EFFECT OF ZEB1 OVEREXPRESSION AND SILENCING ON AN ER-A PROMOTER OF METHYLATION OF BREAST CANCER CELLS AND THE MECHANISM OF ITS TRANSCRIPTIONAL INHIBITION

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ABSTRACT

Objective*: To investigate the effect of zinc finger E-box binding homeobox 1 (ZEB1) overexpression and silencing on the estrogen receptor-α (ER-α) promoter methylation of breast cancer cells and the mechanism of its transcription inhibition.*

Methods: *MDA-MB-231 cell lines were randomly divided into a Ctrl group, an AZA group, a VPA group and an AZA+VPA group. MCF-7 cell lines were selected and randomly divided into a Ctrl group, ZEB1 group, AZA group, VPA group, and AZA+VPA group. The mRNA and protein expression levels of ZEB1 and ER-α in breast cancer cell lines were compared. The expression of DNMT3B and HDAC1 in each group was compared.*

Results: *The expression levels of ZEB1 decreased and the expression levels of ER-α increased. The expression levels of mRNA and ZEB1 proteins in the cells were significantly lower, but the expression levels of ER-α mRNA and proteins in the cells were significantly higher in the h-ZEB1-MDA-MAB-231 group than those in the SC-MDA-MAB-231 group (P<0.05). Moreover, ZEB1 could simultaneously precipitate DNMT3B and HDAC1 proteins, and DNMT3B and HDAC1 antibodies could also correspondingly precipitate ZEB1 protein expression, suggesting that the combination of ZEB1, DNMT3B, and HDAC1 can produce a protein complex. DNMT3B and HDAC1 can also be enriched in the sequence region of E2box elements.*

Conclusions: *ZEB1 can induce the hypermethylation of an ER-α promoter in breast cancer cells and its mechanism may be achieved by recruiting DNMT3B and HDAC1 to the ER-α promoter region.*

Keywords: *ZEB1, breast cancer, DNMT3B, HDAC1.*

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Introduction

Breast cancer is the most common cancer in women worldwide with over two million new cases diagnosed in 2018. It is a leading cause of cancer with an extremely high fatality rate around the world. Its primary treatment is surgical resection, supplemented by systemic treatments such as radiation therapy, chemotherapy, and biological immunotherapy. Breast cancer may also be treated with anti-estrogen therapy after surgery, which can significantly improve the prognosis of patients with breast cancer who have estrogen-dependent tumors[(1)]. However, not all patients with breast cancer are sensitive to anti-estrogen therapy, and some patients may have anti-estrogen resistance due to congenital substitution and acquired drug resistance, which ultimately leads to treatment failure[(2)]. Determining the mechanism of anti-estrogen drug resistance in breast cancer is of great significance to increase the survival time and improve the prognosis of patients with breast cancer. Estrogen can participate in regulating many

physiological and pathological processes of breast cancer tissues. Related studies have shown that about 70% of patients with adenocarcinoma have a positive estrogen receptor alpha (ER-α), and many of these patients are prescribed anti-estrogen hormone therapy[3]. It has been reported that zinc finger E-box binding homeobox 1 (ZEB1) is highly expressed in malignant tumors such as lung cancer and ovarian cancer[4]. Another study confirmed that ZEB1 can regulate the process of epithelial-mesenchymal transition and further lead to the development of a malignant phenotype of a tumor[5].

Moreover, ZEB1 is significantly increased in breast cancer, where it can regulate cell adhesion and polarity changes, promote the formation of breast cancer stem cells, and also mediate breast cancer radiotherapy and chemotherapy resistance[6]. However, the specific action mechanisms of ZEB1 and ER-α has not been elucidated, so this study aims to investigate the effect of ZEB1 overexpression and silencing on the methylation of ER-α promoter in breast cancer cells and the mechanism of its transcription inhibition.

Materials and methods

Experimental materials

Two human breast cancer cell lines, MCF-7 and MDA-MB-231, were purchased from Beijing Sizhengbai Biotechnology Co., Ltd.

Main reagents and instruments

Reagents

Gene expression plasmids and a promoter activity detection kit were purchased from Wuhan Miaoling Biotechnology Co., Ltd.; rabbit ZEB1 and ER-α monoclonal antibodies were purchased from Nanjing Shanben Biotechnology Co., Ltd.; and a BCA protein quantification kit was purchased from Wuhan Yunclo Diagnostic Diagnostics Research Institute Co., Ltd.

