A Positive Tetraspanin 8 (TSPAN8)/β-Catenin Regulatory Loop Enhances the Stemness of Colorectal Cancer Cells

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Background: The expression of TSPAN8 (tetraspanin 8) is upregulated in colorectal cancer; however, its roles in colorectal cancer progression are never been revealed. This work aimed to investigate TSPAN8 effects and the molecular basis in regulating colorectal cancer stemness.

Material/Methods: Real-time quantitative polymerase chain reaction and western blot analysis were used to detect the expression of TSPAN8 expression in clinical samples and the expression of stemness genes in colorectal cancer cells. Sphere forming analysis was performed to detect TSPAN8 effects on sphere forming ability of colorectal cancer cells. Co-IP and ChIP analysis were performed to confirm the molecular basis contributing to TSPAN8-mediated effects on colorectal cancer stemness.

Results: TSPAN8 expression is increased in colorectal cancer tissues. Knockdown of TSPAN8 reduced the expression of stemness genes and sphere forming capacity in colorectal cancer cells. Mechanistically, TSPAN8 directly interacted β-catenin and enhanced its protein expression, which is necessary for TSPAN8-mediated effects on colorectal cancer stemness. Conversely, β-catenin directly bound to TSPAN8 promoter and enhanced TSPAN8 transcription.

Conclusions: TSPAN8 promotes colorectal cancer stemness through a positive TSPAN8/β-catenin regulatory loop.

MeSH Keywords: Colorectal Neoplasms • Neoplastic Stem Cells • Wnt Signaling Pathway

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Background

As one of the most common cancers threatening human life, the recurrence and metastasis of colorectal cancer seriously affect the prognosis of patients [1]. In recent years, the rise of cancer stem cell (CSC) theory provides a new way of thinking for cancer treatment [2]. In colorectal cancer, colorectal CSCs not only participate in the recurrence and metastasis of cancer, but also cause the chemoresistance [3]. Because CSC growth depends on the role of multiple signaling pathways, targeting these signaling pathways will become an important research direction for radical treatment of colorectal cancer in the future. However, the pathways contributing to colorectal CSC progression are still confused.

TSPAN8 (tetraspanin 8) is involved in tumor metastasis, for example, TSPAN8 promotes glioma cell migration through forming a complex with rictor and integrin α3 to mediate mTORC2 activation [4]; TSPAN8 promotes gastric cancer metastasis via activating ERK/MAPK pathway [5]; TSPAN8 is necessary for transcriptional factor AEG-1-mediated metastasis and promotes metastasis in hepatocellular carcinoma cells [6,7]; TSPAN8 enhances breast cancer metastasis accompanied by increased circulating extracellular vesicles [8]. Notably, a recent study showed that TSPAN could facilitate the stemness of breast cancer cells [9]. Additionally, a previous study showed that TSPAN8 could distinguishes spermatogenic stem cells in the prepubertal mouse testis [10]. As CSCs play a critical role in tumor metastasis and evolution process is similar in normal stem cells and CSCs [2], we assume that TSPAN8 might regulate tumor metastasis through modulating CSC progression. Importantly, TSPAN8 expression is increased in colorectal cancer tissues and regarded as a blood marker of cancer stem cell (CSC) theory provides a new way of thinking for cancer treatment [2]. In colorectal cancer, colorectal CSCs not only participate in the recurrence and metastasis of cancer, but also cause the chemoresistance [3]. Because CSC growth depends on the role of multiple signaling pathways, targeting these signaling pathways will become an important research direction for radical treatment of colorectal cancer in the future. However, the pathways contributing to colorectal CSC progression are still confused.

Material and Methods

Tissue samples and cell lines

The colorectal cancer and corresponding adjacent tissues were collected from the First Affiliated Hospital of Nanchang University from July 2015 to August 2018. Colorectal cancer cell lines SW620, HCT-116, HT-29, SW480, SW1116, CT-26, and normal colon epithelial cell line NCM460 were purchased from BeNa Culture Collection (Beijing, China). Cell lines were maintained in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) with 10% FBS (fetal bovine serum, Thermo Fisher Scientific) at 37°C with 5% CO₂.

Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted by TRIzol and then cDNA was synthesized according to Reverse Transcription System. The concentration and purity of RNA solution were determined by ultraviolet spectrophotometer. Ribosome 18s rRNA was used as an internal reference. The amplification conditions were as follows: 95°C 3 minutes, 95°C 30 seconds, 58°C 30 seconds and 72°C 30 seconds. The relative expression of genes was calculated in 35 cycles using 2⁻ΔΔCT method. The result specificity of PCR amplification was detected by 2% agarose gel electrophoresis. The qPCR was carried out on a Step-one Plus system.

Western blot

After 30 minutes of cell lysis on ice, 12 000 rpm, centrifugation at 4°C for 15 minutes, the supernatant was taken, and the total protein of each group was quantified by BCA Quantitative Kit. The protein samples were separated by sodium lauryl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein was transferred to the polyvinylidene difluoride (PVDF) membrane and added with the primary antibodies (1: 1000). After incubating overnight at 4°C, the membrane was washed 5 times the next day with TBST, 5 minutes each time. After the incubation at room temperature for 2 hours with the secondary antibody (1: 1000) and the membranes were washed 10 times with TBST for 5 minutes each time. Electrochemiluminescence (ECL) imaging was performed.

Lentivirus vector package

293T cells in logarithmic growth phase were digested and counted. The cells were inoculated in 10 cm-cell culture dishes, according to the volume of 5×10⁶/disk. The serum-free medium was replaced at 37°C and 5% CO₂ incubator to 70% to induce cells to grow. The empty plasmid and the 3 aforementioned packaging plasmids (pMD2.G, pCMV-VSV-G, pRSV-Rev) into 293T cells. At the same time, empty plasmids and the 3 aforementioned packaging plasmids were co-transfected into 293T cells (control lentivirus). After 6 hours of incubation in a 37°C, 5% CO₂ incubator, the complete medium was replaced. After 72 hours, the supernatant was collected as the viral solution. The supernatant was centrifuged for 10 minutes at 4°C and 3000 g centrifugal force, and then the supernatant was collected. The supernatant after centrifugation was filtered by a 0.45 µm filter and stored in a refrigerator at –80°C after aseptic packing. The lentivirus plasmids were denoted as Len-TSPAN8-kd and Len-β-catenin-oe, respectively.

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**Spheroid forming analysis**

In order to obtain spherical HT-29 cells, basic fibroblast growth factor (bFGF, 20 ng/mL, PeproTech), epidermal growth factor (EGF, 20 ng/mL, PeproTech), insulin (Sigma-Aldrich, 5 g/mL, Sigma-Aldrich) and 0.4% BSA were added to HT-29 cells cultured with the serum-free DMEM/F12 medium (Invitrogen Company) with 0.02×B27 additive (Invitrogen Company) in the super-low adherent 6-well plates at a cell density of 10^5 cells/mL. Then 1.0 mL of culture medium was supplemented every 3 days. After 9 days of culture, spherical cells were photographed and counted.

**Co-immunoprecipitation (Co-IP)**

According to the instructions of Pierce®Direct IP Kit 26148 kit, 20 μL Amino Link Plus Resin and Pierce Control Agarose Resin were incubated with 4 μg TSPAN8 or β-catenin antibody for 90 minutes, and 500 μL protein samples were added. After incubating overnight at 4°C, the above samples were used for 10% SDS-PAGE electrophoresis and western blot. Then 1:1000 diluted TSPAN8 or β-catenin antibody were added, respectively. After 3 times of TBST washing, the samples were incubated at 4°C overnight. The second antibody labeled with HRP was added and incubated at room temperature for 1 hour. After 3 times of TBST washing, ECL imaging was performed.

**Luciferase reporter analysis**

At 24 hours before transfection, the cells in the logarithmic growth were inoculated into a 48-well plate at 2×10^4/well concentration. Topflash (a responsive vector of β-catenin signaling) or FOPflash (a non-responsive vector of β-catenin signaling) and the PRL-TK plasmid (Sea Kidney Luciferase Expressed by pRL-TK) were co-transfected into cells using Lipofectamine 2000 transfection reagent. For validating the promoter activity of TSPAN8 promoter, the promoter sequences of TSPAN8 with or without β-catenin binding sites were inserted into pGL3-promoter vector, named as pGL3-TSPAN8 and pGL3-TSPAN8-mut. The transfection procedure is same with the aforementioned process. After 72 hours of transfection, the luciferase activity was detected by Promega double luciferase detection kit. The results were standardized by comparing with the activity of PRL-TK plasmid.

