# Estrogen Receptor Variant ER-α36 Facilitates Estrogen Signaling Via EGFR Signaling in Glioblastoma

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# Abstract

Glioblastoma is a deadly and common primary brain tumor. Poor prognosis is linked to high proliferation and cell heterogeneity. Sex differences may play a role in patient outcome. Previous studies showed that ER-α36, a variant of the estrogen receptor, mediated non-genomic estrogen signaling and is highly expressed in many ER-negative tumors. ER-α36 also associates with the EGFR. We showed that ER-α36 was highly expressed and confirmed that ER-α36 co-labels with EGFR in glioma specimens. We also investigated the mechanisms of estrogen induced proliferation in ER-α-negative cells U87 and U251. We found that glioblastoma cell varying responsive to mitogenic estrogen signaling which correlated with ER-α36 expression, and knockdown of ER-α36 diminished the response. Exposure to estrogen also caused up-regulation of cyclin protein expression *in vitro*. We also found that low concentrations of estrogen promoted SRC-Y-416 and inhibited SRC-Y-527 phosphorylation, corresponding with activated SRC signaling. Inhibiting SRC or EGFR abolished estrogen-induced mitogenic signaling, including cyclin expression and MAPK phosphorylation. Cumulatively, our results demonstrate that ER-α36 promotes non-genomic estrogen signaling via the EGFR/SRC/MAPK pathway in glioblastoma. This may be important for the treatment of ER-α-negative glioblastomas that retain high level of ER-α36, since estrogen may be a viable therapeutic target for these patients.


# Introduction

Glioblastoma (GBM) is the most common primary brain tumor and is associated with frequent relapse and a high mortality rate [1]. GBM is heterogeneous and the prognosis relies on molecular and biological factors. Current treatments consist of chemotherapy, radiation, and the median survival is ~15 months [2, 3]. Many factors can affect glioblastoma prognosis, including diagnosis stage, molecular and genetic features of the tumor, age, and sex [4, 5]. Estrogens are well known to be neuroprotective in a variety of central nervous system disorders, including Alzheimer's disease [6], Parkinson's disease [7], and ischemic injury caused by stroke [8]. Men are 1.5-2 times more likely to develop glioblastoma than women [9, 10]. Furthermore, the rate of GBM development in women decreases between ages 30-54 years old, then increases and remains constant after 55 years [11]. These trends correspond with estrogen receptor (ER) expression. While normal astrocytes are known to express ER-α, most glioblastomas are ER-α negative [12, 13]. ER-β is also decreased in glioblastoma [14, 15]. Interestingly, despite the decreases in the canonical estrogen receptors, a clinical trial showed that the selective estrogen receptor modulator tamoxifen (TAM) could prolong glioblastoma patients survival, suggesting that estrogen may protect against GBM [16].

Estrogen receptors (ERs) are a nuclear receptor superfamily. The most common nuclear receptors are ER-α66, its splice variant ER-α46, and ER-β. The genomic signaling pathway is mediated via specific binding ligands such as estrogen and TAM. Some ERs are membrane bound and facilitate non-genomic signaling pathways. ER-α36 is mainly localized to the plasma membrane (~50%), and the remainder is within the cytoplasm (~40%) and the nucleus (~10%) [17]. Since its discovery, ER-α36 has been found to be expressed in breast, endometrial, lung, liver, neuroblastoma, and glioblastoma cancers [18–23]. Compared

to ER-α66, ER-α36 retains the DNA binding domain, the partial dimerization region, and an E2 binding domain, but lacks the two transcription-activated domains of activation function AF-1 and AF-2 [24]. However, ER-α36 could induced non-genomic signaling pathway, the rapid estrogen signaling pathway was first reported by Pietras [25]. The non-genomic signaling pathway activates seconds to minutes after estrogen treatment and usually initiates at the plasma membrane. It is mediated by estrogen-binding proteins associated with the plasma membrane such as growth factor receptors and G-protein-coupled-receptor signaling pathway[26]. Estrogen can bind ER-α36 to activate membrane-associated receptors to initiate signaling pathways such as PI3K/Akt, MAPK/ERK, and calcium signaling, which then regulate gene transcription and can promote tumor cells proliferation and anti-estrogen drug resistance [27, 28]. Interestingly, ER-α66 positive tumors usually express low or normal levels of epidermal growth factor receptor (EGFR) initially, then upregulate EGFR during the development of TAM resistance. Recent reports indicate that ER-α36 and EGFR synergize in a positive feedback loop to promote breast cancer cell growth in ER-α-negative breast cancer cells [27, 29]. Furthermore, silencing of ER-α36 in breast cancer cells enhances EGFR protein degradation. ER-α36 also synergizes with HER2 signaling [30, 31].

