ONCOLOGY

ER-α36, a novel variant of estrogen receptor α, is involved in EGFR-related carcinogenesis in endometrial cancer

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OBJECTIVE: To explore the role of estrogen receptor-α36 (ER-α36) in epidermal growth factor receptor (EGFR)-related carcinogenesis in endometrial cancer.

STUDY DESIGN: The expression of ER-α36, EGFR, and phospho-extracellular signal-regulated kinase was analyzed using immunohistochemistry in endometrial cancer samples. The cellular localization of ER-α36 and EGFR was determined using immunofluorescence in the endometrial cancer Hec1A cells. The level of phospho-extracellular signal-regulated kinase of Hec1A cells was determined using Western blotting after treatment with epidermal growth factor.

RESULTS: Positive rate of ER-α36 was increased in high-stage (P = .03) and high-grade (P = .224) endometrial cancer; expression of ER-α36 and EGFR exhibited a significant positive correlation (r = 0.334, P = .025) and they showed substantial colocalization on the plasma membrane of glandular cells; phospho-extracellular signal-regulated kinase positive rate in ER-α36 positive group and EGFR positive group was higher than that of ER-α36 negative group (P = .014) and EGFR negative group (P = .016); finally, ER-α36 mediated epidermal growth factor-stimulated extracellular signal-regulated kinase activation in Hec1A cells.

CONCLUSION: ER-α36 mediates EGFR-related extracellular signal-regulated kinase activation in endometrial cancer.

Key words: endometrial cancer, epidermal growth factor receptor, estrogen receptor-α36, estrogen receptor-α66, MAPK/ERK pathway


Endometrial cancer is one of the most common gynecologic malignancies and is the seventh most common cancer in women worldwide.1 Multiple factors are involved in development and progression of endometrial cancer, such as estrogen,2 testosterone,3 and epidermal growth factor (EGF).4 Recently, a novel variant of estrogen receptor α, termed estrogen receptor-α36 (ER-α36), was cloned.5 ER-α36, which is generated from a promoter located in the first intron of the ER-α66 (the classic estrogen receptor) gene, lacks transcriptional activation domain (AF1 and AF2) of ER-α66, but retains the DNA-binding domain, partial dimerization and ligand-binding domains. ER-α36 possesses unique 27-aa domain to replace the last 138-aa encoded by exons 7 and 8 of ER-α66 gene.6 Current research found that ER-α36 is mainly localized on the membrane and modulates nongenomic signaling pathways, such as PI3K/Akt, MAPK/ERK, and PKC pathway,3,6 which participate in the development and progression of many types of cancers. Clinical studies reported that overexpression of ER-α36 was associated with poorer disease-free survival and disease-specific survival in patients with ER-α66-positive breast cancer who received tamoxifen treatment.7 Breast cancer cell line MCF-7 that constitutively express high levels of recombinant ER-α36 exhibited insensitivity to tamoxifen treatment.8 However, up to now, there was no related clinical data about ER-α36 and endometrial cancer.

Epidermal growth factor receptor (EGFR), the prototypic member of the ErbB/HER receptor tyrosine kinase family, is associated with cancer in general,9,10 and the up-regulation of EGFR contributes to the resistance and progression of cancer. Overexpression of EGFR plays an important role in activating the growth factor signaling.11 Increasing evidence has shown that EGFR is involved in the development of endometrial cancer in particular.4,12 Studies have found that EGF could stimulate the mitogen-activated protein kinase (MAPK) pathway, causing an increase in the level of phosphorylation of AF1 domain of ER-α66, which results in the ex-
expression of estrogen-response genes.\textsuperscript{13-17} Taken together, estrogen receptor and EGFR may act coordinately in the development of gynecologic neoplasm.

As ER-\(\alpha\)36 lacks the transcriptional activation domains of ER-\(\alpha\)66, we thus hypothesized that ER-\(\alpha\)36 might be associated with the EGFR-related carcinogenesis through a different mechanism from ER-\(\alpha\)66. In this study, we aimed to establish the evidence that ER-\(\alpha\)36 plays a role in the EGFR-related endometrial cancer and preliminarily explore its potential mechanism, hoping to provide a new insight to the complicated genesis and development of endometrial cancer.

**MATERIALS AND METHODS**

**Materials and reagents**

Anti-ERK1/2 antibody, anti-phospho-ERK1/2 antibody (Thr<sup>202</sup>/Tyr<sup>204</sup>), and EGFR antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ER-\(\alpha\)36 specific antibody against the 20 unique amino acids at the C-terminal of ER-\(\alpha\)36, was described previously.\textsuperscript{5,6} EGF was obtained from Sigma-Aldrich (St. Louis, MO).

