Transmembrane protein TMEM119 facilitates the stemness of breast cancer cells by activating Wnt/β-catenin pathway

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ABSTRACT

The effects of transmembrane protein 119 (TMEM119) on breast cancer progression have not been elucidated. This study aims to investigate the roles of TMEM119 in breast cancer progression. Clinical samples and online datasets were used to determine TMEM119 expression and its correlation between patients' survival. Wound healing, transwell invasion, mammary spheroid formation, and ALDH activity were performed to detect the effects of TMEM119. RNA-sequencing, Luciferase report analysis, Co-IP, and ChIP analysis were constructed to reveal the underlying mechanisms. We found that TMEM119 was highly expressed in breast cancer tissues and cells compared to that in normal tissues and cells. Additionally, TMEM119 expression was negatively correlated with the survival of breast cancer patients. TMEM119 knockdown reduced the expression of stemness markers, mammary spheroid-formation ability and ALDH activity. RNA-sequencing analysis indicated that Wnt/β-catenin signaling was enriched in cells with TMEM119 overexpression. Further co-IP experiments indicated that TMEM119 interacted with β-catenin and maintained its protein stability. Conversely, β-catenin directly bound to TMEM119 gene promoter and thus increased TMEM119 transcriptional activity and its expression. Finally, we demonstrated that TMEM119-mediated effects depended on Wnt/β-catenin signaling. Thus, this work reveals a novel TMEM119-β-catenin positive feedback loop essential for breast cancer cell stemness.

Introduction

Breast cancer exhibits the highest incidence rate in female malignant tumors and has a poor radical [1,2]. Cancer stem cells (CSCs) have the capability of infinite proliferation and upgradation [3]. An increasing number of results have elucidated that CSCs are critical for the occurrence, recurrence, and metastasis of tumors [3,4]. Thus, the study of breast CSCs and the surface markers can fundamentally facilitate the understanding of the pathogenesis of breast cancer, and seek treatment and prevention methods based on the mechanisms.

Although many markers have been identified in breast CSCs, drugs targeting breast CSCs are still blank in clinical studies; this hints that there might other markers for breast CSCs or other mechanisms contributing to breast CSC progression [5]. The microglia state gene transmembrane protein 119 (TMEM119) is necessary for neuronal development and function [6]. Increasing evidences have revealed that TMEM119 also hold important effects in tumor progression, such as Sun et al. showed that TMEM119 supports ovarian cancer cell proliferation, invasion, and migration [7]; He et al. revealed that the invasion and migration ability was enhanced in gastric ca1960464ncer cells with TMEM119 overexpression [8]. Additionally, TMEM119 could promote osteosarcoma progression [9]. Notably, a recent study identified that TMEM119 could be a CSC marker for liver hepatocellular carcinoma [10]. Although a copy number amplifications of TMEM119 have been identified in anaplastic large cell lymphoma with an association of breast implant [11]; however, the effects of TMEM119 on breast cancer progression need to be explored.

β-catenin is an important transcriptional activator in classical Wnt signaling pathway and plays an important role in promoting the proliferation, differentiation, and metastasis of tumor cells [12].
Abnormal activation of the Wnt signal often leads to β-catenin accumulation in nucleus and nuclear β-catenin can activate T-cell factor/lymphopotentiator, form a complex, and trigger the abnormal expression of downstream target genes, eventually leading to the occurrence of tumors [13]. Abnormal activation of Wnt signaling pathway is involved in the occurrence, development, and metastasis of tumor. Although the upstream and downstream effectors have been revealed in breast cancer cells, such as AXIN2 (Axin-2), c-Myc, and Cyclin-D1, other effectors should be further explored to facilitate the comprehensive understanding of β-catenin signaling.

This work aims to investigate the effects and underlying mechanisms of TMEM119 in breast cancer progression. Here, breast cancer and adjacent tissues were used to determine TMEM119 expression, and it was identified that TMEM119 was highly expressed in breast cancer tissues and cells relative to that in adjacent tissues and breast epithelial cells. Online dataset analysis revealed that a higher TMEM119 expression predicted a poorer overall survival of breast cancer patients. Functional experiment results showed that TMEM119 supported breast cancer cell stemness. Combined RNA sequencing analysis, ChIP (Chromatin immunoprecipitation), and Co-IP (Co-immunoprecipitation) experiments, TMEM119 was identified to interact with β-catenin, enhance β-catenin protein stability and thus increase β-catenin transcriptional activity, this is indispensable for TMEM119-induced effects on breast cancer cell stemness. Therefore, this work reveals a novel TMEM119/β-catenin axis responsible for breast cancer cell stemness, which could be a novel biomarker for breast cancer patients.

