Cytoplasmic expression of estrogen receptor β may predict poor outcome of EGFR-TKI therapy in metastatic lung adenocarcinoma

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Abstract. There is growing evidence that estrogen receptors (ER) are expressed in lung cancer cells, and are able to interact with the epidermal growth factor receptor (EGFR) signaling pathway. However, data on the association between cytoplasmic ER expression and the response to EGFR-tyrosine kinase inhibitors (TKI) treatment are limited. The aim of the present study was to investigate the associations between ERα/ERβ expression and EGFR mutational status and response to TKI treatment in metastatic lung adenocarcinoma. A retrospective study of 126 consecutive patients with lung adenocarcinoma who were diagnosed with stage IV disease and had received EGFR-TKI treatment was conducted. ER expression was detected by immunohistochemistry. EGFR and GTPase KRas (KRAS) mutational statuses were evaluated by denaturing high performance liquid chromatography and PCR-restriction fragment length polymorphism, respectively. In the overall cohort of 126 lung adenocarcinoma samples analyzed, ERα expression in the nucleus of tumor cells was identified in 17 (18.9%) patients, whereas ERβ expression was identified in the nucleus (22/126, 17.5%) and cytoplasm (17/126, 13.5%). The nuclear expression of ERβ was positively associated with the degree of tumor differentiation (P=0.010). EGFR-sensitizing mutations were significantly associated with improved objective response rates (ORR), disease control rates (DCR), median progression-free survival (mPFS) and median overall survival (mOS) (P<0.001; P<0.001; P=0.003; and P=0.026, respectively). Patients with cytoplasmic ERβ expression exhibited non-significant poorer ORR, DCR, mPFS and mOS compared with patients without cytoplasmic ERβ expression (P=0.082; P=0.106; P=0.084; and P=0.119, respectively). However, the significant decrease of ORR, DCR and mPFS was observed in patients with coexisting cytoplasmic ERβ expression and EGFR-sensitizing mutations (P=0.030; P=0.009; and P=0.018, respectively) in comparison with the subgroup with EGFR sensitizing mutations but negative expression of cytoplasmic ERβ. A trend towards shorter mOS was also observed in patients with coexisting cytoplasmic ERβ expression and EGFR-sensitizing mutations (P=0.071). No KRAS mutations were identified in patients with cytoplasmic ERβ expression. Subsequent to adjusting for sex, smoking status and EGFR mutation status, the Cox repression analysis indicated that cytoplasmic expression of ERβ was a negative independent predictor for mPFS in the whole patient cohort (HR=1.870; 95% confidence interval 1.058-3.305; P=0.031). Cytoplasmic ERβ expression was negatively correlated with the efficacy of EGFR-TKI treatment for metastatic lung adenocarcinoma, particularly for patients with coexisting cytoplasmic ERβ expression and EGFR-sensitizing mutations. Cytoplasmic ERβ may be a promising marker to predict the outcome of EGFR-TKI treatment.

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Introduction

Lung cancer is the leading cause of cancer-associated mortality globally (1). Despite recent improvements in its management, the prognosis of patients with lung cancer remains poor. Although smoking is the predominant risk factor for lung cancer, a gradual increase of incidence in the adenocarcinoma subtype has been identified despite a decrease in the size of the smoking population (2). Therefore, etiological factors other than smoking may also serve a role in the development of lung adenocarcinoma.

It has been demonstrated that hormone replacement therapy may increase the risk of mortality for patients with lung cancer, whereas anti-estrogen therapy may reduce the risk of mortality (3-5). Previous laboratory and clinical studies have provided evidence suggesting that estrogen stimulates the proliferation of lung carcinoma cells and tumor growth through estrogen receptor (ER)-mediated signaling (6-8). A

total of 2 forms of ERs have been identified, ERα and ERβ, which are the products of 2 separate genes (9). ERα and ERβ are expressed in non-small cell lung cancer (NSCLC) cell lines and tumor tissues, particularly adenocarcinoma (10).

Epidermal growth factor receptor (EGFR) gene mutational status is the most commonly-used biomarker for EGFR-tyrosine kinase inhibitor (TKI) therapy selection. Certain clinical characteristics, including Asian ethnicity, female sex, adenocarcinoma subtype and non-smoking status were predictive of good responses to therapy (11). Several studies have demonstrated that estrogen may trans-activate growth factor signaling pathways, including the EGFR pathway (6,12). ER protein expression is upregulated in response to gefitinib, an EGFR-TKI, and EGFR expression is upregulated in response to ER antagonists (13,14). The ER-EGFR signaling axis appears to be reciprocal, with ER signaling promoting the activation of EGFR, and EGFR signaling promoting the actvation of ER (15). Therefore, the ER signaling pathway may affect the efficacy of EGFR-TKI treatment. The aim of the present study was to examine the frequency of ER expression and to explore its association with clinicopathological factors, including EGFR mutation status, clinical responses to EGFR-TKI and patient prognosis.

Materials and methods

Patients and tissue specimens. Between January 2011 and July 2016, tissues from 126 consecutive patients with lung cancer with a pathological diagnosis of adenocarcinoma admitted to the Department of Medical Oncology at Peking University International Hospital (Beijing, China) and Beijing Cancer Hospital (Beijing, China) were retrieved by a fine needle aspiration biopsy and reviewed. All patients were diagnosed as stage IV according to the 7th edition of the TNM classification for lung tumors (16) and received EGFR-TKI therapy (250 mg gefitinib or 150 mg erlotinib orally once a day) until disease progression, intolerable toxicity or patient refusal. Inclusion criteria of the present study include a diagnosis of adenocarcinoma, stage IV disease and the patient was receiving EGFR-TKI treatment. Exclusion criteria included indeterminacy of EGFR mutational status and a low treatment compliance. Tumor responses were evaluated according to the Response Evaluation Criteria in Solid Tumors, version 1.1 (17). The present study was approved by the Institutional Ethic Committee of Beijing Cancer Hospital and Peking University International Hospital. Informed consent from the patients or their families was obtained prior to initiation of the study.

Immunohistochemistry. Tissue sections (4-μm thick sections) obtained from the paraffin-embedded specimens were prepared on glass slides. The paraffin-embedded tissue blocks were sectioned (thickness 4 μm) using a microtome and incubated in a 40˚C water bath. The sections were transferred onto glass slides for immunohistochemistry. The samples were incubated in an oven for 2 h at 60˚C. The sections were subsequently deparaffinized in xylene followed by a graded series of alcohol washes (100% ethyl alcohol for 5 min twice, 90, 80 and 70% for 5 min respectively) at room temperature.

