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KRAB-containing zinc finger protein ZNF496 inhibits breast cancer cell proliferation by selectively repressing ERα activity

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ABSTRACT

KRAB-containing zinc finger proteins (KZNF) constitute the largest family of transcriptional regulators in humans and play critical roles in normal development and tumorigenesis. However, the function and mechanism of most KZNFs remain unclear. Here, we report that ZNF496, a KZNF family member, interacts with the DNA binding domain (DBD) of estrogen receptor alpha (ERα) via its C2H2 domain. This interaction decreases ERα binding to chromatin DNA and results in the repression of ERα transactivation, the selective suppression of ERα target genes, and ultimately in a reduction of ERα-positive cell growth in the presence of E2. An analysis of clinical data revealed that the downregulation of ZNF496 expression is observed only in ERα-positive and not in ERα-negative breast cancer tissues when compared with that in matched adjacent tissues. Lastly, we also observed that the downregulation of ZNF496 is associated with poor recurrence-free survival among patients with breast cancer. Collectively, our findings demonstrate that ZNF496 is a novel ERα-binding protein that acts as a target gene-specific ERα corepressor and inhibits the growth of ERα-positive breast cancer cells.

1. Introduction

KRAB-type zinc-finger (KZNF) proteins constitute the single-largest family of transcription regulators in mammals. Their zinc fingers bind to promoters or other transcription factors to repress the transcription of numerous target genes. Most members of this family are involved in multiple important cellular functions, including differentiation, proliferation, apoptosis, and cancer outgrowth [1–5]. Conventionally, KZNF-mediated repression has been shown to depend on the ability of the Krüppel-associated box (KRAB) motif to interact with tripartite motif-containing 28 (TRIM28), a transcriptional corepressor that functions by recruiting histone-modifying enzymes and by promoting the spread of heterochromatin [6–8]. However, emerging evidence has indicated that the molecular mechanisms of KZNF-mediated transcriptional repression are more complex than previously understood.

ZNF496, a KZNF family member, consists of three distinct domains: the N-terminal SCAN domain, the KRAB domain, and the C-terminal domain which consists of five zinc finger regions, including four conventional C2H2 fingers and a novel C2HR motif [9–12]. ZNF496 was first identified through its C2HR motif as a nuclear receptor binding SET domain protein 1 (NSD1)-interacting zinc finger protein that represses transcription in an NSD1-dependent manner [10]. Another study showed that ZNF496 inhibits the transcriptional repression of Jumonji (JMJ) [12]. However, the biological significance and the mechanisms underlying ZNF496 function have not been thoroughly elucidated to date.

Abbreviations: KZNF, KRAB-containing zinc finger; DBD, DNA binding domain; ERα, estrogen receptor alpha; RFS, recurrence-free survival; KRAB, Krüppel-associated box; TRIM28, tripartite motif-containing 28; NSD1, nuclear receptor binding SET domain protein 1; JMJ, Jumonji; IP-MS, immunoprecipitation – mass spectrometry; EREs, estrogen receptor elements; siRNA, small interfering RNA; EMSA, electrophoretic mobility shift assay; EV, empty vector; qChIP, quantitative chromatin immunoprecipitation; KAP1, KRAB-associated protein 1

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We first analyzed the expression profile of ZNF496 using www.proteinatlas.org, and found that it is highly expressed in the female reproductive system and the mammary gland, but there is little or no expression in the corresponding cancer tissues, suggesting that ZNF496 might be associated with the development of breast cancer. Therefore, to understand the role of ZNF496 in breast cancer development, we performed an immunoprecipitation-mass spectrometry (IP-MS) assay to identify ZNF496-binding proteins in MCF-7 breast cancer cells and found that ERα was pulled down by ZNF496. The interaction between ZNF496 and ERα was then confirmed by immunoprecipitation.

ERα is normally expressed in breast tissues and mediates hormonal functions in the female reproductive physiology and development. Approximately 70% of breast cancers are ERα-positive. ERα is involved in the regulation of tumor cell proliferation and serves as a prognostic marker and therapeutic target in the management of hormone-dependent tumors [13–16].

ERα is a ligand-dependent transcription factor and its activity is strictly regulated by the E2 concentration and cofactors under physiological conditions [17,18]. In addition to ERα activators, ERα repressors have been proposed to counterbalance the estrogen-induced ERα transactivation and represent a cellular mechanism that may be employed for tumor suppression [19]. Nuclear receptor corepressor 1, silencing mediator of retinoid and thyroid hormone receptors, receptor-interacting protein 140, progesterone receptors, and repressor of estrogen action are classical corepressors that negatively regulate the ERα-mediated transcriptional activity [20–23]. Many ERα corepressors are frequently deregulated in breast cancer, and targeting these deregulated corepressors can effectively repress the development of breast cancer and predict clinical outcomes.

In this study, we characterized the role of ZNF496 as a corepressor of ERα in breast cancer cells. ZNF496 interacts with the DBD domain of ERα and suppresses the binding of ERα to estrogen response elements (EREs) in response to E2, resulting in a selective reduction in the expression of ERα target genes and the suppression of ERα-positive breast cancer cell growth.

2. Methods

2.1. Cell culture, treatment and transfection

MCF-7, T-47D, MDA-MB-231 and HEK293T cells were maintained in DMEM media supplemented with 10% fetal bovine serum (Zhejiang Tianhang Biotechnology, Hangzhou, China).

For the E2 treatment experiments, cells were cultured in estrogen-free media, which is Phenol red free DMEM (Kccell, Beijing, China) supplemented with charcoal stripped 5% certified fetal bovine serum (FBS) (04-201-1A, BIOLOGICAL INDUSTRIES, Kibbutz Beit Haemek, Israel) for at least 3 days, and then treated with 10 nM 17-β-estradiol (E2, Sigma-Aldrich, St. Louis, MO).