All cases presented no metastasis. Written informed consent has been obtained from each subject. The research use of these clinical materials was approved by the Shandong Cancer Hospital Ethics Committee (20,200,713). All experiments conformed to the Declaration of Helsinki. Kaplan – Meier (KM) Plotter online tool was utilized to analyze the relationship between the expression of TMEM119 and relapse-free survival, overall survival, and post-progression survival of breast cancer patients following the concrete parameters [15]: patients were split by the median expression of TMEM119; All periods were followed up the threshold; User selected probe set were used for analysis; The patients included all types of breast cancer patients.

Cell culture

Human TNBC line MDA-MB-231, luminal B type of breast cancer cell-line BT474, luminal A type of breast cancer cell-line T47D and MCF-7, and MCF-10A cells (human mammary epithelial cells) were purchased from COBIOER (Nanjing, China), L-15 medium (Thermo Fisher Scientific, Waltham, MA, USA) and DMEM medium with 10% FBS (fetal bovine serum, Thermo Fisher) were used to culture MDA-MB-231 and other cell lines at 37°C under a circumstance containing 5% CO₂, respectively.

Real-time quantitative PCR (Polymerase chain reaction) (RT-qPCR)

Total RNA was extracted from cells and frozen tissues, and purified using the MolPure® TRIeasy RNA Kit (Cat # 19211ES60, YEASEN, Shanghai, China) and cDNA was synthesized using First-Strand cDNA Synthesis SuperMix (Cat # AT301, Transgen Biotech, Beijing, China). RT-qPCR was carried out on a Light Cycler 480 machine (Roche, Basel, Switzerland) using Hieff UNICON® qPCR SYBR Green Master Mix (Cat # 11184ES03, YEASEN). The relative expression levels of transcripts were calculated using 2−ΔΔct method and normalized to GAPDH expression.

Western blot

RIPA lysate (Cat # KGP702, KeyGen, Nanjing, China) was used to extract the total protein from

Material and methods

Clinical samples and online datasets

Forty-three breast tumors (including 14 luminal A subtypes, 14 luminal B subtypes and 15 TNBC (triple-negative breast cancer) sub-types) with 21 adjacent mammary epithelial clinical samples were collected from 43 patients who accepted surgery at the Shandong Cancer Hospital from January 2018 to September 2020 and aged from 37 to 71 years-old.
cells and frozen tissues which was quantified through BCA (Bicinchoninic acid) protein quantitative kit (Cat # KGP902, KeyGen) and electrophoresed by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). PVDF (Polyvinylidene fluoride) membranes (Cat # 162-0177, Bio-Rad, Hercules, CA, USA) were used for protein transfer and then incubated with 5% non-fat milk at room temperature for 1.5 h. The membranes were incubated with primary antibody at 4°C over night. After being washed with TBST (Tris-Buffered Saline and Tween 20) for three times, the membranes were added with secondary antibody and incubated at room temperature for 1 h. After then, TBST was used to wash the membranes for three times. Protein expression was detected using ECL (Electrochemiluminescence) detecting reagents (Cat # KGP1121, KeyGen).

**Plasmid construction, the synthesis of siRNAs (Small interfering RNA) and transfection**

The coding sequences of β-catenin and TMEM119 were inserted into pcDNA3.1 vector, named as β-catenin-oe and TMEM119-oe, respectively. The negative control (NC) and siRNAs against β-catenin and TMEM119 were synthesized by Biomics (Nantong, China). The Sinofection® Reagent (Cat # STF02, SinoBiological, Beijing, China) was used for transfection.

**Luciferase reporter analysis**

To detect β-catenin activity, cells were seeded into 24-well plates at 8 × 10⁴ cells/well. A responsive vector of β-catenin signaling TopFlash or A non-responsive vector FOPFlash and the Sea Kidney Luciferase expressed by pRL-TK PRL-TK plasmid were co-transfected into 293 T cells using Sinofection® (SinoBiological). For examining TMEM119 promoter activity, the promoter sequences of TMEM119 containing β-catenin binding sites or not were inserted into pGL3-promoter vector, denoted as pGL3-TMEM119-wt and pGL3-TMEM119-mut. Then pGL3-TMEM119-wt or pGL3-TMEM119-mut was cotransfected with PRL-TK plus β-catenin overexpression or knockdown. After 72 h of transfection, the double luciferase detection kit (Cat # E1910, Promega) was used to measure the luciferase activity. The results were normalized to PRL-TK plasmid activity.

