Original Article

TSPAN13 is overexpressed in ER-positive breast cancers and contributes to tumor progression

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Abstract: Tetraspanin-13 (TSPAN13) is a largely uncharacterized member of the tetraspanin superfamily and it plays an important role in the process of human tumorigenesis. In this study, the mRNA levels of TSPAN13 in human normal and breast cancer tissues were analyzed and found to be upregulated by using public Oncomine microarray datasets. Therewith, lentivirus-mediated shRNA was employed to silence TSPAN13 expression in ZR-75-30 breast cancer cells to study the phenotypic and molecular changes. We found that suppression of TSPAN13 expression significantly reduced the growth rate of breast cancer cells in vitro by MTT and colony formation assays. Cell cycle arrest at the G0/G1 phase was monitored by flow cytometry. Downstream signaling study revealed that TSPAN13 knockdown downregulated Bcl-2, p-Akt, and p-mTOR expressions and promoted Caspase-3 cleavage. In a word, our data provided novel and compelling evidences that TSPAN13 might be an anti-cancer target against breast cancer.

Keywords: TSPAN13, breast cancer, shRNA, cell proliferation, cell cycle

Introduction

Breast cancer (BC) is currently the most frequently diagnosed cancer and the leading global cause of cancer-related death in women, accounting for 23% of cancer diagnoses (1.38 million women) and 14% of cancer deaths (458,000 women) each year [1]. Although the five-year relative survival rate for BC in women has improved from 63% in the early 1960s to 90% currently, BC survivors still have a far high risk of recurrence [2]. Gene expression profiling can understand and identify different subsets of genes with differential expression during cancer progression [3]. This understanding has led to gene-targeted therapies against breast cancer.

Tetraspanin-13 (TSPAN13) gene, which located on chromosome 7p21.1, encodes a 204 amino acid membrane protein that belongs to the transmembrane 4 superfamily of proteins, also known as the tetraspanin family. Based on protein sequence similarities, there are at least 33 putative family members in human characterized by their four transmembrane regions, two extracellular and one intracellular loops, and two short N- and C-terminal cytoplasmic domains [4]. Although the function of most members of the family is currently unknown, a similar feature of the tetraspanin is their potential to associate with other transmembrane proteins forming the so-called tetraspanin web [5, 6]. Tetraspanin-enriched micro-domain scan provides a scaffold for the transmission of external stimuli to intracellular signaling components [7]. In addition, it has been reported that tetraspanins are involved in a variety of cellular processes as diverse as cell migration, intracellular trafficking and also play an increasingly prominent role in the pathogenesis of human neoplasia [8, 9]. However, the exact biological function of TSPAN13 in breast cancer cells is still unclear.

Through a lentivirus shRNA library-based screening, we identified TSPAN13 gene as a putative oncogene in breast cancer cells. To further study the function of TSPAN13, we conducted a data mining by using the public Oncomine microarray database to compare the expression pattern of TSPAN13 in normal and breast cancer tissues. Meanwhile, the effects of TSPAN13 silencing were studied in ER positive ZR-75-30...
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breast cancer cells, and the downstream signaling molecules were determined.

Materials and methods

Data mining of oncomine gene expression microarray datasets

To determine the expression of TSPAN13 in breast cancers, three datasets (Curtis Breast, TCGA Breast, and Ma Breast 4) in Oncomine database (https://www.oncomine.org) were studied. First, to present the differential expression of TSPAN13 between breast cancer and normal tissues, a combination of filters was applied to show the corresponding datasets. The Analysis Type was defined as Cancer vs. Normal Analysis, and Data Type was mRNA. Each dataset revealed by the filters could be analyzed separately. Curtis et al. (EGA accession EGAS000000000083) [10], The Cancer Genome Atlas-Invasive Breast Carcinoma Gene Expression Data (TCGA, http://tcga-data.nci.nih.gov/tcga/) and Ma et al. (GEO accession GSE14548) [11] were used to compare TSPAN13 expression levels between different types of breast cancer and normal tissues. The log-transformed and normalized expression values of TSPAN13 were extracted, analyzed and read from the bar chart.

