Original Paper

Increased Expression of Estrogen Receptor α -36 by Breast Cancer Oncogene IKK ϵ Promotes Growth of ER-Negative Breast Cancer Cells

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Key Words

IKK ϵ • ER- α 36 • ER-negative breast cancer

Abstract

Background/Aims: The expression of estrogen receptor- α (ER α) is one of the most important diagnostic and prognostic factors of breast cancer. Recently, ER α -36 has been identified as a novel variant of ER- α . ER α -36 lacks intrinsic transcription activity and mainly mediates non-genomic estrogen signaling. The noncanonical IKK family member IKK ϵ is essential for regulating antiviral signaling pathways and is recently discovered as a breast cancer oncogene. IKK ϵ interacts with and phosphorylates ER α on serine 167, induces ER α transactivation activity and enhances ER α binding to DNA in ER-positive breast cancer cells. However, the correlation between IKK ϵ and the ER α -36 signaling pathway in ER-negative breast cancer cells remains unclear. **Methods and Results:** In this study, we show that IKK ϵ interacts with ER α -36 and increases its expression in breast cancer cells. As shown by western blot assays, the upregulation of ER α -36 by IKK ϵ was significant. In MDA-MB-231 cells which are ER-negative, IKK ϵ was able to increase the expression of ER α -36 in a dose-dependent manner, and the RNA interference assay indicated the correlation between IKK ϵ and ER α -36 expression. Moreover, IKK ϵ enhanced the growth of MDA-MB-231 and MDA-MB-436 cells. **Conclusions:** These results suggest that IKK ϵ increases ER α -36 expression and is involved in ER α -36 mediated non-genomic estrogen signaling.

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Introduction

Breast cancer is the most common cancer among women, the ER signaling pathway plays a pivotal role in the development of different types of breast cancer [1, 2]. Two types of ERs have been identified, ER α and ER β [3, 4]. There are 3 types of ER α isoforms identified: 66-KD, 46-KD and 36-KD [5]. Tumors expressed ER α -66 were considered as ER α -positive [6]. Previous studies indicated that approximately 70% of these tumors express estrogen receptor α [7]. Patients with ER α -positive tumors are associated with a well differentiated phenotype and have a better prognosis; ER-negative tumors had a shorter disease-free interval and overall survival than patients with ER-positive tumors [8]. Although significant progress has been made in the development of endocrine therapies that target estrogen or ER for ER-positive tumors, there is no effective therapy for the 30% of breast cancers that are ER negative [9, 10]. Recently, a 36-kDa variant of ER α -66, named ER-a36 has been identified, it is mainly expressed in the cytoplasm and plasma membrane and has minimal or no nuclear expression in breast cancer cells [11-13]. ER α -36 was expressed in ER-negative tumor samples and ER-negative breast cancer cell lines [14]. Some studies have suggested that ER α -36 is involved in nongenomic estrogen signaling, and ER α -36 promotes cell proliferation via the MAPK/ERK pathway in ER-negative breast cancers [13, 14].

IKK ϵ is one of noncanonical IKK family member that plays a critical role in the regulation of interferon signaling pathways [15-17]. When IKK ϵ was activated, interferon regulatory factors 3 and 7 (IRF3 and IRF7) were phosphorylated, allowing their nuclear translocation and the transcriptional upregulation of genes involved in the type I interferon response. IKK ϵ also phosphorylates STAT1 following activation by IFN β [18]. IKK ϵ had been identified as a breast cancer oncogene amplified and overexpressed in over 30% of breast carcinomas and breast cancer cell lines [19]. Recently, it has also been demonstrated that IKK ϵ phosphorylates Ser-167 of ER α , and induction of ER α transactivation contributes to tamoxifen resistance in breast cancer [20].

In this study, we show for the first time that IKK ϵ increases the expression of ER α -36 in MDA-MB-231 and MDA-MB-436 breast cancer cell lines. We further show that IKK ϵ interacts with ER α -36. The results from our study also show that IKK ϵ is involved in ER α -negative breast cancer cell growth through regulation of ER α -36 mediated mitogenic estrogen signaling, identifying a novel mechanism by which IKK ϵ are constitutively activated in ER α -negative breast cancer cells.

