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Estrogen Receptor β Is Involved in Acquired Resistance to EGFR-tyrosine Kinase Inhibitors in Lung Cancer

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Abstract. *Background/Aim: Acquired resistance to epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) has posed serious clinical problems in the treatment of lung adenocarcinoma (LADC) patients harboring relevant EGFR mutations. In this study, we explored the role of estrogen receptor β (ERβ) in the development of acquired resistance to EGFR-TKIs in human LADC. Materials and Methods: First, the role of ERβ in erlotinib resistance of LADC cell lines (PC9/ER) was examined. Then, the immunolocalization of ERβ in 28 LADC patient samples treated with EGFR-TKIs was investigated. Results: Cytoplasmic ERβ was upregulated in erlotinib resistant cell lines. EGFR-TKIs sensitivity increased with ERβ inhibition in PC9/ER cells. ERK1/2 and AKT activities were both markedly increased by specific ERβ agonists even under erlotinib treatment of PC9/ER cells. Cytoplasmic ERβ immunoreactivity was significantly associated with clinical response to EGFR-TKIs. Conclusion: Cytoplasmic ERβ in LADC cells was involved in the development of resistance to EGFR-TKIs.*

Non-small cell lung cancers (NSCLCs) account for approximately 80% of all lung cancer patients but their five-year survival rate is still 15% despite recent employment of new target specific therapy (1). Recently, epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (EGFR-TKIs) such as erlotinib, gefitinib, and afatinib have been clinically used as a first-line therapy for NSCLCs, especially adenocarcinoma patients harboring *EGFR*-activating mutations (2-5). However, it is also true that 20-30% of patients with these *EGFR* mutations have primary intrinsic resistance known as '*de novo*' resistance (6, 7). In addition, practically all patients who clinically responded to EGFR-TKIs developed therapeutic resistance to EGFR-TKIs (6, 7). This acquired resistance has been postulated to be induced by the activation of alternative proliferative pathways, for example, through HGF overexpression and amplification of the MET gene as well as through the acquisition of secondary *EGFR* gene mutations, such as T790M and C797S (8-10). However, the details of the mechanism used for developing resistance to EGFR-TKIs have remained virtually unknown at this juncture.

Estrogens are key signaling molecules regulating various physiological processes such as cell growth, development, and differentiation by binding to two receptors: ERα and ERβ (ERs will refer to both subtypes), and also play a major role in many pathological processes of hormone-dependent diseases (11-13). ERs have also been reported to be expressed in NSCLC cell lines, tumor tissues, and cells derived from the normal lung (14-17). In particular, ERβ status of carcinoma cells has been reported to be correlated with prognosis and postoperative survival rate of patients with NSCLC (18-21). Estrogen has been also known to act as a mitogen for NSCLC cells both *in vitro* and *in vivo* and to modulate the expression of various genes in NSCLC cell lines, which are important for the regulation of cell proliferation through binding to ERs (22-24). In addition, a combination of anti-estrogen with gefitinib, both of which could suppress the G1-S cell cycle transition by utterly different mechanisms, has been reported to be beneficial with additive therapeutic effects (25-27).

One of the signaling pathways activated by plasma membrane ERβ binding of estrogen has been shown to be that of the proline-directed, serine/threonine kinase, extracellular-regulated kinase (ERK) in breast carcinoma cell line (28, 29). ERβ has also been reported to be correlated

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with the development of '*de novo*' resistance to EGFR-TKIs in lung adenocarcinoma. Therefore, the correlation between estrogen signaling through ERβ and resistance to EGFR-TKI has been reasonably postulated but has not been studied yet. In addition, this cross-talk between the ERβ/EGFR pathways has not been explored in lung adenocarcinoma cells. Therefore, in this study, we hypothesized that ERβ could be involved in the process of 'acquired' resistance to EGFR-TKIs in lung adenocarcinoma cells and that it could also serve as a potential predictor of clinical response to EGFR-TKIs in patients with lung adenocarcinoma.

Materials and Methods

Reagents and antibodies. The following materials were obtained as follows: gefitinib, was from Biaffin, Kassel, Germany; erlotinib, was kindly provided from Roche Diagnostics, Mannheim, Germany; 17β-estradiol, Diarylpropionitrile (DPN), Propylpyrazoletriol (PPT), and ICI 182,780, were from Tocris Bioscience, St Louis, MO, USA. The antibodies employed in this study were obtained from the following sources: β-actin from Sigma-Aldrich, St. Louis, MO, USA; phospho-EGFR, phospho-ERK1/2, and phospho-AKT from Cell Signaling Technology (Beverly, MA, USA); ERβ from Santa Cruz Technology (Santa Cruz, CA, USA).

Cell culture. The human lung adenocarcinomas cell line used in this study was PC9 cells (Immuno-Biological Laboratories, Gunma, Japan). The cells were grown in RPMI-1640 (Sigma-Aldrich) containing 10% fetal bovine serum (Nichirei, Tokyo, Japan). The PC9 cells were exposed to increasing concentrations of erlotinib in order to establish those with acquired resistance to each of drugs (PC9/6m; 0→0→0 μM, PC9ER; 0.01→1→5 μM, PC9/ERr; 0.5→1→5 μM) (30). The breast carcinoma cell line T47D was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). In this study, PC9/6m, PC9/ER and PC9/ERr cells had the same passage numbers (between 39-44). The passage number of T47D cells was 11-16.

Patients and tissue specimens. A total of 28 lung adenocarcinomas cases were retrieved from surgical pathology files at the Department of Pathology, Tohoku University Hospital (Sendai, Japan) and Miyagi Cancer Center (Natori, Japan). These samples had been fixed in 10% neutral formalin and embedded in paraffin. Assessment of clinical response to EGFR-TKIs treatment in the tumor was based on computed tomography (CT) evaluation every 2 months. Research protocols were approved by the Ethics Committee at Tohoku University School of Medicine (2009-380) and Miyagi Cancer Center (H21-No.34).