The sections were placed in 0.1 mol/l citrate buffer (pH 6.0) and incubated in a pressure cooker for 3 min at 125˚C for antigen retrieval, then treated with 3% H_2O_2 for 5 min at room temperature. Samples were incubated with the following primary antibodies at room temperature for 30 min. A rabbit monoclonal antibody SP1 (cat. no. MA5-14501; Lab Vision Corporation, Fremont, CA, USA) against ERα at a dilution of 1:50 and mouse monoclonal antibody 14C8 (cat. no. ab288; Abcam, Cambridge, UK) against ERβ at a dilution of 1:100 were used. A two-step polymer-horseradish peroxidase method (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) was used for detection. Positive controls for ERα and ERβ were obtained from breast cancer cases from the Department of Breast Surgery of Peking University International Hospital in December 2016. Negative controls were performed as array sections without the primary antibody. Cytoplasm or nucleus staining intensity and pattern were evaluated using a scale from 0 to 3+: 0, completely negative; 1+, faint positivity; 2+, moderate positivity; 3+, strong positivity. Samples were scored as positive when >10% of tumor cells exhibited specific, positive staining in the nucleus or cytoplasm with at least 1+ staining. The immunohistochemistry analysis was performed independently by 2 pathologists (from the Department of Pathology, Peking University International Hospital and Beijing Cancer Hospital, Beijing, China), a light microscope at x200 magnification, in 10 randomly selected field of view was used.

EGFR and GTPase KRas (KRAS) mutation. Genomic DNA was extracted from paraffin-embedded biopsy tissues using the E.Z.N.A® formalin-fixed paraffin-embedded (FFPE) DNA kit (Omega Bio-Tek, Inc., Norcross, GA, USA). EGFR-sensitizing mutations (in exon 19del746-750 and 21L858R) were detected by denaturing high performance liquid chromatography and KRAS mutations (codon 12 and 13 in exon 1) by polymerase chain reaction-restriction fragment length polymorphism. The detailed methods and procedures have been described in our previous studies (18,19).

Statistical analysis. All statistical analyses were performed with SPSS software (version 16.0; SPSS, Inc., Chicago, IL, USA). All data are expressed as the mean ± standard deviation. The association between patient characteristics and ER expression and also clinical characteristics were analyzed using Pearson's χ^2 test or the Fisher's exact test. The time-to-event variables, for example overall survival (OS), progression-free survival (PFS), median OS (mOS) and median PFS (mPFS) were calculated using a Kaplan-Meier estimation. Comparisons between different groups were performed using log-rank tests. The Spearman's rank correlation was used to estimate the correlation between immunohistochemistry markers. Cox proportional hazards regression analysis was used to examine the effects of EGFR mutations, expression of ERβ and clinical variables on survival rates. $P<0.05$ (two-sided) was considered to indicate a statistically significant difference. The 95% confidence intervals (CIs) for odds ratios and frequencies were calculated as exact CIs.

Results

Clinical variables. The clinicopathological characteristics of patients are summarized in Table I. The 126 patients comprised

Table I. Patient cohort characteristics.

Characteristics	Frequency (%)	EGFR mutation (%)	ERα (%) (n=90)	Cyto-ERβ (%)	Nuclear-ERβ (%)
Sex					
Female	70 (55.6)	38 (54.3)	11 (22.0)	7 (10.0)	10 (14.3)
Male	56 (44.4)	26 (46.4)	6 (15.0)	10 (17.9)	12 (21.4)
Age, years					
≥60	76 (60.3)	33 (43.4)	14 (23.7)	11 (14.5)	13 (17.1)
<60	50 (39.7)	31 (62.0)[a]	3 (9.7)	6 (12.0)	9 (18.0)
Smoking status					
Ever or current	40 (31.7)	17 (42.5)	5 (15.6)	7 (17.5)	8 (20.0)
Never or light	86 (68.3)	47 (54.7)	12 (20.7)	10 (11.6)	14 (16.3)
Differentiation					
Undifferentiated + poor	30 (23.8)	15 (50.0)	4/22 (18.2)	4 (13.3)	1 (3.3)
Moderate	58 (46.0)	35 (60.3)	6/40 (15.0)	6 (10.3)	10 (17.2)
Well	34 (27.0)	12 (35.3)	6/28 (21.4)	7 (20.6)	11 (32.4)[a]
Unknown	4 (3.2)	2 (50.0)			
ORR					
CR + PR	46 (36.5)	34 (73.9)	6 (20.0)	3 (6.5)	9 (19.6)
SD + PD	80 (63.5)	30 (37.5)[b]	11 (18.3)	14 (17.5)	13 (16.2)
DCR					
CR + PR + SD	91 (72.2)	58 (63.7)	9 (14.8)	9 (9.9)	16 (17.6)
PD	35 (27.8)	6 (17.1)[b]	8 (27.6)	8 (22.9)	6 (17.1)

ORR, objective response rate; DCR, disease control rate; ER, estrogen receptor; EGFR, epidermal growth factor receptor; Cyto-ERβ, cytoplasmic ERβ. [a]P<0.05.

56 males and 70 females, with a median age of 62 years (range, 31-81 years). There were 76 patients aged >60 years, and the majority of patients were never/light smokers (defined as patients who had smoked less than 100 cigarettes in their lifetime) (86/126, 68.3%). All patients had tissue sample assessable for EGFR mutation and ERβ expression detection, whereas only 90 samples were assessable for ERα detection as cancer tissues were obtained by fine needle aspiration biopsy, therefore a large amount was not collected. All patients received EGFR-TKI therapy, with 46 patients receiving EGFR-TKI treatment as first-line therapy.

ERs expression in lung adenocarcinoma. ERα was only expressed in the nucleus, whereas ERβ staining was detected in the cytoplasm and nucleus. A total of 18.9% (17/90) lung tumors were positive for nuclear ERα expression, 17.5% (22/126) cases were positive for nuclear ERβ expression, 13.5% (17/126) exhibited positive staining for cytoplasmic ERβ expression and 3.2% (4/126) cases exhibited positive nuclear and cytoplasmic ERβ staining. There was no statistically significant difference in ERs protein expression associated with sex, age or smoking status. However, the nuclear expression of ERβ was positively correlated with the degree of tumor differentiation (χ^2=9.127; P=0.010). No significant associations between the expression levels of the different ERs were identified in the cohort of the present study. Fig. 1 demonstrates representative immunohistochemical staining of ERα in the nucleus and ERβ in the nucleus and cytoplasm in NSCLC cells.