Materials and Methods

Chemicals and antibodies

The 17 β -estradiol (E $_2$) was obtained from Sigma. The IKK ϵ (I-4907), HA(H-6908), Flag(F-1804), β -actin(A-1978) antibodies were obtained from Sigma. The ER α -36 antibody (CY1109) was obtained from Cell Applications. Cyclin D1(H-295), c-Myc(9E10) were obtained from Santa Cruz Biotechnology. IKK ϵ (sc-39056) siRNA was obtained from Santa Cruz Biotechnology.

Cell lines, treatment and growth assay

MDA-MB-231, MDA-MB-436 cells and human embryonic kidney cell line (HEK293) were obtained from the American Type Culture Collection (ATCC). All cells were maintained in Dulbecco's modified Eagle's medium and 10% fetal calf serum at 37°C in a 5% CO $_2$ incubator. For E $_2$ treatment, cells were maintained in phenolred-free media with 2.5% charcoal-stripped fetal calf serum (HyClone, Logan, UT, USA) for 3 days and then in serum-free medium for 24 h before experimentation.

To cell growth assays, cells were treated with indicated concentrations of E $_2$ or vehicle (ethanol) as a control. The cells were seeded at 1 $\times$ 10 4 cells per dish in 60mm dishes and the cell numbers were determined using the ADAM automatic cell counter (Invitrogen, USA) after 12 days. Five dishes were used for each treatment and experiments were repeated more than three times.

siRNA transfection

MDA-MB-231 and MDA-MB-436 cells were seeded at 2×10^5 cells/dish in 60mm culture dishes 24 h before transfection. IKK ϵ or control siRNA weighing 1 mg was mixed with siRNA transfection medium and siRNA transfection reagent (Santa Cruz Biotechnology) and incubated for 30 min at room temperature before added into cultured cells. The efficiency of siRNA knock-down was assessed with western blot analysis.

DNA Transfection and Luciferase Assays

For transient transfection assays, cells were seeded in six-well dishes and grown to 60–70% confluence in phenol-red-free medium plus 2.5% steroid-free FCS. Cells were washed and transiently transfected with a total of 5 μ g of plasmid with the FuGene6 reagent (Roche Molecular Biochemicals). The luciferase assays were performed by using the Luciferase Assay kit from Promega according to the manufacturer's recommendations. The Gal4 L-ELK plasmid was purchased from Stratagene. At 48 h after transfection, cell extracts were prepared and luciferase activities were determined and normalized using the Dual-Luciferase Assay System (Promega).

Immunoprecipitation and immunoblot analysis

For immunoprecipitation assays, cells were washed twice with ice-cold phosphate buffered saline and lysed with the lysis buffer (150mM NaCl, 20mM Tris-HCl, pH 7.4, 0.1% NP-40) supplemented with protease and phosphatase inhibitors (Roche). Cell lysates were then incubated with indicated primary antibodies or pre-immune serum and immunoprecipitated with protein A/G plus agarose. The precipitates were then washed, separated on SDS-polyacrylamide gel electrophoresis and analyzed with western blot analysis.

For Western blot analyses, cells were disrupted with RIPA buffer, and the cell lysates were boiled in a gel-loading buffer and separated by SDS-PAGE in 10% gels. After PAGE, the proteins were transferred to a poly vinylidene difluoride membrane (Millipore), and visualized with the appropriate species-specific peroxidase-conjugated secondary Abs (Santa Cruz Biotechnology) and enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia).

Immunohistochemistry

Tumor tissues were collected from 25 cases of patients with ER-negative breast carcinomas with informed consent in Jinan Military General Hospital (Jinan, China). The design of the study was approved by the Hospital Ethical Committee. Immunohistochemistry (IHC) was carried out based on the method described before. Briefly, antigen retrieval was performed using 10 mM sodium citrate buffer (pH 6.0) in a 95°C water bath for 15 minutes. Then primary antibody was used overnight at 4 °C in a humidified chamber, followed by secondary antibody.