Cell proliferation assays. Cells grown in RPMI-1640 with FBS were seeded into 96-well plates (2.5×10^3 cells per well) and treated with different concentrations of various chemotherapeutic agents (erlotinib, gefitinib, 17β-estradiol, ICI 182,780) for 72 h. Cell proliferation was analyzed using Cell counting kit-8 (Dojindo Laboratories, Tokyo, Japan).

Quantitative real-time RT-PCR. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA synthesized using a Quanti Tect Reverse Transcription kit (Qiagen, Hilden, Germany). Reverse transcription was performed using the LightCycler System (Roche Diagnostics) and QuantiFast SYBR Green PCR kit (Qiagen). As an endogenous control, RPL13A was amplified in each sample. The primer sequences used in this study were as follows: ERα forward: 5'-AGA CAC TTT GAT CCA CCT GA-3'; ERα reverse: 5'-CAA GGA ATG CGA TGA AGT AG-3'; ERβ forward: 5'-TGG CAA CTA CTT CAA GGT TTC-3'; ERβ reverse: 5'-GCT GTG ACC AGA GGG TAC ATA-3'; RPL13A forward: 5'-CCT GGA GGA GAA GAG GAA AG-3'; RPL13A reverse: 5'-TTG AGG ACC TCT GTG TAT TT-3'.

Immunoblotting. The cells treated with 17β-estradiol, PPT and DPN were pre-exposed to serum-free RPMI-1640 medium for 48 h before treatment. Following treatment, cells were washed with PBS. Protein was extracted by adding 100 μM M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, Waltham, MA, USA) with Halt Protease Inhibitor Cocktail, Phosphatase Inhibitor and EDTA (all from Pierce Biotechnology, Rockford, IL, USA) and scraped into a microtube. After centrifugation at 13,000 rpm for 15 min at 4˚C, protein concentration was determined using Protein Assay Kit Wako (Wako Pure Chemical Industries, Osaka, Japan) and heated in SDS sample buffer at 98˚C. Proteins were resolved by SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membrane. After blocking with tris-buffered saline containing 0.05% Tween 20 and 5% non-fat dry skim milk for 1 h at room temperature, the membrane was incubated overnight at 4˚C with primary antibody. The following antibodies were used: anti-pEGFR monoclonal antibody, 1:500 dilution; anti-pAkt monoclonal antibody, 1:500 dilution; anti-pErk1/2 monoclonal antibody, 1:500 dilution (all from Cell Signaling Technology); anti-ERβ polyclonal antibody 1:250 dilution (Santa Cruz Biotechnology, Inc.). The reacted membrane was then washed and incubated for 1h with horseradish peroxidase-conjugated goat anti-mouse/rabbit IgGs (GE Healthcare, Buckinghamshire, UK) at room temperature. Immunoreactive bands were visualized by Molecular imager Chemi Doc XRS Plus with Image lab software (Biorad, Hercules, CA, USA).

RNA interference. Cells were cultured in 6 well plates at 1.5×10^5 cells per well and grown overnight in RPMI + 10% FBS. Cells were then transfected with validated small interference ERβ RNA (s4826; Life Technology, Carlsbad, CA, USA) or negative control Silencer siRNA (Ambion, Austin, TX, USA) using Lipofectamine RNAi MAX reagent (Invitrogen, Carlsbad, CA, USA). Knockdown efficiency was assessed by RT-PCR.

Nuclear protein extraction. Nuclear proteins were isolated using the CelLytic NuCLEAR Extraction Kit (Sigma-Aldrich). Cells were incubated in 10x Lysis Buffer, hypotonic (100 mM HEPES pH7.9, 15mM $MgCl_2$, 100mM KCl) and destructed using IGEPAL CA-630 10% Solution. Then, the sample was centrifuged at 10,000 g for 30 s. The supernatant was fractionated and used as a cytoplasmic fraction for immunoblotting analysis. Extraction Buffer with dithiothreitol and Protease Inhibitor Cocktail (all from Sigma-Aldrich) was added to the pellet after centrifugation, resuspended and shaken for 10 min. The sample was centrifuged at 20,000 g, and the supernatant was used as a nuclear fraction for immunoblotting analysis.

Immunohistochemistry. The specimens for immunohistochemistry had been embedded in paraffin. Anti- ERβ monoclonal antibody (14C8;