**Figure 1.** TSPAN8 expression is upregulated in colorectal cancer and negatively correlated with patient overall survival. (A) the qPCR assay on TSPAN8 expression in colorectal cancer and corresponding normal tissues. (B) Western blot analysis on TSPAN8 expression in colorectal cancer and corresponding normal tissues, the represented pictures are shown. (C, D) TSPAN8 expression was examined in colorectal cancer and normal colon epithelial cells. (E) Online datasets analysis on the correlation between TSPAN8 expression and the overall survival of colorectal cancer patients. **P<0.01.
Statistical analysis

SPSS 3.0 software was used for data analysis. The measurement data were expressed by mean ± standard error of the mean (SEM) and compared by t-test. The difference was statistically significant with \( P < 0.05 \).

Results

**TSPAN8 expression is upregulated in colorectal cancer and negatively correlated with patient overall survival**

First, TSPAN8 expression was detected in colorectal cancer tissues and normal adjacent tissues. It was shown that TSPAN8 expression was upregulated in colorectal cancer tissues compared with that in normal adjacent tissues (Figure 1A, 1B). A consistent result was obtained in colorectal cancer cells and normal colorectal epithelial cells (Figure 1C, 1D). Additionally, TSPAN8 expression was negatively correlated with the overall survival of colorectal cancer patients using the online bioinformatics dataset analysis (Figure 1E).

**Knockdown of TSPAN8 reduces the stem cell-like traits of colorectal cancer cells**

We chose HT-29 cells as the research object since TSPAN8 expression displayed the highest level in HT-29 cells. The knockdown efficiency of TSPAN8 was validated via qPCR and western blot assays (Figure 2A, 2B). It was found that TSPAN8 knockdown decreased the expression of stemness genes (Figure 2C, 2D). Notably, knockdown of TSPAN8 attenuated the spheroid size and number formed by HT-29 cells (Figure 2E, 2F). Collectively, these effects demonstrate that TSPAN8 promotes the stemness of colorectal cancer cells.

**TSPAN8 directly interacts with \( \beta \)-catenin and enhances its protein expression**

Then the signaling onsets contributing to TSPAN8-induced effects were explored. Since TSPAN8 has been confirmed to interact with \( \beta \)-catenin in other microenvironment and \( \beta \)-catenin-mediated signaling is an important signaling contributing to CSC progression, we wonder whether this TSPAN8/\( \beta \)-catenin axis exists in HT-29 cells. As shown in Figure 3A, TSPAN8 knockdown decreased the luciferase activity of TOP\(_{\text{flash}}\) reporter but the nonresponsive FOP\(_{\text{flash}}\) control was unaffected. Indeed, TSPAN8 directly interacted with \( \beta \)-catenin in HT-29 cells through Co-IP assay (Figure 3B). Additionally, TSPAN8 knockdown decreased the expression of \( \beta \)-catenin and its downstream effectors (c-Myc, AXIN2, and cyclin-D1), which was rescued by \( \beta \)-catenin overexpression (Figure 3C, 3D).
-catenin directly binds to TSPAN8 promoter and enhances its transcription

Notably, we found that TSPAN8 promoter holds the potential binding sites of β-catenin through bioinformatics prediction (Figure 4A). We speculate that whether β-catenin could conversely regulate TSPAN8 expression by directly binding to TSPAN8 promoter. As expected, luciferase reporter analysis indicated that overexpression of β-catenin promoted the promoter activity of TSPAN8, while the promoter activity with β-catenin mutated binding sites was unaltered (Figure 4B). Additionally, ChIP assay revealed that β-catenin directly bound to TSPAN8 promoter region (Figure 4C). Indeed, overexpression of β-catenin increased TSPAN8 expression in HT-29 cells (Figure 4D, 4E).

