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ZEB1 stimulates breast cancer growth by up-regulating hTERT expression

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ABSTRACT

Dysfunctional cell proliferation and death are the foundation of the malignant biological characteristics of cancers. In this study, we discovered that ZEB1 was positively correlated with hTERT in breast invasive ductal carcinoma samples at both the mRNA and protein levels. Further, our in vitro study in breast cancer cell lines confirmed that ZEB1 regulates hTERT expression at the mRNA and protein levels; thus, hTERT promotes or inhibits telomerase activity, and telomere length is either protected or reduced. Finally, we verified that ZEB1, which mostly functions as a transcriptional repressor, can recruit the co-activator YAP to enhance the transcriptional activation of hTERT. Fascinatingly, instead of acting on E-boxes, the ZEB1/YAP complex tends to function as a transcriptional activator by binding with sequences potentially located in the hTERT promoter. Consequently, our research revealed a new ZEB1-hTERT signaling pathway involved in cell proliferation regulation that has never before been illuminated in breast cancer.

1. Introduction

Breast cancer accounted for 30% of the new cancer diagnoses in women in 2017 [1] and was the leading cause of cancer death among young Chinese women in 2015 [2]. As early as 50 years ago, scientists found that immortality was an essential hallmark of cancer [3] and the cornerstone of many malignant characteristics of cancer, such as rapid growth and distant metastasis. Thus, the mechanisms underlying the unlimited growth of cancer cells urgently need to be elucidated in order to identify potential strategies for breast cancer treatment.

Zinc finger E-box binding homeobox 1 (ZEB1) is a transcription factor belonging to the ZEB protein family that binds to specific DNA sequences known as E-boxes (5′-CACGTG-3′) to control target gene expression [4]. ZEB1 has been shown to act as a transcriptional repressor [5,6] or activator [7] of gene expression in various cancer types. Because one of its best-known targets is the epithelial-mesenchymal transition (EMT)-related gene E-cadherin [8], many researchers have concentrated on its functions in migration and invasion of various cancer types [9,10]. However, accumulating evidence has shown that ZEB1 is also involved in cancer cell proliferation and apoptosis [11,12].

Telomerase is one of the best-known enzyme complexes that protects telomeres. Telomerase contains several subunits, such as human telomerase reverse transcriptase (hTERT) and human telomerase RNA (hTR), and hTERT is a limiting factor for telomerase activity [13]. High hTERT levels have been detected in more than 85% of human cancers but are not detected in somatic tissues; thus, elevated hTERT expression may represent the mechanism by which cancer cells prevent telomere shortening and become immortal [14]. A transcription factor-related approach is one prominent mechanism involved in hTERT activity. In a 1998 study by Jing Wang et al., up-regulation of c-MYC expression increased telomerase activity by stimulating hTERT expression [15].

In this study, we discovered a positive correlation between hTERT and ZEB1 expression in breast invasive ductal carcinoma (IDC) patients. In follow-up analyses, we examined the influence of ZEB1 on hTERT expression and telomerase function in the breast cancer cell lines MDA-MB-231 and MCF-7 and revealed the involvement of ZEB1 in hTERT-mediated cell proliferation and apoptosis. In addition, we investigated the mechanism by which ZEB1 regulates hTERT using chromatin immunoprecipitation (ChIP), co-immunoprecipitation (co-IP) and luciferase-based reporter assays.

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2. Materials and methods

2.1. Patients and tissue specimens

In total, 133 patients with breast IDC (stage I–III) who underwent a modified radical mastectomy or breast-conserving therapy at Taizhou Hospital (Taizhou, China) between January 2001 and August 2004 were enrolled. All patients agreed to the use of their tumor tissues for research purposes, under the condition that their participation had no effect on their pathological diagnoses. A portion of each sample was used for immunohistochemistry (IHC), and the remainder was stored in liquid nitrogen until further use.

2.2. Tissue microarray construction

A portion of the 133 breast tumor specimens was formalin-fixed, paraffin-embedded, and then used to construct a tissue microarray with 0.6-mm-diameter cores using a tissue-arraying instrument (Beecher Instruments). Then, 4-μm-thick microarray sections were cut and placed into one recipient block for IHC.

2.3. IHC

The rabbit polyclonal antibody against ZEB1 and the monoclonal antibody against hTERT used for IHC analyses were purchased from Bethyl Laboratories (UK) and Abcam Biotechnology (UK), respectively. IHC was performed as previously described [16].

