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The EZH2- H3K27me3-DNMT1 complex orchestrates epigenetic silencing of the *wwc1* gene, a Hippo/YAP pathway upstream effector, in breast cancer epithelial cells

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ABSTRACT

It is well known that epithelial-mesenchymal transition (EMT) can confer cancer cells with invasive and migratory capabilities associated with distant metastasis. As a key upstream factor in the Hippo pathway, Kibra (*wwc1* gene) has been shown to suppress EMT in breast cancer cells, and we have found that its expression is reduced or lost completely in both human breast cancer cell lines and clinical tissue samples, particularly in triple negative breast cancer (TNBC). Unfortunately, the molecular mechanisms underlying this progression-associated event remain to be elucidated. Epigenetic gene silencing is one of the most common causes of suppressed expression of tumor suppressor genes. Furthermore, recent studies have demonstrated that EZH2 can recruit DNA methyltransferases, resulting in DNA methylation and subsequent gene silencing in certain circumstances. Thus, we hypothesized that there may exist a link between EZH2 and DNA methylation in association with *wwc1* silencing in breast cancer. To test this hypothesis, we performed bisulfite sequencing, shRNA, co-IP, ChIP, MeDIP and ChIP-qPCR. As expected, RG108 or 5-Aza treatment improved the *wwc1* gene transcription and Kibra protein expression. Both bisulfite sequencing and MeDIP demonstrated higher CpG methylation of the *wwc1* promoter the TNBC cells (MDA-MB-231) than in luminal breast cancer cells (MCF7). It is noteworthy that ChIP and co-IP assays showed that EZH2, H3K27me3 and DNMT1 are enriched at the *wwc1* promoter, and there exist physiologically relevant protein-protein interactions between them. We also found that EZH2 knockdown leads to a partial increase in Kibra expression and a considerable reduction in H3K27 and DNMT1 trimethylation. Moreover, ChIP-qPCR revealed more DNA fragments containing the *wwc1* promoter in MDA-MB-231 than in MCF7 cells after immunoprecipitation with EZH2, DNMT1 and H3K27me3 antibodies. Collectively, our results reveal crosstalk between H3K27me3 inhibition catalyzed by EZH2 and CpG island methylation mediated by DNMT1 within the *wwc1* promoter, which synergistically silence *wwc1* gene expression in TNBC. Based on these results, we conclude that EZH2 shows promise as a potential anti-tumor target.

1. Introduction

Breast cancer is one of the leading causes of cancer-related deaths in women worldwide [1,2], with an estimated 1.7 million cases and 521,900 deaths in 2012. Triple-negative breast cancer (TNBC) one a molecular subtype of breast cancer [3–5], representing about 10% to 20% of all cases, which is characterized by a lack of estrogen receptors, progesterone receptors, and amplification of human epidermal growth factor receptor 2 (HER2).

Due to the high rate of metastasis, which is responsible for approximately 90% of all cancer-associated mortalities, TNBC displays more aggressive biological behavior and generally shows poorer clinical outcomes including short durations of relapse-free survival (RFS) and overall survival (OS) than non-TNBC subtypes [6,7]. Additionally,

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TNBC is a highly heterogeneous subtype associated with multiple molecular events and multiple signaling pathways. Thus, therapy for TNBC has been a challenge for clinicians.

The epithelial-mesenchymal transition (EMT) is a critical biological process related to the dynamic plastic phenotype of cells during embryonic development and carcinoma progression. Emerging evidence has challenged that EMT is a key mechanism triggering cancer metastasis; nonetheless, it is becoming increasingly clear that EMT endows epithelial malignant tumors with the increased motility, invasiveness, chemoresistance and radioresistance [8–10]. Moreover, the latest study revealed that EMT and its intermediate states have been identified as crucial drivers of organ fibrosis and tumor progression [11].

Kibra, also known as *wwc1*, encodes a scaffold protein that positively regulates the Hippo/YAP tumor suppressor pathway [12], which mainly controls organ size and maintains tissue homeostasis by acting on cell growth, proliferation, and apoptosis in adult life. It appears that loss of Kibra protein expression is associated with mitosis related cell-cycle events [13] and increased EMT [14,15] in cutaneous squamous cell carcinoma. Our published data has shown that Notch3 can inhibit EMT by activating the tumor suppressor Kibra, which mediates Hippo/YAP signaling in breast cancer epithelial cells [16]. We further observed that the decreased expression and even total loss of Kibra in MDA-MB-231 and BT549 that are characteristic of TNBC.

It is well known that epigenetic gene silencing is one of the most common causes leading to the lost expression of tumor suppressors. Regarding reduced Kibra expression, we noted that it has only been reported in both acute and chronic lymphocytic leukemia, which could be caused by *wwc1* (Kibra) promoter methylation [17,18]. Unfortunately, the underlying mechanism of Kibra reduction and loss in TNBC is poorly understood.