Cell lines

Human breast cancer cell line ZR-75-30 and human embryonic kidney cell line 293T were used in this study. They were all purchased from the Shanghai Cell Bank of Chinese Academy of Sciences. ZR-75-30 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS; Biowest, France). 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Hyclone) with 10% FBS.

Lentivirus production and transfection

We designed one shRNA (5'-GTCCGAAGTGT-TAACCCAAACTCGAGTTGGTTAACACTTGC-GAACTTTTTT-3') targeting the TSPAN13 (NM_014399.3) and one negative control (5'-TTCTCCGAACGTGTCACGT-3') targeting the TSPAN13. They were cloned into the lentiviral expression vector pLV-G418 (Shanghai Hollybio, China), respectively and confirmed by DNA sequencing. The reconstructed pLV-G418 and pLV-G418 were then co-transfected into 293T cells together with the helper plasmids pCMVΔR8.92 (Shanghai Hollybio) using Lipofectamine 2000 (Invitrogen, USA) to generated lentiviruses, according to the manufacturer's instructions. After 48 h of incubation, supernatant was collected and lentiviral particles were harvested by high-speed centrifugation at 12000×g for 10 min in order to remove cell debris.

For lentiviral transfection, ZR-75-30 cells (5 × 10⁶ cells/well) were seeded into 6-well plates and transduced with lentiviruses containing TSPAN13 shRNA (shTSPAN13) and control shRNA (shCon) at a multiplicity of infection (MOI) of 35, respectively. The infection efficiency was determined by assessing GFP-expressing cells under fluorescence microscopy 96 h after infection.

Quantitative PCR

Total RNA was isolated from ZR-75-30 cells after 6 d of transfection using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The quality of RNA was examined by A260 absorption, and then 1 μg of total RNA were used for first-strand DNA synthesis by M-MLV cDNA synthesis kit (Promega, USA). Real-time PCR was performed in triplicate on a Bio-Rad Connect Real-Time PCR platform using GoTaq Kit qPCR Green (Bio-Rad, USA). Each PCR reaction mixture contained 10 μl of 2 × SYBR premix ex taq, 0.8 μl of forward and reverse primers (2.5 μM), 5 μl of cDNA and 4.2 μl of ddH₂O. The condition for PCR was run for 40 cycles with initial denaturation at 95°C for 1 min, denaturation at 95°C for 5 s and extension at 60°C for 20 s. The primers were synthesized by Sangon (China): forward primer 5'-CCTCTAGCCAGCAACCTTCC-3' and reverse primer 5'-GACCCTCCACCCTCCCTCAG-3' for TSPAN13; forward primer 5'-GTGGACATCCGCAAAGAC-3' and reverse primer 5'-AAAGGGTGTAACGCAACTA-3' for β-actin used as the reference. The relative mRNA expression levels were calculated using 2^ΔΔCt method.

Western blot

At 6th day after Lv-shRNA infection, approximately 3 × 10⁶ cells were homogenized with lysis buffer (RIPA; Beyotime, China) containing 1 mM of phenylmethylsulfonyl fluoride (PMSF; Beyotime) on ice. Protein lysates were dissolved in 2 × SDS sample buffer (100 mM Tris-
HCl, pH 6.8, 10 mM EDTA, 4% SDS, 10% glycine). 30 μg total proteins were loaded in each lane on the 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and electrophoresed at 50 V for 3 h. The gel was transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA) at 300 mA for 1.5 h. After being blocked in 5% nonfat milk, the membranes were incubated with rabbit anti-TSPAN13 antibody (1:1000, Proteintech, USA, number 13570-1-AP), Caspase 3 (1:500, Cell Signaling, USA, number 9661), Bcl-2 (1:1000, Cell Signaling, USA, number 2876), p-Akt (1:1000, Cell Signaling, USA, number 13038), p-mTOR (1:500, Cell Signaling, USA, number 5536), mTOR (1:1000, Cell Signaling, USA, number 2983), ERK (1:1000, Santa Cruz, USA, number sc-154), p-ERK (1:1000, Cell Signaling, USA, number 4370) and mouse anti-Akt (1:1000, Santa Cruz, USA, number sc-55523) overnight at 4°C and then with HRP-labeled anti-rabbit (1:5000, Santa Cruz, USA, number SC-2054), HRP-labeled anti-mouse (1:5000, Santa Cruz, USA, number SC-2005) secondary antibody for 2 h at room temperature. As for Tubulin, the membranes were incubated with anti-Tubulin-HRP antibody (1:6000, Proteintech, number HRP66031) for 2 h at room temperature. Finally, all the blots were visualized by super ECL detection reagent (Beyotime).

