Additionally, the Transwell assays were employed to explore the effects of Tspan1 knockdown on the migration and invasion of PCC cells in vitro.

Materials and methods

Ethical aspects. This study complied with the International Ethical Declaration and was approved by the Human Ethics Committee and the Research Ethics Committee of Shaanxi Province of China. Through the surgery consent form, patients were informed that the resected specimens were kept by our
Quantitative reverse-transcription PCR. The expression of Tspan1 in the PCC specimens and the cell lines were detected by quantitative real-time PCR (qRT-PCR). Tumor samples (50 mg) were ground under liquid nitrogen, lysed with 1 ml of TRIzol (Takara, Japan), and total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). Total RNA (2 µg) was added to the tumor extract with Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT; Takara) was added to the tumor extract with Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT; Takara) and RiboMAX-Prep kit (Promega). cDNA was synthesized using 2X Mix SYBR green I (10 µl; Biosea, USA), primer (0.25 µl, 10 pmol/l), template DNA (1 µl), and sterile water (8.5 µl). All PCR reactions included initial denaturation and multiple cycles at 95°C for 3 min; 39 cycles at 95°C for 10 sec, 55°C for 10 sec, and 72°C for 30 sec; followed by 95°C for 10 sec, 65°C for 5 sec, and a final 95°C for 15 sec. The primer for each gene was synthesized by Invitrogen. The real-time PCR primers used to quantify GAPDH expression were: forward, 5'-CGAGATCCCCCTCATAATC-3' and reverse, 5'-TTTCACACCATTGACGAA-3'; and for Tspan1 were: forward, 5'-GGTGCTGATGTGCTA-3' and reverse, 5'-GCAAGTTTCATTGCGT-3'. Expression of Tspan1 was normalized to endogenous GAPDH expression.

Western blot analysis. Tspan1 protein levels both in PCC tissues and cell lines were determined by western blot analysis. Briefly, samples were lysed for 30 min in Cytobuster Protein Extraction Buffer (Novagen, USA) and centrifuged at 12,000 rpm. The supernatant was collected, total protein was measured, and 50 µg was used for 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein was then transferred to a nitrocellulose (NC) membrane and sealed with Tris-buffered saline Tween-20 (TBST) containing 5% non-fat milk powder. The membrane was subsequently incubated with goat anti-human Tspan1 polyclonal antibody (1:500, sc-81545; Santa Cruz Biotechnology) at 4°C overnight. After washing with PBS, the sections were incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-rat IgG (HRP-IgG) at 37°C for 30 min and colored with 3,3'-diaminobenzidine (DAB) at room temperature. PBS was substituted for the anti-Tspan1 antibody in negative control subjects. All sections were then blindly analyzed by three experienced pathologists under a light microscope. The clinicopathologic data and patient outcomes were not known by the pathologists. The results of IHC staining were evaluated as described (13). The cases were classified into positive groups by the intensity and proportion of the immunostained cancer cells or Tspan1. The proportion of positive cells was assessed as low (5-25% cells stained), medium (25-50% cells stained), and high (>75% cells stained).

Cell culture. Human PCC cell lines AsPC-1 and PANC-1, as well as normal human pancreatic hTERT-HPNE cell lines were all purchased from the Cell Bank of the Chinese Academy of Sciences. All the above cells were cultured in specific medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% antibiotics at 37°C in a humidified incubator under 5% CO2 condition (14).

Tspan1 knockdown. According to the CDS of Tspan1 recorded in neuclopeptide, we predesigned siRNA targeting the human Tspan1 gene (gene ID, 10103) (http://RNAiDesigner.invitrogen.com). The siRNA sequences targeting Tspan1 were as follows: si-1, 5'-CCCTGCGGTCTCCTTTTT-3'; si-2, 5'-GCC TTCGTGTAC-ACCACA-3'; and si-3, 5'-GCCGCGCATC AAGAAGAT-3'. Lentivirus was packaged in 293T cells using Lipofectamine 2000 (Invitrogen) and virus titers were determined. Target cells, including AsPC-1 and PANC-1 cells, were infected with 1x106 recombinant lentivirus-transducing units in the presence of 6 µg/ml polybrene (Sigma), respectively. The efficiency of knockdown was tested by qRT-PCR and western blot analysis. All experiments were performed in triplicate.