**Cell culture and cell lines**

Human Hec1A endometrial cancer cells were provided by Dr Li-Hui Wei (Peking University People’s Hospital, Beijing, China). It has been reported that endometrial cancer Hec1A cell is an ER-\(\alpha\)66-negative cell line.\textsuperscript{3,18} Hec1A cells were grown at 37°C with 5% CO\(_2\) in DMEM supplemented with 10% fetal calf serum. We established stable Hec1A cell lines transfected with an ER-\(\alpha\)36 shRNA expression vector (Hec1A/RNAi) and the empty expression vector (Hec1A/V) as described elsewhere.\textsuperscript{3}

**Immunofluorescence and confocal microscopy**

The cellular colocalization of ER-\(\alpha\)36 and EGFR was determined by indirect immunofluorescence. Hec1A cells cultured on sterile glass coverslips were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes. After being permeabilized with 0.4% Triton X-100 for 10 minutes at room temperature, cells were blocked in 4% bovine serum albumin (BSA)-supplemented DMEM for 1 hour and incubated overnight at 4°C with anti-ER-\(\alpha\)36-specific antibody against the 20 unique amino acids at the C-terminal of ER-\(\alpha\)36. After 3 washes in PBS, the cells were labeled with TRITC-conjugated secondary antibody. The cells were again blocked in 1% BSA-supplemented PBS for 1 hour at room temperature, followed by staining with EGFR antibody. After 3 washes in PBS, the cells were labeled with FITC-conjugated secondary antibody. The DNA dye Hoechst 33258 was used for nuclear staining. Microscopic analyses were performed using a Confocal Laser-Scanning Microscope (Zeiss LSM 510 META; Carl Zeiss, LLC, Berlin, Germany).

**Western blotting analysis**

Cells were grown in phenol-red-free medium with 2.5% dextran charcoal-stripped FCS (Biochrom AG, Berlin, Germany) for 48-72 hours and then switched to medium without serum 12 hours before stimulation by the agents indicated. The cells were collected in ice-cold PBS, and the cell extracts were prepared in RIPA buffer with proteinase inhibitor cocktail from Sigma-Aldrich (St. Louis, MO). Cell lysates were boiled with gel-loading buffer for 5 minutes at 100°C, resolved on 10% SDS-PAGE, transferred to polyvinylidene fluoride membrane. After transfer, the membranes were blocked in TBST (TBS containing 0.1% Tween 20) containing 5% skimmed milk for 2 hours, followed by incubation overnight at 4°C with appropriate primary antibodies. After washing 3 times in TBST, 10 minutes each, the membranes were incubated for 1 hour at 37°C with 1:2000 horseradish peroxidase-conjugated appropriate secondary antibodies. Finally, the membranes were processed and visualized using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotechnology, Piscataway, NJ).

**Immunohistochemistry**

Endometrial tissues were collected for immunohistochemistry when patients with endometrial cancer underwent hysterectomy in Peking University Third Hospital. The study was approved by the Ethics Committee of Peking University Third Hospital and all written informed consent documents were signed by all patients. The mean age of the patients was 56.94 ± 1.48 years old. All the samples were type I endometrial cancers with histology of endometrioid cancer. We used International Federation of Gynecology and Obstetrics 2000 to define the stage. Stage Ia (low-stage) was defined as the cancer confined in the endometrium without metastasized to myometrium or other position else. Stage above Ia (high-stage) was defined as cancer metastasized...
outward endometria. Tissue slides were deparaffinized with xylene and rehydrated through a graded alcohol series. Antigen retrieval was carried out by immersing the slides in sodium citrate buffer and boiling in a water bath at 100°C for 30 minutes. The endogenous peroxidase activity was blocked by incubation in a 3% hydrogen peroxide buffer for 10 minutes. The slides were rinsed in PBS and incubated with normal goat serum to block nonspecific staining. The slides were then incubated with the primary antibody overnight at 4°C in a humidified chamber. The sections were incubated with the second antibody for 30 minutes. A diaminobenzidine was used as a chromogen, and sections were counterstained with hematoxylin. The staining intensity in the plasma membrane was evaluated. Duplicate sections were immunostained without exposure to primary antibodies and were used as negative controls. Samples were evaluated by 3 observers who were unaware of the identity of the sections. Scoring for staining was graded as follows: no staining or staining observed in less than 10% of glandular cells was scored as 0; faint/barely perceptible staining detected in ≥10% of glandular cells was scored as 1; a moderate or strong complete staining observed in ≥10% of glandular cells was scored as 2+ or 3+, respectively. Scores of 0 were considered negative, whereas 1+, 2+, and 3+ were considered positive. The χ² tests were used to analyze the data.