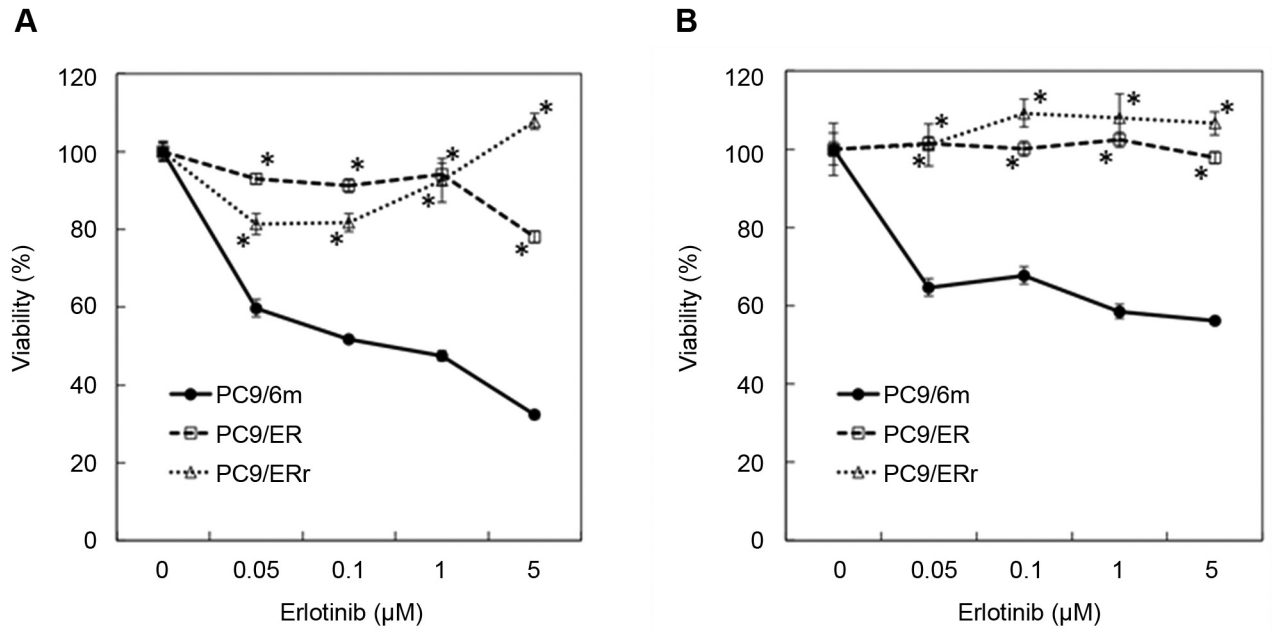

Figure 1. *Effect of TGFR-TKIs on erlotinib resistant PC9. Both erlotinib-resistant and control cells were treated with Erlotinib (0.05, 0.1, 1, 5 μM) (A) or Gefitinib (0.05, 0.1, 1, 5 μM) (B) for three days, and their viability was evaluated using Cell counting kit-8. PC9/6m, control PC9 cells; PC9/ER, erlotinib resistant PC9 cells were produced following incubation with increasing doses of erlotinib (0.01→1→5 μM); PC9/ERr, erlotinib resistant PC9 cells were produced following incubation with increasing doses of erlotinib (0.5→1→5 μM); *p<0.05 vs. PC9/6m.*

GeneTex Inc., Irvine, CA, USA) (31, 32) was used as primary antibody (1:1,000). In this study, we used a Histofine Kit (Nichirei Bioscience, Tokyo, Japan) based on the streptavidin-biotin amplification method. The antigen-antibody complex was visualized with 3,3'-diaminobenzidine (1 mM diamino benzidine, 50 mM Tris–HCl buffer, pH 7.6, and 0.006% H_2O_2). The evaluations were made independently by two of the investigators using multiheaded light microscopy. More than 10% of ERβ-positive cancer cells showing any intensity of positive nuclear staining were defined as positive cases.

Statistical analysis. Statistical analysis was performed using Student's *t*-test. Results are expressed as mean±SD. A *p*-value less than 0.05 was determined to be significant. The statistical calculations were performed using JMP Pro 11.2.0 (SAS Institute, Cary, NC, USA).

Results

Effect of EGFR-TKI on resistant PC9 cells. We first confirmed the effect of EGFR-TKIs on the resistant cells (Figure 1). After erlotinib administration, the survival of PC9/ER and PC9/ERr cells was significantly higher at each concentration than that of control PC9/6m cells (Figure 1A). These erlotinib-resistant PC9/ER and PC9/ERr cells were also found to be resistant to gefitinib (Figure 1B).

Cytoplasmic ERβ status of EGFR-TKI resistant PC9 cells. ERβ and ERα mRNA expression levels in EGFR-TKI-resistant PC9 cells examined using quantitative PCR are shown in Figure 2A. In PC9/ER and PC9/ERr cells, both ERβ and ERα mRNA expression levels were significantly higher than those in the control PC9/6m cells. The expression of ERβ was remarkable; its expression in EGFR-TKI-resistant PC9 cells was 8 (PC9/ERr) and 12 (PC9/ER) times higher than that in PC9/6m. The expression of ERs in PC9/6m, which had been cultured for 6 months as a control, was not significantly different from that of the parental PC9 cell line.

Next, we examined ERβ protein expression in EGFR-TKI-resistant PC9 cells (Figure 2B). Relatively higher levels of ERβ were observed in both PC9/ER and PC9/ERr cells compared to PC9/6m cells. We also examined ERβ expression in the nuclear and cytoplasmic fractions of PC9/ER and PC9/6m cells (Figure 2C). While ERβ expression was observed in both the nuclear and cytoplasmic fractions, its expression in the cytoplasmic fraction was relatively higher in both PC9/ER and PC9/6m cells.

The effect of estrogen treatment (10 nM estradiol for 12 h) on ERβ protein expression was further investigated (Figure 2D). In breast cancer T47D cells employed as positive control for ERβ, the addition of estrogen reduced ERβ levels in the cytoplasmic fraction. Conversely, no marked change in cytoplasmic ERβ was observed in PC9/ER cells upon estrogen addition.