ER-α36 and EGFR may play key roles in the development of resistance to anti-estrogen therapy in a variety of tumors. EGFR is expressed in most parts of central nervous system and is highly expressed in developing astrocytes [32]. EGFR expression is decreased and becomes absent in mature astrocytes [33, 34]. Interestingly, EGFR is up-regulated in astrocytes in many CNS diseases including ischemia, brain tumors, and neurodegenerative diseases. The effects of EGFR and ER-α36 on the progression and development of glioblastoma have yet to be reported. In this study, we reveal the contribution of ER-α36 to glioblastoma development and its interaction with EGFR, showing that ER-α36 promotes the SRC/ERK pathway via EGFR in glioblastoma cells.

## Materials And Methods

# Reagents

17β–estradiol (E2) was purchased from Abcam (US). The MEK1/2 inhibitor U0126, the Src inhibitor PP2, the EGFR inhibitor AG1478 were purchased from Sigma Chemical Co (US). pSrc-Y416, pSrc-Y527, β-tubulin, p-ERK, ERK, p-P38 and P38 antibodies purchased from Cell Signaling Technology (US); Anti-ER-α and ER-β purchased from abcam; CyclinD1, CyclinE, CyclinB, GAPDH, CDK4, EGFR and Src antibodies purchased from Proteintech (Wuhan, China); KI67 antibody purchased from Zhongshan Goldenbridge Biotechnology (Beijing, China). anti-ER-α36 antibody, ER-α36 expression vector and ER-α36 shRNA were from Dr. Wang ZY (Creighton University Medical School).

# Tumor specimens and Immunohistochemistry

Thirty-one formalin-fixed paraffin embedded glioma tissue specimens (five low grade and twenty-six high grade glioma specimens) were retrieved from the First Affiliated Hospital of Dalian Medical University.

The patients were ages between 25-83 with infiltrative glioma. No patients received any radiation, chemotherapy, or endocrine therapy before surgical resection. Their relatives gave written informed consent which was approved by the Ethics Committee on the Use of Human Subjects. Immunohistochemical assay for ER-α36, ER-α66 and ER-β were performed using the commercially available detection kits (Zhongshan Goldenbridge Biotechnology) and DAB staining procedures (Solarbio, Beijing, China).

# Cell culture, Treatment and Growth Assay

Glioblastoma cells U251 were obtained from Shanghai Cell Bank (Shanghai, China). Likely glioblastoma cell line U87 obtained from ATCC (Shanghai, China). These cells were maintained in DMEM with 10% fetal calf serum (FBS) at 37°C in a 5% CO2 incubator. For MAPK and EGFR/Src signaling activation, cells were maintained in phenol red-free media (Gibco, US) with 2.5% charcoal-stripped fetal calf serum (HyClone, Logan, UT) for 72 h, and then in serum free medium for 12 hours and then E2 treatment. To test the effects of U0126, PP2, AG1478, all inhibitors were added 10 min before E2 treatment.

To assess cell growth, cells were treated with different concentrations of E2, or vehicle (ethanol) as a control. The cells were seeded at 1 X $10^4$ cells per well in 6 well plates and were maintained in phenol red-free media with 2.5% charcoal-stripped fetal calf serum. The cell numbers were determined using the cell counting methods after 8 days. Three wells were used for each treatment and experiments were repeated three times.

# Establishment of Stable Cell Lines

Glioblastoma U251 cells were maintained in the culture medium containing increasing concentrations of TAM for several weeks until all cells grew well in the medium containing 10 μM TAM. The established TAM resistant U251 cell line was named U251/TAM.

