ERβ1 Expression Patterns Have Different Effects On EGFR TKIs Treatment Response in EGFR Mutant Lung Adenocarcinomas.

Lijuan Zhang
Shandong Provincial Hospital
Meng Tian
First Affiliated Hospital of Nanjing Medical University
Jiamao Lin
Shandong Cancer Hospital and Institute
Jianbo Zhang
Shandong Cancer Hospital and Institute
Haiyong Wang
Shandong Cancer Hospital and Institute
Zhenxiang Li (lizx0108@163.com)
Shandong Cancer Hospital and Institute https://orcid.org/0000-0002-9648-8869

Primary research

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Abstract

Background: Estrogen receptor β (ERβ) can regulate cellular signaling through non-genomic mechanisms, potentially promoting resistance to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs). However, the mechanisms underlying the ERβ-mediated resistance to EGFR TKIs remain poorly understood.

Methods: qRT-PCR was performed to investigate ERβ1 and ERβ5 expression levels in cell lines. The localization of ERβ and ERβ1 within cells was assessed using immunocytochemistry and immunofluorescence. The effect of estradiol and/or gefitinib on EGFR signaling pathways was determined by western blot. Cell viability and colony formation assays were used to assess gefitinib response for different cell lines. The apoptosis was verified by tunel and western blot. Immunohistochemistry was used to assess the expression of ERβ1 in lung adenocarcinoma tissues. Patient survival was estimated using the Kaplan-Meier method, and comparisons between groups were conducted using log-rank tests.

Results: PC9 cell lines stably overexpressing ERβ1 or ERβ1/ERβ5 were established successfully. Immunofluorescence revealed that ERβ5 overexpression partly retained ERβ1 in the cytoplasm. Immunoblotting analyses revealed that EGFR pathway activation levels were higher in PC9/ERβ1/5 cells than those in PC9/ERβ1 or control PC9 cells. In the presence of estradiol, PI3K/AKT/mTOR pathway activation levels were higher in ERβ1/5-expressing cells than those in ERβ1-expressing cells. Additionally, PC9/ERβ1/5 cells were less prone to the cytotoxic and pro-apoptotic effects of gefitinib compared with PC9/ERβ1 or control PC9 cells.

Conclusion: Cytoplasmic ERβ1 was associated with poor progression-free survival in lung cancer patients treated with EGFR TKIs. These results suggest that anti-estrogen therapy might reverse EGFR TKI treatment resistance to some extent in selected patients.

Background

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) have revolutionized non-small cell lung cancer (NSCLC) personalized treatment, and have improved the survival and quality of life of EGFR-mutant NSCLC patients [1–3]. However, the development of primary and acquired resistance to TKIs remains a significant clinical challenge. Several mechanisms underlying acquired resistance to EGFR TKIs have been identified, including the acquisition of EGFR T790M mutation, c-MET amplification, PIK3CA mutations, and phenotypic transformation into small cell lung cancer [4, 5]. Nevertheless, the mechanisms involved in primary resistance are poorly understood [5–8].

Estrogen receptor β (ERβ) is the primary ER subtype expressed in lung cancer; upon binding to estrogen in the cytoplasm, ERβ activates non-genomic signaling pathways, including PI3K/AKT/mTOR and RAS/RAF/MEK/MAPK pathways, promoting cancer cell proliferation and apoptosis evasion [9, 10]. Importantly, significant overlap exists between ERβ- and EGFR-regulated signaling pathways [11].

Preclinical studies have shown that EGFR expression was downregulated in response to estradiol (E2); in contrast, ERβ antagonists upregulated EGFR expression, highlighting the crosstalk between ERβ and EGFR signaling [12]. Hence, it is believed that non-genomic signaling events may modulate EGFR TKI resistance. ER belongs to the nuclear receptor superfamily of ligand-activated transcription factors. Since a nuclear localization of ERβ in cancer cells has been reported [13], the relevance of cytoplasmic ERβ in non-genomic signaling activation in cancer cells has attracted increasing attention in recent years.

Studies on endocrine-related cancers suggested that certain ERβ isoforms are associated with ERβ protein localization and patient prognosis [14–17]. For example, ERβ1 (also known as wild-type ERβ) was primarily found in the nucleus of prostate cancer cells, whereas ERβ5 localized both in the cytoplasm and nucleus [16, 18, 19]. Although ERβ5 lacks the ability to bind estrogen or form homodimers due to the absence of helix 12 in its C-terminal, it can heterodimerize with ERβ1 in the presence of estrogen [20].

The aim of this study was to assess the role of the interaction between ERβ1 and ERβ5 in non-genomic signaling in lung adenocarcinoma. To this end, we overexpressed ERβ1 and ERβ5 in EGFR exon 19 deletion-harboring lung adenocarcinoma cells and assessed their ability to form heterodimers, as well as the relevance of ERβ1/ERβ5 heterodimerization in non-genomic signaling and response to EGFR TKIs.

Materials And Methods

Cell culture and chemicals

The EGFR-mutant lung adenocarcinoma cells PC9, HCC827, H1975, and H1650 were kindly provided by Peking University Cancer Hospital. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and maintained at 37°C in a humidified 5% CO_2 atmosphere.

Gefitinib was purchased from Selleck Chemicals (Selleck, USA) and diluted in dimethyl sulfoxide (DMSO) at a concentration of 10 mmol/L. Estradiol (E2) was purchased from Sigma-Aldrich (Sigma-Aldrich, Germany) and diluted in pure ethanol at a concentration of 10 mmol/L. Both drugs were aliquoted and stored at -80°C.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using the RNAsimple Total RNA kit (Tiangen, China), and first-strand cDNA synthesis was performed using the PrimeScript™ RT Master Mix (Takara, Japan). The relative mRNA levels of ERβ1 and ERβ5 were measured using SYBR green PCR assays (Thermo Fisher Scientific, USA). The sequences of the primers used in qRT-PCR were as follows: ERβ1 forward primer: 5'-GTCAGGCATGCGAGTAACAA-3', reverse primer: GGGAGCCCTCTTTGCTTTTA; ERβ5 forward primer: 5'-TGGTCACAGCGACCCAGGATG-3', reverse primer: 5'-TTAGGGCGCGTACCTCGCATG-3'; GAPDH forward primer: 5’-GACCCCTTCATTGACCTCAAC-3’, reverse primer: 5’-CTTCTCCATGGTGGTGAAGA-3’. Cycle threshold (Ct) values were determined using the system and analysis software. The relative mRNA levels were determined by normalizing to the GAPDH mRNA levels.