Recent studies have demonstrated that Enhancer of Zeste Homolog 2 (EZH2), a subunit of the Polycomb repressive complex 2 (PRC2), plays important roles in epigenetic gene silencing by catalyzing di- and tri-methylation of H3K27. In addition, in certain circumstances, EZH2 can recruit the DNA methyltransferases to a target promoter, resulting in DNA methylation and subsequent gene silencing. Herein, we provide solid evidence that there exists crosstalk between H3K27me3 inhibition mediated by EZH2 and CpG island methylation catalyzed by DNMT1 within the *wwc1* promoter, which synergistically silence *wwc1* gene expression in TNBC.

2. Materials and methods

2.1. Human breast cancer samples, ethics approval and consent to participate

Human breast cancer specimens were obtained from total 123 patients who underwent breast cancer surgery at the Cancer Hospital of Shantou University Medical College, China between 2010 and 2011. This study was approved by the Shantou University Medical Cancer Hospital Research Ethics Committee, and our study was in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). Written informed consent was obtained from each patient.

2.2. Cell lines, antibodies, reagents and plasmids

MDA-MB-231 and MCF7 breast cancer cell lines were purchased from the Committee on Type Culture Collection of the Chinese Academy of Science (Shanghai, China). Cells were routinely grown at 37 °C in 95% air/5% CO2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were passaged every 4–5 days and the medium was changed once between passages. After attaining confluence, cells were harvested and used for subsequent experimentation.

Human breast cancer specimens were obtained from 16 patients who underwent breast cancer surgery at the Cancer Hospital of Shantou University Medical College, China between 2013 and 2014. Written informed consent was obtained from each patient, and the study was approved by the Hospital Research Ethics Committee.

Antibodies used in this study are shown in Table 4. Reagents include RG108 (Catalog No. S2821; Selleckchem, Houston, TX, USA) and 5′-Azadeoxycytidine (5-aza-dC; Catalog No.A3656-10MG; Sigma-Aldrich, St. Louis, MO, USA).

2.3. Vectors, transient transfection and stable cell line construction

EZH2 and *wwc1*-interfering plasmids were made using pGPU6/GFP/Neo (Shanghai GenePharma Co., Ltd., Shanghai, China) as a backbone. Cells were plated in a six-well plate at a density of 1×10^5 cells per well in 2 ml of the appropriate growth medium supplemented with serum. For each transient transfection, 2.5 μg DNA was diluted into 125 μl serum-free medium, and 5–10 μl lipofectamine reagent was diluted into 125 μl serum-free medium. The two solutions were combined, gently mixed, and incubated at room temperature for 15–45 min. Cells were then washed once with 2 ml serum-free medium. For stable transfection, cells were then allowed to grow and express the protein for G418 resistance under non-selective conditions for at least 24 h. To select stably-expressing cells, cells were cultivated in standard medium supplemented with the appropriate amount of G418 for at least 3 weeks under selection pressure to avoid contamination with non-resistant cells; the G418 concentration was reduced after 1–2 weeks to maintain stable lines.

2.4. CpG island predictions, MSP and BSP primer design, and DNA extraction

A specific search for CpG enrichment in the *wwc1* promoter was performed using the CpG Island Searcher online tool (http://urogene.org/methprimer/index.html). Methylation-specific PCR (MSP) and bisulfite-sequencing PCR (BSP) primers were designed using MethPrimer. Genomic DNA was extracted from MCF-7 and MDA-MB-231 cell lines as well as breast cancer tissue samples using standard methods. The TIANamp Genomic DNA Kit (Cat. #DP304–03; TIANGEN Biotech Co., Beijing, China) according to the manufacturer's instructions.

2.5. Bisulfite genomic sequencing

Bisulfite genomic sequencing was performed as previously described [19]. For a complete listing of PCR primer sequences used for BGS, refer to Table 5. TaqGold (ABI) thermostable DNA polymerase was used for all reactions. Bands were purified from agarose gels using the Qiaex II gel extraction kit (150; Qiagen, Hilden, Germany) and cloned using the TA Cloning Kit (Cat. # K4580-01; Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions. Products from at least two independent PCR reactions were cloned and sequenced in a 96-well plate format using M13 reverse and/or forward primers. All sequencing was performed at BBILife Sciences Corporation (Shanghai, China).

2.6. Quantitative real-time PCR

In brief, total RNA was isolated with Trizol reagent according to the manufacturer's instructions. DNA contaminants were removed using the TURBO DNA-freeTM Kit (Ambion, Austin, TX, USA) and cDNAs were synthesized from 1 μg total RNA using PrimeScriptTM RT reagent Kit (TaKaRa Bio, Tokyo, Japan) in a 20 μl reaction mixture following the manufacturer's instructions. Real-time PCR was carried out in the Bio-Rad 5-Color System (Hercules, CA, USA). Primers were designed with IDT Scitools and the sequences were listed in Table 5. To confirm the specificity of PCR products, melting curves were determined using iCycler software (Hercules, CA, USA) and samples were run on an agarose gel. Expression changes in target genes in RCS-p + rats relative