Association between EGFR-sensitizing mutations and ERs expression. EGFR-sensitizing mutations were detected in 50.8% (64/126) patients, including 31 with exon19 alone, 29 with exon21 alone and 4 double mutations. The expression levels of any of the ERs were not significantly different between mutant and wild-type EGFR groups.

Biomarker-associated clinical outcomes. All patients who received EGFR-TKI treatment were evaluated for tumor response according to the Response Evaluation Criteria in Solid Tumors (version 1.1) (17). A total of 46 (36.5%) patients experienced partial response (PR), 45 (35.7%) exhibited stable disease (SD), and 35 (27.8%) exhibited progressive disease (PD). No complete response (CR) was observed in the cohort of the present study. In the overall cohort, the ORR was 36.5% (46/126) and the DCR was 72.2% (91/126). The mPFS was 7.5 months. The mOS was 22.5 months. As expected, patients with EGFR-sensitizing mutations exhibited a significantly increased ORR (53.1 vs. 19.4%; P<0.001), DCR (90.6 vs. 53.2%; P<0.001) and longer mPFS survival (10.7 vs. 2.1 months; P=0.003; Fig. 2A) compared with the wild-type patients. It was also identified that the presence of EGFR-sensitizing mutations was associated with an improved prognosis (35.4 vs. 25.9 months; χ^2=4.968; P=0.026; Fig. 2B). Several previous studies have demonstrated that patients with advanced NSCLC with EGFR exon 19 del746-750 exhibited a longer mPFS following treatment with gefitinib or erlotinib compared

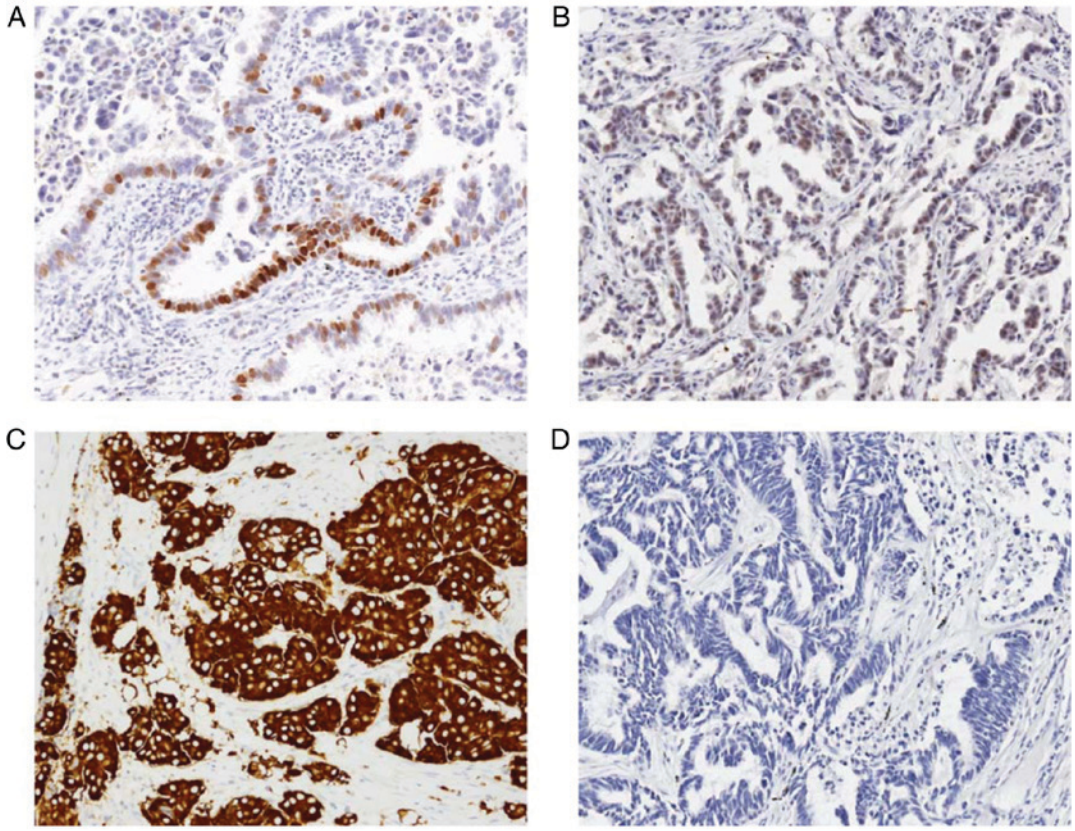

Figure 1. Representative immunohistochemical staining of ERα and ERβ in lung adenocarcinoma tissue. All images were captured at magnification, x200. (A) positive nuclear ERα staining, (B) positive nuclear ERβ staining, (C) positive cytoplasmic ERβ staining, and (D) negative control. ER estrogen receptor.