Establishment of cell lines stably expressing ERβ1 and ERβ1/5

Lentiviral vectors expressing ERβ1 and ERβ5 were purchased from GenePharma (Shanghai, China). PC9 cells were infected with lentiviruses (MOI = 50) for three days. Subsequently, transduced cells were selected with 2 μg/mL of puromycin for one week. ERβ1-overexpressing single-cell clones were established (hereafter referred to as PC9/ERβ1 cells), and stable ERβ1 overexpression was confirmed by Western blot and qRT-PCR. PC9/ERβ1 cells were then infected with viruses carrying ERβ5 open reading frame (ORF), followed by selection with neomycin (600 mg/mL) for one week.

Immunocytochemistry and immunofluorescence

ERβ expression was assessed by immunofluorescence (IF) and immunocytochemistry (ICC). For IF, cells were fixed with 4% paraformaldehyde at room temperature for 20 minutes, followed by incubation with 0.5% Triton X-100 for 15 minutes. Non-specific binding was blocked with 5% bovine serum at 37°C for 30 minutes, before incubating with anti-ERβ (1:100; GeneTex, USA) and anti-ERβ1 (1:200; Santa Cruz, USA) primary antibodies. After incubation at 4°C overnight, cells were incubated with a secondary antibody conjugated with Alexa Fluor® 488 (1:500, Cell Signaling Technology, USA) for 1 hour at room temperature. Subsequently, cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI), and samples were imaged using a confocal laser scanning microscope (Zeiss, Germany). For ICC, a two-step polymer-HRP detection method (Dako, Carpinteria, CA) was used.

Immunoblotting analysis

Cells were cultured in serum-free medium for 24 hours and then treated with gefitinib (40 nM) or/and estradiol (20 nM) for another 8 hours. Total protein was extracted from cells using cell lysis buffer (Beyotime, China) supplemented with a protease inhibitor cocktail (Roche, Germany); protein concentration was determined using the BCA protein assay (Beyotime, Beijing, China). The following primary antibodies (1:1000) were used: anti-EGFR, anti-phospho-EGFR (Tyr1068), anti-AKT, anti-phospho-AKT (Ser473), anti-RPS6, anti-phosphor-RPS6 (Ser235/236), anti-P21, anti-CyclinD3, anti-cleaved-PARP (cPARP), and anti-beta-actin (Cell Signaling Technology, USA). Membranes were then incubated with peroxidase-linked anti-mouse or anti-rabbit secondary antibodies (1:5000; Cell Signaling Technology, USA) for 2 hours at room temperature.

Cell viability and colony formation assays

Cells were treated with estradiol (20 nM) during the experiment. Cell viability was assessed using a cell counting kit-8 (CCK8; Dojindo, Japan). Briefly, cells were seeded (3×10^3 cells/ well) in sextuplicate in 96-well plates containing 100 μL medium and incubated for 24 hours. Subsequently, cells were treated with increasing concentrations of the indicated drugs for an additional 72 hours. After treatment, 10 μL of water-soluble tetrazolium salt (WST-8) was added to each well and incubated for 2 hours. Optical absorbance at 450 nm was measured using a microplate reader. Relative viability was calculated using

the following formula: Relative viability (%/control) = [A450 (treated)- A450 (blank)]/[A450 (control) - A450 (blank)].

For colony formation assays, cells were seeded into 6 cm cell culture dishes (500 cells/dish) and treated for two weeks with 40 nM gefitinib or DMSO (1/1000 dilution). After washing twice with phosphate-buffered saline (PBS), cells were stained with crystal violet (Beyotime, China) for 20 min and washed with PBS.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

Cells were seeded on glass slides in 6-well plates 3×10^4 cells/well. After a 24 hour treatment with 40nM gefitinib or DMSO, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. Then, 50 μL of a freshly prepared TUNEL solution (Keygene, China) was added onto each slide. Subsequently, cell nuclei were counterstained with DAPI, and samples were imaged using a fluorescence microscope (Zeiss, Germany). The percentage of apoptotic cells normalized to the control group (set to 100%) was calculated after counting cells in five representative fields. The data were expressed as mean ± standard deviation (SD).

Patients

The data from 103 Chinese patients with advanced lung adenocarcinoma were retrospectively reviewed. The inclusion criteria used for patient enrollment were as follows: (1) Pathological diagnosis of adenocarcinoma; (2) sufficient tissue for both EGFR and KRAS mutation detection and ERβ1 immunohistochemistry; (3) presence of EGFR mutations associated with sensitivity to EGFR TKIs, including 19 exon deletion and 21 exon point mutation, and absence of EGFR T790M or KRAS mutations; (4) patients treated with EGFR TKIs, including erlotinib, gefitinib, and icotinib; (5) available clinicopathological characteristics, including sex, age, disease stage, and smoking history. Treatment responses were classified according to the response evaluation criteria in solid tumors (RECIST), version 1.1. Progression-free survival (PFS) time was defined as the time between the first day of EGFR TKI treatment until radiologic progression or death. The study was approved by the Ethics Review Committee of the Shandong Cancer Hospital.

EGFR and KRAS mutation detection and immunohistochemistry for ERβ1

Amplification refractory mutation system (ARMS) was employed to detect different genetic variants, including EGFR (exon 19 deletions, L858R, and T790M) and KRAS mutations.

ERβ1 expression in lung adenocarcinoma tissue samples was assessed by immunohistochemistry (IHC). Informed consent to use biopsy tissues was obtained from all patients. Briefly, formalin-fixed, paraffin-embedded tissue sections (3-μm) were deparaffinized and stained according to standard procedures. Sections were probed with anti-ERβ1 mouse antibody (1:200; Abcam, USA); a biotinylated anti-mouse IgG secondary antibody was used. Brown staining in cytoplasm or/and nucleus was considered positive. No

staining was observed in negative controls, including lung tissues probed with a non-immune primary antibody. Based on the localization of “positive” immunoreactivity in the cytoplasm, nucleus, or both, patients were grouped as cERβ1-, n/cERβ1-, or nERβ1-positive. IHC staining was evaluated independently by two investigators (Lijuan Zhang and Meng Tian) and a pathologist (Jianbo Zhang).