Statistical analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences 13.0 (SPSS). Data are presented as the means $\pm$ SEM. Statistical significance was determined by a one-way analysis of variance or the t-test. P-values <0.05 were considered to indicate statistically significant differences.

Results

IKK ϵ interacts with ER α -36

Because IKK ϵ interacts with the ER α -66, which has conserved domains similar to those of ER- α , we next examined whether ER α -36 interacts with IKK ϵ . The coimmunoprecipitation was performed in HEK-293T cells, which had been transfected with HA-ER α -36 and Flag-IKK ϵ . ER α -36 was detected in flag-IKK ϵ immunoprecipitates (Fig. 1A). Furthermore, endogenous ER α -36 and IKK ϵ were able to form a complex in MDA-MB-231 cells (Fig. 1, B and C), which express ER α -36 as well as high levels of IKK ϵ . These findings indicate that ER α -36 interacts with IKK ϵ . To further explore the molecular basis of the interaction between IKK ϵ and ER α -36, the regions of IKK ϵ were defined. Results of co-immunoprecipitation experiment showed that the kinase domain (1–290 amino acids) of IKK ϵ were indispensable for their interaction (Fig. 1D). In order to obtain evidence of direct IKK ϵ phosphorylation of ER α -36, recombinant GST-ER α -36 was incuba-

Fig. 1. IKK ϵ and interacts with ER α -36. (A) IKK ϵ interacts with ER α -36. HEK-293T cells were transfected with Flag-IKK ϵ and HA-ER α -36. After a 24-h incubation, cells were lysed and immunoprecipitated with anti-Flag antibody. The immunoprecipitates were immunoblotted with anti-HA antibody and anti-Flag antibody. (B and C) endogenous ER α -36 binds to IKK ϵ . MDA-MB-231 cells, expressing ER α -36 and IKK ϵ , were immunoprecipitated with anti-ER α -36 and detected with anti-IKK ϵ antibody (B) and vice versa (C). IgG was used as a control for coimmunoprecipitation. (D) 293T cells were co-transfected with the indicated plasmids, and anti-Flag immunoprecipitates were analyzed by immunoblotting with anti-HA or anti-Flag antibody. (E) Recombinant GST-ER α -36 was incubated with IKK ϵ immunoprecipitates from transfected 293T cells in the presence of [γ - 32 P]-ATP. The reaction products were analyzed by SDS-PAGE and autoradiography.

ted with IKK ϵ immunoprecipitates from transfected cells in the presence of [γ - 32 P]-ATP. Analysis of the reaction products by SDS-PAGE and autoradiography showed that incubation of IKK ϵ with GST-ER α -36 resulted in phosphorylation of ER α -36. These findings indicate that IKK ϵ can phosphorylates ER α -36 (Fig. 1E).

IKK ϵ increase the expression of the ER α -36 protein

To explore the possible effects of IKK ϵ on ER α -36, we first examined whether IKK ϵ affects ER α -36 expression. HEK-293T cells were cotransfected with Flag-tagged IKK ϵ and HA-ER α -36. The protein levels of ER α were greatly increased by WT IKK ϵ . In contrast, kinase dead mutant of IKK ϵ (K38A) resulted in a complete abolished IKK ϵ -mediated ER α -36 upregulation (Fig. 2A). Furthermore, overexpression of IKK ϵ in ER α -negative breast cancer cell lines (MDA-MB-231 and MDA-MB-436) leads to increase the endogenous protein levels of ER α -36. Similarly, overexpression of IKK ϵ (K38A) mutation abolished IKK ϵ -mediated endogenous ER α -36 upregulation in these cell lines (Fig. 2B). In addition, decreasing the endogenous amount of IKK ϵ in human MDA-MB-231 and MDA-MB-436 cells with IKK ϵ siRNA oligos led to decrease the endogenous protein levels of ER α -36, and this phenotype was reversed by cotransfected with IKK ϵ (res) plasmid and IKK ϵ siRNA oligos (Fig. 2C). Therefore, we suggest that IKK ϵ -mediated increase of ER α -36 protein expression, and this effect probably mediated by IKK ϵ kinase activity. Moreover, deletion of the kinase domain of IKK ϵ abolishes the IKK ϵ -induced increase the expression of ER α -36 (Fig. 2D). In order to explore whether IKK ϵ regulates ER α -36 at transcriptional level, Realtime-PCR analysis was done. Realtime-PCR analysis showed no significant difference in the transcriptional level of ER α -36 by IKK ϵ overexpression (Fig. 2E).