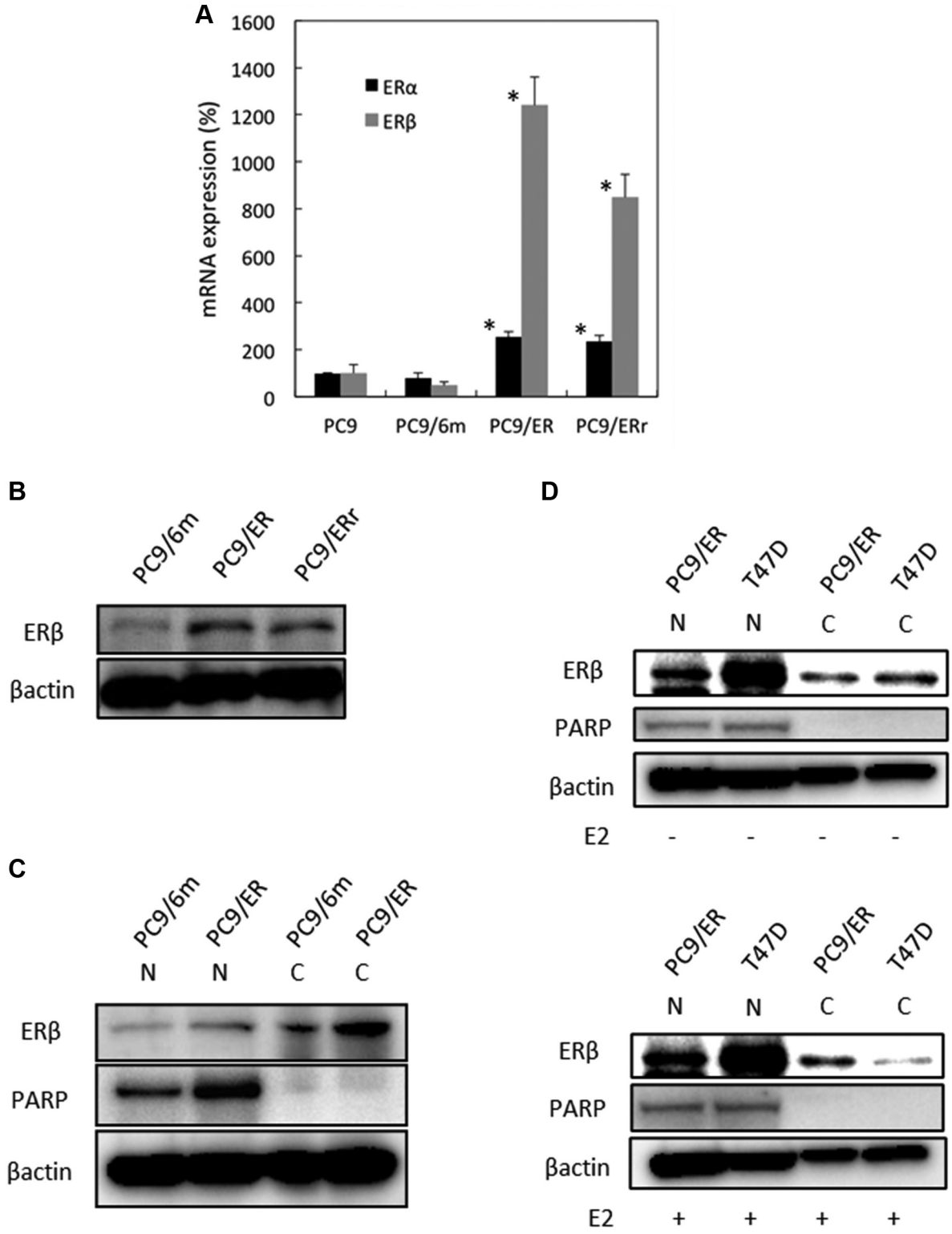

Figure 2. *Expression of ERβ in erlotinib-resistant PC9. (A) Quantitative real-time PCR of ERα and ERβ in erlotinib-resistant (PC9/ER, PC9/ERr) and control (PC9/6m) PC9 cells. PC9, parental cell line; *p<0.05 vs. PC9/6m. (B) Expression of ERβ protein in PC9/ER, PC9/ERr, and PC9/6m. (C) Expression of ERβ protein in the nucleus and cytoplasmic fractions of PC9/ER, PC9/ERr, and PC9/6m. N: Nuclear fraction; C: cytoplasmic fraction. (D) ERβ protein expression in nuclear and cytoplasmic fractions with (lower panel) or without (upper panel) estrogen treatment. N: Nuclear fraction; C: cytoplasmic fraction. E2: estradiol (10 nM, 12 h); –: absence of E2; +: presence of E2.*