Statistical analysis

Differences in the relative mRNA levels, cell viability, and apoptosis between different cell lines were analyzed using two-tailed Student’s t-tests. Patient survival was estimated using the Kaplan-Meier method, and comparisons between groups were conducted using log-rank tests. All statistical tests were two-tailed, and P-values < 0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism 8.0. (Prism Software Inc., San Diego, USA).

Results

ERβ5 affects ERβ1 localization in EGFR-mutant lung adenocarcinoma cancer cells

ERβ5 has been identified as the predominant ERβ splice variant in non-malignant lung tissues. In this study, we found that ERβ5 mRNA levels were elevated in four lung adenocarcinoma cell lines harboring EGFR mutations (Figure 1A). We further assessed the role of ERβ5 in lung adenocarcinoma using PC9 cells, which harbor EGFR exon 19 deletions. Immunocytochemistry and immunofluorescence analyses revealed that endogenous ERβ predominantly localized in the cell cytoplasm, and only low ERβ levels were detected in the nucleus (Figure 1B, 1C).

Next, we overexpressed ERβ1 in PC9 cells (PC9/ERβ1); we also overexpressed ERβ5 in PC9/ERβ1 cells (hereafter referred to as PC9/ERβ1/5). ERβ1 and ERβ5 overexpression were confirmed at the mRNA and protein levels by qRT-PCR and immunoblotting, respectively (Figure 1D; Table 1). Immunofluorescence using a non-variant specific antibody revealed that ERβ levels were elevated both in PC9/ERβ1 and PC9/ERβ1/5 cells; however, ERβ localization differed between the two cell lines. Although ERβ primarily localized in the cell nucleus in PC9/ERβ1 cells, in PC9/ERβ1/5 cells, it was found both in the cytoplasm and nucleus (Figure 2A).

ERβ1 has the highest affinity for estradiol among all ERβ splice variants. Hence, we used an ERβ1-specific antibody to determine ERβ1 localization. ERβ1 predominantly localized in the cell nucleus in PC9/ERβ1 cells. However, in PC9/ERβ1/5 cells, we observed that ERβ1 was partly detained in the cytoplasm, suggesting that the expression of ERβ5 suppressed ERβ1 translocation from the cytoplasm to the nucleus (Figure 2B).

The interaction between ERβ1 and ERβ5 regulates downstream signaling events in the presence of estradiol

Next, we assessed the role of nuclear and cytoplasmic ERβ in transcriptional regulation and non-genomic signaling, respectively. The expression of the cell cycle regulator P21 is induced by the nuclear ERβ [18].

In this study, we found that P21 expression levels were profoundly higher in PC9/ERβ1 cells compared to those in PC9/NC or PC9/ERβ1/5 cells, especially after stimulation with estradiol (Figure 3A).

PI3K/AKT/mTOR signaling pathway is regulated by both EGFR and ERβ [11]. To determine the PI3K/AKT/mTOR pathway activation status, we assessed the levels of EGFR, AKT, and RPS6. We found that phospho-EGFR levels were lower in PC9/ERβ1/5 cells than those in PC9/NC or PC9/ ERβ1 cells. Although total and phospho-EGFR levels decreased in all groups after estradiol treatment, the decrease in phospho-EGFR levels was stronger in PC9/ERβ1 and PC9/ERβ1/5 cells than that in PC9/NC cells. The phospho-AKT levels were higher in PC9/ERβ1/5 cells than those in PC9 or PC9/ERβ1 cells, both at baseline and after estradiol treatment. Although ERβ1 overexpression had a limited impact on baseline phospho-AKT levels, the increase in phospho-AKT levels after estradiol treatment was stronger in ERβ1-overexpressing cells compared with that in PC9/NC cells. The levels of phospho-RPS6, which functions downstream of mTOR, were similar among the groups (Figure 3A).

We also found that ERβ1 but not ERβ5 was upregulated in PC9/NC cells after estradiol treatment. Interestingly, qRT-PCR showed no changes in the ERβ1 mRNA levels after estradiol treatment, suggesting that the estradiol-mediated ERβ1 upregulation occurs at the post-transcriptional level (Figure 3B).

When cells were treated with gefitinib in addition to estradiol, phospho-EGFR levels were decreased in all groups, whereas phosphor-AKT levels were increased, especially in PC9/ERβ1/5 cells. Similar to phospho-EGFR, phospho-RPS6 levels were decreased in all groups after gefitinib treatment. P21 was also downregulated in gefitinib-treated cells (Figure 3C).

PC9/ERβ1/5 cells are less prone to the cytotoxic effects of gefitinib

To determine the effects of different ERβ splice variants on response to gefitinib, we performed cell viability and colony formation assays. We found that PC9/ERβ1/5 cells were less prone to the cytotoxic effects of gefitinib (40 nM) compared with PC9/NC or PC9/ERβ1 cells, although we found no significant differences in cell viability at low concentrations of gefitinib (Figure 4A). Additionally, the ability of gefitinib (40 nM) to inhibit colony formation was stronger in PC9/ERβ1 and PC9/NC than in PC9/ERβ1/5 cells (Figure 4B).

Gefitinib treatment induced apoptosis in all three groups. However, the pro-apoptotic effects of gefitinib were more potent in PC9/ERβ1 and PC9/NC cells compared with those in PC9/ERβ1/5 cells (Figure 4C). We also measured cleaved-PARP and cyclin D3 levels, which are commonly used as markers of apoptosis. We found that cPARP levels were increased in all three groups after gefitinib treatment. However, the increase in cPARP levels was more substantial in PC9/NC and PC9/ERβ1 cells than in PC9/ERβ1/5 cells. Consistently, the decrease in cyclin D3 levels was more profound in PC9/NC and PC9/ERβ1 cells, while almost no change in cyclin D3 levels was observed in PC9/ERβ1/5 cells after gefitinib treatment (Figure 4D). These results suggest that PC9/ERβ1/5 cells are less sensitive to EGFR TKIs than PC9/NC and PC9/ERβ1 cells.