MTT assay. Cell viability was determined using the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were plated into 96-well culture plates at an optimal density of 5x103 cells/ml in 200 µl of culture medium/well. After 24-96 h of culture, 20 µl of 5 mg/ml MTT was added to each well and incubated at 37°C for 4 h. The medium was then gently aspirated and 150 µl of dimethyl sulfoxide (DMSO) was added to each well to solubilize the formazan crystals. The optical density of each
sample was immediately measured using a microplate reader (Bio-Rad, Hercules, CA, USA) at 490 nm.

**Apoptosis assay.** A propidium iodide (PI) and Annexin V-FITC flow cytometry assay (BD Pharmingen) was used to detect the apoptosis rate in the cells after Tspan1 transfection. Briefly, 1x10^6 cells/well were cultured in 6-well plates in the absence of 10% FBS for 48 h. Adherent cells were detached with 0.25% trypsin without EDTA in 1X PBS. Cells were harvested in complete RPMI-1640 medium and centrifuged at 1,000 rpm for 5 min. Each of the cell lines was washed with 1X PBS and stained with 50 µg/ml PI and Annexin V-FITC, following the manufacturer's instructions.

**Cell migration and invasion assay.** We employed BioCoat Matrigel invasion chambers (BD Biosciences, Bedford, MA, USA) to compare the effect of Tspan1 knockdown on *in vitro* invasion of AsPC-1 and PANC-1 cells as previously described (15). Briefly, for the invasion assay, Costar Transwell 8-µm inserts were coated with 50 µg reduced serum Matrigel (BD Biosciences). Invasion chambers were coated with Matrigel, and 1x10^6 cells were added per chamber. Medium supplemented with 10% FBS was used in the lower chamber. Following incubation the cells that had invaded through the membrane were fixed and stained before the membrane was removed and mounted on a slide for microscopic assessment. Invasive cells were visualized at x40 magnification and the number of cells in five random fields was counted and an average calculated. For migration assays, the same procedure was used excluding the Matrigel. After 12 h, non-invading cells and media were removed, and cells on the lower surface of the membrane were fixed with polyoxymethylene and stained with 0.1% crystal violet (both from Sigma) for 0.5 h. Stained cells were counted under a microscope in five randomly selected fields, and the average was used to indicate cell migration and invasion. All experiments were performed in triplicate (15,16).

**Statistical analysis.** SPSS v11.5 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Data are presented as means ± standard deviation. The unpaired t-test was used for comparison between groups. Association between Tspan1 expression and other clinicopathological factors of the tumor were assessed by the Fisher’s exact test (two-sided) for categorical variables and χ^2 test were used to compare ordinal variables. The grading-related data were analyzed by Spearman’s test. A P<0.05 was considered statistically significant.

### Results

**Upregulation of Tspan1 expression in human PCC tissues.** Tspan1 staining in normal adjacent tissue was weak relative to PCC tissues. The IHC positive files of Tspan1 exhibited light yellow to brown staining (Fig. 1).

Either qRT-PCR or western blot analysis showed that the expression of Tspan1 in PCC tissue was significantly stronger than that of normal tissues (P<0.05, Fig. 1).

