Original Article
TET3 and BRCA1 co-repress EZH2 to inhibit the aggressive behavior of breast cancer cells

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Abstract: The functions of Ten-eleven translocation 3 (TET3) in breast cancer are still unclear. Here, we reported that TET3 was downregulated in highly metastatic breast cancer cell lines such as MDA-MB-231 cells and MDA-MB-453 cells compared with non-metastatic MCF-7 cells and T47D cells. In 32 breast cancer clinical samples, the expression of TET3 was reduced in lymph node metastatic tissues compared with primary tumor tissues. Moreover, knockdown of TET3 in breast cancer cell lines increased the proliferation and invasion of breast cancer cells. Furthermore, we shown that TET3 protein expression was increased by co-expression with BRCA1, and BRCA1 could stabilize the TET3 protein level. We declared the mechanism that TET3 could bind to the EZH2 promoter and negatively regulates EZH2 transcription. Finally, we revealed TET3 and BRCA1 negatively regulated breast cancer cells aggressive behavior through the inhibition of EZH2. Taken together, these findings indicated that TET3 may contribute to the inhibition of breast cancer proliferation and metastasis through the EZH2 repression.

Keywords: BRCA1, TET3, EZH2, proliferation, invasion, breast cancer

Introduction
Breast cancer is one of the most commonly diagnosed cancer among women all over the world and is considered as the leading cause of cancer death [1, 2]. Breast cancer is a heterogeneous disease which can be classified with distinct intrinsic subtypes such as luminal subtypes A and B, basal-like and HER2-positive breast cancer Johnson [3-5].

BRCA1 was the first identified susceptibility gene with frequently germ line mutations in hereditary breast cancers and BRCA1 mutation usually have a distinctive basal-like phenotype [6, 7] BRCA1 protein has been assigned as a tumor suppressor gene with multifunction in DNA damage repair, cell cycle regulation, transcriptional regulation, chromatin remodeling and ubiquitination [8], it has been shown to interact with variety of proteins such as RNA polymerase II complex [9] and enzymes involved in chromatin remodeling [10].

EZH2 (enhancer of zeste homologue 2) is the enzymatic subunit of PRC2, which contains a SET domain and the mainly function is histone H3 lysine 27 trimethylation [11]. EZH2 is often overexpressed in human breast cancer and has been implicated to promote mammary stem cell expansion, tumorigenesis and metastasis [12, 13]. EZH2 is commonly associated with differentiation genes silence and has key roles in embryonic stem (ES) cell self-renewal and organism developmental patterning Bardot [14, 15]. So understand the regulation of EZH2/PRC2 was of desperately needed.

Ten-eleven translocation 3 (TET3) is a member of the Tet family proteins, the function of which is conversion 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), Although decreased TETs were reportedly in various cancers such as esophageal squamous cell carcinoma [16, 17], mechanism of TET3 in transcription regulation and the functions of TET3 in breast cancer are still unclear.

In this study we demonstrate the importance of our study by exploring that TET3 contributes to the inhibition of breast cancer tumorigenesis and we describe a novel mechanism in which...
TET3 represses EZH2. Finally, we show TET3 is stabilized by BRCA1, define that TET3 and BRCA1 corepress EZH2 to inhibit the aggressive behavior of breast cancer cells.

Materials and methods

Patients and tissue specimens

Tissue specimens were obtained from 32 patients who were diagnosed as primary breast cancer and 32 breast cancers with lymph node metastasis in Qilu Hospital of Shandong University from April 2008 to November 2013. The tumor tissues were received after surgery immediately and stored at -80°C until use. The tumors were classified by the Department of Pathology in Qilu hospital according to the guidelines of the American Society of Clinical Oncology (ASCO). None of the patients were subjected to preoperative chemotherapy. Written consent to allow leftover tissue samples for scientific research use was obtained from all participants prior to the study. Ethical approval was given by the medical ethics committee of Qilu Hospital.

RNA isolation and cDNA synthesis

Total RNA was extracted using TRIZOL reagent (Invitrogen, Thermo Fisher Scientific Inc. Waltham, USA), complementary RNA was reverse transcribed using the Transcriptor First Stand cDNA synthesis kit (Roche, Burgess Hill, UK) according to manufacturer’s instructions.