ERβ1 expression and intracellular distribution affect PFS in patients with advanced EGFR-mutant lung adenocarcinoma

In this study, we retrospectively analyzed the data from 103 stage IIIb-IV lung adenocarcinoma patients treated with EGFR TKIs at the Shandong Cancer Hospital between January 2014 and November 2017. All patients harbored EGFR mutations affecting response to EGFR TKIs, including exon 19 deletions (47; 45.6%) and exon 21 point mutations (55; 53.4%); one patient had G719X mutations, whereas no EGFR T790M or KRAS mutations were detected. The clinicopathological characteristics of the patients are summarized in Table 2. Most patients were never/light smokers (79; 76.7%) and women (65; 63.1%).

ERβ1 expression was detected in 80.6% (80/103) of the patients, and the intracellular distribution pattern differed significantly (Figure 4A); strong nuclear accumulation (nERβ1) was observed in 37.9% (39/103) of the patients, nuclear/cytoplasmic accumulation (n/cERβ1) in 23.3% (24/103), and strong cytoplasmic accumulation (cERβ1) in 19.4% (20/103) of the patients (Table 2).

At the time of data collection (Nov 20, 2017), four patients were lost to follow-up, and 79 patients (76.7%) presented with progressive disease. The median survival of the 99 patients was 13.3 months (Figure 4B). There was no significant difference in PFS between patients with ERβ1-positive and ERβ1-negative tumors (13.3 months vs. 14 months, P = 0.794) (Figure 4C). Interestingly, we also found significant differences in the median PFS of patients with different intracellular ERβ1distribution pattern (nERβ1, 17.5 months; n/cERβ1, 11.4 months; cERβ1, 6.5 months; negative, 14 months, P = 0.008) (Figure 4D). Because cytoplasmic ERβ1 is key for non-genomic signaling activation, we combined patients with n/cERβ1 expression and those with cERβ1 (cytoERβ1). Survival analysis showed that patients with cytoERβ1 expression (n = 43) had a shorter median PFS after EGFR TKI treatment (9.5 months) compared to those with nERβ1 expression (n =37; PFS, 17.5months; P = 0.0003) (Figure 4E).

Discussion

Approximately 20%-30% of patients with EGFR activating mutations exhibit primary resistance to EGFR TKIs. The mechanism underlying resistance to EGFR TKIs, and primary resistance, in particular, are extremely complex and remain poorly understood. ERβ expression has been associated with response to EGFR TKIs. Notably, in a Japanese cohort study, strong ERβ expression predicted favorable clinical outcomes in patients with lung adenocarcinoma after treatment with EGFR TKIs. In contrast, we previously identified high cytoplasmic ERβ expression as a predictor of poor PFS [21, 22]. Therefore, further elucidation of the expression pattern and intracellular distribution of ERβ is required to determine the effects of non-genomic signaling on EGFR signal transduction and clinical outcomes.

Several ERβ splicing variants have been identified, the most important of which are ERβ1 (wild-type ERβ), and ERβ2–5 [20, 23]. ERβ1 is the only fully functional receptor in the ERβ family, and has the highest affinity for estradiol; other ERβ family members have weak to no ligand binding capacity, despite maintaining their ability to heterodimerize with ERβ1 [20]. Therefore, assessing the function of ERβ splice

variants other than ERβ1 is equally important. Notably, the crucial role of ERβ5 in lung cancer is becoming increasingly evident [17, 24].

In this study, we focused on the role of ERβ1 and ERβ5 in lung adenocarcinoma. Previous studies demonstrated that ERβ1 was predominantly localized in the cell nucleus and exerted anti-proliferative effects. In contrast, ERβ5 was found both in the cytoplasm and nucleus, and it has been implicated in cancer cell migration and invasion [17, 22, 25]. Our results confirmed the elevated ERβ5 levels in EGFR-mutant lung cancer cells; in contrast, ERβ1 was lowly expressed. These results were consistent with those of a previous study showing that ERβ5 was the primary ERβ isoform expressed in non-malignant lung cells, and heterodimerized with ERβ1 [20]. Similarly, we previously showed that ERβ5 formed complexes with ERβ1, confirming their ability to interact [22].

In this study, we also found that ERβ1 was predominantly localized in the cell nucleus. However, the forced overexpression of ERβ5 partly retained ERβ1 in the cytoplasm. Hence, the presence of ERβ5 can explain previous findings of ERβ1 localization in the nucleus and cytoplasm in cancer cells.

Total and phospho-EGFR levels were decreased after estradiol treatment, highlighting the crosstalk between EGFR and ERβ signaling pathways [12]. P21 is an essential cell cycle regulator, playing important tumor-suppressing roles [26]. Importantly, P21 expression was induced by ERβ [18, 25]. In this study, we found that confirmed that ERβ1 increased P21 levels, suggesting a role of ERβ1 in transcriptional regulation in lung cancer cells. Consistently, ERβ1 exerted anti-proliferative effects in other cancer cells [15, 18]. However, when ERβ1 and ERβ5 were co-expressed, P21 levels were lower compared with those in PC9/ERβ1 cells, suggesting that ERβ5 impairs the transcriptional abilities of ERβ1. However, in the presence of estradiol, PI3K/AKT/mTOR signaling pathway activation levels were higher in ERβ1/5-expressing lung cancer cells than those in ERβ1-expressing cells, suggesting that the interaction between ERβ1 and ERβ5 potentiated the effects of ERβ1 in non-genomic signaling. Hence, we believe that ERβ1 translocation from the nucleus to cytoplasm in the presence of ERβ5 was essential in determining its biological function, reflecting the bi-faceted role of ERβ in cancer [27].

mTOR signaling was inhibited by gefitinib treatment in all groups, although phospho-AKT levels were increased. Consistently, gefitinib treatment exerted cytotoxic effects in all cell groups. However, ERβ1/ERβ5 co-expression rendered cells less prone to the cytotoxic and pro-apoptotic effects of gefitinib. These results confirmed the critical role of ERβ1/ERβ5 complexes in estrogen receptor-mediated non-genomic signaling. We also found that estradiol upregulated ERβ1 but not ERβ5 at the post-transcriptional level, confirmed the high affinity of ERβ1 for estradiol.