Instruments

A constant temperature cell incubator was purchased from Beijing Qinye Yongwei Technology Co., Ltd.; an inverted ordinary light microscope was purchased from Shenzhen Xinbosheng Biotechnology Co., Ltd.; a low-speed tabletop centrifuge was purchased from Shanghai Chenlian Biotechnology Development Co., Ltd.; a high-speed tabletop centrifuge was purchased from Shenzhen Saijin Biological Technology Co., Ltd.; an ultra-clean workbench was purchased from Dongguan Biaobiao Experimental Equipment Technology Co., Ltd.; and a gel imaging exposure system was purchased from Wuxi Lefusi Biological Experiment Equipment Co., Ltd.

Methods

• The liminal type cell line MCF-7 and basal type cell line MDA-MB-231 were tested by real-time fluorescence quantitative PCR and western blot to observe and analyze the expression levels of ZEB1 and ER-α mRNA and proteins in these breast cancer cell lines.

• MCF-7 and MDA-MB-231 were selected for conducting ZEB1 overexpression and silencing tests. The ZEB1 gene expression plasmid ZEB1-pLV-EF1α-MCS-IRES-Bsd and the ZEB1 gene silencing plasmid shZEB1-pLV-H1-EF1α-Puro were transfected with lentivirus before initiating the study, to establish a stable expression cell line SC-MDA-MAB-231 group, sh-ZEB1-MDA-MAB-231 group, MCS-MCF-7 group, and ZEB1-MCF-7 group. Real-time fluorescence quantitative PCR and western blot were used to detect the mRNA and protein expression levels of ZEB1 and ER-α in these breast cancer cell lines.

• The remaining MDA-MB-231 cell lines were randomly divided into a Ctrl group that received no treatment, an AZA group that was treated with methylation transferase inhibitor AZA, a VPA group that was treated with VPA, and an AZA+VPA group that received both therapies. Some MCF-7 cell lines were randomly divided into a Ctrl group, ZEB1 group, AZA group, VPA group, and AZA+VPA group. The treatment methods of these cell lines in each group were the same as the MDA-MB-231 cell lines. Real-time fluorescence quantitative PCR and western blot were used to detect the mRNA and protein expression levels of ZEB1 and ER-α in these breast cancer cell lines.

• The truncated series plasmids of an ER-α promoter were established according to the original E2box sequence position and named ER-seg-I, ER-seg-II, and ER-seg-III. After sequencing verification, MCF-7 cells were transiently transfected and the promoter activity of the truncated plasmids was measured.

• Some proteins, which were collected from 1mL of freshly extracted proteins diluted with RIPA, were stored in an ultra-low temperature refrigerator and defined as the input positive control group. In

addition, an experimental group (comprised of the protein centrifuge and discard supernatant) and an IgG negative control group (using the protein supernatant after protein centrifugation) were set up. The expression of DNMT3B and HDAC1 in each group were detected using immunoprecipitation.

Statistical analysis

All data were analyzed using software SPSS23.0 and the measurement data were expressed by ($\bar{x}\pm s$). A t-test was used for comparison between two groups and multivariate analysis of variance was used for comparison between multiple groups. P<0.05 was regarded as statistically significant.

Results

Comparison of expression levels of ZEB1 and ER-α in the breast cancer cell lines

In the MDA-MB-231 and SUM-159 cell lines, the expression levels of ZEB1 increased, but the expression levels of ER-α decreased; while in the MCF-7 and ZR75-1 cell lines, the expression levels of ZEB1 decreased, while the expression levels of ER-α increased. See Figures 1 and 2.

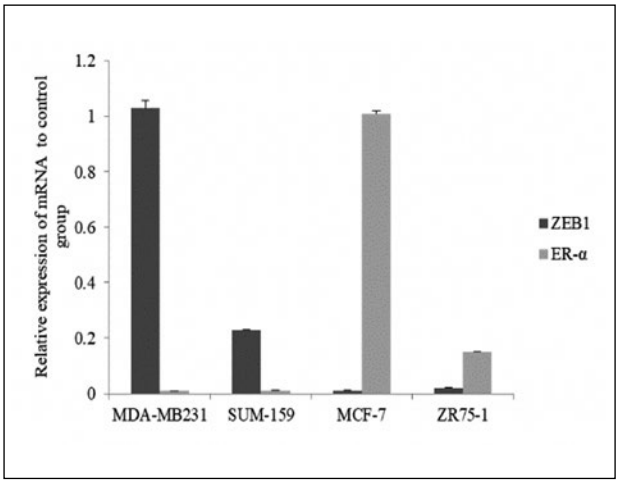

Figure 1: Comparison of ZEB1 and ER-αmRNA expression levels in the breast cancer cell lines.