IKK ϵ enhance ER α -36 mediated mitogenic estrogen signaling in ER-negative breast cancer cells

Previous reports have indicated that ER α -36 mediates mitogenic estrogen signaling in ER-negative breast cancer cells. E $_2$ stimulates a rapid activation of the MAPK/ERK pathway mediated by ER α -36. Consequently, E $_2$ was also able to induce expression of the growth-promoting genes c-Myc and cyclin D1. We then decided to determine whether IKK ϵ impact the estrogen-

Fig. 2. IKK ϵ increase the expression of the ER α -36 protein. (A) HEK-293T cells were cotransfected with HA-tagged ER α -36 and Flag-tagged IKK ϵ or IKK ϵ kinase dead mutant (K38A). Whole-cell lysates were analyzed by immunoblotting with anti-Flag or anti-HA antibody, β -actin was used as equal loading control. Columns represent the means of three experiments; bars represent the s.e. * $P < 0.05$. (B) MDA-MB-231 and MDA-MB-436 cells were transiently transfected with Flag-tagged IKK ϵ or IKK ϵ kinase dead mutant (K38A). Whole-cell lysates were analyzed by immunoblotting with anti-Flag or anti-ER α -36 antibody. Columns represent the means of three experiments; bars represent the s.e. * $P < 0.05$. (C) MDA-MB-231 and MDA-MB-436 cells transfected with siIKK ϵ and a siRNA-resistant IKK ϵ (IKK ϵ -res) expression plasmid. Whole-cell lysates were analyzed by immunoblotting with anti-Flag or anti-ER α -36 antibody, β -actin was used as equal loading control. Columns represent the means of three experiments; bars represent the s.e. * $P < 0.05$. (D) MDA-MB-231 cells were co-transfected with the indicated plasmids. Whole-cell lysates were analyzed by immunoblotting with anti-Flag or anti-ER α -36 antibody, β -actin was used as equal loading control. (E) MDA-MB-231 cells transfected with vector or IKK ϵ expression plasmids. RNA was extracted 24 hr later, ER α -36 mRNA level was analyzed by quantitative realtime-PCR.

activated MAPK pathway mediated by ER α -36, we use a luciferase reporter construct containing the ERK-responsive transactivation domain of human Elk, which is a downstream effector of the MAPK/ERK signaling pathway. The HEK-293T cells were transfected with ERK-responsive GAL-Elk chimeric transcription factor and GAL4 fused ERK-responsive transactivation domain of Elk1. The activity of a luciferase reporter gene containing GAL4-binding sites in the presence of E $_2$ /Gal4 fusion protein-mediated transactivation of the reporter gene than transfected with ER α -36 alone, whereas no effect on the transcription activity of the Elk/Gal4 fusion protein in the control cells transfected with empty vector or E $_2$ absence cells (Fig. 3A). Furthermore, IKK ϵ transfected cells also increase transcription activity of the Elk/Gal4 than transfected with empty vector in ER-negative breast cancer cells (MDA-MB-231 and MDA-MB-436), whereas no effect on the transcription activity of the Elk/Gal4 in the cells transfected with siRNA oligos or E $_2$ absence cells (Fig. 3B and C). Consequently, IKK ϵ was also able to enhance expression of the growth-pro-