Quantitative real-time PCR

Quantitative real time PCR was carried out on the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using Syber green Real-time PCR Master Mix (Roche) with 2.0 μg of cDNA, according to manufacturer’s instructions. PCR amplification was performed at 95°C for 60 s followed by at 95°C 15 s, 60°C 15 s, 72°C 45 s for 40 cycles. Data were collected and analyzed using SDS2.4 Software (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as an internal control. Primer sequences qRT-PCR are listed as follows:

Cells lines and culture: The human non-tumorigenic mammary epithelial cell line MCF-10A and human breast cancer cell lines MCF-7, T47D, MDA-MB-453 and MDA-MB-231 were obtained from American Type Culture Collection (Manassas, VA, USA). All the cell lines were maintained in a humidified incubator at 37°C with 5% (v/v) CO2/air. The culturing of all cell lines was performed as described previously [18].

Antibodies and reagents: Antibodies used are as follows: rabbit polyclonal anti-BRCA1 antibody (Santa Cruz; Santa Cruz, CA, USA, sc-6954); anti-TET3 antibody (Cell Signaling Technology, Danvers, MA, USA); anti-EZH2 antibody (BD Biosciences, Bedford, MA, USA); and anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA). Non-targeting siRNA was used as a control (NC), small interfering RNAs (siRNAs) targeting the sequences human TET3 (siTET3) or BRCA1 (siBRCA1) were synthesized (Genechem, shanghai, China). The transfection of plasmids or siRNAs was performed using Lipofectamine 2000 Reagent (Invitrogen) according to manufacturer’s instructions. RNA or Protein was collected 72 h following treatment with 2.5 μg plasmid or 100 nM siRNA.

Western blot: Cells were collected and solubilized using EDTA lysis buffer. The proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membrane (Millipore). The membranes were incubated in primary antibodies at 4°C overnight, the primary antibodies were anti-BRCA1 (1:1000); anti-EZH2 (1:2000); anti-TET3 (1:500) and anti-β-actin (1:2000). Followed by incubation with the secondary antibody (Santa Cruz). The immunoreactive proteins were detected using the enhanced chemiluminescence kit (Santa Cruz).

Chromatin immunoprecipitation: The Flag-TET3 plasmid was transfected into and MCF-7 or MDA-MB-231 cells for 48 hours, and chromatin immunoprecipitation (ChIP) assays were performed using a ChiP assay kit (Millipore, Billerica, MA, USA) according to manufacturer’s instructions. Anti-Flag antibody-enriched EZH2 promoter fragments were PCR amplified. Isotype IgG (Abcam) was used as a negative control. PCR Products were visualized on a 2% TAE (Tris/borate/EDTA) agarose gel or detected by ABI 7500 system.

Luciferase assays: TET3 was amplified from human genomic DNA by PCR and the PCR products were then inserted into the pcDNA3.1 vector (Promega, Madison, WI, USA). The pcDNA3.1-Flag-TET3 plasmid expressing Flag-TET3 was obtained. Cells were seeded into six-well plates (20000 cells per well) until growth to 80% con-
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flucence, siTET3 or Flag-TET3 as well as their controls were transfected, then transfected with control (pGL3-Basic empty vector) or EZH2 promoter with firefly luciferase and co-transfected with Renilla expression construct. After 48 hours, cells were lysed with Passive Lysis Buffer (Promega) the luciferase and renilla activities of the cells were assessed using a dual-luciferase reporter assay kit (Promega), according to manufacturer’s structures. Reporter luciferase activity was normalized to that of the Renilla.

Figure 1. Expression of TET3 in breast cancer. A. qRT-PCR was used to determine the mRNA levels of TET3 in four breast cancer cell lines. GAPDH was used as the internal control. B. The mRNA levels of TET3 were measured in the transwell pre-invasion and post-invasion MDA-MB-231 cells and MDA-MB-453 cells, respectively. GAPDH was used as the internal control. Data were presented as mean ± sd, of three independent experiments. C. qRT-PCR were used for TET3 mRNA levels in the breast cancer primary tissues and their paired metastatic tumor tissues in lymph node, while the bars indicated the SD. n=32.
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**MTT assay:** Cells were seeded into the 96-well plate at a density of 10,000 cells per well and assessed by MTT assay. At 24, 48, 72 or 96 h, 10 µL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/mL in PBS) (Sigma-Aldrich) was added to the wells and incubated for 4 h at 37°C. 100 µL/well dimethyl sulfoxide (Sigma-Aldrich) was replaced to terminate the reaction. The optical absorbance was read at 570 nm, the quantification of the cell viability was determined according to the optical density values.