**RESULTS**

**The positive rate of ER-α36 was increased in high-stage endometrial cancer**

The cellular localization of ER-α36 was determined by immunohistochemistry using anti-ER-α36 antibody. As shown in Figure 1, A, ER-α36 was mainly localized on the plasma membrane of the glandular cells in endometrial cancer tissue, though there was also weaker staining in the cytoplasm. The expression of ER-α36 and its association with endometrial cancer characteristics was shown in Table 1. The ER-α36 positive rate in the Ia stage group of endometrial cancer (41.4%, 12/29) was significantly lower than that of the above Ia stage group (75%, 12/16) (P = .03, χ² = 4.683). The ER-α36 positive rate in the low-grade group (40%, 6/15) was also lower than that of the high-grade group (58.8%, 20/34), and no statistical significance was observed (P = .224, χ² = 1.481).

**ER-α36 and EGFR are colocalized on the plasma membrane of endometrial cancer Hec1A cells**

To examine ER-α36 and EGFR localization in Hec1A cells, immunofluorescence was performed with anti-ER-α36 antibody and anti-EGFR antibody. Figure 2, A showed the immunofluorescent staining of EGFR (green) and ER-α36 (red) on the Hec1A cells. The cells were counterstained with Hoechst 33258 (blue) to show the cell nuclei. Merged images of ER-α36 and EGFR indicated substantial colocalization signals (yellow) on the plasma membrane.

**There is a correlation between expression of ER-α36 and EGFR**

EGFR was mainly localized on the plasma membrane of the glandular cells in endometrial cancer tissue (Figure 1, B). The expression of ER-α36 and EGFR in 45 endometrial cancer samples was shown in Table 2. The EGFR positive rate in the ER-α36 positive group (83.3%, 20/24) was higher than that of ER-α36 negative group (52.4%, 11/21) (P = .025). The ER-α36 positive rate in the EGFR positive group (64.5%, 20/31) was also higher than that of EGFR negative group (28.6%, 4/14) (P = .025). In addition, the expression of the 2 receptors exhibited a significant positive correlation (r = 0.334, P = .025).

**TABLE 1**

The association between ER-α36 expression and endometrial cancer characteristics

<table>
<thead>
<tr>
<th>Endometrium cancer characteristics</th>
<th>ER-α36</th>
<th>P value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage Ia</td>
<td>1</td>
<td>0.03</td>
<td>4.683</td>
</tr>
<tr>
<td>Above Ia</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade Low-grade</td>
<td>41.2</td>
<td>0.224</td>
<td>1.481</td>
</tr>
<tr>
<td>Grade High-grade</td>
<td>41.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade above Ia</td>
<td>58.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade above Ia</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> P < .05 has the significant difference.

**TABLE 2**

The expression of ER-α36 and EGFR in endometrial cancer samples

<table>
<thead>
<tr>
<th>EGFR</th>
<th>ER-α36</th>
<th>Total</th>
<th>P value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>10</td>
<td>14</td>
<td>.025</td>
<td>5.007</td>
</tr>
<tr>
<td>Positive</td>
<td>11</td>
<td>31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>21</td>
<td>45</td>
<td></td>
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</tbody>
</table>

<sup>a</sup> P < .05 has the significant difference.

Table 4 showed the expression of EGFR and phospho-ERK in 28 endometrial cancer samples. The phospho-ERK positive rate in the EGFR positive group (66.7%, 14/21) was higher than that of EGFR negative group (14.3%, 1/7) ($P = .016$).

In addition, the ER-α36 positive rate in the 14 EGFR-positive and phospho-ERK-positive samples was 71.4% (10/14). The EGFR positive rate in the 10 ER-α36-positive and phospho-ERK-positive samples was 100% (10/10).

**ER-α36 mediates EGF-stimulated ERK activation**

Next, we examined the phosphorylation levels of ERK in EGF-stimulated endometrial cancer Hec1A cells. As shown in Figure 2, B, EGF treatment induced phosphorylation of ERK1/2 in Hec1A cells, reaching the peak after 10 minutes. Reprobing the same membrane with a total ERK1/2 antibody indicated that the total ERK1/2 content was not changed. We next examined the changes in ERK1/2 phosphorylation in Hec1A cells after treatment with different doses of EGF for 10 minutes. As shown in Figure 2, C, EGF induced a dose-dependent increase in ERK1/2 phosphorylation. Reprobing the same membrane with a total ERK1/2 antibody indicated that the total ERK1/2 content was not changed. However, EGF failed to induce ERK1/2 phosphorylation in Hec1A/RNAi cells (Figure 2, D). Our results indicated that ER-α36-mediated activation of MAPK pathway is involved in EGF signaling.