2.4. Cell culture

The human breast cancer cell lines MCF-7 and MDA-MB-231 were purchased from the Cell Bank of the Chinese Academy of Sciences (CAS) for our study. MCF-7 cells were cultured in minimum essential medium (MEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) in a humidified 37 °C incubator containing 5% CO_2, and MDA-MB-231 cells were cultured in Leibovitz's 15 medium (L15, Gibco) supplemented with 10% FBS under the same conditions. The medium was exchanged every 2 days with fresh medium to maintain cell activity.

2.5. Transfection

siRNAs for ZEB1 (ZEB1-si), hTERT (hTERT-si) and YAP (YAP-si) as well as overexpression plasmids for ZEB1 (ZEB1-OE) and YAP (YAP-OE) and corresponding negative controls (OE-NC, si-NC) were purchased from GenePharma (Suzhou, China). For the negative control (NC) groups, we transfected OE-NC or si-NC separately or together into the cells. We used Lipofectamine 3000 (Invitrogen, USA) for transfection according to the manufacturer's protocol. All siRNA sequences are listed in Supplementary Table 1.

2.6. qRT-PCR

Tissues were homogenized using a tissue grinder, and then, total RNA was extracted using RNAiso Plus (TaKaRa, Japan). Total RNA was also extracted from cells using the same method. First, target mRNAs were reverse-transcribed to cDNAs with a PrimeScript RT Master Mix Perfect Real-Time Kit (TaKaRa). Then, quantitative real-time fluorescence PCR (qRT-PCR) analysis was performed using a Bio-Rad CFX96 system with a SYBR Premix Ex Taq II Kit (TaKaRa) to detect the mRNA levels. All primers are listed in Supplementary Table 1.

2.7. Western blot

Western blot analysis was performed as previously described [17]. The antibodies used in this study were rabbit anti-hTERT (1:500) purchased from Abcam Biotechnology and rabbit anti-ZEB1 (1:500), anti-YAP (1:500), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:1000) purchased from Proteintech Group (USA).

2.8. Cell proliferation assay

Cell proliferation assays were performed with the MTT Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, China) according to the manufacturer's protocol.

2.9. Cell apoptosis assay

Treated cells in logarithmic phase were collected for flow cytometry with a Cell Apoptosis Analysis Kit (Beyotime) to analyze cell apoptosis according to the manufacturer's instructions.

2.10. Telomerase activity and telomere length detection

According to the protocol of the TeloTAGGG Telomerase PCR ELISA PLUS Kit purchased from Roche (Switzerland), we first performed telomeric repeat amplification using PCR and then performed ELISA for non-quantitative detection of telomerase activity. For telomere length measurements, 10^4 transfected cells in logarithmic phase were collected, subjected to a hypotonic treatment with 75 mmol/L KCl for 20 min and fixed with a liquid mixture containing 3 vol of methanol and 1 volume of glacial acetic acid for 30 min. Then, one drop of the cell suspension was added to a wet slide, which was rinsed in detergent for 1 h to yield chromosome smear samples. Next, these chromosome smear samples were hybridized with a Cy3-conjugated peptide nucleic acid (PNA) probe provided in the Telomere PNA FISH Kit/Cy3 (Dako, Denmark) according to the manufacturer's instructions. Fluorescence microscopy was used to visualize the Cy3 signals of the telomeres and the 4,6-diamidino-2-phenylindole dihydrochloride (DAPI)-labeled cell nuclei.

2.11. Bioinformatics analysis

The hTERT promoter sequence was identified 2000 bp upstream of the transcription start site using UCSC Human Gene Sorter (http://genome.ucsc.edu/cgi-bin/hgNear). Binding sites were predicted with the help of the online bioinformatics software Multigenome Analysis of Positions and Patterns of Elements of Regulation (MAPPER2, http://genome.ufl.edu/mapper/mapper-main) and PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3).

2.12. ChIP

ChIP assays were performed with an EZ ChIP Kit (Millipore, USA) according to the manufacturer's protocol. The PCR primers are listed in Supplementary Table 1.

2.13. Luciferase-based reporter assay

We performed luciferase-based reporter assays using a dual-luciferase assay kit (Promega, USA). All groups included three replicates, and the assays were repeated 3 times.

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