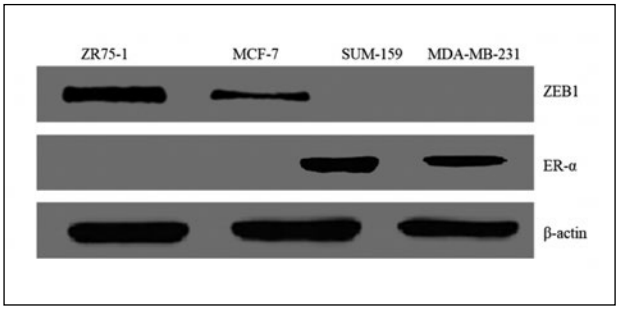

Figure 2: Comparison of ZEB1 and ER-α protein expression levels in the breast cancer cell lines.

Comparison of expression levels of ZEB1 and ER-α in stably transfected cell lines of breast cancer

The expression levels of mRNA and proteins of ZEB1 in the cells were significantly lower, but the expression levels of ER-α mRNA and proteins in the cells were significantly higher in the h-ZEB1-MDA-MAB-231 group than those in the SC-MDA-MAB-231 group (P<0.05), as shown in Figure 3.

The expression levels of ZEB1 mRNA and proteins in the cells were significantly higher, but the expression levels of ER-α mRNA and proteins were significantly lower in the ZEB1-MCF-7 group than those in the MCS-MCF-7 group (P<0.05), as shown in Figure 4.

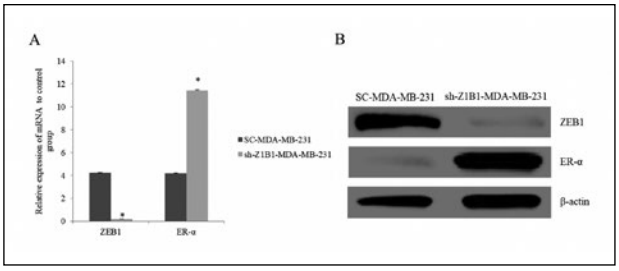

Figure 3: Comparison of mRNA and protein expression levels of ZEB1 and ER-α in the MDA-MB-231 cell line. *Note: *indicated that compared with SC-MDA-MAB-231 group, P<0.05; Panel A: SC-MDA-MAB-231 group; Panel B: sh-ZEB1-MDA-MAB-231 group.*

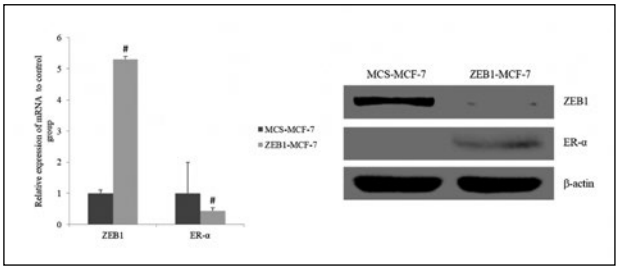

Figure 4: Comparison of mRNA and protein expression levels of ZEB1 and ER-α in the SUM-159 cell line. *Note: #indicated that compared with the MCS-MCF-7 group, P<0.05; Figure A: MCS-MCF-7 group; Figure B: ZEB1-MCF-7 group.*

Effect of ZEB1 overexpression and silencing on the methylation of ER-α promoter in breast cancer cells

The expression levels of ER-α mRNA and proteins in the MDA-MB-231 cell line of the AZA group, VPA group, and AZA+VPA group were significantly higher than those in the Ctrl group (P<0.05); and the expression levels of ER-α mRNA and proteins in the MDA-MB-231 cell line were significantly higher in the AZA+VPA group than those in the AZA group (P<0.05). The expression levels of ER-α mRNA and proteins in the MDA-

MB-231 cell line in the AZA group were not statistically different from those in the VPA group (P>0.05), as shown in Figure 5. The expression levels of ER-α mRNA and proteins in the MCF-7 cell lines of the ZEB1 group, AZA group, and VPA group were significantly lower than those of the Ctrl group (P<0.05); the expressions of ER-α mRNA and proteins in the MCF-7 cell line of the AZA+VPA group were significantly higher than those in the ZEB1 group (P<0.05), as shown in Figure 6.