**Mammary spheroid formation analysis**

Spheroid formation assay was performed to determine cell stemness. Briefly, cells were seeded into ultra-low attachment 24-well plates (Cat # 174930, Corning, Union City, CA) at 1000 cells/well. And MammoCult™ Human Medium Kit (Cat # 05620, Stemcell Technologies, Vancouver, BC, Canada) was utilized to culture cells for 10 days, and followed by measuring spheroid number and size (more than 50 μm) under a microscope.

**Wound healing analysis**

The migration capability of cells was determined using wound-healing analysis. The detailed procedure was referred to the previous study [16]. Briefly, cells were seeded in six-well plates and transiently transfected with pc-TMEM119 or siRNA against TMEM119. Cells were allowed to grow up to 90% confluency in complete medium. A vertical wound was made to cells with a sterile pipette tip, and the wounded monolayer was washed with PBS to remove cell debris, and serum-free medium was used to maintain cells. Phase-contrast images were taken of each sample at 0 h and 48 h at the same position with respect to the wound. The distances that cells migrated into wound surface were calculated for three different times. The migration rate was taken as (D₀ − Dₜ)/D₀, where D₀ stands for the distance measured at 0 h and Dₜ refers to the distance measured at 48 h.

**Transwell invasion analysis**

The invasion capability of cells was evaluated using transwell invasion assay referring to the previous study [17]. Briefly, the invasion assay was performed using a BD Biosciences Matrigel invasion chambers (a pore size of 8 μm; BD Biosciences). After transfection, 10³ cells in serum-free medium were placed into the upper chamber, and the lower chamber was filled with complete medium containing 20% FBS. For the invasion assay, cells were allowed to invade at 37°C for
24 h (MDA-MB-231) or 48 h (MCF-7). The invaded cells were fixed in methanol for 30 min and stained with 0.1% Crystal Violet for 30 min. The stained cells were photographed, and the dye was eluted with glacial acetic acid and quantified by measuring with Microplate Reader [optical density (OD) at 570 nm].

**ALDH (aldehyde dehydrogenase) activity analysis**

ALDH activity was measured through an Aldehyde Dehydrogenase Activity Colorimetric Assay Kit (Cat # MAK082, Sigma-Aldrich) following the manufacturer’s protocols. Briefly, 1 × 10^6 cells were homogenized in 200 μL of ice-cold ALDH Assay Buffer. The samples were centrifuged at 13,000 g for 10 min to remove insoluble material. Add 50 μL of the appropriate Reaction Mix to each of the wells. After 5 min, take the initial measurement (T_{initial}). Measure the absorbance at 450 nm at the initial time (A_{450})_initial. Continue to incubate the plate at room temperature taking measurements (A_{450}) every 2–3 min. Continue taking measurements until the value of the most active sample is greater than the value of the highest standard (10 nmole/well). At this time the most active sample is near or exceeds the end of the linear range of the standard curve. The final measurement [(A_{450})_final] for calculating the enzyme activity would be penultimate reading or the value before the most active sample is near or exceeds the end of the linear range of the standard curve. The time of the penultimate reading is T_{final}.

**Chromatin immunoprecipitation (ChIP) assay**

ChIP assay was carried out to detect TMEM119 promoter abundance in the DNA pulled down by anti-β-catenin. This experiment was performed using a ChIP Kit (Cat # 17–10086, Merck Millipore, Billerica, MA, USA) according to the manufacturer’s recommendation.

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

Protein A/G beads (Cat # 1614833, Bio-Rad) were incubated with TMEM119 or β-catenin antibody for 90 min, and followed by incubating with protein samples. After incubating overnight at 4°C, SDS-PAGE electrophoresis and Western Blot were utilized to analyze protein abundance.

**RNA sequencing assay**

MCF-7 cells with or without TMEM119 overexpression were subjected to RNA sequencing analysis. This experiment and related assays were conducted by Novogen (Beijing, China).

**Statistical analysis**

Data were shown as the mean ± SD (standard deviation). The student’s t-test was used to analyze the significance of datasets with only two groups. One-way ANOVA with the Tukey–Kramer posttest were used for the groups, and P < 0.05 was considered to be significant.

**Results**

**TMEM119 is highly expressed in breast cancer tissues and cells**

TMEM119 expression was initially detected in breast cancer and adjacent tissues and it was shown that TMEM119 expression was significantly increased in breast cancer tissues (Figure 1a and Figure 1b). The different online dataset analysis showed that a high TMEM119 expression level predicted a poor overall survival (Figure 1c), relapse-free survival (Figure 1d) and post-progression survival (Figure 1e) of patients. Analysis of breast cancer cells obtained a consistent result indicating that TMEM119 was highly expressed in breast cancer cells relative to the normal mammary epithelial cells (Figure 1f and Figure 1g). These results suggest that TMEM119 might be an oncogene in breast cancer.