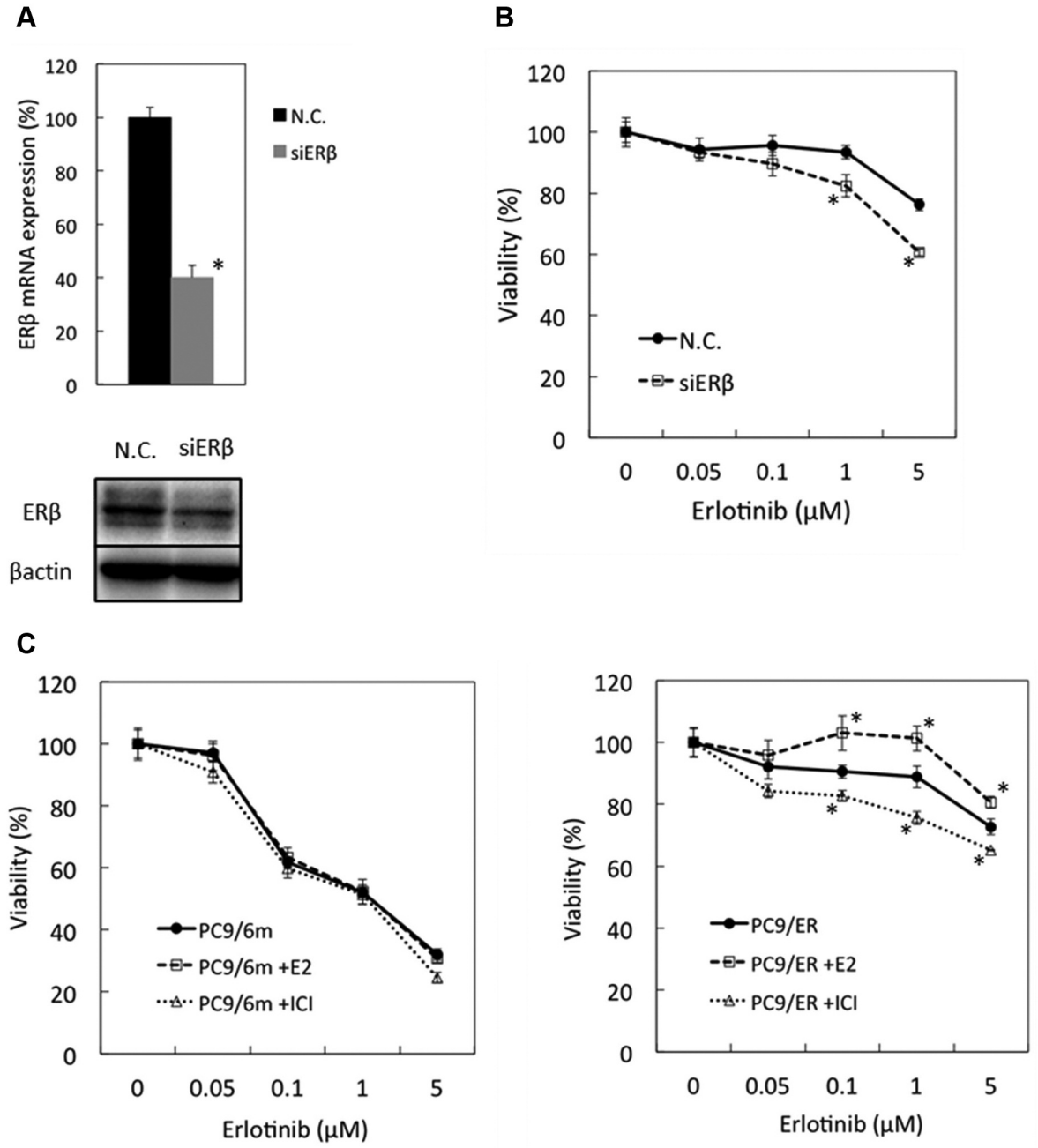

Figure 3. *Effect of estrogen on cell viability of EGFR-TKI resistant PC9. (A) Effect of ERβ knock down in PC9/ER. After 3 days of transfection, total mRNA and proteins were extracted. Efficacy of ERβ knock down was evaluated by quantitative real-time RT-PCR (Upper) and immunoblotting (Lower). (B) Erlotinib was added to the PC9/ER 3 days after transfection with siRNAs and the viability of ER cells was evaluated by Cell counting kit-8 3 days later. (C) The effect of co-addition of erlotinib with estrogen on PC9/6m (Left) and PC9/ER (Right) was evaluated. E2: 10 nM estradiol; ICI: 5 μM ICI18,780, p<0.05 vs. PC9/ER (erlotinib alone). N.C.: Transfection of negative control siRNA; siERβ, transfection of siRNA for ERβ; *p<0.05 vs. N.C.*

ERβ expression is related to EGFR-TKI sensitivity in EGFR-TKI resistant cells. We treated PC9/ER cells with ERβ siRNA to further study the effects of ERβ knockdown on EGFR-TKI sensitivity in these cells (Figure 3). ERβ siRNA reduced the expression of both ERβ mRNA and protein in PC9/ER cells (Figure 3A). The control siRNA-transfected PC9/ER cells showed resistance to erlotinib, while ERβ siRNA-transfected PC9/ER cells displayed significantly

restored sensitivity to erlotinib (1 and 5 μM) (Figure 3B).

We subsequently examined the effect of estrogen (estradiol, 10 nM) on erlotinib sensitivity (Figure 3C). In control PC9/6m cells, no significant effect of either estrogen or the estrogen receptor down regulator ICI 182,780 was observed at any concentration of erlotinib. In PC9/ER cells, the effect of erlotinib (0.1, 1.0, and 5.0 μM) was significantly inhibited in estrogen-treated cells compared to non-estrogen-treated cells. ICI 182,780 significantly enhanced the effects of erlotinib compared to non-estrogen-treated PC9/ER cells.

Estrogen related to EGFR-TKI resistance. We treated EGFR-TKI resistant cells with estradiol for different time periods and evaluated EGFR, ERK1/2, and AKT activation to further determine if there was activation of the EGFR signaling pathway (Figure 4). In the estrogen-treated PC9/6m cells, pEGFR expression decreased between 30 and 60 min, whereas pERK expression decreased 15 min post estradiol treatment (Figure 4A). pAKT expression gradually decreased from 5 to 30 min of estradiol treatment; however, the expression was restored after 60 min of estradiol treatment (Figure 4A). In PC9/ER cells, the expression of pEGFR was relatively higher than in PC9/6m cells; however, no significant change in its expression after estrogen addition was observed (Figure 4A). In contrast, a remarkable increase in pAKT and pERK expression levels was observed after 15 and 30 min of estrogen treatment (Figure 4A).

In PC9/6m cells, erlotinib treatment markedly reduced both pEGFR and pERK levels (Figure 4B). Erlotinib also attenuated pEGFR expression in PC9/ER cells, but recovery was observed 3 h after its addition (Figure 4B). In PC9/ER cells treated with erlotinib, pERK expression showed changes similar to that of pEGFR (Figure 4B).

Next, we treated the cells first with estrogen and subsequently with erlotinib and evaluated the EGFR activation status (Figure 4C). In PC9/6m cells, pEGFR and pERK expression levels were reduced, even after co-treatment with erlotinib and estrogen (Figure 4C). In PC9/6m cells, pAKT recovered 60 min after co-treatment, but an evident decrease was observed 5-30 min after co-treatment (Figure 4C). In PC9/ER cells, co-treatment with erlotinib and estrogen did not show distinct suppression of pEGFR, pERK, or pAKT expression (Figure 4C).