In this study, we also investigated the effect of the ERβ1 expression pattern on PFS in EGFR-mutant lung adenocarcinoma patients. We found that patients with nuclear ERβ1 expression exhibited a relatively longer PFS after EGFR TKI treatment, whereas cytoplasmic ERβ1 was associated with shorter PFS after EGFR TKI treatment. These results highlight the clinical relevance of our findings from in vitro experiments in EGFR-mutant lung cancer cell lines. Importantly, nuclear ERβ1 expression in lung cancer tissues was associated with tumor-suppressing effects, whereas cytoplasmic ERβ1 promoted EGFR TKI

resistance to some extent. Although cytoERβ1 was associated with a lower response to EGFR TKIs, the median PFS of patients with cytoERβ1 was 9.5 months, suggesting that EGFR mutations remain the most powerful predictor for EGFR TKI treatment response. The findings reported here need to be confirmed in large cohort prospective studies. Additionally, the relationship between ERβ1/ERβ5 ratio and response to EGFR TKIs merits further investigation.

Conclusion

We showed that ERβ1 localized in the cell cytoplasm by interacting with ERβ5, inducing non-genomic signaling activation, and promoting EGFR TKI treatment resistance in EGFR-mutant lung adenocarcinoma. Hence, anti-estrogen therapy might reverse EGFR TKI treatment resistance to some extent in certain patients.

Abbreviations

EGFR TKI, epidermal growth factor receptor tyrosine kinase inhibitor; ER, estrogen receptor; qRT PCR, quantitative real time PCR; PFS, progression free survival; ORF, open reading frame; CCK, cell counting kit; WST, water-soluble tetrazolium; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; RECIST, response evaluation criteria in solid tumors.

Declarations

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Shandong Cancer Hospital and Institute. The research has been carried out in accordance with the World Medical Association Declaration of Helsinki. All patients provided written informed consent.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used during this research are available from the corresponding author upon reasonable request.

Competing interests

The authors have no conflict of interest.

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Not applicable.

Authors' contributions

ZXL: design the study, analyze the data, write and revise the manuscript. LJZ and MT: performed the cell experiments. JML and JBZ conducted the immunohistochemistry. HYW collected the information of the patients. All authors read and approved the final manuscript.

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Tables

Table 1 ΔCT value of ERβ1 and ERβ5 in indicated cell lines.

Cell lines	ERβ1 ΔCT（mean±SD）	P value	ERβ5 ΔCT（mean±SD）	P value
PC9/NC	22.59±1.33		13.33±0.37	
PC9/ERβ1	2.73±0.05	＜0.01*	13.15±0.27	
PC9/ERβ1/5	2.25±0.03	＜0.01*	5.84±0.14	＜0.01#

*The value was compared to PC9/NC. #The value was compare to PC9/NC and PC9/ERβ1 respectively.

Table 2 Clinical and pathological characteristics of 103 patients with EGFR mutant lung adenocarcinomas.

Variables	Number of cases (%)
Age, Years	55
Median	33-77
Range	38(36.9)
Gender	65(63.1)
Male	24(23.3)
Female	79(76.7)
Smoking status	6(5.8)
Ever or current	97(94.2)
Never	47(45.6)
Stage	55(53.4)
III	1(1)
IV	56(54.4)
EGFR mutation type	43(41.8)
19del	2(1.9)
L858R	2(1.9)
Other	39(37.9)
Response evaluation	24(23.3)
PR	20(19.4)
SD	20(19.4)
PD	
No Evaluation	
ERβ expression pattern	
Nuclear	
Cytoplasmic+Nuclear	
Cytoplasmic only	
Negative	