**MTT assay**

After 96 h of lentiviral treatment, three different groups (shTSPAN13, shCon and control) were collected and seeded into 96-well plates at a density of 2 × 10^3 cells per well and then incubated at 37°C for 1, 2, 3, 4 or 5 d. At indicated time points, 20 μl of 5 mg/ml 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT; Sigma, USA) solution was added to each well for 4 h. Acidic isopropanol (10% SDS, 5% isopropanol and 10 M M HCl) was added to stop the reaction and measured with an ELISA reader (Bio-Rad) at a wavelength of 595 nm. Each experiment was repeated three times and performed in quintuplicate.

**Colony formation assay**

Lentivirus-transduced cells for four days were seeded into six-well plates in triplicate at an initial concentration of 500 cells per well. The culture media was changed every two days. After ten days of culturing, the cells were washed and fixed in 4% paraformaldehyde. The fixed cells were stained with Crystal Violet Staining Solution (Beyotime) for 5 min. After rinsed with distilled water three times, colonies with more than 50 cells were counted under light/fluorescence microscopy.

**Cell cycle analysis**

The cell cycle distribution (G0/G1, S or G2/M phase) was analyzed by flow cytometry using propidium iodide (PI) staining. After 96 h of transfection, ZR-75-30 cells were reseeded in 6-cm dishes at a density of 2 × 10^6 cells/dish and cultured for 40 h to 80% confluence. Then the cells were harvested, washed with ice cold PBS and fixed in 75% ethanol at 4°C overnight. The fixed cells were resuspended in PI/RNase/PBS buffer (Beyotime) for incubation in dark (37°C, 60 min). Next, samples were detected by flow cytometer.

**Statistical analysis**

GraphPad Prism 5 (GraphPad Prism software, La Jolla, CA, USA) was used for data analysis. All data were presented as mean ± standard deviation (SD) of three independent experiments. The statistical analysis used Student’s t-test and P < 0.05 was considered to be statistically significant.

**Results**

TSPAN13 mRNA was significantly upregulated in breast cancers

The expressions of TSPAN13 mRNA levels in human breast cancer were investigated using data-mining of the publicly available Oncomine microarray gene expression datasets (www.oncomine.org). We found that TSPAN13 expression was significantly elevated in tubular breast carcinoma (n = 67, P = 5.19E-27), invasive ductal and invasive lobular breast carcinoma (n = 90, P = 1.43E-26), mucinous breast carcinoma (n = 46, P = 6.78E-15) and invasive lobular breast carcinoma (n = 148, P = 9.69E-33) comparing with normal breast tissues as revealed in Curtis Breast dataset; and was slightly upregulated in most types of other breast cancer types, including breast carcinoma (n = 14), ductal breast carcinoma in situ (n = 10), invasive breast carcinoma (n = 21), invasive ductal breast carcinoma (n = 1,556), except medullary breast carcinoma (n = 32) (Figure 1A). But the expression between the benign breast tumors and...
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Figure 1. TSPAN13 mRNA expression in human breast cancers using the Oncomine database. A. Gene expression of TSPAN13 was revealed in the normal breast tissues, various Breast Carcinoma tissues by using the Curtis Breast dataset from Oncomine database, including tubular breast carcinoma (P = 5.19E-27), invasive...
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and normal breast tissue showed no significant difference.

