**Human Cancer Biology**

**Immunohistochemical Expression of Estrogen and Progesterone Receptors Identifies a Subset of NSCLCs and Correlates with EGFR Mutation**


**Abstract**

**Purpose:** To determine the frequency of estrogen receptor α and β and progesterone receptor protein immunohistochemical expression in a large set of non–small cell lung carcinoma (NSCLC) specimens and to compare our results with those for some of the same antibodies that have provided inconsistent results in previously published reports.

**Experimental Design:** Using multiple antibodies, we investigated the immunohistochemical expression of estrogen receptors α and β and progesterone receptor in 317 NSCLCs placed in tissue microarrays and correlated their expression with patients’ clinicopathologic characteristics and in adenocarcinomas with EGFR mutation status.

**Results:** Estrogen receptors α and β were detected in the nucleus and cytoplasm of NSCLC cells; however, the frequency of expression (nucleus, 5-36% for α and 42-56% for β; cytoplasm: <1-42% for α and 20-98% for β) varied among the different antibodies tested. Progesterone receptor was expressed in the nuclei of malignant cells in 63% of the tumors. Estrogen receptor α nuclear expression significantly correlated with adenocarcinoma histology, female gender, and history of never smoking ($P = 0.0048$ to $<0.0001$). In NSCLC, higher cytoplasmic estrogen receptor α expression significantly correlated with worse recurrence-free survival (hazard ratio, 1.77; 95% confidence interval, 1.12, 2.82; $P = 0.015$) in multivariate analysis. In adenocarcinomas, estrogen receptor α expression correlated with EGFR mutation ($P = 0.0029$ to $<0.0001$). Estrogen receptor β and progesterone receptor but not estrogen receptor α expressed in the normal epithelium adjacent to lung adenocarcinomas.

**Conclusions:** Estrogen receptor α and β expression distinguishes a subset of NSCLC that has defined clinicopathologic and genetic features. In lung adenocarcinoma, estrogen receptor α expression correlates with EGFR mutations. (Clin Cancer Res 2009;15(17):5359-68)

**Lung cancer** is the most common cause of cancer mortality worldwide, with >1 million deaths each year (1). Lung cancer includes several histologic types, the most frequently occurring of which are two types of non–small cell lung carcinoma (NSCLC): adenocarcinoma and squamous cell carcinoma (2). During the last two decades, mortality rates associated with cancer have continued to decrease across all major sites in men and women; however, the rates for lung cancer in females have continued to increase (3, 4). Despite global statistics estimating that 15% of lung cancer in men and 53% in women are not attributable to smoking (1), smoking remains the primary risk factor for lung cancer. The higher proportion of lung cancer in females who have never smoked compared with males who have never smoked suggests a possible role for gender-dependent hormones in the development of lung cancer (5).

Estrogen receptors α and β are expressed in normal lung tissue and in lung tumors in men and women (6), yet the data are inconsistent about whether estrogen receptor expression is
A better understanding of the signaling pathways that lead to tumor growth may help in the development of new and more effective strategies for targeted chemoprevention and treatment of lung cancer. Our finding of frequent overexpression of estrogen receptor and progesterone receptor in non–small cell lung carcinoma suggests that the activation of these pathways is an attractive novel target for lung cancer chemopreventive and therapeutic strategies. The correlation between estrogen receptor and EGFR mutation in lung adenocarcinoma suggests that it might be important to target both pathways simultaneously for lung cancer therapy.

gender biased (6–9) or associated with NSCLC overall survival (9–11). The data reported on the immunohistochemical expression for both estrogen receptors in NSCLC remain controversial. Estrogen receptor α has been reported to be expressed in the nucleus (0-45%) and cytoplasm (0-73%) of malignant lung cancer cells in the cases examined (9, 10, 12, 13). The percentages for estrogen receptor β are more consistent, with 46% to 60% of NSCLC cases showing only nuclear expression (9–14). Similarly, two reports suggested that progesterone receptor is frequently (47%) expressed in NSCLC tumor cells, and this expression correlated with better patient outcome (12, 15).