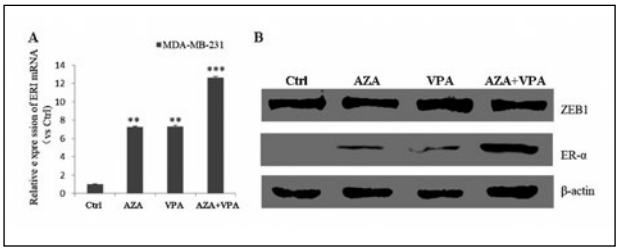

Figure 5: Effects of ZEB1 overexpression and silencing on the methylation of ER-α promoter in the MDA-MB-231 cell line.
*Note: **means compared with the Ctrl group, P<0.05; ***means compared with the AZA group, P<0.05.*

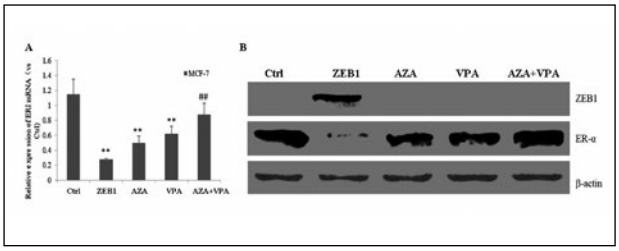

Figure 6: Effects of ZEB1 overexpression and silencing on the methylation of ER-α promoter in the MCF-7 cell line.
*Note: **means compared with the Ctrl group, P<0.05; ##means compared with ZEB1 group, P<0.05.*

Activity detection of ER-α truncated promoter in the breast cancer cells

ER-seg-II and ER-seg-III could initiate ER-α transcription and the activity of the truncated plasmid ER-seg-III promoter without E2box elements was not inhibited by ZEB1, as shown in Figure 7AB.

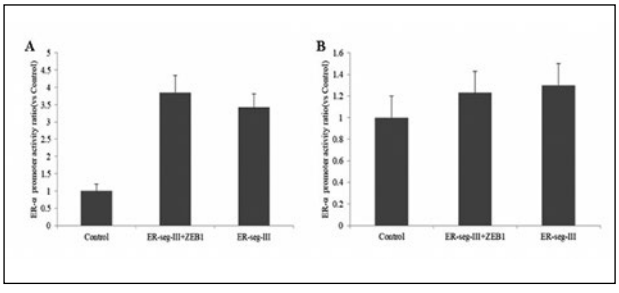

Figure 7: Activity detection of ER-α truncated promoter in the breast cancer cells.
Note: Figure A: ER-seg-II promoter activity; Figure B: ER-seg-III promoter activity.

ZEB1 recruits DNMT3B and HDAC1 to ER-α promoter to conduct its transcription

ZEB1 could simultaneously precipitate DNMT3B and HDAC1 proteins, and DNMT3B and HDAC1 antibodies could also correspondingly precipitate ZEB1 proteins, suggesting that the combination of ZEB1, DNMT3B, and HDAC1 could produce a protein complex. DNMT3B and HDAC1 can be enriched in the sequence region of E2box elements, as shown in Figure 9.

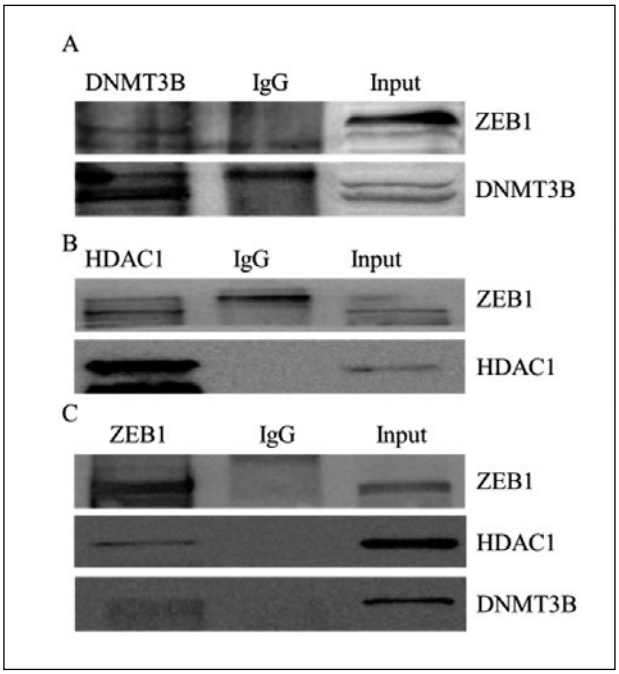

Figure 8: ZEB1 recruits DNMT3B and HDAC1 to ER-α promoter to conduct its transcription.