**TMEM119 promotes breast cancer cell stemness**

As CSCs have been regarded as one of the roots for tumor occurrence, we firstly investigated the effects of TMEM119 on breast cancer cell stemness. TMEM119 was knocked down in MDA-MB-231 cells and overexpressed in MCF-7 cells, respectively, as MDA-MB-231 cells are shown to hold a higher while MCF-7 cells hold a lower
stemness, and TMEM119 exhibited a lower level in MCF-7 cells and a higher level in MDA-MB-231 cells. As expected, TMEM119 knockdown decreased stemness marker expression, while TMEM119 overexpression increased it (Figure 2a–d). Additionally, the mammary spheroid formation capability was attenuated in MDA-MB-231 cells with TMEM119 knockdown, and enhanced in MCF-7 cells with TMEM119 overexpression, as evident by the alteration of spheroid number and size (Figure 2e and Figure 2f). Furthermore, ALDH activity was reduced by TMEM119 knockdown, and increased by TMEM119 overexpression (Figure 2g).
**Figure 2.** TMEM119 supports the stemness of breast cancer cells. (a and b) The mRNA levels of stemness markers were detected in breast cancer cells with TMEM119 knockdown or overexpression. (c and d) The protein levels of stemness markers were examined in breast cancer cells with TMEM119 knockdown or overexpression. (e and f) The spheroid number and size were assessed in breast cancer cells with TMEM119 knockdown or overexpression. (g) ALDH activity was evaluated in breast cancer cells with TMEM119 knockdown or overexpression. n ≥ 3, **P < 0.01 vs. control.

**TMEM119 enhances the migration, invasion, and EMT (epithelial–mesenchymal transition) process of breast cancer cells?**

Since CSCs contribute to cancer metastasis, the effects of TMEM119 on the metastatic ability were further investigated in breast cancer cells. As Figure 3a–Figure 3d results showed that TMEM119 knockdown suppressed the migration and invasion ability of MDA-MB-231 cells, while TMEM119 overexpression promoted it. Additionally, the EMT process was facilitated by TMEM119 overexpression in MCF-7 cells, while TMEM119 knockdown inhibited the EMT process in MDA-MB-231 cells, which was characterized as the alternated expression of EMT markers (Vimentin, a mesenchymal marker, E-cadherin, an epithelial marker) (Figure 3e–g). Herein, these results demonstrate that TMEM119 can promote the stemness of breast cancer cells.

**TMEM119 activates Wnt/β-catenin signaling pathway by interacting with β-catenin**

Furthermore, the mechanisms by which TMEM119 promotes the stemness of breast cancer
cells were explored. RNA-sequencing analysis showed that Wnt/β-catenin signaling is mostly enriched in cells with TMEM119 overexpression (Figure 4a). Consistently, TMEM119 knockdown decreased the luciferase activity of TOP\textsuperscript{flash} reporter and TMEM119 overexpression increased it, while the nonresponsive FOP\textsuperscript{flash} control was unaffected (Figure 4b). We wondered whether TMEM119 interacted with β-catenin. Co-IP experiments indicated that TMEM119 indeed interacted with β-catenin in breast cancer cells (Figure 4c). Additionally, it was found that TMEM119 enhanced the stability of β-catenin protein (Figure 4d). Moreover, the expression of β-catenin downstream effectors (AXIN2, c-Myc, and Cyclin-D1) was increased by TMEM119.
overexpression in MCF-7 cells (Figure 4e). These results indicate that TMEM119 can activate Wnt/β-catenin pathway in breast cancer cells.

**β-catenin directly binds to TMEM119 promoter and thus increases TMEM119 expression**

Furthermore, it was shown that three potential binding sites of β-catenin existed in TMEM119 promoter (Figure 5a). We then assumed that β-catenin could regulate TMEM119 promoter activity and thus its expression conversely. As expected, β-catenin overexpression indeed increased the luciferase activity of pGL3-TMEM119, while β-catenin knockdown decreased it (Figure 5b and Figure 5c). The increased activity was disappeared upon all of the binding sites were mutated (Figure 5b and Figure 5c). ChIP analysis showed that β-catenin bound to the three binding sites...
**Figure 5.** β-catenin directly binds to TMEM119 promoter and thus increases TMEM119 expression. (a) The diagram showing the potential β-catenin binding sites on TMEM119 promoter sequences. (b and c) Luciferase reporter analysis was carried out to detect the effects of β-catenin on TMEM119 promoter activity. (d and e) ChIP analysis was constructed to evaluate TMEM119 promoter abundance in DNA pulled down by anti-β-catenin. (f–h) TMEM119 expression was examined in breast cancer cells with β-catenin overexpression or knockdown. n ≥ 3, **P < 0.01 vs. control, ##P < 0.01 vs. NC.