ERβ-specific agonist associated with EGFR-TKI resistance. We further examined the effects of ERα- and ERβ-specific agonists (PPT and DPN, respectively) on EGFR-TKI resistant cells (Figure 5). The expression of phosphorylated EGFR was suppressed 30 min after the addition of erlotinib, and PPT could not rescue this suppression (Figure 5A). PPT also did not affect the expression of pERK and pAKT (Figure 5A). Co-treatment with the ERβ–specific agonist DPN largely protected against the inhibition of EGFR phosphorylation by erlotinib in PC9/ER cells but not in PC9/6m cells (Figure 5B). DPN also markedly blocked the inhibitory effect of erlotinib on the expression of pERK and pAKT in PC9/ER cells (Figure 5B). These findings were similar to the results of estrogen treatment for EGFR signal suppression exerted by erlotinib in EGFR-TKI-resistant PC9 cells (Figure 4C).

ERβ in lung adenocarcinoma tissues. ERβ immunoreactivity was detected in the nucleus and/or cytoplasm of lung adenocarcinoma cells (Figure 6A). A significant inverse correlation was established between the clinical response rate to EGFR-TKI treatment and the cytoplasmic ERβ positive rate of the patients (p=0.0018) (Figure 6B). There was no association between ERβ expression and other clinico-pathological factors in this study (data not shown).

Discussion

The results of our study demonstrated that cytoplasmic ERβ induced EGFR-TKIs resistance, activated EGFR downstream pathways including ERK1/2 and AKT, and was involved in the development of acquired resistance to EGFR-TKIs in lung carcinoma patients.

ERβ expression was also found to be increased in the cytoplasm of TKI resistant cells using CelLytic NuCLEAR Extraction Kit, but this method could not differentiate between cytoplasmic and membrane proteins. Therefore, we further evaluated whether membrane or cytoplasmic ERβ was translocated to the nucleus. In the presence of E2, cytoplasmic ERβ was translocated from the cytosol to the nucleus of T47D cells, a breast carcinoma cell line used as a control, but not in PC9/ER cells examined in this study. This result suggested that cytoplasmic ERβ may not be involved in transcriptional activities. Therefore, we then evaluated the possible effects of induced ERβ on the sensitivity to EGFR-TKIs and the activation of EGFR downstream proteins in TKI resistant cells. Dextran-coated charcoal-stripped FBS (c-FBS), which is depleted of steroid hormones, has been used in order to increase the sensitivity to steroid hormones. However, c-FBS is also known to be depleted of several growth factors including EGF. Therefore, we treated cells with E2 and/or EGFR-TKIs and evaluated the effects of induced-ERβ on EGFR downstream signaling using normal FBS. In this *in vitro* system, cytoplasmic ERβ activated the EGFR downstream proteins ERK1/2 and AKT, in the resistant cells.

Estrogens are known to bind to ERα and ERβ and regulate various physiological processes. Therefore, we evaluated the different estrogenic effects mediated through ERα and ERβ receptors on EGFR downstream factors using EGFR-TKIs and PPT (selective agonist to ERα) or DPN (to ERβ). We found that the effects of E2 on EGFR downstream