Likewise, expression of TSPAN13 was also significantly higher in the invasive lobular breast carcinoma tissues than the normal breast tissues by using TCGA Breast dataset (Figure 1B, n = 36, P = 1.84E-9). Furthermore, the above results were similar to another independent dataset, Ma Breast 4, where TSPAN13 was up-regulated in two types of ductal breast cancers (n = 9, P = 0.004) comparing with normal breast tissues (Figure 1C). It is noteworthy that the expression level of TSPAN13 and estrogen receptor is negatively correlated (Figure 1D). These data which showed a consistent upregulation of TSPAN13 expression in breast cancers suggested that TSPAN13 played a potential carcinogenesis in breast, possibly in estrogen receptor-positive breast cancer.

Effective knockdown of TSPAN13 by shRNA in ZR-75-30 cells

To identify the function of TSPAN13 in breast cancer cells, shTSPAN13 and shCon were used to infect ZR-75-30 cells respectively. The expression of GFP showed the high percentage by transduction of lentiviruses (Figure 2A), suggesting that the lentivirus infections are successful and highly efficient. Silencing efficiency was determined by quantitative PCR and Western Blot analysis. Compared with shCon group, endogenous TSPAN13 mRNA was significantly reduced (Figure 2B) by almost up to 99.3% and its protein level was also decreased
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(Figure 2C) in the shTSPAN13 cells, indicating lentivirus-mediated shRNA could efficiently suppress the expression of endogenous TSPAN13 in ZR-75-30 cells.

Knockdown of TSPAN13 inhibited cell proliferation in ZR-75-30 cells

To examine the effect of TSPAN13 for breast cell growth, cell proliferation was performed following shRNA treatment for 5 days using MTT assay. The growth curve of shTSPAN13 infected cells declined from the third day, compared to non-infected cells and shCon infected cells (Figure 3A). On the fifth day, the difference was significantly wider (**P < 0.001), manifesting knockdown of TSPAN13 could remarkably inhibit the cell proliferation in ZR-75-30 cells. Furthermore, an assay of colony formation was performed for ZR-75-30 cells to determine the role of TSPAN13 in tumourigenesis. The results showed the size of single colony of shTSPAN13 transfected cells was much smaller than control's (Figure 3B) and the number of colonies in the shTSPAN13 group was apparently lower than in the shCon group (5 ± 1 versus 329 ± 10, P < 0.001; Figure 3C), strongly demonstrating that reduced TSPAN13 levels might inhibit...
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Knockdown of TSPAN13 induced cell cycle arrest in ZR-75-30 cells

The influence of TSPAN13 silencing on cell cycle was analyzed using a flow cytometer. The cell cycle distribution of TSPAN13 shRNA-infected cells was different from that in the shCon group (Figure 4A). The cell proportion in G0/G1 phase was significantly increased from 57.31% in the shCon group to 77.25% in the shTSPAN13 group (P < 0.001), meanwhile, the cell population of the S phase showed a compensatory decrease (P < 0.001) compared to shCon infected cells (Figure 4B). Taken together, the results indicated that TSPAN13 knockdown seriously affected the cell cycle distribution of ZR-75-30 cells and caused cell cycle arrest at G0/G1 phase.

Knockdown of TSPAN13 altered the downstream signaling molecules in ZR-75-30 cells

To explore the function of TSPAN13 in breast cancer cells, we screened for several cellular proteins in signaling pathways after TSPAN13 knockdown in ZR-75-30 cells. Compared with shCon group, knockdown of TSPAN13 in ZR-75-30 cells resulted in downregulation of p-ERK (Thr202/Tyr204), p-mTOR (Ser2448), p-Akt (Thr308) and BCL-2 and upregulation of cleaved caspase-3 (Figure 5). These results indicated an underlying mechanism how TSPAN13 knockdown affected the proliferation and malignant transformation of ZR-75-30 breast cancer cells.

Discussion

Treatment of early-stage breast cancer requires a multimodality approach to eradicate residual cancer and prevent recurrent disease. Targeting the pathways that promote or sustain growth and invasion of carcinoma cells is critical to the effective treatment of breast cancer [12]. Targeting the estrogen receptor (ER) is the oldest molecular targeted therapy, and widespread use of the selective ER modulator tamoxifen in breast cancer is responsible for major improvements in cure rates, quality of life and disease prevention during the past 25 years. In breast cancer, the expression of the tetraspanin superfamily, especially the third tetraspanin family member of TSPAN13 [13], is related to estrogen receptor level and human epidermal growth factor receptor tyrosine kinase 2 (HER2) status. However, its function has not been fully understand.