# Transfection

The cells were seeded at 3 X $10^5$ in 6 well plates. Cells were cultured for 24 h before transfection (Cell confluence reaches 90%). 1 μg ER-α36 shRNA and 4 μg ER-α36 expression vector was mixed with Lipofectamine 2000 reagent (Thermo Fisher Scientific, US ) and incubated for 20 min at room temperature before added into cultured cells. The cells were changed normal medium after 4 h. The efficiency of shRNA knock-down was assessed with Western blot and qPCR analysis.

# Western blotting

Cells were harvested and then lysed in a cold lysis buffer (20 mmol L−1 Tris-HCl, pH 7.5, 70 mmol $L^{-1}$ NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100 and 1% PMSF) to extract protein. The concentration of total protein was determined by the Bradford method. The protein samples were then subjected to 10% SDS-PAGE. After electrophoresis, protein bands were transferred to a polyvinylidene fluoride (PVDF) membrane, and then blocked in PBS-T (pH 7.4) containing 5% dried skim milk. Then the PVDF membrane was probed with the specified primary antibody, followed by the appropriate secondary

antibody, and finally visualized using the $ECL_{TM}$ which is a Western blotting chemiluminescent reagent kit (Amersham biosciences) according to the manufacturer's instructions. Immunoblot data were quantified using ImageJ software (NIH, Bethesda, MD). The region of interest was marked and measured in every lane, and the background was subtracted to give the final band intensity [35, 36].

# Immunofluorescence

For ER-α36, KI67 and EGFR detection in glioma tissues, slides were deparaffinized with xylene, rehydrated with ethanol, and then antigen retrieval in 10 mM citrate buffer (pH, 6.0) at 120 $^0$C for 5 min. After blocking, the samples were then incubated with anti-ER-α36 (1:300), anti-EGFR (1:100) and KI67 (1:100) at 4 $^0$C overnight, followed by incubation with fluorescein-conjugated goat anti-mouse antibodies (1:200, Zhongshan Goldenbridge Biotechnology, Beijing, China) or Rhodamine-conjugated goat anti-rabbit antibodies (1:200, Zhongshan Goldenbridge Biotechnology, Beijing, China). Nuclei were stained with DAPI (Solarbio, Beijing, China). The fluorescent signals were detected and photographed with a fluorescence microscope (Olympus IX73, Japan).

# RNA purification and qPCR

Total RNA was prepared with the TRIzol RNA purification reagent (Invitrogen, US). A total of 1 μg RNA

was reversely transcribed using the RT-PCR kit (Promega, US). RT-PCR analysis of ER-α36, EGFR and GAPDH was performed using gene specific primers as the following. ER-α36: forward primer: 5'-TTTTCTCACTTCCCTCACTCCTTC; reverse primer: 5'-TCCCTGCCATTCTCCTTATCC-3'; EGFR: forward primer: 5'-TCCTGCTCCTCAACCTCCTC-3'; reverse primer: 5'-TTATCTGCTCCTTACGCCCTTC-3'

GAPDH :5'-GGCACAGTCAAGGCTGAGAATG-3'; reverse primer:5'-ATGGTGGTGAAGACGCCAGTA-3'. PCR procedure was carried out as SYBR green II kit (Tarkara, Dalian, China).

# Cell cycle and cell death analysis

The cells were fixed with 70% ethanol. Ethanol-fixed cells were treated with 50 mg/ml PI (propidiumiodide) in dark for 30 min at room temperature. Flow cytometry analysis of cell cycle distribution (BD-Biosciences, C6). Cell death was detected using the annexinV-FITC apoptosis kit (BD-Biosciences) according to the manufacturer's instruction. Data acquisition was analyzed with the ModFitsoftware.

# Statistical analysis

Data were summarized as the mean ± standard error (SE) using the GraphPad InStat software program. Unpaired Student's t-test was used to test for statistical significance between the control and test groups. Comparisons of multiple groups were analyzed using a one- or two-way ANOVA followed by post hoc Tukey's test. Significance was determined for $P<0.05$.