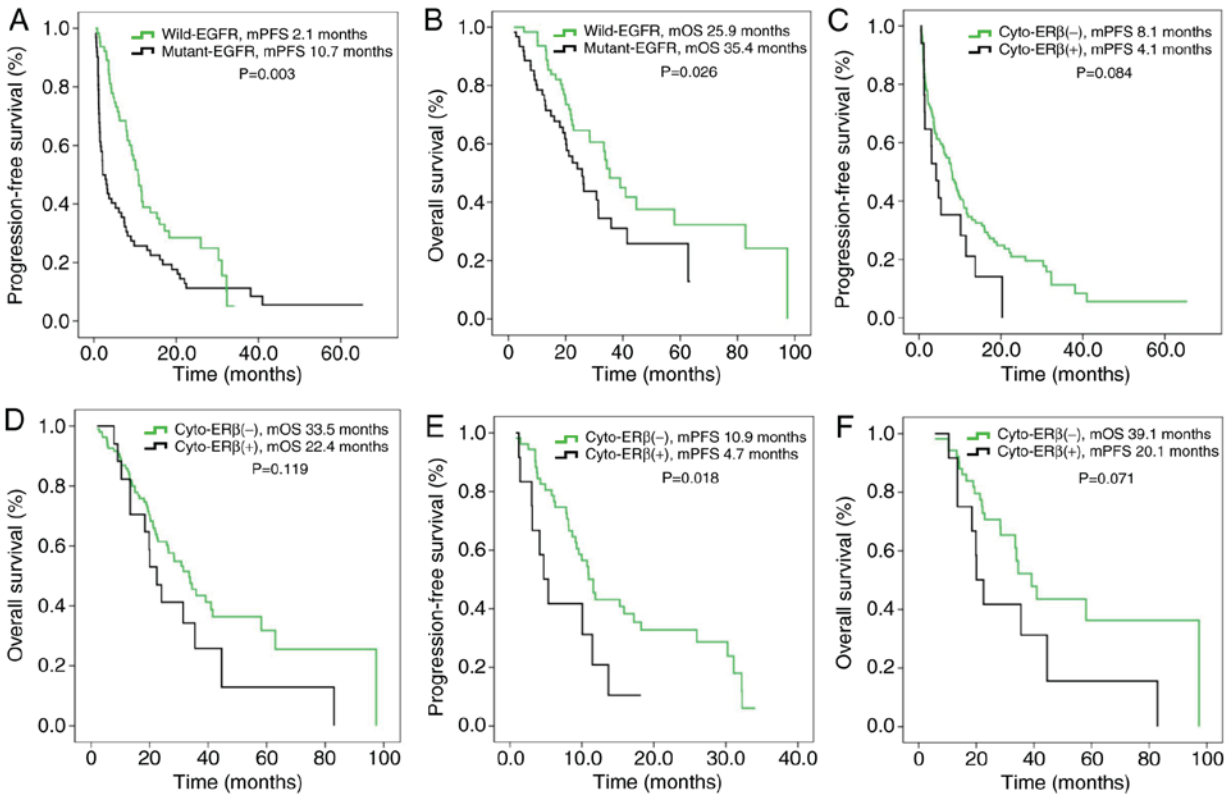

Figure 2. Kaplan-Meier curves stratified by EGFR mutation and cytoplasmic ERβ expression. (A) PFS according to EGFR mutation status. (B) OS according to EGFR mutation status. (C) PFS according to cytoplasmic ERβ expression in all patients. (D) OS according to cytoplasmic ERβ expression in all patients. (E) PFS of patients with EGFR-sensitizing mutations according to cytoplasmic ERβ expression. (F) OS of patients with EGFR-sensitizing mutations according to cytoplasmic ERβ expression. EGFR, epidermal growth factor receptor; ER, estrogen receptor; PFS, progression-free survival; mPFS, median PFS; OS, overall survival; mOS, median OS.

Table II. Univariate analysis of PFS in all patients.

Characteristics	mPFS, months	95% CI	χ^2	P-value
Sex				
Male	4.7	0.429-8.904	4.375	0.036
Female	9.7	6.061-13.339		
Age, years				
≥60	7.9	5.420-10.314	0.653	0.419
<60	5.8	1.251-10.282		
Smoking status				
Ever or current	5.3	0.876-9.657	1.214	0.271
Never or light	8.2	4.869-11.531		
EGFR mutation				
Mutant	10.7	8.400-13.000	8.735	0.003
Wild-type	2.1	0.728-3.472		
Nuclear-ERα				
Positive	6.2	0.000-13.180	0.690	0.406
Negative	7.8	4.420-11.114		
Cyto-ERβ				
Positive	4.1	1.859-6.341	2.988	0.084
Negative	8.1	5.707-10.493		
Nuclear-ERβ				
Positive	7.3	3.584-11.016	0.954	0.329
Negative	7.8	4.771-10.762		

ER, estrogen receptor; EGFR, epidermal growth factor receptor; 95% CI, 95% confidence interval; Cyto-ERβ, cytoplasmic-ERβ; mPFS, median progression-free survival.

with those with exon 21 L858R mutation (20,21). However, this significant difference was not observed in the cohort of the present study (11.6 vs. 10.1 months; P=0.325), which consistent with the results from Sequist *et al* (22). Table II summarizes the association between clinicopathological characteristics, ER expression and PFS in all patients.

In the present study, no significant association between tumor response or mPFS and the expression of nuclear ERα or nuclear ERβ was observed. A trend toward a decreased tumor response rate was observed in patients with cytoplasmic expression of ERβ when compared with cases without cytoplasmic expression (ORR, 17.6 vs. 39.4%, P=0.082; DCR, 52.9 vs. 75.2%, P=0.106). Similarly, patients with cytoplasmic ERβ expression exhibited a poorer PFS (4.1 vs. 8.1 months; χ^2=2.988; P=0.084; Fig. 2C) and exhibited a trend toward a poorer mOS (22.4 vs. 33.5 months; χ^2=2.428; P=0.119; Fig. 2D) following EGFR-TKI treatment compared with those without cytoplasmic ERβ expression, although this did not reach a statistical significance. In light of the predictive value of EGFR-sensitizing mutations in EGFR-TKI treatment, the associations between cytoplasmic ERβ expression and clinical response and mPFS following EGFR-TKI treatment categorized by EGFR mutations were analyzed. No significant difference was observed in clinical response and mPFS in patients with wild-type EGFR genotypes, regardless of whether cytoplasmic ERβ was expressed or not. However, in the subgroup with EGFR-sensitizing mutations, patients with cytoplasmic ERβ expression exhibited a significantly decreased ORR (25.0 vs. 59.6%; χ^2=4.691; P=0.030) and DCR (66.7 vs. 96.2%; χ^2=6.809; P=0.009), and exhibited a poorer mPFS (4.7 vs. 10.9 months; χ^2=5.602; P=0.018; Fig. 2E) compared to patients without cytoplasmic ERβ expression. It was also identified that patients with coexisting cytoplasmic ERβ expression and EGFR sensitizing mutations tended to exhibit a shorter median OS, but the difference was not significant (20.1 vs. 39.1 months; P=0.071; Fig. 2F). In the Cox regression analysis adjusted for sex, smoking status and EGFR mutation status, the results indicated that cytoplasmic expression of ERβ was an independent negative predictor for PFS in the whole group (Hazard ratio=1.870; 95% CI, 1.058-3.305; P=0.031; Table III). In order to exclude the effect on clinical response of EGFR-TKI treatment and survival by KRAS mutation, KRAS mutations were detected and no mutant KRAS was observed in any patients with cytoplasmic ERβ expression. No significant difference was observed in OS between patients according to nuclear ERα or nuclear ERβ expression.