**Cell invasion assays:** The invasion abilities of cells were assessed using Matrigel-coated Transwell inserts (Chemicon, USA). The assays and counting of invading cells were performed according to the manufacturer's structures.

**Statistical analysis**

All observations were confirmed by at least three independent experiments. Data were presented as mean ± standard deviation (SD). Student’s t-test was used to compare the differences.
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**Results**

It is still unclear that how TET3 expresses in the progress of breast cancer. To address whether the expression of TET3 was attenuated in breast cancer cell lines, we collected four kinds of breast cancer cell lines with different metastatic capacity to detect the mRNA level of TET3. As shown in **Figure 1A**, the expression of TET3 was significantly down-regulated in metastatic breast cancer cell lines MDA-MB-231 and MDA-MB-453 compared with MCF-7 and T47D. To further confirm the argument, we develop two cell invasion models using MDA-MB-231 cells or MDA-MB-453 cells, as shown in **Figure 1B**, the expression of TET3 decreased in post-invasion cells than the pre-invasion cells. Since the TET3 mRNA level differs between different metastatic breast cancer cells, further, we detected the TET3 mRNA level in 32 breast cancer samples and breast cancer with lymph node metastasis tissues. As shown in **Figure 1C**, TET3 mRNA levels were significantly down-regulated in breast cancer with lymph node metastasis tissues compared with their primary breast cancer tissue.

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The expression of TET3 led us to investigate the role of TET3 during breast cancer progression. Firstly, MCF-7 cells or MDA-MB-231 cells were transfected with two different TET3 siRNAs, the silence efficiency was evaluated by qRT-PCR.

**Figure 3.** TET3 protein expression is increased by co-expression with BRCA1. A. Nuclear extracts were prepared from MCF-7 cells transiently transfected with vector, or TET3, or TET3 and BRCA1. Immunoblotting was carried out with an antibody against TET3. B. MDA-MB-231 cells transiently transfected with TET3 or co-transfected with BRCA1. The nuclear extracts were immunoblotted for TET3 protein. C. MCF-7 cells were transiently transfected with TET3 in the absence of BRCA1, after treated with cycloheximide (10 mg/ml), cells were harvested at time points up to 24 hours. D. MCF-7 cells were co-transfected with TET3 and BRCA1, after treated with cycloheximide (10 mg/ml), cells were harvested and nuclear extracts were separated by SDS-PAGE and immunoblotted for TET3 protein.
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Then, MTT assay was performed in MCF-7 cells to detect whether TET3 was involved in breast cancer cell proliferation, as shown in Figure 2B, the knockdown of TET3 significantly promotes the MCF-7 cells and MDA-MB-231 cells proliferation, when compared with the control groups. To determine whether TET3 may influence breast cancer cells invasion, transwell assay were performed in MDA-MB-231 cells transfected with TET3 siRNA or negative control, as expected, siTET3 resulted in an obviously increase effect in the number of MDA-MB-231 cells migrating across the filter (Figure 2C).

**TET3 protein expression is increased by co-expression with BRCA1**

In MCF-7 and MDA-MB-231 breast cancer cells, the greater expression of TET3 was observed when TET3 was cotransfected with BRCA1 compared to cells transfected with TET3 alone,
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the nuclear protein lysates for TET3 protein expression was monitored by Western immunoblotting with a TET3 antibody (Figure 3A and 3B). This suggested that BRCA1 might be stabilizing the expression of TET3 protein. Therefore, we further performed experiments to investigate whether BRCA1 could stabilize TET3. We transiently transfected TET3 expression plasmid either alone or in combination with BRCA1 expression plasmid into MCF-7 cells, after added cycloheximide into the culture medium, the nuclear proteins were harvested at intervals over a 24 hours period. Cells transfected with TET3 alone, the TET3 protein half-life was approximately 8 hours (Figure 3C). By comparison, cells cotransfected with BRCA1, the half-life of TET3 protein was greater than 24 hours (Figure 3D). Therefore, these results suggested that BRCA1 could indeed stabilize the TET3 protein expression.