Fig. 3. IKK ϵ enhance ER α -36 mediated mitogenic estrogen signaling. (A) HEK-293T cells were cotransfected with Flag-IKK ϵ , HA-ER α -36, Gal4-LUC luciferase reporter plasmid and the Gal-ELK expression vector. The cell cultures were maintained in estrogen-free medium for 36 h before E $_2\beta$ (10 nM) was added for 12 h. Luciferase activities with SDs representative of more than three experiments performed in duplicate. MDA-MB-231 (B) and MDA-MB-436 (C) cells were cotransfected with Flag-IKK ϵ , siER α -36 oligo, Gal4-LUC luciferase reporter plasmid and the Gal-ELK expression vector. The cell cultures were maintained in estrogen-free medium for 36 h before E $_2\beta$ (10 nM) was added for 12 h. Luciferase activities with SDs representative of more than three experiments performed in duplicate. (D) MDA-MB-231 cells transfected with Flag-IKK ϵ . The cell cultures were maintained in estrogen-free medium for 36 h before E $_2\beta$ (10 nM) was added for 12 h. Whole-cell lysates were analyzed by immunoblotting with anti-c-Myc or anti-CyclinD1 antibody, β -actin was used as equal loading control. (E) MDA-MB-231 cells transfected with indicated plasmids or siER α -36 oligo. The cell cultures were maintained in estrogen-free medium for 36 h before E $_2\beta$ (10 nM) was added for 12 h. Whole-cell lysates were analyzed by immunoblotting with anti-c-Myc or anti-CyclinD1 antibody, β -actin was used as equal loading control. (F) MDA-MB-231 cells transfected with indicated plasmids or siER α -36 oligo. The cell cultures were maintained in estrogen-free medium for 36 h before E $_2\beta$ (10 nM) was added for 12 h. Whole-cell lysates were analyzed by immunoblotting with anti-p-ERK or anti-ERK antibody, β -actin was used as equal loading control.

moting genes c-Myc and cyclin D1 in MDA-MB-231 cell lines with E $_2\beta$ treated (Fig. 3D). We also found that IKK ϵ failed to enhance expression of c-Myc and cyclin D1 in MDA-MB-231 cell lines with E $_2\beta$ treated by transfected with ER α -36 siRNA oligos (Fig. 3E). To determine whether IKK ϵ induced phosphorylation of the MAPK/ERK1/2 mediated by ER α -36, we transfected cells with IKK ϵ and ER α -36 siRNA oligos to decrease the endogenous protein levels of ER α -36. The signal

Fig. 4. IKK ϵ promote ER-negative breast cancer cell growth mediated by ER α -36. (A, B) MDA-MB-231 and MDA-MB-436 cells were transfected with IKK ϵ were seeded at a density of 1×10^4 per well in six-well plates. The cell cultures were maintained in estrogen-free medium for 36 h before E $_2$ (10 nM) was added for 12 h. Cell viability was assessed at the indicated times. Bars represent the s.e. * $P < 0.05$. (C) MDA-MB-231 transfected with IKK ϵ or cotransfected with IKK ϵ and ER α -36 siRNA were seeded at a density of 1×10^4 per well in six-well plates. The cell cultures were maintained in estrogen-free medium for 36 h before E $_2$ (10 nM) was added for 12 h. Cell viability was assessed at the indicated times. Bars represent the s.e. * $P < 0.05$. (D) Scatter plot of IKK ϵ and ER α -36 expression in ER α -negative breast cancer from 25 patients. IKK ϵ shows positively correlated with ER α -36 in breast cancer samples using Spearman's correlation analysis ($P = 0.036$, $r = 0.384$).

for p-ERK was significantly enhanced by overexpressed IKK ϵ , whereas this increased phosphorylation was greatly reduced by ER α -36 siRNA oligos (Fig. 3F). These results demonstrated that IKK ϵ enhance ER α -36 mediated mitogenic estrogen signaling.