Spearman’s analysis showed that the Tspan1 level was correlated with the lymph node metastasis (r=0.311, P<0.05) and the pathological tumor node metastasis (pTNM) stages (r=0.295, P<0.05) in these 45 cancer samples (Table I). Within a period of 60 months of the follow-up, 15 cancer-related deaths occurred, all of the deaths come from patients with
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Tspan1 positive tumors. The 5-year survival rate of pTNM stage I is >95%, while it is <10% in patients with pTNM stage III-IV. The Kaplan-Meier estimates of overall survival rate were based on cell Tspan1 expression in the patients with...
In the entire cohort, the overall survival rate of patients with Tspan1-negative tumors was significantly higher than that in Tspan1-positive tumors (85.71 vs. 35.71%; log-rank test: $\chi^2 = 19.08$, $P = 0.0001$). The relationship between Tspan1 expression pattern (IHC staining) and survival rate was also determined. Results revealed that Tspan1 higher-expression group had significantly shorter survival than the Tspan1 lower-expression group (Tspan1-medium vs. Tspan1-high, $P < 0.05$; Tspan1-negative vs. Tspan1-high, $P < 0.05$; Tspan1-medium vs. Tspan1-negative, $P < 0.05$; Fig. 2).

**Interference expression of Tspan1 by siRNA transfection.** The PCC cell lines, AsPC-1 and PANC-1 cells, as well as the hTERT-HPNE cell stably transfected with Tspan1-siRNA-expressing vector (named as AsPC-1-Tspan1-si-1/2/3, PANC-1-Tspan1-si-1/2/3 and normal-Tspan1-si1/2/3, respectively). Control AsPC-1, PANC-1 and hTERT-HPNE cells were transfected with empty vectors. They were recorded as AsPC-1-Empty, PANC-1-Empty and normal-Empty, respectively. Tspan1 mRNA levels detected by RT-PCR were significantly lower in Tspan1-siRNA-expressed AsPC-1, PANC-1 and hTERT-HPNE cells than the matched control, respectively ($P < 0.05$). Western blot analysis found that the level of immunoreactive protein was significantly downregulated in Tspan1-siRNA-transfected cells relative to the controls cells ($P < 0.05$; Fig. 3). Stable expression of three Tspan1-siRNA (si-1, si-2 and si-3) in AsPC-1, PANC-1 and hTERT-HPNE cells resulted in >60% decrease in Tspan1 expression (Fig. 3). Considering the highest rates of inhibition of expression in Tspan1, AsPC-1-Tspan1-si-2 and PANC-1-Tspan1-si-2 were chosen as the target for further investigation.

**Effects of Tspan1 on PCC cells proliferation.** We assessed the effect of Tspan1 expression silencing on the regulation of PCC cells viability. MTT assay showed that silencing of Tspan1 expression caused significant decrease in cell viability in AsPC-1 and PANC-1 cells, but not in hTERT-HPNE cells (Fig. 4).

**Downregulation of Tspan1 induces increased apoptosis of PCC cells.** There was a significant increase in the apoptosis rate in Tspan1-siRNA-infected cells relative to empty-infected ones (Fig. 5). There were more apoptotic PCC cells in AsPC-1-Tspan1-sh2 and PANC-1-Tspan1-sh1 groups, when compared with that of AsPC-1-Empty and PANC-1-Empty groups, respectively ($P < 0.05$, Fig. 5). However, Tspan1-siRNA showed no significant effects on the apoptosis of human normal hTERT-HPNE cells ($P > 0.05$).

**Effect of Tspan1 knockdown on PDAC cell migration and invasion.** Following knockdown, we compared the migration...
of control non-transduced innocent cells (blank), non-targeting scrambled siRNA-transduced cells (negative control), as well as the Tspan1 knockdown cells transduced with Tspan1 targeting siRNA. The migration assay showed that the crystal violet stained cells significantly decreased in the Tspan1-siRNA-treated cells, compared with that of the matched WT and non-targeting siRNA control groups (P<0.01, Fig. 6A). The Tspan1 knockdown treatment significantly decreased the migration of the two cell types compared to the negative and blank control. Through the whole experimental duration, migration was not significantly different between AsPC-1 and PANC-1 blank cells and the negative control cells transduced with non-targeting scrambled siRNA, as shown in Fig. 6A.