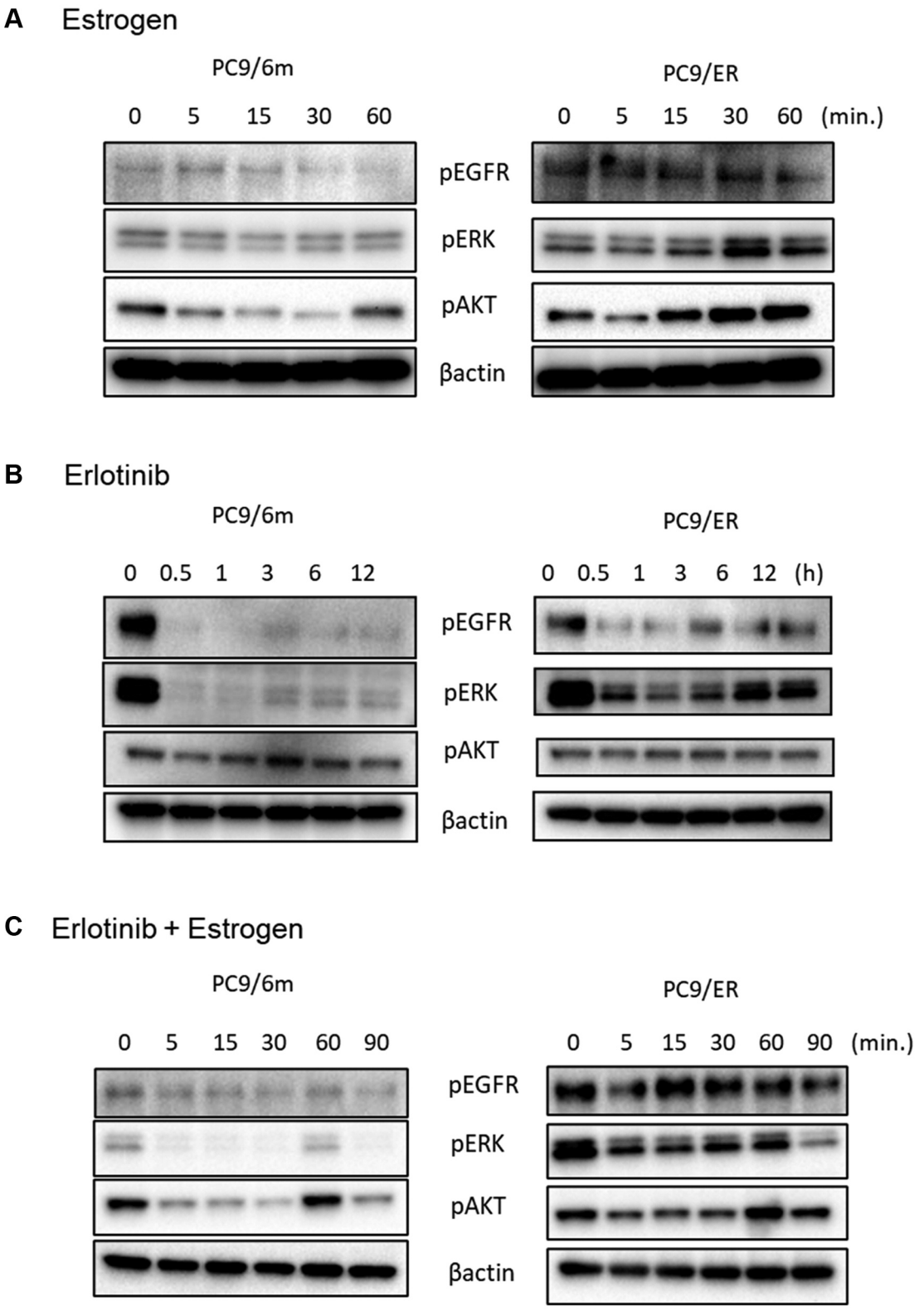

Figure 4. *Effects of estrogen on the expression of phospho-EGFR and its downstream signaling molecules. (A) PC9/6m (Left) and PC9/ER (Right) cells were treated with estrogen (10 nM estradiol). (B) PC9/6m (Left) and PC9/ER (Right) cells were treated with erlotinib (5 μM). (C) PC9/6m (Left) and PC9/ER (Right) cells were co-treated with erlotinib (5 μM) and estrogen (10 nM estradiol). pEGFR: phosphor-EGFR; pERK: phospho-ERK1/2; pAKT: phospho-AKT.*

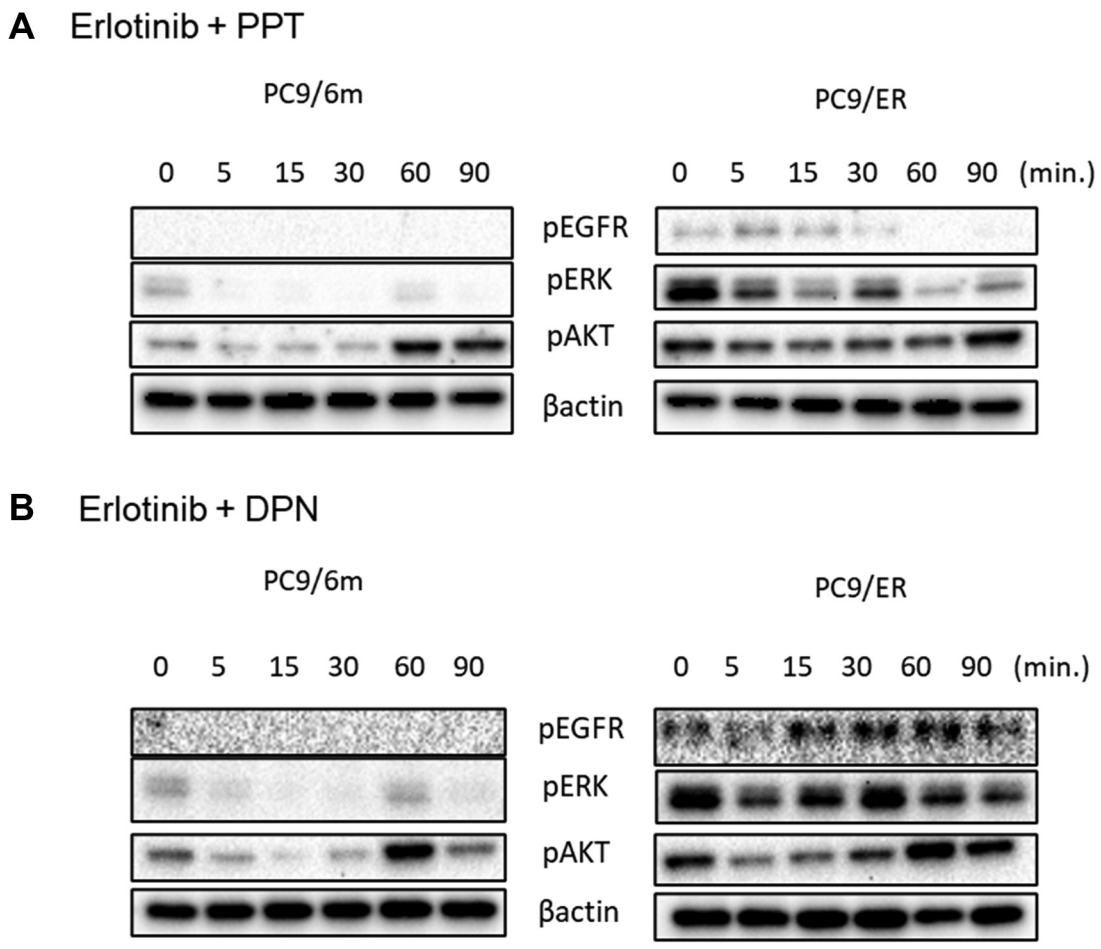

Figure 5. *Effects of estrogen receptor specific ligands on the expression of phospho-EGFR and its downstream signaling molecules. (A) PC9/6m (Left) and PC9/ER (Right) cells were co-treated with erlotinib (5 μM) and ERα specific ligand PPT (10 nM). (B) PC9/6m (Left) and PC9/ER (Right) cells were co-treated with erlotinib (5 μM) and ERβ specific ligand DPN (10 nM). pEGFR: phosphor-EGFR; pERK: phospho-ERK1/2; pAKT: phospho-AKT.*

factors were dependent upon the ERβ status of carcinoma cells. E2 has also been recently reported to activate EGFR downstream cascades in lung adenocarcinoma cells, A549 and PC9 cells, *via* ERβ, and contributed to '*de novo*' resistance to EGFR-TKIs (33, 34). However, these studies did not evaluate cells that acquired therapeutic resistance to EGFR-TKIs. Therefore, this is the first study to demonstrate a role of cytoplasmic ERβ in 'acquired' resistance to EGFR-TKIs in lung adenocarcinoma cells.