TET3 binds to the EZH2 promoter and negatively regulates EZH2 transcription

In order to investigate the role of TET3 in EZH2 transcription, qChIP was performed in MCF-7 cells as well as MDA-MB-231 cells, as data shown (Figure 4A), the binding of TET3 on the promoter of EZH2 was obviously higher than the normal IgG. We therefore wanted to further
define the mechanism through which BRCA1 regulate EZH2, luciferase assays were performed in the above two cell lines, cells were transfected with a TET3-directed siRNA combined with the EZH2 promoter luciferase construct. As shown (Figure 4B), the activity of the EZH2 were decreased when cells transfected with increasing TET3-directed siRNA. Thus, TET3 does indeed regulate EZH2. We also analyzed protein extracts from TET3 siRNA-transfected MCF7 cells and demonstrated the reduction of TET3 was accompanied by the increase of EZH2 protein expression (Figure 4C).

**TET3 and BRCA1 negatively regulates breast cancer cells aggressive behavior through the inhibition of EZH2**

To further investigate the role of EZH2 in TET3/BRCA1 inhibition of breast cancer cell progression, upon treatment with siRNA targeting EZH2 in the siTET3-transfected MCF-7 cells, the effect of TET3 on breast cancer cell proliferation was identified, similarly, we transfected siEZH2 in the siBRCA1-treated MCF-7 cells to detect the function of BRCA1 on breast cancer cell proliferation. The data indicated the knockdown of EZH2 could significantly reduce the effect of TET3 or BRCA1 in MCF-7 cells (Figure 5A). Further, we observed that when siTET3 combined with siBRAC1, the effect on the proliferation was greater than siTET3 or siBRAC1 alone (Figure 5B). While the effect of siTET3 or siBRCA1 on the invasive potential of the invasive MDA-MB-231 cells, could be offset by siEZH2 (Figure 5C). The combination of siTET3 and siBRCA1 indicated the positive effect on the invasive potential of MDA-MB-231 cell line compared with siTET3 or siBRAC1 alone (Figure 5D) (P < 0.05). These results revealed that TET3/BRCA1 inhibited breast cancer cell proliferation and invasion through the inhibition of EZH2.

**Discussion**

Although the TET family such as TET3 was indicated as tumor suppressor in regulating cancer growth and invasion [19], the function of TET3 was still poorly understood. Here, we firstly reported the TET3 expression was reduced and was inversely correlated with high metastatic breast cancer cells as well as metastatic patient tissues, which indicated TET3 might be as a valuable diagnosis in the breast cancer patients with metastasis. Furthermore, depletion of TET3 resulted in enhanced proliferation ability in MCF-7 cells and enhanced invasive ability in MDA-MB-231 cells. TET3 could bind to the EZH2 promoter and negatively regulated EZH2 transcription.

Approximately 10% of women diagnosed with breast cancer had a family history, BRCA1 was the first reported breast cancer susceptibility gene. Subsequent to its identification and mutations, more and more groups focused their attention on the function of BRCA1, although the exact mechanism of BRCA1 in cell cycle regulation and transcriptional regulation remains to be defined, it is well accepted that BRCA1 carries out diverse roles through interact with a wide range of different proteins. In our study, we find BRCA1 could increase the TET3 protein expression and stabilize the TET3 protein expression. As reported before, BRCA1 is a key negative modulator of EZH2 and that loss of BRCA1 enhances the aggressive breast cancer phenotype by affecting PRC2 function [20]. Our data revealed another mechanism that through the stabilization of TET3, TET3 and BRCA1 negatively regulates breast cancer cells aggressive behavior through the inhibition of EZH2. In further studies, more detailed reason about why the expression of TET3 is down regulated in high metastatic ability of breast cancer cells are required, and more proteins that could be stabilized by BRCA1 need to be investigated.

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**Disclosure of conflict of interest**

None.

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