Figures

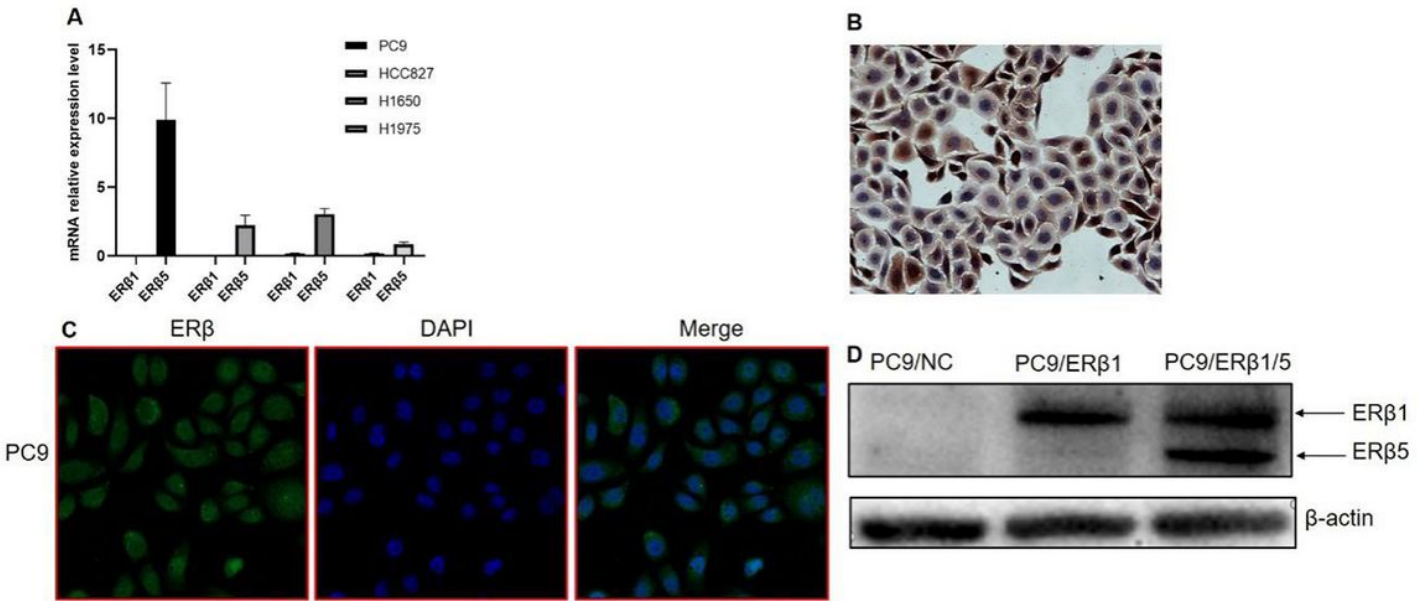

Figure 1

Construction of a EGFR mutant lung cancer cell lines stably expressing ERβ1 and ERβ1/5. A. mRNA level of ERβ1 and ERβ5 in four lung adenocarcinoma cell lines harboring EGFR mutation using specific primers determined by qRT PCR. Data shown as mean±SD. B. ERβ expression pattern was assessed by immunocytochemistry in PC9. C. ERβ expression pattern was assessed by immunofluorescence in PC9. D. Western blot for detecting the expression level of ERβ after stable transfection of ERβ1 and ERβ5. The upper stand refers to ERβ1 while the lower is ERβ5.

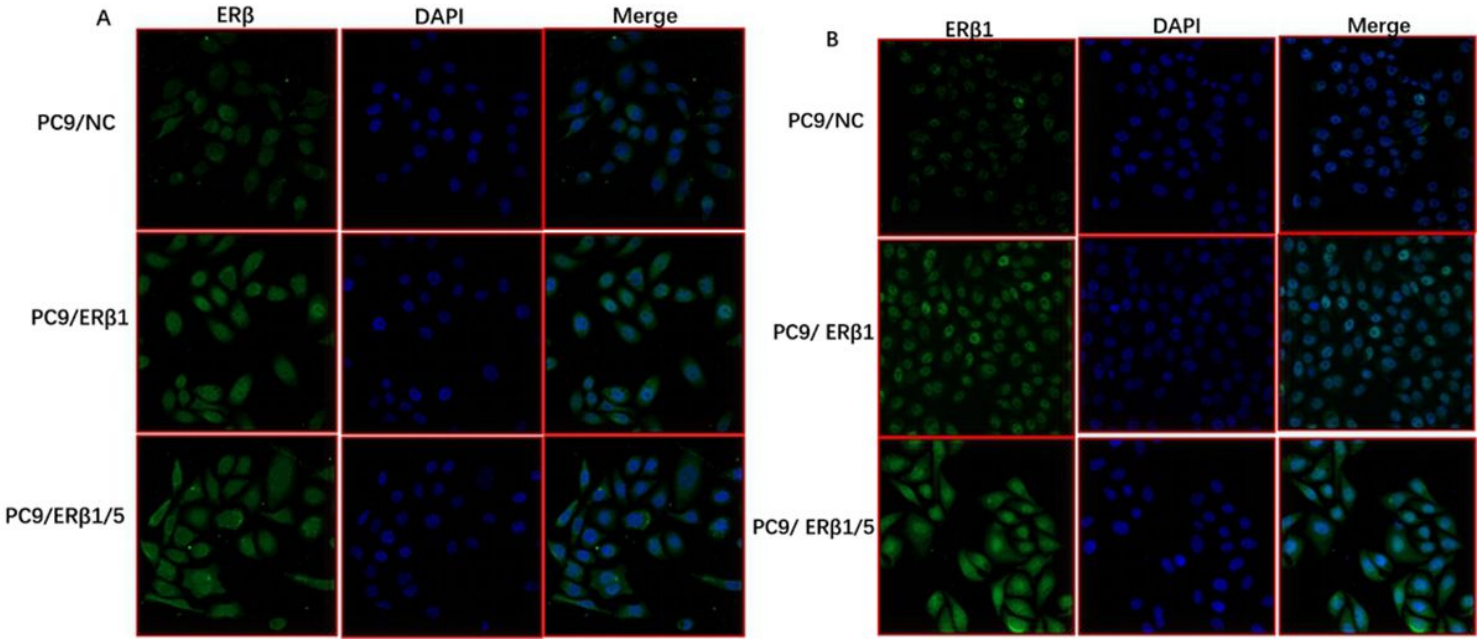

Figure 2

Different intracellular localization of ERβ in three cell lines (PC9/NC, PC9/ERβ1 and PC9/ ERβ1/5. A. ERβ expression was analyzed in established cell lines using immunofluorescence by using primary antibodies against ERβ. B. ERβ1 expression was detected in the cell lines using immunofluorescence by using primary antibodies against ERβ1.