Discussion

Although the lungs were not previously considered as a target organ for sex steroids, increasing evidence clearly indicates the importance of estrogen signaling in the initiation and progression of lung cancer (7). In addition, the cross-talk between ER and EGFR signaling pathways has been confirmed (15), but there have been few studies examining the association between ER expression and response to EGFR-TKI treatment in metastatic lung adenocarcinoma.

The frequency of ERα and β expression in NSCLC has been demonstrated to be inconsistent in previous studies (23-26). ERα expression cannot be detected in lung cancer using antibodies commonly used in breast tumors including clone number 6F11 or 1D5, which target full-length or the N terminus of ERα, respectively, due to the existence of ERα variants (26). Previous studies examining ERα immunohistochemical expression in FFPE NSCLC specimens using 6 different antibodies have identified in frequencies ranging from 0-38% for nuclear ERα expression and from 0-73% for cytoplasmic expression. In the present study, an ERα positive rate of 18.9% was identified using a monoclonal antibody against the COOH terminus of ERα, but all staining occurred in the nucleus alone. Unlike ERα expression in the present study, ERβ has been demonstrated to be expressed in the nucleus and cytoplasm of cancerous cells (27), and the expression level in lung cancer tissues is significantly increased compared with normal lung tissues (28). Consistent with Skov *et al* (27), ERβ expression was also observed in the nucleus and cytoplasm in lung tumors, and the positive rates were 17.5 and 13.5%, respectively. The distribution of ERβ was more widespread compared with ERα in the immunohistochemical analysis of the present study. The positive rate of cytoplasmic ERβ expression was similar (13.5 vs. 10.0%), but the nuclear expression of ERβ was decreased in the present study (17.5 vs. 69.0%), compared with that of Skov *et al* (27). The reason for these conflicting results on the expression frequency and localization of ERs between different studies in lung cancer may be

Table III. Results of the multivariate analysis Cox proportional hazards model for progression-free survival.

Characteristics	Wald	HR	95% confidence interval	P-value
Cyto-ERβ	4.640	1.870	1.058-3.305	0.031
Sex	4.334	0.575	0.342-0.968	0.037
EGFR mutation	11.925	0.487	0.324-0.733	0.001
Smoking status	0.137	0.902	0.524-1.554	0.711

Wald, Wald statistic for logistic regression algorithms; HR, hazard ratio; ER, estrogen receptor; EGFR, epidermal growth factor receptor; cyto-ERβ, cytoplasmic-ERβ.

due to lack of standardization of multiple aspects, including the antibodies used, interpretation of the staining and differences in the study population.

Differences in sex are an important characteristic of ER expression in lung cancer, which has been identified in several previous studies (29,30). ERα expression was exhibited in a significantly higher proportion of female patients compared with male patients (31), while the positive rate for ERβ expression was increased in males (26). A trend toward a higher percentage of men exhibiting positive cytoplasmic ERβ expression when compared with women [10 of 56 (17.9%) males vs. 7 of 70 (10.0%) females] was observed in the present study, which was similar to a study performed by Toh *et al* (30). In the present study, there were no significant associations between sex and the expression of ERα, or nuclear or cytoplasmic ERβ. ERβ expression has been demonstrated to be positively correlated with the degree of lung cancer differentiation (32), and a positive association between nuclear ERβ expression and tumor differentiation was also observed in the present study. This suggests that ERβ expression may, to a certain extent, reflect the degree of malignancy and therefore may be used as a predictor of prognosis.

A Japanese study investigating surgically-resected adenocarcinoma revealed a high expression frequency of ERα and ERβ among 447 patients, and the authors identified a significantly positive correlation between strong nuclear expression of ERβ and EGFR mutations (33). Similarly, Raso *et al* (34) also demonstrated that EGFR mutant adenocarcinoma exhibited a significantly increased expression of nuclear ERα and ERβ compared with wild-type tumors. However, inconsistent with these studies, Deng *et al* (35) suggested that the percentage of samples with detectable expression of ERβ was increased in patients with wild-type EGFR compared with a mutant EGFR group. The EGFR mutational status was available for all patients in the present study, but no significant association between EGFR mutations and expression of any ER was observed.

ERα and cytoplasmic ERβ expression has been demonstrated to exhibit a significant association with poor outcome in patients with NSCLC (28,36), whereas nuclear ERβ expression has been indicated to be a favorable prognostic factor (37), although only occasionally for male patients or patients with EGFR mutations (6,33). In the present study, a trend toward a poorer mOS was noted in patients with cytoplasmic expression of ERβ; however, the survival difference was non-significant, regardless of whole group or subgroups categorized by EGFR mutational status or sex. Similarly, no significant difference of OS was identified in terms of nuclear ERα or nuclear ERβ expression, regardless of sex or EGFR mutational status.

Previous studies have suggested a cross-talk between EGFR and the ER signaling pathway in the development of lung cancer (12,38-40). Estrogen depletion induced by endocrine therapy may activate EGFR signaling pathway, while ERβ expression was increased following EGFR-TKI treatment in NSCLC cells (13). Therefore, expression of ERs may affect the outcome of EGFR-TKI treatment in NSCLC. In the cohort of the present study, an inverse correlation between cytoplasmic estrogen receptor β expression and clinical response of EGFR-TKI treatment was observed; however, the difference was not significant. Based on the predictive value of EGFR-sensitizing mutations in EGFR-TKI treatment, the association between cytoplasmic ERβ expression and clinical response to EGFR-TKI therapy categorized by EGFR mutational status was analyzed and it was observed that patients with cytoplasmic ERβ expression exhibited significantly decreased ORR, DCR and a poorer PFS compared with cases without cytoplasmic ERβ expression in the mutant EGFR subgroup. Strong nuclear ERβ expression has been demonstrated to predict a good clinical response and prolonged PFS of EGFR-TKI treatment for patients with lung adenocarcinoma (41), but this conclusion was not reached in the present study. Due to the controversial results on survival indicated by these studies (41-43), the present study analyzed the combined effect of positive cytoplasmic ERβ staining/negative nuclear ERβ staining compared with negative cytoplasmic ERβ staining/positive nuclear ERβ staining on survival, and did not identify a significant difference between these two groups. The present study suggests that cytoplasmic signal transduction may serve a more important role compared with nuclear signal transduction of ERβ in lung cancer. To the best of our knowledge, the present study is the first to describe the association between cytoplasmic ERβ expression and clinical responses of EGFR-TKI treatment in metastatic lung adenocarcinoma. Unfavorable clinical response rates and PFS were identified in patients with EGFR mutations and patients with metastasis with coexisting cytoplasmic ERβ expression, but the reason remains unclear. The primary inclusion criteria of the present study were as follows: Adenocarcinoma diagnosis, stage IV and previous EGFR-TKI treatment. There have been a small number of previous studies including all these three criteria concomitantly (41-43). Therefore, there was discrepancy of cohort selection between our study and previous studies.