Estrogen receptors are differentially expressed in human glioma specimens

The expression of ER-α and ER-β are low or negative in GBM [37]. ER- α36 is overexpressed in glioblastoma cells line U87 cells. However, their role in the pathogenesis of GBM is unclear. We examined the expression of ER-α66 and ER-β in 10 cases of glioma and found that six of ten exhibited ER-α66 expression. ER-β was expressed in all glioma cases. The expression of ER-α66 and ER-β is lower than WHO grade I and grade II and predominantly stained in a cytoplasmic and nuclear pattern (Figure 1A). Contrary to ER-α66 and ER-β expression patterns, ER-α36 was over-expressed in 25 of 26 (96.3%) of the glioma samples and was barely detectable in grade I tumors brain [38]. In this study, we found have no difference in ER-α36 between male and female patients ($P$=0.2) (Figure 1B-1D).

Previous studies suggested that EGFR and ER- α36 may interact in a positive feedback loop to promote tumor development [27]. In this study, we tested whether this may occur in human glioblastoma specimens. We found that both ER- α36 and EGFR have lower expression levels in grade I glioma compared to high grade glioma. Furthermore, in high grade glioma specimens showed the co-expression ER-α36 and EGFR is65.4 % (Figure 2A). To test the effect of ER-α36 expression on proliferation, we performed immunofluorescence staining of ER-α36 and the proliferation marker KI67 on human glioma specimens. We found that the rate of KI67 positive cells is 40% in ER-α36 positive glioma cells, suggesting that ER-α36 may be related to GBM cells proliferation (Figure 2B and 2C).

Low concentration Estrogen stimulated glioblastoma cells proliferation through ER-α36

Our lab has reported ER-α36 expression is lower in U251 cells than U87 cells [22]. To determine the function of E2 on glioblastoma cells proliferation, we first determined the proliferation rate of U87 and U251 in response to different concentrations of E2. The U87 cells treated with low concentrations (<10 nM) E2β exhibited an increased growth rate compared with cells treated with vehicle, and the best concentration is 1nM (Figure 3A). The U251 cells treated with E2, showed that10 nM E2 increased cells proliferation and low concentrations slightly increased cell proliferation but no significance (Figure 3C). We found Cyclin D1, Cyclin E and Cyclin B expression increased and CDK 4 have no difference in the U87 cells treated with 1nM E2 (Figure 3B). 1 nM E2 could not increase Cyclin D1 and CDK 4 expression but increased Cyclin B expression in the U251 cells (Figure 3D). Knockdown of ER-α36, E2 could not stimulate cell growth at any concentration in U87 cells (Figure 3E). The cell cycle protein also has no change after E2 treatment (Figure 3F). These results suggested that E2 exhibited a biphasic pattern in ER- α36 over-expression cells U87; increasing concentrations initially stimulated cell growth but failed to do so at higher concentration.

ER-α36 mediates mitogenic estrogen signaling

To test whether ER-α36 mediated mitogenic estrogen pathway signaling in glioblastoma cells, we treated U87 cells with varying concentrations of E2 across different time periods. Figure 4A-4C illustrates that E2 induced ERK phosphorylation within 10 mins after E2 treatment, peaked at 45 mins, declined at 60 mins. E2 induced p38 phosphorylation within 30 mins after E2 treatment, declined at 45 mins, then exhibited another more sustained activation at 60 mins, and subsequently declined at 120 mins. ERK

phosphorylation was induced after 10 mins following treatment with 1 and 10 nM E2. At very high concentrations (10 nM, 100 nM), E2 did not elicit ERK or P38 phosphorylation ($p$=0.089 ) (Figure 4D-4F). In U87/36KD glioblastoma cells, which have a low expression of ER-α36, 1 nM E2 did not induce ERK or P38 phosphorylation ($p$=0.3)(Figure 4G-4H).

In this study, 1 nM E2 were used to treated U251 cells for 10 min, and found that it could not stimulate phosphorylation of MAPK ($p$=0.071)(Figure 5A-5C). However, following ER-α36 overexpression via transfection with ER-α36 vector, U251 cells responded to E2 and showed increased ERK phosphorylation ($p$=0.002) and p38 phosphorylation ($p$=0.031) (Figure 5D-5F). In a previous study, we established a TAM-resistant U251 cell line which overexpressed ER- α36. In this cell line, treatment with 1 nM of E2 promoted ERK phosphorylation ($p$=0.0012) and p38 phosphorylation ($p$=0.02)(Figure 5G and 5H).