TSPAN8 increases the stem cell-like traits of colorectal cancer cells through β-catenin

Finally, we investigate whether TSPAN8 regulates the stemness of colorectal cancer cells through β-catenin. β-catenin was overexpressed in HT-29 cells with TSPAN8 knockdown, the overexpression efficiency of β-catenin was confirmed by western blot (Figure 5A). As expected, the decreased expression of stemness genes led by TSPAN8 knockdown was partially reversed by β-catenin overexpression (Figure 5B, 5C).

**Figure 3.** TSPAN8 directly interacts with β-catenin and enhances its protein expression. (A) Luciferase reporter analysis on the TOPflash and FOPflash in HT-29 cells with or without TSPAN8 knockdown. (B) Co-IP analysis on the TSPAN8-β-catenin interaction in HT-29 cells. (C, D) The qPCR and western blot analysis on the expression of c-Myc, AXIN2, and cyclin-D1 in HT-29 cells with or without TSPAN8 knockdown as well as β-catenin overexpression. **P<0.01.
Control Len-β-catenin-oe

Figure 4. β-catenin directly binds to TSPAN8 promoter and enhances its transcription. (A) The diagram of the wild-type and mutant binding sites of β-catenin on the promoter region of TSPAN8. (B) Luciferase reporter analysis on pGL3-TSPAN8 and pGL3-TSPAN8-mut in HT-29 cells infected with or without len-β-catenin-oe. (C) ChIP analysis on TSPAN8 promoter abundance in HT-29 cells with or without β-catenin overexpression. (D, E) TSPAN8 expression was detected in HT-29 cells with or without TSPAN8 knockdown. ** P<0.01.

Discussion

Colorectal cancer is one of the most common malignant tumors of digestive tract. It ranks among the most common malignant tumors in the world. Every year, there are more than 1 million new cases, showing an increasing trend. In recent years, with the changes of people’s lifestyle and diet structure, the incidence of colorectal cancer tends to be younger.

In consistent, β-catenin overexpression rescued the inhibition of TSPAN8 knockdown on spheroid forming ability, characterized as the recovery of sphere size and number (Figure 5D, 5E). As a result, our results suggest that TSPAN8 positively regulates the stemness of colorectal cancer cells through interacting with β-catenin.

Recurrence, metastasis and drug resistance of colorectal cancer are leading to its death. The study of cell-specific markers of colorectal cancer and related signaling pathways will help to elucidate the mechanism of colorectal cancer occurrence and development, and it will provide new ideas for targeted therapy of colorectal cancer.

Although the promoting roles of TSPAN8 have been elucidated before, in the current study, it is first shown that TSPAN8 expression is negatively correlated with the overall survival of colorectal cancer patients. Additionally, TSPAN8 expression is significantly increased in colorectal tissues and cells. Since CSCs have been considered as the origin of tumor progression, we focus on TSPAN8 roles in regulating colorectal cancer stemness. As expected, we found that knockdown of TSPAN8 reduces the expression of stemness genes and spheroid forming ability.
Furthermore, we explored the underlying mechanisms contributing to TSPAN8-mediated effects. As the TSPAN8-β-catenin interaction was identified on other tumors before [14] and β-catenin-induced signaling is a critical signaling during CSC progression [15], we wondered whether this TSPAN8-β-catenin interaction exists in colorectal cancer. Additionally, although the TSPAN8-β-catenin interaction has been confirmed before, it is unclear whether TSPAN8 directly binds to β-catenin. Indeed, it was found that TSPAN8 directly binds to β-catenin and increased its expression and transcription activity via Co-IP and luciferase reporter analysis. This work supplements aforementioned previous report. Conversely, further luciferase reporter assay was constructed and indicated that β-catenin could directly bind to TSPAN8 promoter and increase its expression. Finally, our results demonstrated that TSPAN8 regulates the stemness of colorectal cancer cells dependent on β-catenin expression. Collectively, these results suggest a TSPAN8-β-catenin interaction in regulating colorectal CSC progression. However, we recommend further in vivo experiments should be conducted to confirm this conclusion. Notably, it is still unclear whether β-catenin could promote colorectal cancer stemness dependent on TSPAN8.

**Conclusions**

This work identifies a novel TSPAN8-β-catenin interaction during colorectal CSC progression, and provides a potential target for colorectal cancer therapy.
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