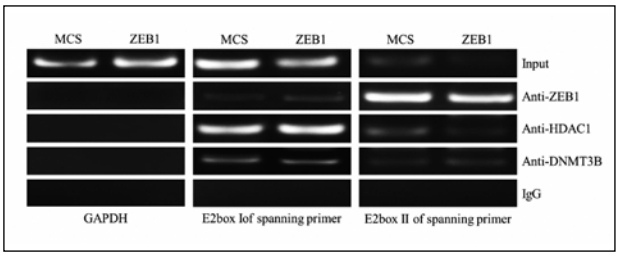

Figure 9: DNMT3B, HDAC1, and ZEB1 can specifically enrich the E2box sequence as an ER-α promoter of breast cancer development.

Discussion

Anti-estrogen resistance is one of the most important factors leading to treatment failure of estrogen-dependent breast cancer and ER-α expression level is an important reference index for the selection of anti-estrogen therapy[7]. However, studies have confirmed that ER-α is silent during the development and progression of breast cancer and the mechanism of anti-estrogen resistance in treating

breast cancer is not yet clear[8]. ZEB1 is a member of the zinc finger homeodomain transcription factor family and is primarily composed of the homology domain and the zinc finger nodes on both sides. It can participate in the transcriptional regulation of target genes through its zinc finger structure and plays a major role in embryonic growth and tissue and organ development[9].

Clinical studies have shown that ZEB1 in patients with breast cancer is negatively correlated with ER-α expression levels. Many studies have found that ZEB1 can regulate epigenetics in tumor progression[10]. In addition, ZEB1 can cooperate with HDAC1 and HDAC2 to control the promoter activity of E-cadherin through either the dependent or independent miR-200 pathway and further hinder E-cadherin transcription, regulate tumor EMT process, and eventually lead to tumorigenesis, but its specific mechanism of action in regulating cell dynamic epigenetic modification is not yet clear[11-12]. Therefore, this study explored the important regulatory role of ZEB1 in anti-estrogen behaviors.

Clinical studies have shown that ZEB1 overexpression in breast cancer cells can lead to anti-estrogen resistance in breast cancer therapy and ZEB1 silencing can increase the sensitivity of anti-estrogen therapy. This result shows that ZEB1 can be used as an important indicator for evaluating anti-estrogen resistance, therefore it may become a new target for the treatment of breast cancer[13-14]. In this study, we found that ZEB1 can transcriptionally regulate ER-α, which increases in breast cancer cells as the expression levels of ER-α decreases accordingly. Furthermore, the abnormal expression of ER-α is regulated by ZEB1 transcription, which is mainly manifested as ER-α negative and anti-estrogen resistance, suggesting that ZEB1 overexpression may be an important factor leading to the loss of ER-α expression in invasive breast cancer, which is helpful to elucidate the reasons for ER-α negative expression and the mechanism of anti-estrogen resistance during the progression of breast cancer. Many studies have confirmed that ER-α inactivation is caused by a variety of factors[15].

In this study, we found that ZEB1 overexpression can reduce the expression levels of ER-α in breast cancer, and the sensitivity of anti-estrogen therapy is also reduced; ZEB1 can recruit DNMT3B and HDAC1 to the ER-α promoter region and is a hypermethylated ER-α promoter in breast cancer cells. To further confirm this result, we used a methyltransferase inhibitor AZA and a deacetylase inhibitor VPA to transform the process. ZEB1 can control the expression level of ER-α by inducing promoter methylation and chromosomal remodeling. In this study, we found that ZEB1 and ER-α induced antiestrogen therapy resistance in treating breast cancer may result from ZEB1-induced hypermethylation of the ER-α promoter. ZEB1 expression may occur when estrogen regulates the progression of breast cancer. We also identified the function of ZEB1 in regards to the anti-estrogen effect in treating congenital resistance. The copper drum hinders ZEB1 from promoting the expression of ER-α, and combined with AZA and VPA, may become a new method to improve anti-estrogen resistance in treating certain breast cancers.

In summary, ZEB1 can induce the hypermethylation of an ER-α promoter in breast cancer cells and its mechanism of action may be achieved by recruiting DNMT3B and HDAC1 to the ER-α promoter region.

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