EGFR relationship with ER-α36 in glioblastoma cells

To determine if ER- α36 influences EGFR expression, we first examined the expression of ER- α36 and EGFR in U87 and U251 glioblastoma cell lines. Both ER-α36 (p=0.009) and EGFR(p=0.0076) are lower in U251 cells compared to U87 cells (Figure6A and 6B). Following ER-α36 overexpression via ER-α36 expression vector in U251 cells, EGFR was upregulated (p=0.0032) (Figure 6C and 6D). Conversely, EGFR was reduced in U87 cells following knockdown via shRNA (p=0.0013) (Figure 6E and 6F). We also observed significant changes in the mRNA expression of EGFR in these cells through Qpcr method (Figure 6G and 6H). Cumulatively, these results suggest that ER-α36 regulates EGFR expression in human glioblastoma.

SRC/EGFR is involved in estrogen induced cell cycle regulation

We next tested whether ER-α36 is involved in SRC/EGFR signaling by inhibiting SRC and EGFR signaling with the inhibitors PP2 and AG1478 for 24 hours. We found that SRC/EGFR inhibition resulted in reduced ER-α36 protein expression in U87 cells, while EGFR expression was stable. The MAPK inhibitor U0126 reduced EGFR expression (p=0.0043) (Figure 7A-7C). Next, we tested if the mitogenic effects of E2 were facilitated by the SRC/EGFR pathway. We found that the increases in Cyclin D1 and B expression following 1 nM E2 treatment were ablated with SRC/EGFR inhibition by PP2 and AG1478 (Fig. 7D-7F). We use flow cytometry to isolate cell cycle fractions. Consistent with increases in cyclin expression, U87 cells treated with E2 showed an enrichment in the S phase of the cell cycle. After inhibition with U0126, PP2, or AG1478, the fraction of cells in the S phase was reduced after E2 stimulation (Figure 8A and 8B). However, in U251 cells which have low expression of ER-α36, E2 stimulation failed to increase the percentage of cells in the S phase, and AG1478 could not reduced the number of S phase cells (Figure 8C and 8D).

SRC/EGFR is involved in estrogen signaling

Next, we tested whether SRC is directly involved in E2 signaling. We first examined the phosphorylation levels of Src-Y416 and Src-Y527 in cells treated with 1 nM E2 for 10 min. In U87 cells, E2 treatment

elicited SRC-Y416 phosphorylation (p=0.028) and reduced SRC-Y527 phosphorylation (p=0.034)(Figure 9A and 9B). Conversely, in U251 cells, E2 exposure did not increase SRC-Y416 phosphorylation (p=0.2), but did increase SRC-Y527 phosphorylation (p=0.031) (Figure 9C and 9D). In TAM resistant U251 cells, E2 failed to reduce SRC-Y527 phosphorylation, however, SRC-Y416 phosphorylation was increased (p=0.0025) (Figure 9E and 9F). In U251 cells overexpressing ER-α36, E2 treatment increase SRC-Y416 phosphorylation and reduced SRC-Y527 phosphorylation (p=0.0067) (Figure 9G and 9H).

We continued to dissect the effects of E2 on EGFR signaling. Following inhibition with PP2 and AG1478 and E2 exposure for 10 mins, E2-induced ERK activation was reduced in both U87 and TAM resistant U251 cells (Figure 10). Furthermore, PP2 treatment reduced phosphorylation of both SRC-Y416 and SRC-Y527 in both U87 and U251/TAM cells, while AG1478 increased SRC-Y527 phosphorylation and reduced SRC-Y416 phosphorylation (Figure10E-H).

# Discussion

It has been reported that hormones, including hormone replacement therapy (HRT) have effect on glioblastoma. Some studies have shown that hormone replacement therapy can reduce glioma risk in females, while other studies did not get similar conclusions, and some even reached the opposite result [39, 40]. One possible explanation for these discrepancies is potential differences in estrogen receptor expression. In this study, we found that 25 of 26 cases of glioma samples expressed ER-α36, predominantly on the plasma membrane, and also in the cytosol and the nucleus, and have no difference between men and women. ER-α66 expression was positive in 6 of 10 cases. While all the 10 cases expressed ER-β, however, ER-β expression was lower in grade IV compared with grade I glioma. It has been reported that ER-α and ER-β are localized to the nucleus and act as transcription factors. We found that ER-α and ER-β were also found in the cytoplasm in glioblastoma cells. Others have shown that ER-α and ER-α36 may oppose each other and that ER-α36 can inhibit ER-α nuclear translocation [41, 42]. Cumulatively, these results suggest that dysregulated genomic and non-genomic estrogen signaling play a role in the carcinogenesis of glioblastoma.