For the plasmid or siRNA transient transfection, TurboFect transfection reagent was used following the manufacturer's protocol (R0532, Thermo Fisher Scientific, Waltham, MA).

2.2. Plasmids, antibodies, siRNA, and reagents

The plasmids of ZNF496 and its truncates were constructed by PCR followed by subcloning into a pCMV-Myc expression plasmid (PT3282-5, CLONTECH Laboratories, Inc., Mountain View, CA). The plasmids of ERα and its truncates were constructed by PCR followed by subcloning into pFlag-CMV2 plasmids (E3762, Sigma-Aldrich). E2F1 plasmids are kind gifts from Dr. Pingkun Zhou. The anti-Myc-HRP antibodies were purchased from Medical Biological Laboratories (60004-1, International Corporation, Woburn, MA). Anti-ZNF496 (SAB1405312), anti-Myc (M4439), anti-Flag (F7425), and anti-Flag-HRP (SL12445) were purchased from Sigma-Aldrich, and anti-ERα antibodies were purchased from LifeSpan BioSciences (LS-C118606, Seattle, WA). siRNA was purchased from Gene Pharma (Suzhou, China). The following sequences were used: ZNF496–1 (5′-GGUCUUAAUAACAAGAACA-3′), ZNF496–2 (5′-GCUUCACGCAGAACUAUGA-3′), ERα-1 (5′-GAGGGAGAAUGUUGAAACA-3′), ERα-2 (5′-GGCUAGAGAUCCUGAUGAU-3′), non-targeting siRNA (5’-UUCUCCGAACGUGUCACGUTT-3′).

2.3. Establishing breast cancer cells stably expressing ZNF496

The ZNF496 lentiviral expression vector was constructed by inserting ZNF496 cDNA into the pLV-Neo vector (VL3002, Yingmaoshengye Biotechnology, Beijing, China). MCF-7, T-47D, and MDA-MB-231 cells that stably expressed ZNF496 were established according to the manufacturer's protocol. In brief, 293 T cells were transfected with lentiviral and helper plasmids after reaching 80% confluence. Medium was replaced after 18–24 h. After 48 h, supernatant was collected and filtered through a 0.22 μm filter. The cells were inoculated in culture dishes supplemented with the same volume of retroviral supernatants for 24 h; and then, the medium was replaced with fresh medium. The cells were screened with G418 after infection for 3 days, and after 10 days, single clones were selected and detected by Western blotting.

2.4. RT-PCR and quantitative PCR

Total RNA was extracted using a TRIzol kit (Sigma-Aldrich). cDNA was generated from 500 ng of total RNA using a ReverTra Ace qPCR RT Kit (TOYOTO, Osaka, Japan) according to the manufacturer's instructions. Fluorescence quantitative PCR was performed using KAPA SYBR FAST qPCR Master Mix (2×) and qTOWER 3 (Analytik Jena AG, Jena, Germany). PCR was performed in triplicate, each experiments were repeated three times, and standard deviations representing experimental errors were calculated. All data were analyzed using qPCRsoft 3.2 software. The following PCR primers were used: ZNF496 (5′-CTCCAGCCATTCCAGGTAG-3′, 5′-CCATGCTGGGAGTCCTCGT-3′), ERα (5′-TCCAAACCCATCGTCAGTGC-3′, 5′-TCAAGTCTCTATAACCAATGACCT-3′), pS2 (5′-TTGTGGTTTTCCTGGTGTCA-3′, 5′-CCGAGCTCTGGGACTAATCA-3′), Greb1 (5′-CCTGCCGCGACCATCGGCTT-3′, 5′-GACATGCCTGCGCTCTCATACTTA-3′), Wisp2 (5′-CAGGGGTCGCAGTCCACAAAA-3′, 5′-AGGCAGTGAGTTAGAGGAAAGG-3′), Serpina3 (5′-CTGACCTGTCAGGGATCACA-3′, 5′-TGCAGAAAGGAGGGTGATTT-3′), XBP1 (5′-GCGCCTCACGCACCTG-3′, 5′-GCTGCTACTCTGTTTTTCAGTTTCC-3′), Sgk1 (5′-TCGGACTCTGCAAGGAGAAC-3′, 5′-GGCAGGCCATACAGCATCTC-3′), and GAPDH (5′-GGGAAGGTGAAGGTCGGAGT-3′, 5′-TTGAGGTCAATGAAGGGGTCA-3′).

2.5. Anti-ZNF496-immunoprecipitation and LC-MS/MS

MCF-7 nuclear lysates were extracted using a nuclear and cytoplasmic protein extraction kit from 2×10^8 cells and centrifuged at 100,000*g* for 30 min at 4 °C. For immunoprecipitation, nuclear lysates were incubated with 1 μg anti-ZNF496 antibodies or normal IgG for 3 h, and 30 μL protein A/G PLUS agarose was later added and incubated for 8 h. Agarose was collected by centrifugation at 3000*g* for 2 min at 4 °C and washed with NETN lysis buffer 3–4 times for 5 min. Precipitated protein was resolved in 2 × protein loading buffer and boiled for 10 min. The protein was loaded onto SDS-PAGE, immunoblotted with the indicated antibody, and stained with Coomassie blue. The full lanes were cut into small bands regardless of whether the protein band was visible, and were subjected to LC-MS/MS sequencing and data analysis as previously described [24].

2.6. Co-immunoprecipitation, immunoblotting and cellular localization analysis

Co-immunoprecipitation, immunoblotting, and a cellular localization analysis were performed as previously described [25].