Nose *et al* (41) demonstrated that a strong expression of nuclear ERβ was able to predict an improved clinical response and longer PFS following treatment with EGFR-TKI for lung adenocarcinoma; however, these data referred to the nuclear expression, and did not consider the clinical implications of cytoplasmic ERβ, which was different to the protocols of the present study. The predictive discrepancy between these two studies additionally strengthened the concept of a distinct function between cytoplasmic and nuclear ERβ. Increasing evidence indicated that ERβ, but not ERα, served the predominant role in the initiation and development of lung cancer (6,44). ERβ protein was detected in the cytoplasm of NSCLC cells, suggesting that the cytoplasmic component of ERβ may be biologically significant (45). The cytoplasmic ERβ compartment has not usually been considered or measured with nuclear staining together in previous studies, and therefore the clinical significance of cytoplasmic ERβ staining may have been underestimated. Nuclear ERβ was considered to serve an important role in tumor suppression in breast cancer, but increasing evidence has indicated that cytoplasmic ERβ serves the opposite role in cancer cells (38,39). Similar with that in breast cancer, the clinical significance of ERβ was also suggested to be diverse due to ERβ localization in lung cancer cells through genomic, non-genomic and mitochondrial mechanisms (38-40,46). The estrogen-ERβ complex binds to the nuclear estrogen response elements of target genes to stimulate gene transcription. The non-genomic mechanisms of ERβ by which estrogen regulate cell functions are membrane-initiated, or 'pre-genomic' signaling pathways involving the activation of intracellular protein kinases, including phosphatidylinositol-3-kinase (PI3K), mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase, which are considered common downstream substrates of EGFR signaling and usually occur in the cytoplasm (47,48). Estrogen may stimulate rapid activation of MAPK, PI3K, Proto-oncogene tyrosine-protein kinase Src and protein kinase B kinases that are associated with subsequent stimulation of cell proliferation, angiogenesis and tumor metastasis in NSCLC through the cytoplasmic ER-mediated signaling pathway (38-40). ERβ present in cytoplasm was demonstrated to fail to translocate to the nucleus in the presence of estrogen in lung cancer cells, instead functioning through non-genomic mechanisms (49), which involved bidirectional crosstalk with growth factor receptor pathways, in particular the EGFR pathway (6,12). This ER-EGFR signaling axis appears to be reciprocal: ER signaling promotes the activation of EGFR, while EGFR signaling promotes the activation of ER (6,12). Therefore, the ER signaling pathway may affect the effect of EGFR-TKI therapy. These data also suggest that ERβ demonstrates oncogenic features. The present study additionally indicated that the non-genomic ERβ pathway in lung cancer also has important clinical significance. Based on the interaction between ER and the EGFR signaling pathway, anti-estrogen combined with anti-EGFR treatment may be a potential therapeutic option. Our previous results demonstrating an association between these 2 pathways additionally strengthened the possibility of combined therapy for a selected group of patients (42). Laboratory studies have indicated an robust synergistic effect on tumor growth when the two inhibitors were simultaneously used *in vitro* and *in vivo* in an NSCLC model (6,42). A phase I study designed to assess the safety and tolerability of gefitinib combined with fulvestrant in 22 postmenopausal female patients with lung cancer demonstrated that the combination of these drugs was well-tolerated and exhibited anti-tumor activity in patients with stage IIIB/IV NSCLC (50). A phase II trial of erlotinib or erlotinib + fulvestrant in previously-treated patients with advanced NSCLC suggested that the clinical benefit rate was significantly increased among patients treated with the combination regimen (51). Two phase II clinical trials (trail nos. NCT 00100854 and NCT 01556191; clinicaltrails.gov) are currently ongoing to explore their effects on advanced NSCLC, mostly in a second-line setting and combined with the EGFR-TKI. Therefore, anti-estrogen therapy may be a novel strategy to reverse the resistance to EGFR-TKI treatment for patients harboring cytoplasmic ERβ in metastatic lung adenocarcinoma.

However, due to the limitation of the small size sample and cohort selection in the present study, a larger cohort will be required to examine and validate the predictive value of these markers.

In summary, the results of the present study indicate that ERα and β were frequently expressed in metastatic lung adenocarcinoma, and that cytoplasmic ERβ expression was identified to be a negative predictor for clinical response of EGFR-TKI treatment in patients with EGFR mutations. The ER and EGFR pathways together may contribute to the progression of lung cancer, and ER antagonists may become an alternative treatment for patients with acquired resistance to EGFR inhibitors.

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Availability of data and materials

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

Authors' contributions

JL designed the project and was responsible for conducting the study. XD and LL performed the histological examination of the tumors samples and were major contributors in writing the manuscript. CT contributed to data analysis and interpretation. CM, WX, XWe, ZG, TZ, YF, LZ, XWa and LL were responsible for the acquisition of data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethical Review Board of Peking University International Hospital (Beijing, China). Informed consent from the patients or their families was

obtained prior to initiation of the study. Ethical approval and informed consent was also obtained for the use of the breast cancer samples as controls.

Consent for publication

Identifying data, including names, initials, date of birth or hospital numbers, images or statements were not included in the manuscript. Informed consent for publication was obtained prior to initiation of the study.