IKK ϵ promote ER-negative breast cancer cell growth mediated by ER α -36

To further pursue the significance of IKK ϵ -ER α -36 interaction, we tested whether IKK ϵ impact ER α -36 mediates estrogen-stimulated cell proliferation. In assays of anchorage-dependent growth, MDA-MB-231 cells and MDA-MB-436 cells transfected with IKK ϵ grew faster than those transfected with empty vector with E $_2$ treated, whereas IKK ϵ had no effect on the growth of the control MDA-MB-231 cells without E $_2$ treated (Fig. 4A and B). ER α -36 knockdown almost completely abolished IKK ϵ -mediated growth stimulation of MDA-MB-231 cells (Fig. 4C). In order to further support the role of IKK ϵ in ER α -negative breast cancer as well as to substantiate the functional link between IKK ϵ and ER α -36 and extend the physiological relevance of this link, we collected 25 ER α -negative breast cancer samples from breast cancer patients. The expression of IKK ϵ and ER α -36 was analyzed by immunohistochemistry staining with an antibody of IKK ϵ and ER α -36. The results suggested that IKK ϵ had a significant positive correlation with ER α -36 expression ($P = 0.036$, $r = 0.384$) (Fig. 4 D).

Discussion

Breast cancer is the most common malignant tumor which is the leading cause of cancer-related deaths in women worldwide [21]. ER α expression is one of the most important responses to endocrine therapy [22]. Approximately 70% of breast cancer cases are ER α -positive, and the remaining 30% are ER-negative [10]. ER α -36 is a variant of conventional ER α , which lacks both transcriptional activation domains of ER α -66, retains DNA-binding domain, partial dimerization and ligand-binding domains, and possesses a unique 27 amino acid domain that replaces the last 138 amino acid of ER α -66 [11, 13]. It was found that ER α -36 expression levels were higher in ER-negative tumors and lower in ER-positive tumors [12]. ER α -36 mediated mitogenic estrogen signaling in ER-negative breast cancer cells, such as MDA-MB-231 and MDA-MB-436 cells that lack

expression of ER-α66 but highly express ER-α36. It was reported that E₂β induced the MAPK/ERK activation through a mechanism that involves ER-α36 and the EGFR/Src/Shc complex and exhibited a potent mitogenic estrogen signaling *in vitro* and *in vivo* [11, 12, 14].

In the present study, we identified that IKKε interacts with ERα-36, increases the expression of ERα-36 in MDA-MB-231 and MDA-MB-436 breast cancer cells. Additionally, IKKε enhances ERα-36 mediated mitogenic estrogen signaling and promotes ER-negative breast cancer cell growth mediated by ERα-36.

IKKε is a member of IKK family, and shares 30% amino acid identity with IKKα and IKKβ in their kinase domains [15]. IKKε plays a central role in regulating innate immunity by activating the NF-κB signaling pathway and phosphorylating IRF3 and IRF7 to regulate the type I interferon response [16]. IKKε also phosphorylates the tumor suppressor CYLD and promotes cell transformation [23]. In addition, recent study indicated that IKKε is amplified in a substantial percentage of human breast tumors and acts as an oncogene in breast cancers [19]. Especially, IKKε phosphorylates ERα on Ser-167 site, leading to activation of ERα and up-regulation of cyclin D1. Furthermore, overexpression of IKKε in ERα-positive breast cancer cells induces tamoxifen resistance [20]. This effect mainly required kinase activity of IKKε. In this study, we found that kinase lethal mutant of IKKε (K38A) resulted in a complete abolished IKKε-mediated ERα-36 upregulation, indicating that ERα-36 may be a new IKKε kinase substrate.

In summary, we have shown that breast oncogene IKKε interacts with ERα-36, and increases ERα-36 expression in ER-negative breast cancer cells. In addition, IKKε increases ERα-36 mediated MAPK/ERK signaling pathway, and enhances nongenomic estrogen signaling, contributing to development and progression of ER-negative breast cancer cells. Therefore, IKKε plays important roles in breast cancer tumorigenesis, and may be a very effective target for breast cancer therapy through regulating multiple signaling pathways.

Conflict of Interest

None.

Acknowledgements

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