We then studied the ERβ status and its correlation with the clinical response of EGFR-TKIs in lung adenocarcinoma patients using immunohistochemistry. Accurate evaluation of membrane ERβ positivity was difficult because only archived materials were available. Therefore, we tentatively classified ERβ localization as nucleus and/or cytoplasmic in all patients examined and a significant inverse correlation was detected between clinical response rate to EGFR-TKIs treatment and cytoplasmic ERβ positive rate. Wang *et al.* have reported that co-expression of cytoplasmic and nuclear ERβ could be a potential molecular indicator of EGFR-TKI resistance. In our present study, this status of "co-expression" was by no means related to the therapeutic resistance in lung adenocarcinoma patients treated with EGFR-TKIs. Further investigations are required to clarify this difference.

In conclusion, cytoplasmic ERβ contributed to the 'acquired' resistance to EGFR-TKIs *via* regulation of EGFR downstream pathways in lung adenocarcinoma cells. These results also indicated that inhibition of ERβ could be a novel therapeutic strategy for overcoming EGFR-TKIs resistance in lung adenocarcinomas patients, and a possible molecular predictor of the response to EGFR-TKIs.

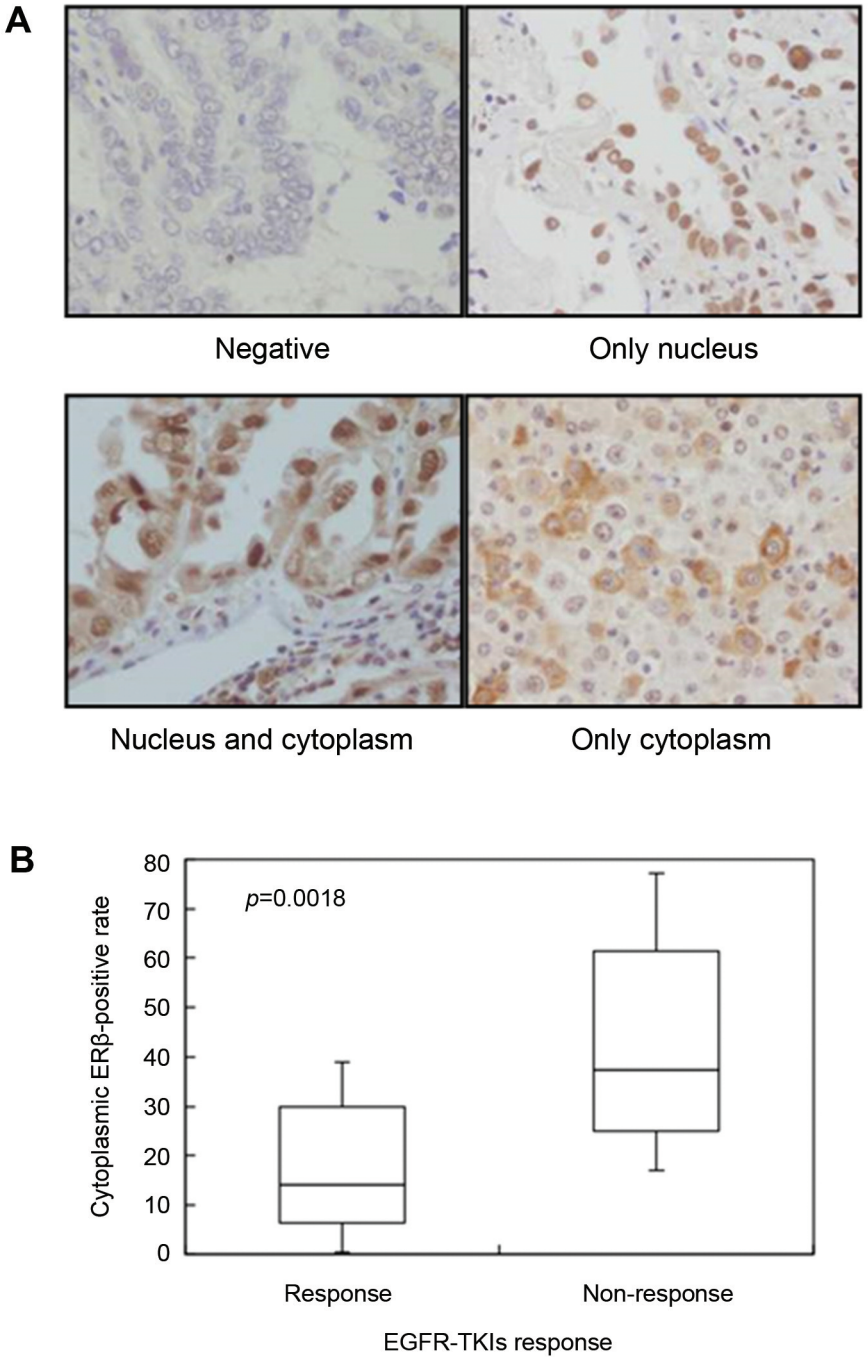

Figure 6. *Immunohistochemistry of ERβ in lung adenocarcinoma cases. (A) ERβ immunoreactivity was detected in the nucleus and/or cytoplasm of cancer cells. (B) The EGFR-TKI non-response group had a significantly higher cytoplasmic ERβ-positive rate than the response group.*

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Authors' Contributions

HSu and YM conceptualized and designed the study. HSu, YM, EI, and KO performed the experiments. HSu, YM and EI analyzed the

data. RS and IS reviewed the pathological analyses. YO provided clinical data and evaluated them. HSu wrote the manuscript. HSa supervised all experiments. All Authors have revised the manuscript, and read and approved the final manuscript.

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