Previously, it was thought that estrogen did not play a major role in signaling for cells which lacked ER-α66 expression. Over the last decades, non-genomic estrogen signaling via ER-α36 has gained traction as an alternate mechanism of action [43, 44]. In this study, we tested the effect of E2 on cell proliferation in the glioblastoma cell lines U87 and U251 which lack ER-α66 expression, however, we found that ER-β expression is positive, and higher expressed in U251 cells than U87 cells. Our lab has reported that ER-α36 expression is higher in U87 cells than U251 cells [22]. Here, we found that E2 stimulation at low concentrations promoted cell proliferation and increased cell cycle protein expression in U87 cells. Furthermore, high concentrations of E2 reduced proliferation. Following knockdown of ER-α36 in U87 cells, the effect of E2 on proliferation is decreased, although have significance compared with control. In U251 cells with low ER-α36 expression, only high concentrations of E2 (100 nM and 1 μM) was able to

promote cell proliferation. These results are consistent with recent reports which show that cells with high expression of ER-α36 exhibit the typical biphasic response to E2 [29, 45].

The MAPK pathway is commonly dysregulated in many glioblastomas and plays numerous pathophysiological roles in carcinogenesis [46, 47]. In this study, we found that E2 exposure in GBM cell lines produces a dose- and time-dependent increase in ERK1/2 phosphorylation. However, in cells with low expression or knockdown of ER-α36 and U251 cells, E2 failed to activate this pathway. These found strongly demonstrated that non-genomic estrogen signaling pathway contributed to glioblastoma cells development which ER-α is negative and ER-a36 is positive.

The epidermal growth factor receptor (EGFR) pathway is also often dysregulated in human glioblastoma [48]. Previous reports have shown that EGFR co-localizes with ER-α36 in multiple ER-α negative tumor cells [29]. In this study, we also revealed a novel cross-talk mechanism in which EGFR and ER-α36 positively regulate each other's expression, which may play an important role in malignant growth of glioblastoma cells. EGFR expression was detected in 26 cases, 17 of which co-labled with ER-α36, suggested that a subset of glioblastoma co-expressed ER-α36 and EGFR. Here we also found ER-a36 and EGFR have regulation of positive feedback in the glioblastoma cells U87 and U251. It is suggested that E2 induced cell proliferation in glioblastoma that is associated with EGFR/ER-a36.

In this study, we also found that ER-α36 mediated the glioblastoma cell growth through non-genomic estrogen signaling pathway MAPK and associated with EGFR pathway. We found that 1 nM E2 induced Src phosphorylation at Tyr-416 and inhibited Src phosphorylation at Tyr-527, consistent with other studies report that E2 induced Src-Y416 phosphorylation in these ER-negative and ER-a36 positive tumor cells [29, 49]. The Src inhibitor PP2 and EGFR inhibitor AG1478 blocked E2-induced Src-Tyr-416 phosphorylation and inhibited E2-stimulated cyclin protein expression and cells proliferation in U87 cells. These results thus indicated that EGFR/Src signaling plays a key role in mitogenic estrogen signaling in glioblastoma cells U87 that highly express ER-α36.

In summary, our study demonstrated that ER-α36 expression is highly expressed in grade IV glioblastoma and co-labels with EGFR in human samples. ER-α36 is involved in mitogenic estrogen signaling in the U87 glioblastoma cell line which have a high expression level of ER-α36. Furthermore, EGFR/SRC signaling is positively regulated by ER-α36 and E2 signaling pathway in glioblastoma cells. These results may provide insight into the development of novel treatments for glioblastoma.

# Abbreviations

Glioblastoma GBM

Epidermal growth factor receptor EGFR

Estrogen receptors ERs

Tamoxifen TAM

Central nervous system CNS

17β-estradiol E2

quantitative real time polymerase chain reaction Qpcr

# Declarations

### Data availability statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

### Ethics approval and consent to participate

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Human Ethics Committee of First Affiliated Hospital of Dalian Medical University . Written informed consent was obtained from individual or guardian participants.