Competing interests

The authors declare that they have no competing interests.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin 61: 69-90, 2011.
2. Lam B, Lam WK, Lam CL, Ooi GC, Ho JC, Wong MP and Tsang KW: Adenocarcinoma of the lung in Chinese patients: A revisit and some perspectives from the literature. Postgrad Med J 77: 708-712, 2001.
3. Liu Y, Inoue M, Sobue T and Tsugane S: Reproductive factors, hormone use and the risk of lung cancer among middle-aged never-smoking Japanese women: A large-scale population-based cohort study. Int J Cancer 117: 662-666, 2005.
4. Chlebowski RT, Schwartz AG, Wakelee H, Anderson GL, Stefanick ML, Manson JE, Rodabough RJ, Chien JW, Wactawski-Wende J, Gass M, *et al*: Oestrogen plus progestin and lung cancer in postmenopausal women (Women's Health Initiative trial): A post-hoc analysis of a randomized controlled trial. Lancet 374: 1243-1251, 2009.
5. Bouchardy C, Benhamou S, Schaffar R, Verkooijen HM, Fioretta G, Schubert H, Vinh-Hung V, Soria JC, Vlastos G and Rapiti E: Lung cancer mortality risk among breast cancer patients treated with anti-estrogens. Cancer 117: 1288-1295, 2011.
6. Márquez-Garbán DC, Chen HW, Fishbein MC, Goodglick L and Pietras RJ: Estrogen receptor signaling pathways in human non-small cell lung cancer. Steroids 72:135-143, 2007.
7. Pietras RJ, Márquez DC, Chen HW, Tsai E, Weinberg O and Fishbein M: Estrogen and growth factor receptor interactions in human breast and non-small cell lung cancer cells. Steroids 70: 372-381, 2005.
8. Hershberger PA, Vasquez AC, Kanterewicz B, Land S, Siegfried JM and Nichols M: Regulation of endogenous gene expression in human non-small cell lung cancer cells by estrogen receptor ligands. Cancer Res 65: 1598-1605, 2005.
9. Enmark E, Pelto-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjöld M and Gustafsson JA: Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. J Clin Endocrinol Metab 82: 4258-4265, 1997.
10. Stabile LP, Davis AL, Gubish CT, Hopkins TM, Luketich JD, Christie N, Finkelstein S and Siegfried JM: Human non-small cell lung tumors and cells derived from normal lung express both estrogen receptor alpha and beta and show biological responses to estrogen. Cancer Res 62: 2141-2150, 2002.
11. Mok TS, Wu YL, Thongprasert S, Yang CH, Chu DT, Saijo N, Sunpaweravong P, Han BH, Margono B, Ichinose Y, *et al*: Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Engl J Med 361: 947-957, 2009.
12. Stabile LP and Siegfried JM: Estrogen receptor pathways in lung cancer. Curr Oncol Rep 6: 259-267, 2004.
13. Stabile LP, Lyker JS, Gubish CT, Zhang W, Grandis JR and Siegfried JM: Combined targeting of the estrogen receptor and the epidermal growth factor receptor in non-small cell lung cancer shows enhanced antiproliferative effects. Cancer Res 65: 1459-1470, 2005.
14. Siegfried JM, Gubish CT, Rothstein ME, Henry C and Stabile LP: Combining the multitargeted tyrosine kinase inhibitor vandetanib with the antiestrogen fulvestrant enhances its antitumor effect in non-small cell lung cancer. J Thorac Oncol 7: 485-495, 2012.
15. Pietras RJ and Márquez-Garbán DC: Membrane-associated estrogen receptor signaling pathways in human cancers. Clin Cancer Res 13: 4672-4676, 2007.
16. Detterbeck FC, Boffa DJ and Tanoue LT: The new lung cancer staging system. Chest 136: 260-271, 2009.
17. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, Arbuck S, Gwyther S, Mooney M, *et al*: New response evaluation criteria in solid tumours: Revised RECIST guideline (version 1.1). Eur J Cancer 45: 228-247, 2009.
18. Bai H, Mao L, Wang HS, Zhao J, Yang L, An TT, Wang X, Duan CJ, Wu NM, Guo ZQ, *et al*: Epidermal growth factor receptor mutations in plasma DNA samples predict tumor response in Chinese patients with stages IIIB to IV non-small-cell lung cancer. J Clin Oncol 27: 2653-2659, 2009.
19. Wang S, An T, Wang J, Zhao J, Wang Z, Zhuo M, Bai H, Yang L, Zhang Y, Wang X, *et al*: Potential clinical significance of a plasma-based KRAS mutation analysis in patients with advanced non-small cell lung cancer. Clin Cancer Res 16: 1324-1330, 2010.
20. Jackman DM, Yeap BY, Sequist LV, Lindeman N, Holmes AJ, Joshi VA, Bell DW, Huberman MS, Halmos B, Rabin MS, *et al*: Exon 19 deletion mutations of epidermal growth factor receptor are associated with prolonged survival in non-small cell lung cancer patients treated with gefitinib or erlotinib. Clin Cancer Res 12: 3908-3914, 2006.
21. Zhang Y, Sheng J, Kang S, Fang W, Yan Y, Hu Z, Hong S, Wu X, Qin T, Liang W and Zhang L: Patients with exon 19 deletion were associated with longer progression-free survival compared to those with L858R mutation after first-line EGFR-TKIs for advanced non-small cell lung cancer: A meta-analysis. PLoS One 9: e107161, 2014.
22. Sequist LV, Martins RG, Spigel D, Grunberg SM, Spira A, Jänne PA, Joshi VA, McCollum D, Evans TL, Muzikansky A, *et al*: First-line gefitinib in patients with advanced non-small-cell lung cancer harboring somatic EGFR mutations. J Clin Oncol 26: 2442-2449, 2008.
23. Jala VR, Radde BN, Haribabu B and Klinge CM: Enhanced expression of G-protein coupled estrogen receptor (GPER/GPR30) in lung cancer. BMC Cancer 12: 624, 2012.
24. Tanaka K, Shimizu K, Kakegawa S, Ohtaki Y, Nagashima T, Kaira K, Horiguchi J, Oyama T and Takeyoshi L: Prognostic significance of aromatase and estrogen receptor beta expression in EGFR wild-type lung adenocarcinoma. Am J Transl Res 8: 81-97, 2016.
25. Gomez-Fernandez C, Mejias A, Walker G and Nadji M: Immunohistochemical expression of estrogen receptor in adenocarcinomas of the lungg: The antibody factor. Appl Immunohistochem Mol Morphol 18: 137-141, 2010.
26. Schwartz AG, Prysak GM, Murphy V, Lonardo F, Pass H, Schwartz J and Brooks S: Nuclear estrogen receptor beta in lung cancer: Expression and survival differences by sex. Clin Cancer Res 11: 7280-7287, 2005.
27. Skov BG, Fischer BM and Pappot H: Oestrogen receptor beta over expression in males with non-small cell lung cancer is associated with better survival. Lung Cancer 59: 88-94, 2008.
28. Stabile LP, Dacic S, Land SR, Lenzner DE, Dhir R, Acquafondata M, Landreneau RJ, Grandis JR and Siegfried JM: Combined analysis of estrogen receptor beta-1 and progesterone receptor expression identifies lung cancer patients with poor outcome. Clin Cancer Res 17: 154-164, 2011.
29. Fasco MJ, Hurteau GJ and Spivack SD: Gender-dependent expression of alpha and beta estrogen receptors in human nontumor and tumor lung tissue. Mol Cell Endocrinol 188: 125-140, 2002.
30. Toh CK, Ahmad B, Soong R, Chuah KL, Tan SH, Hee SW, Leong SS, Tan EH and Lim WT: Correlation between epidermal growth factor receptor mutations and expression of female hormone receptors in east-Asian lung adenocarcinomas. J Thorac Oncol 5: 17-22, 2010.
31. Sun HB, Zheng Y, Ou W, Fang Q, Li P, Ye X, Zhang BB, Yang H and Wang SY: Association between hormone receptor expression and epidermal growth factor receptor mutation in patients operated on for non-small cell lung cancer. Ann Thorac Surg 91: 1562-1567, 2011.
32. Chen XQ, Zheng LX, Li ZY and Lin TY: Clinicopathological significance of oestrogen receptor expression in non-small cell lung cancer. J Int Med Res 45: 51-58, 2017.
33. Nose N, Sugio K, Oyama T, Nozoe T, Uramoto H, Iwata T, Onitsuka T and Yasumoto K: Association between estrogen receptor-beta expression and epidermal growth factor receptor mutation in the postoperative prognosis of adenocarcinoma of the lung. J Clin Oncol 27: 411-417, 2009.