**COMMENT**

The current study found that ER-α36, a novel variation of estrogen receptor, was localized on the plasma membrane of glandular cells in both endometrial cancer tissues and cell lines, which was consistent with recent findings.\(^3,19\) The immunohistochemistry and immunofluorescence results also indicated that EGFR expressed mainly on the plasma membrane of glandular cells in both endometrial cancer tissues and cell lines. The current results demonstrated that both ER-α36 and EGFR were mainly localized on the plasma membrane and...
might function concordantly with each other in their roles in malignance.

The classic estrogen receptor, ER-α66, is a ligand-activated transcription factor whose transcriptional activities are influenced by various second messenger signaling pathways. EGF has been reported to produce ligand-independent transcriptional activation of ER-α66. However, as ER-α36 lacks transcriptional activation domain of AF1 and AF2, the above mechanism of EGFR and ER-α66 crosstalk could not be referred to ER-α36. Our current study demonstrated that ER-α36 and EGFR were colocalized on the plasma membrane of glandular cells in endometrial cancer Hec1A cells, and their expression levels in endometrial cancer tissues had a positive correlation, suggesting that ER-α36 and EGFR can form a complex on the membrane. It is well known that MAPK/ERK pathway participates in the development and progression of many types of cancers, including endometrial cancer. In the current report, we found that EGF treatment induced time-dependent and dose-dependent increase in ERK1/2 phosphorylation in ER-α66-negative Hec1A cells. However, EGF failed to stimulate phosphorylation of the ERK1/2 in Hec1A cells with ER-α36 knockdown. It is possible that EGF promotes endometrial cancer cell proliferation through the ER-α36 and EGFR complex-mediated MAPK/ERK pathway.

Furthermore, the result of ER-α36 expression in human endometrial cancer tissues revealed that ER-α36 was implicated in invasion and differentiation of endometrial cancer. Several recent researches also demonstrated that ER-α36 was positively involved in the genesis and development of carcinoma, such as gastric cancer and breast cancer. Previous studies have also shown that EGFR played an important role in the genesis and development of endometrial cancer with the underlying mechanism that EGFR stimulated MAPK pathway. And the current study found for the first time that ER-α36 was essential in EGF-mediated MAPK pathway activation in endometrial cancer Hec1A cells and further associated with the condition of endometrial cancer. However, how EGFR and ER-α36 interaction needs further explore in the future in endometrial cancer.

In summary, we have found that ER-α36 was colocalized with EGF on the plasma membrane of glandular cells in endometrial cancer cells. We have demonstrated that ER-α36 mediated EGF-stimulated ERK activation and the expression of ER-α36 was negatively associated with the stage of endometrial cancer. The current study has put a new insight into understanding ER-α36-related endometrial carcinogenesis. Further research of ER-α36 functions and its role in malignance may provide novel information for designing new drugs for the treatment of endometrial cancer.

ACKNOWLEDGMENTS
We thank Dr Li-Hui Wei for kindly providing the Hec1A cells and Professor Taiping Wang for revising the report.

REFERENCES
8. Lin SL, Yan LY, Zhang XT, et al. ERalpha36, a variant of ER-alpha, promotes tamoxifen agonist action in endometrial cancer cells via the

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**TABLE 3**

<table>
<thead>
<tr>
<th>ER-α36</th>
<th>Phospho-ERK</th>
<th>P value</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative, %</td>
<td>68.7 (11/16)</td>
<td>31.3 (5/16)</td>
<td>.014</td>
</tr>
<tr>
<td>Positive, %</td>
<td>23.1 (3/13)</td>
<td>76.9 (10/13)</td>
<td></td>
</tr>
</tbody>
</table>

ER-α36, estrogen receptor-α36; ERK, extracellular signal-regulated kinase.

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**TABLE 4**

<table>
<thead>
<tr>
<th>EGFR</th>
<th>Phospho-ERK</th>
<th>P value</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative, %</td>
<td>85.7 (6/7)</td>
<td>14.3 (1/7)</td>
<td>.029</td>
</tr>
<tr>
<td>Positive, %</td>
<td>33.3 (3/7)</td>
<td>66.7 (1/7)</td>
<td></td>
</tr>
</tbody>
</table>

EGFR, epidermal growth factor receptor; ER-α36, estrogen receptor-α36; ERK, extracellular signal-regulated kinase.

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