### Consent for publication

Not applicable

### Availability of data and materials

All data generated or analysed during this study are included in this published article.

### Competing interests

The authors declare that they have no competing interests.


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The study funders do some experiments, such as YL tested the cell proliferation through cell counting and flow cytometry and collected the human samples. However, they had no role in the design.


### Author's contribution

Chao Qu, Cui Wang write the manuscript and did the Western blot, Qpcr and analyzed the data; Hongyan Li did the western blot and immunohistochemistry of estrogen receptors, Chao Han and Ying Li tested the

cell proliferation through cell counting and flow cytometry; Xiaofeng Tao, Xin Guan and Yejun Zhang collected the human samples; Meng Chen stained the ER-α36 and Ki67; Jing Liu, Wei Zou devised the experiment scheme.

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# Figures

### Figure 1

**Estrogen receptor expression in human glioma tissue.** A) Immunohistochemical (IHC) staining of ER-a and ER-b. Red arrows indicate ER expressed in the cytoplasm. B) IHC staining of ER-a36, demonstrating strong nuclear and cytoplasmic staining in high grade glioma specimens C and D) are the qualification of B. Bar=50 μm (n=5-26).

### Figure 2

**ER-α36 and EGFR, Ki67 co-expression in glioma specimens.** The glioma specimens were used to tested ER-α36, EGFR and Ki67 expression. A) IF analysis of the ER-α36 (green) and EGFR (red) expression. B) IF analysis of the ER-α36 (green) and Ki67 (red) expression. C) are the qualification of A and B. Bar=50 μm (n=5-20).

### Figure 3

**The effect of E2 on glioblastoma cell growth.** The cells were cultured in phenol red-free media with 2.5% dextran-charcoal-stripped fetal calf serum for 3 days. And then different concentrations of E2 were used to treat cells for 8 days. A). examination of the effect of E2 on cells growth through cell counting method. B). U87 cells were treated with 1 nM E2 for 24 h, Western blot method tested the expression of cell cycle proteins CyclinB, CyclinD1 and CyclinE. GAPDH is housekeeping protein. C). effect of E2 on U251 cells growth were examined using cell counting method. D). U251 cells were treated with 1 nM E2 for 24 h, Western blot method to examine the expression of cell cycle proteins CyclinB, CyclinD1 and CDK4. GAPDH is housekeeping protein. E). effect of E2 on U87-36KD cells growth were examined using cell counting method. F). U87-36KD cells were treated with 1 nM E2 for 24 h, Western blot method analysis of cell cycle proteins CyclinB, CyclinD1 and CDK4 expression. GAPDH is housekeeping protein. (n=3-6, **P<0.01).

## Figure 4

**E2 activated MAPK pathway through ER-α36 in U87cells.** Cells were cultured in phenol red-free media with 2.5% dextran-charcoal-stripped fetal calf serum for 3 days, and then cultured in serum-free medium for 12 h. A). U87 cells were treated with 1 nM E2 for different time periods (5, 10, 15, 30, 45, 60, 120 min). Western blot analyzed the level of ERK and p38 phosphorylation. B). The qualification of p-ERK/ERK. C). The qualification of p38/GAPDH. D). Different concentration (1 nM, 10 nM, 100 nM, 1 μM, 5 μM) of E2 treated cells for 10 min. Activation of P38 and ERK was tested through Western blot. E). The qualification of p-ERK/ERK. F). The qualification of p38/GAPDH. G). 1 nM E2 treated U87-36KD cells for 10 min, Western blot analysis of ERK and p38 activation. H). The qualification of p-ERK/ERK and p-P38/P38.

## Figure 5

**The effect of estrogen on MAPK activation in U251 cells.** Cells were cultured in phenol red-free media with 2.5% dextran-charcoal-stripped fetal calf serum for 3 days, and then cultured in serum-free medium for 12 h. A). 1 nM E2 was used to treat U251 cells for 10 min, Western blot analysis of the P38 and ERK activation in U251 cells. B). The qualification of p-ERK/ERK. C). The qualification of p-P38/P38. D). U251 cells were transfected with ER-α36 expression vector, empty vector as control. 1 nM treated U251/OE cells for 10 min. Western blot tested the level of phosphorylation of ERK and P38. E). The qualification of p-ERK/ERK. F). The qualification of p-P38/P38. G). 1 nM treated U251/TAM, which is tamoxifen resistance cell line and ER-α36 over expression. Western blot analysis of ERK and P38 activation. H). The qualification of p-ERK/ERK and p-P38/P38.