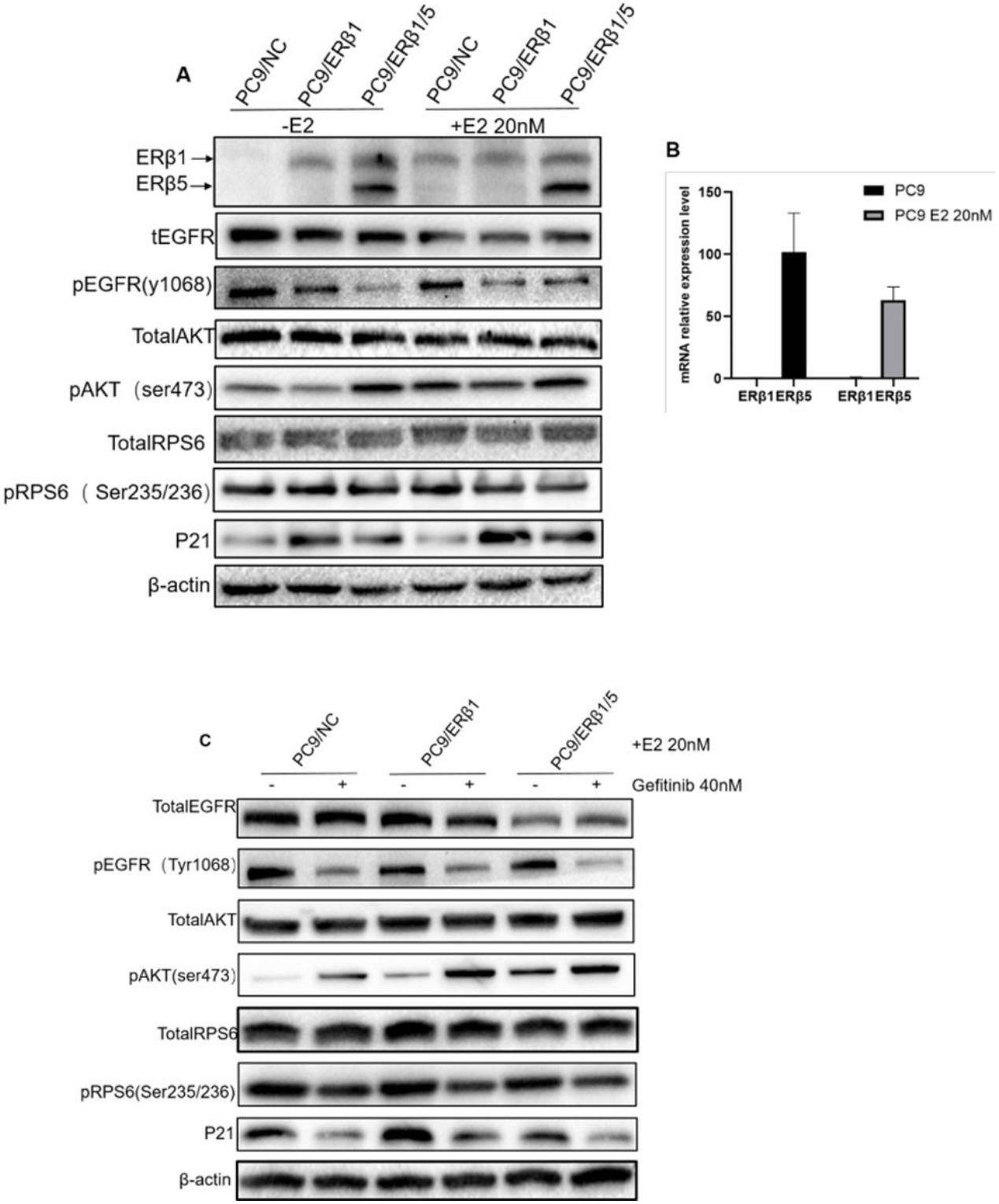

Figure 3

Interaction between ERβ1 and ERβ5 have complex impact on downstream signaling under stimulation of Estradiol. A. Western blot was used to detect the indicated proteins involved in PI3K/AKT/mTOR signal pathway in the presence or absence of Estradiol (20nM). P21 was selected as marker for detecting ERβ nuclear transcriptional activity. B. Relative mRNA expression level of ERβ1 and ERβ5 in the presence or absence of Estradiol for PC9/NC. C. The indicated cell lines were treated with gefitinib (40nM) for 8 hours in the presence of estradiol. Cell extracts were immunoblotted to detect the indicated proteins.