34. Raso MG, Behrens C, Herynk MH, Liu S, Prudkin L, Ozburn NC, Woods DM, Tang X, Mehran RJ, Moran C, *et al*: Immunohistochemical expression of estrogen and progesterone receptors identifies a subset of NSCLCs and correlates with EGFR mutation. Clin Cancer Res 15: 5359-5368, 2009.
35. Deng F, Li M, Shan WL, Qian LT, Meng SP, Zhang XL and Wang BL: Correlation between epidermal growth factor receptor mutations and the expression of estrogen receptor-β in advanced non-small cell lung cancer. Oncol Lett 13: 2359-2365, 2017.
36. Olivo-Marston SE, Mechanic LE, Mollerup S, Bowman ED, Remaley AT, Forman MR, Skaug V, Zheng YL, Haugen A and Harris CC: Serum estrogen and tumor-positive estrogen receptor-alpha are strong prognostic classifiers of non-small-cell lung cancer survival in both men and women. Carcinogenesis 31: 1778-1786, 2010.
37. Wu CT, Chang YL, Shih JY and Lee YC: The significance of estrogen receptor beta in 301 surgically treated non-small cell lung cancers. J Thorac Cardiovasc Surg 130: 979-986, 2005.
38. Hammes SR and Levin ER: Extranuclear steroid receptors: Nature and actions. Endocr Rev 28: 726-741, 2007.
39. Fox EM, Andrade J and Shupnik MA: Novel actions of eatrogen to promote proliferation: Intergration of cytoplasmic and nuclear pathways. Steroids 74: 622-627, 2009.
40. Fan S, Liao Y, Liu C, Huang Q, Liang H, Ai B, Fu S and Zhou S: Estrogen promotes tumor metastasis via estrogen receptor beta-mediated regulation of matrix-metalloproteinase-2 in non-small cell lung cancer. Oncotarget 8: 56443-56459, 2017.
41. Nose N, Uramoto H, Iwata T, Hanagiri T and Yasumoto K: Expression of estrogen receptor beta predicts a clinical response and longer progression-free survival after treatment with EGFR-TKI for adenocarcinoma of the lung. Lung Cancer 71: 350-355, 2011.
42. Wang Z, Li Z, Ding X, Shen Z, Liu Z, An T, Duan J, Zhong J, Wu M, Zhao J, *et al*: ERβ localization influenced outcomes of EGFR-TKI treatment in NSCLC patients with EGFR mutations. Sci Rep 5: 11392, 2015.
43. Fu S, Liu C, Huang Q, Fan S, Tang H, Fu X, Ai B, Liao Y and Chu Q: Estrogen receptor β1 activation accelerates resistance to epidermal growth factor receptor-tyrosine kinase inhibitors in non-small cell lung cancer. Oncol Rep 39: 1313-1321, 2018.
44. Zhang G, Yanamala N, Lathrop KL, Zhang L, Klein-Seetharaman J and Srinivas H: Ligand-independent antiapoptotic function of estrogen receptor-beta in lung cancer cells. Mol Endocrinol 24: 1737-1747, 2010.
45. Hershberger PA, Stabile LP, Kanterewicz B, Rothstein ME, Gubish CT, Land S, Shuai Y, Siegfried JM and Nichols M: Estrogen receptor beta (ERbeta) subtype-specific ligands increase transcription, p44/p42 mitogen activated protein kinase (MAPK) activation and growth in human non-small cell lung cancer cells. J Steroid Biochem Mol Biol 116: 102-109, 2009.
46. Swedenborg E, Power KA, Cai W, Pongratz I and Ruegg J: Regulation of estrogen receptor beta activity and implications in health and disease. Cell Mol Life Sci 66: 3873-3894, 2009.
47. Levin ER: Bidirectional signaling between the estrogen receptor and the epidermal growth factor receptor. Mol Endocrinol 17: 309-317, 2003.
48. Kato S, Endoh H, Masuhiro T, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, *et al*: Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science 270: 1491-1494, 1995.
49. Zhang G, Liu X, Farkas AM, Parwani AV, Lathrop KL, Lenzner D, Land SR and Srinivas H: Estrogen receptor beta functions through nongenomic mechanisms in lung cancer cells. Mol Endocrinol 23: 146-156, 2009.
50. Traynor AM, Schiller JH, Stabile LP, Kolesar JM, Eickhoff JC, Dacic S, Hoang T, Dubey S, Marcotte SM and Siegfried JM: Pilot study of gefitinib and fulvestrant in the treatment of post-menopausal women with advanced non-small cell lung cancer. Lung Cancer 64: 51-59, 2009.
51. Garon EB, Siegfried JM, Dubinett SM, Elashoff RM, Park DJ, Parikh RJ, Patel R, Hu EH, Reckamp KL, Adams B, *et al*: Abstract 4664: Result of TORI L-03, a randomized, multi-center phase II clinical trial of erlotinib (E) or E + fulvestrant (F) in previously treated advanced non-small cell lung cancer (NSCLC). In: AACR 104th Annual Meeting Washington DC. Philadelphia (PA): AACR 73 Apr 6-10, 2013.