## Figure 6

**ER-α36 positively regulates EGFR expression in glioblastoma cells** A). Western blot analysis of ER-α36 and EGFR expression in U87 and U251 cells. B). The qualification of ER-α36 and EGFR. C). ER-α36 expression vector was transfected into U251, and empty vector as control, Western blot analysis of ER-α36 and EGFR expression. D). The qualification of ER-α36 and EGFR. E). ER-α36 shRNA was transfected into U87 cells, Western blot tested the expression of EGFR and ER-α36. F). The qualification of EGFR and ER-α36. G). The expression of ER-α36 and EGFR was compared in the U87 and U251 cells by QPCR method. H). The expression of ER-α36 and EGFR was compared in the U87 and U87-36KD cells by QPCR method (n=3-5,**P<0.01 VS CTRL).

## Figure 7

**EGFR/Src signaling was involved in E2-induced cell growth through ER-α36.** Cells were cultured in phenol red-free media with 2.5% dextran-charcoal-stripped fetal calf serum for 3 days, and then the MEK inhibitor U0126, the Src inhibitor PP2, the EGFR inhibitor AG1478 treated cells for 24 h. A). Western blot analysis of ER-α36 and EGFR expression. B). is the qualification of ER-α36. C). is the qualification of EGFR. D). Cells were cultured in phenol red-free media with 2.5% dextran-charcoal-stripped fetal calf serum for 3 days, E2 combined with U0126，PP2，and AG1478 treating U87 cells for 24 h. E). Western blot tested the expression of cell cycle protein CyclinB and CyclinD expression. E is the qualification of CyclinD1. F). is the qualification of the CyclinB.

## Figure 8

**EGFR/Src signaling pathway was involved in ER-α36-regulated cell cycle.** A). Cells were cultured in phenol red-free media with 2.5% dextran-charcoal-stripped fetal calf serum for 3 days, E2 combined with the MEK inhibitor U0126, the SRC inhibitor PP2, the EGFR inhibitor AG1478 treating U87 and U251 cells for 24 h. A) and B). Flow cytometery analysis of cell cycle change in U87 cells. C) and D). Flow cytometery analysis of cell cycle change in U251 cells (n=3,*$P$<0.05,**$P$<0.01).

## Figure 9

**E2 activates EGFR/Src signaling pathway through ER-α36.** Cells were cultured in phenol red-free media with 2.5% dextran-charcoal-stripped fetal calf serum for 3 days, serum-free for 12 h. A). U87 cells were treated with 1 nM E2 for different time periods (5, 10, 15, 30, 45, 60, 120 min), Western blot analysis of Src

phosphorylation. B). The qualification of Src-Y416 and Src-Y527. C). 1 nM E2 treated U251 cells for 10 min. Western blot analyzed the level of Src-Y416 and Src-Y527 phosphorylation. D). The qualification of Src-Y416 and Src-Y527. E). 1 nM E2 treated U251/TAM cells for 10 min. Western blot analyzed the level of Src-Y416 and Src-Y527 phosphorylation. F). The qualification of Src-Y416 and Src-Y527.. Forced ER-α36 expression through transfecting ER-α36 expression vector, and the empty vector was used to as a control. 1 nM E2 treated U251/OE cells for 10 min. G) Western blot analyzed the level of Src-Y416 and Src-Y527 phosphorylation. H). The qualification of Src-Y416 and Src-Y527.

## Figure 10

**The effect of EGFR/Src on E2-mediated MAPK activation.** E2 combined with the PP2, U0126 and AG1479 treated U87 cells and U251/TAM for 10 min. A). Western blot tested ERK and Src pathway activation in U87 cells. B), C) and D) is the qualification of Figure A. E). Western blot tested ERK and Src pathway activation in U251/TAM cells. F), G) and H) is the qualification of Figure E.