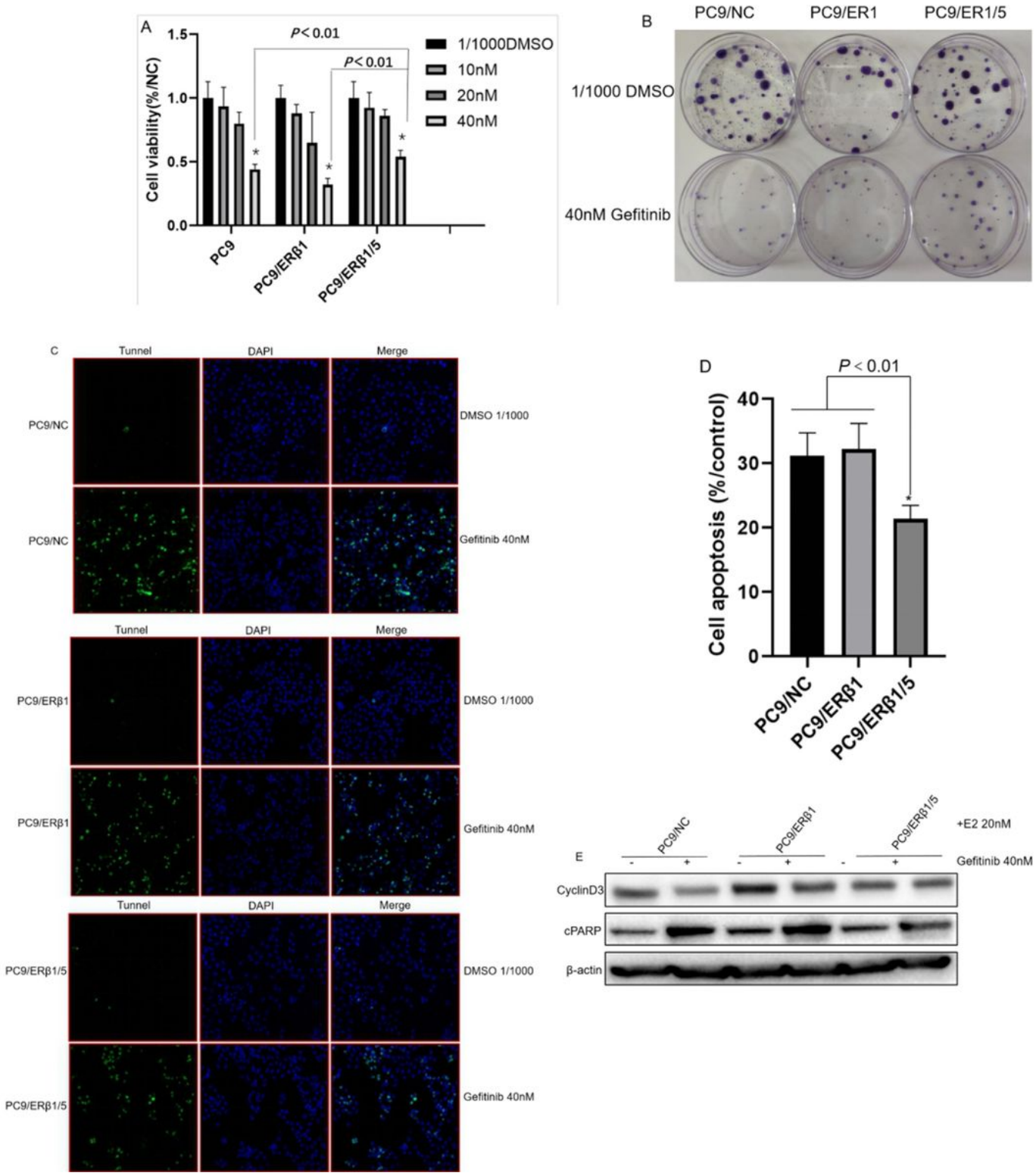

Figure 4

PC9/ERβ1/5 showed less effective to gefitinib compared to that of PC9/NC and PC/ERβ1 through anti-apoptosis effect. A. Cell viability test for 72 hours treatment at indicated concentration of Gefitinib (0, 10nM, 20nM, 40nM) in the presence of estradiol (20nM). PC9/ERβ1/5 cells were less effective to gefitinib compared to PC9/NC and PC9/ ER1 cells at concentration 40nM (*P□0.01). B. Colony formation assay for 2 weeks in the presence of gefitinib 40nM or 1/1000 DMSO for three cell lines. C. Tunnel analysis for three cells in the presence or absence of Gefitinib 40nM for 24 hours with estradiol (20nM). D. Cell apoptosis percentage after treatment of gefitinib (40nM) for 24 hours in the presence of estradiol (20nM). Data shown as mean±SD (* P□0.01). E. Western blot for detecting cleaved PARP and CyclinD3 for three cell lines with or without Gefitinib (40nM) treatment under the stimulation of estradiol (20nM).

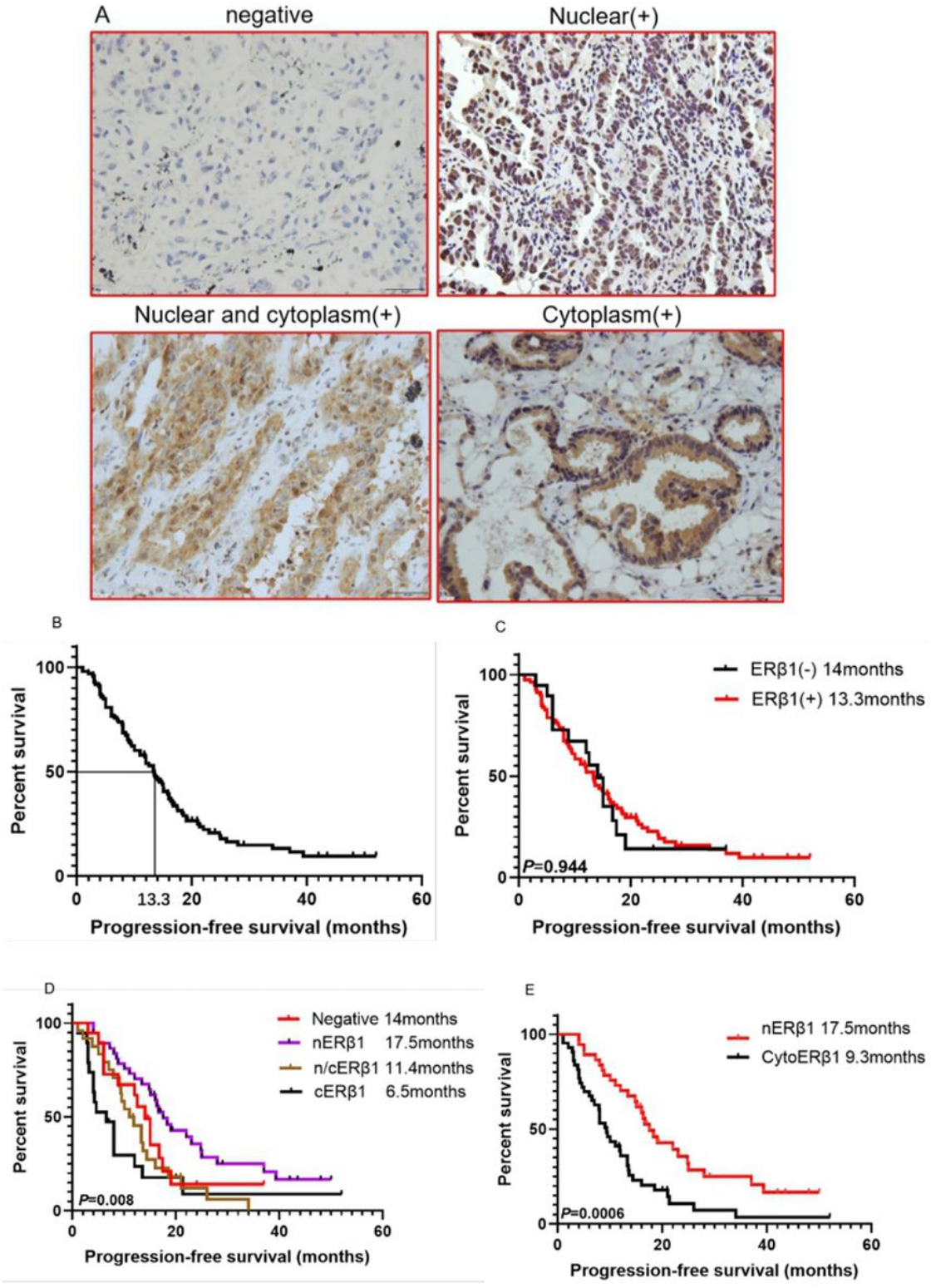

Figure 5

ERβ1 expression pattern had different effect on progression-free survival in advanced EGFR mutant lung adenocarcinomas. A. Representative ERβ1 expression within lung adenocarcinoma tissues (Negative, nuclear, nuclear and cytoplasm, cytoplasm). B. Kaplan-Meier curve showed progression free survival for all the patients (median survival: 13.3 months). C. Kaplan-Meier curves illustrated progression-free survivals in the groups with ERβ1 positive (median survival:13.3 months) and ERβ1 negative (median

survival: 14 months) (log-rank test, P=0.944). D. Comparison of progression free survival in four groups using log-rank test (P=0.008). E. Comparison of progression free survival between nuclear ERβ1 positive group(median survival: 17.5 months) and cytoERβ1 positive group (median survival:9.3 months) (log-rank test, P=0.0006).