(Figure 5d and Figure 5e). Additionally, β-catenin positively regulated TMEM119 expression in breast cancer cells (Figure 5f–Figure 5h). Thus, our results demonstrate that TMEM119 and β-catenin can form a positive feedback loop in breast cancer cells.

**TMEM119 promotes the stemness of breast cancer cells dependent on Wnt/β-catenin signaling pathway**

Finally, we explored whether TMEM119 promotes the stemness of breast cancer cells dependent on Wnt/β-catenin signaling. MCF-7 cells with TMEM119 knockdown were overexpressed by β-catenin-oe and MDA-MB-231 cells with TMEM119 overexpression were knocked down by si-β-catenin, respectively. As shown in Figure 6a–Figure 6c, the inhibition of TMEM119 knockdown on the expression of stemness markers was partially abrogated through β-catenin overexpression. Additionally, TMEM119 knockdown-mediated suppression on spheroid-formation ability was attenuated by overexpressing β-catenin (Figure 6d and Figure 6e). Furthermore, the decreased ALDH activity led by TMEM119 knockdown was rescued by
overexpressing β-catenin (Figure 6f). Consistently, TMEM119 overexpression-induced promoting effects on breast cancer cell stemness were attenuated by β-catenin knockdown, which was evidenced by the alteration of stemness marker expression, spheroid formation ability, and ALDH activity (Figure 6a–Figure 6f). All in all, our results support that TMEM119 promotes the stemness of breast cancer cells dependent on β-catenin.

Discussion

In the current work, the expression level of TMEM119 was firstly detected in breast cancer cells and tissues, and TMEM119 was shown to hold a high expression level in breast cancer cells and tissues although clinical samples are limited. These results hint that TMEM119 might hold critical roles in breast cancer progression. To our knowledge, this is the first study demonstrating the expression of TMEM119 in breast cancer tissues and correlation between TMEM119 expression and the survival of breast cancer patients.

CSC is considered to be the root of tumor progression, then we focused on the effects of TMEM119 on breast cancer cell stemness. We confirmed that TMEM119 positively regulated the stemness of breast cancer cells through examining the expression of stemness markers, mammary spheroid formation ability and ALDH activity. However, TMEM119 effects on the cell proliferation, apoptosis and other biological activities were not evaluated here. Moreover, in vivo experiments should be carried out to consolidate the conclusion made in this work in the future.

To investigate the mechanisms underlying TMEM119-mediated effects on the stemness of breast cancer cells, RNA-sequencing analysis was performed and we showed that the Wnt/β-catenin, Hippo, PI3K-Akt, and MAPK-signaling pathways were enriched in breast cancer cells with TMEM119 overexpression. Given that the Wnt/β-catenin pathway was located on the top, we attached importance to Wnt/β-catenin pathway although other signaling pathways are also shown to engaged in CSC progression [18–20]; thus, we speculate that TMEM119 might regulate
the stemness of breast cancer cells through other signaling pathway. We found that TMEM119 physically interacted with β-catenin, enhanced β-catenin protein stability and thus increased β-catenin activity through Co-IP, luciferase reporter, and protein stability analysis. However, the domains in TMEM119 and β-catenin responsible for the TMEM119-β-catenin interaction are still unclear, and this should be explored in the future.

β-catenin is a transcriptional factor that can bind to the promoter of the downstream effectors, such as Cyclin-D1, c-Myc, and AXIN2 [21]. Then we wondered whether there are potential binding sites on the promoter of TMEM119 of β-catenin using bioinformatic prediction, and identified that there are three potential binding sites on the promoter sequences of TMEM119. Subsequently, the binding of β-catenin to TMEM119 promoter was confirmed using luciferase reporter and ChIP analysis. Finally, we showed that TMEM119 promotes the stemness of breast cancer cells dependent on β-catenin. As β-catenin roles in CSC progress are strongly established [22–24], we did not re-evaluate the effects of β-catenin on breast cancer cell stemness here.

Conclusions

Taken together, we identified a novel TMEM119-β-catenin positive feedback loop responsible for breast cancer cell stemness, which can be novel biomarkers for breast CSCs.

Data availability statement

All data generated or analyzed during this study are included in this published article (http://dx.doi.org/10.1080/21655979.2021.1960464).

Disclosure statement

No potential conflict of interest was reported by the author(s).

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