There were significant reductions in the invasion of AsPC-1 and PANC-1 cells following Tspan1 knockdown, in comparison with that of the control cells, respectively (P<0.005, Fig. 6B). The invasion of control cells transduced with non-targeting siRNA, which had unchanged levels of Tspan1, was not significantly different from the non-transduced WT PCC cells.

Discussion

The present study focused on the possible roles of Tspan1 involved in the tumorigenic process of PCC. Our results revealed that Tspan1 was elevated in human PCC tissues and cell lines. The increased Tspan1 in PCC tissues was associated with the clinicopathological features and survival rate. Interference of Tspan1 expression by special siRNA introduction induced significant decline in proliferative capacity, increase in apoptosis and reduced migration and invasion of AsPC-1 and PANC-1 cells, compared with that of hTERT-HPNE cells. These data indicated that Tspan1 may be involved in the pathological changes and development of PCC.

Our results revealed that expression of Tspan1 in PCC cells displayed cytoplasmic patterns, which showed the distribution and functional sites of the Tspan1 molecule in cells (12). The molecule may accept extracellular signals when located on the membrane and carry out functions in the cytoplasm, like other tetraspanins such as CD9, CD82 and CD63 (12,17,18). In the present study, we found that the expression of Tspan1 in PCC tissues was significantly higher than the normal adjacent tissues.

Tetraspanins, a large family of ubiquitously expressed membrane proteins, have been identified and implicated in the regulation of cell development, differentiation, proliferation, motility and tumor cell invasion (19-21). In many human cancers, tumor progression was found to be associated with an altered expression of tetraspanins (22). Tspan1, a new member of the tetraspanins group, was found to be elevated in some tumors (12,23-25). Recent studies also suggested Tspan1 gene may play a role in the proliferation of cancer cells and be associated with cancer cell motility, implying a function of the gene in the development of various cancer (12,23,26). In this study, our results revealed that Tspan1 immunopositive staining was significantly correlated with the lymph node metastasis, pTNM stages and poor prognosis of PCC. Our data also showed that there was a significant correlation between the Tspan1 level and overall survival rate. Similarly, other reports show that there was a significant correlation between the Tspan1 expression and overall survival rate, disease stages
as well as the pathological features of other various tumors, such as colorectal cancer and cervical carcinoma (13, 23).

The present data also demonstrated that siRNA-mediated Tspan1 expression knockdown significantly inhibiting the growth, proliferation, migration and invasion of PCC cells in vitro, which was supported by earlier reports in different cancer cells (10, 12). We speculated that siRNA-mediated downregulation of Tspan1 inhibited the proliferation of PCC cells in vitro by inhibiting cell cycle progression from G1 to S phase (26). Therefore, we postulated that: i) Tspan1 overexpression status may yield poor prognosis for PCC; and ii) Tspan1 may play a critical role in the progression of tumor growth and proliferation in human PCC.

In conclusion, our results show that Tspan1 was elevated in human PCC tissues and cell lines. Interference of Tspan1 expressions by siRNA introduction induced significant decline in proliferative capacity and increase in apoptosis of AsPC-1 and PANC-1 cells. This finding suggests that Tspan1 plays an

Figure 5. Tspan1 knockdown induces apoptosis in PCC cells. AsPC-1 cells, PANC-1 and hTERT-HPNE cells were transfected with Tspan1-siRNA or empty vector as control. (A-F) The influence of silencing Tspan1 on the apoptosis of AsPC-1 cells, PANC-1 and hTERT-HPNE was detected by FCM, respectively. (G) Quantification shows that the percentage of apoptotic cells in Tspan1-siRNA-transfected group is significantly higher compared to that in the Empty control group, except that in hTERT-HPNE cells. Values plotted are means ± SD (n=3). Tspan1, tetraspanin 1; PCC, pancreatic cancer; FCM, flow cytometry.
important role in PCC progression, and siRNA targeting of Tspan1 may be a potential therapeutic strategy for the treatment of PCC. Identifying the patients with high-risk PCC by Tspan1 expression detection would be of great benefit for improving treatment strategies.

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References