Oncomine is an unique biomedical research community which collects, standardizes, analyzes and delivers cancer transcriptome data for the public [14]. In the present study, overexpression of TSPAN13 in breast cancer was proved by data mining of three independent microarray datasets in the Oncomine database, with a sample sizes of 2,136 (Curtis Breast), 97 (TCGA Breast), 66 (Ma Breast 4), respectively. The results suggested that TSPAN13 might function as a proto-oncogene in breast cancers. In consistent with our findings, it has also been reported that TSPAN13 is significantly overexpressed in endometrioid adenocarcinomas of the ovary [15], testicular germ cell tumors [16, 17], bladder carcinoma [18], B-cell acute lymphoblastic leukemia [19] and prostate cancer [20], implying a role for TSPAN13 in the carcinogenesis or malignant transformation of tumors.

Interestingly, Curtis Breast dataset also indicated that TSPAN13 was upregulated in ER positive breast cancers compared to normal breast tissues, whereas downregulated in ER negative breast cancers compared to ER positive breast cancers (Figure 1D). The data was in a certain part in consistent with the findings reported by Huang et al., showing that TSPAN13 was negatively correlated with ER/HER-2 expression [13, 21]. However, unfortunately, they did not study the expression of TSPAN13 in normal breast tissues or normal breast cell lines.
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And although ER/HER-2 deficiency is normally found in advanced breast cancers, negative correlation of TSPAN13 to ER/HER-2 does not necessarily support the proposal made by Huang et al. that TSPAN13 is a tumor suppressor in breast cancer, as the occurrence and development of tumor is a complex process. On the contrary, large population based analysis through oncomine data mining should give a more objective view of how TSPAN13 functions in breast cancer.

Certain genes may switch between oncogene or tumor suppressor under certain circumstances. For example, it is well known that TGF-β is not only a tumor suppressor gene but also a cancer-promoting gene. TGF-β can inhibit tumor cell proliferation via inducing the expression of 4EBP1 [22] and Cyclin-dependent kinase (CDK) inhibitor (p15, p21 and p57) [23, 24] and real-ize the spread of the tumor cells by induced the expression of transcription factors such as Snail1/2, ZEB1/2 and HMG2 to obtain a migration and invasion phenotype [25]. Accordingly, TSPAN13 might not simply be an oncogene or a tumor suppressor, it’s function might switch in a certain stage of the tumorigenesis, e.g., depending on ER or HER states.

To study why TSPAN13 was upregulated in ER positive breast cancers, we use the ZR-75-30 ER-positive cell line for endogenous TSPAN13 study. TSPAN13 knockdown led to G0/G1-phase arrest and growth inhibition. The expression levels of phosphorylated p-ERK, p-mTOR, and pAkt were found to be decreased upon TSPAN13 knockdown. These results indicated an important role of TSPAN13 in maintaining breast cancer cell survival. Meanwhile, Bcl-2, the anti-apoptotic protein was reduced, whereas cleaved Caspase 3 was increased in ZR-75-30 cells after TSPAN13 silencing, suggesting activation of the intracellular apoptotic pathway. These results add the evidence that depletion of TSPAN13 could suppress ZR-75-30 cancer cell growth via cell cycle arrest and apoptosis.

In conclusion, our study provides more insight into the role of TSPAN13 in breast cancer. The above data suggested that TSPAN13 is a critical molecular in breast cancer. TSPAN13 mediates cell survival through cell-cycle arrest and apoptosis. TSPAN13 promotes breast cancer growth by regulating intracellular ERK, mTOR, Akt, Bcl-2 and Caspase 3 signaling pathways. Therefore, TSPAN13 might be a biomarker and therapeutic target for breast cancer.

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Disclosure of conflict of interest

None.

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