Several in vitro and in vivo studies have provided evidence supporting a biological role for estrogens in lung carcinogenesis by direct promotion of cell-proliferation estrogens stimulate the proliferation of NSCLC cells through estrogen receptor–mediated signaling, whereas antiestrogens inhibit the growth of NSCLC cells (6, 7, 13, 16, 17). Estrogen can directly stimulate the transcription of estrogen-responsive genes in the nucleus of lung cells and can also transactivate growth factor–signaling pathways, the epidermal growth factor receptor (EGFR) pathway in particular (13, 18). In estrogen stimulation of lung cancer cells, EGFR ligands are rapidly released, activating the EGFR and mitogen-activated protein kinase 1 growth pathways (19). Activation of the EGFR pathway seems to play an important role in the pathogenesis and progression of NSCLC (20). In lung cancer cells, the constitutive activation of EGFR is achieved by several mechanisms, including increased production of ligands, increased levels of the receptor, and mutation of the EGFR tyrosine kinase domain (20–22). Of interest, EGFR protein expression is down-regulated in response to estrogens and up-regulated in response to antiestrogens, suggesting that a reciprocal control mechanism exists between the EGFR and estrogen receptor pathways (19).

The purpose of the current study was to determine the frequency of estrogen receptor α and β and progesterone receptor protein immunohistochemical expression in a large set of NSCLCs placed in tissue microarray specimens and to compare our results with those for some of the same antibodies that have provided inconsistent results in previously published reports (9–14). In addition, the receptor-expression results were correlated with patients’ clinicopathologic features, including NSCLC histology, gender, smoking history, and patient outcome, and in adenocarcinoma with tumors’ EGFR activating mutation status. Finally, to understand estrogen receptor α and β and progesterone receptor protein expression in the early pathogenesis of lung cancer, we investigated the characteristics of estrogen receptor α and β and progesterone receptor protein expression in the nonmalignant respiratory epithelium adjacent to tumors taken from a subset of our retrospectively reviewed lung adenocarcinoma cases.

**Materials and Methods**

**Case selection and tissue microarray construction.** We obtained archived, formalin-fixed, paraffin-embedded tissue from surgically resected (with curative intent) lung cancer specimens (lobectomies and pneumonectomies) containing tumor and adjacent normal epithelium tissues from the Lung Cancer Specialized Program of Research Excellence Tissue Bank at The University of Texas M.D. Anderson Cancer Center, which has been approved by the Institutional Review Board. The tissue had been collected from 1997 to 2001, and the tissue specimens were histologically examined and classified using the 2004 WHO classification system (2). We selected 317 NSCLC tissue samples (201 adenocarcinomas and 116 squamous cell carcinomas) for our tissue microarrays. Tissue microarrays were constructed using triplicate 1-mm diameter cores per tumor, and each core included central, intermediate, and peripheral tumor tissue. Detailed clinical and pathologic information, including demographics, smoking history (never and ever smokers), and smoking status (never, former, and current), clinical and pathologic tumor-node-metastasis stage, overall survival duration, and time to recurrence, were available for most cases (Supplementary Table S1). Patients who had smoked at least 100 cigarettes in their lifetime were defined as smokers, and smokers who quit smoking at least 12 months before their lung cancer diagnosis were defined as former smokers. Tumors were pathologic tumor-node-metastasis stages I to IV according to the revised International System for Staging Lung Cancer (23).

To assess the immunohistochemical expression of estrogen receptor α and β and progesterone receptor markers in the nonmalignant respiratory epithelium adjacent to lung tumors, we selected whole histology sections containing tumor and adjacent lung tissue from 64 adenocarcinomas that were included in our tissue microarrays.

**Immunohistochemical staining and evaluation.** The following antibodies against estrogen receptors α and β and progesterone receptor were purchased: (a) estrogen receptor α-1, clone 6F11, Novocastra, Leica Microsystems, Inc.; (b) estrogen receptor α-2, clone 6F11, Chemicon, Millipore Corporate; (c) estrogen receptor α-3, clone HC20, Santa Cruz Biotechnology, Inc.; (d) estrogen receptor α-4, clone 1D5, Lab Vision Corporation; (e) estrogen receptor β-1, clone H150, Santa Cruz Biotechnology; (f) estrogen receptor β-2, clone 14C8, GeneTex, Inc.; and (g) progesterone receptor, clone SP2, Lab Vision Corporation. Details on immunohistochemistry conditions and characteristics of the antibodies are listed in Supplementary Table S2. Immunohistochemical staining was done as follows: 5-μmol/L formalin-fixed, paraffin-embedded tissue sections were deparaffinized, hydrated, heated in a steamer for 10 min with 10 mmol/L sodium citrate (pH 6.0) for antigen retrieval, and washed in Tris buffer. Peroxide blocking was done with 3% H2O2 in methanol at room temperature for 15 min, followed by 10% fetal bovine serum in TBS-Tween for 30 min. The slides were incubated with primary antibody at an ambient temperature for 60 min for all antibodies; the exception was estrogen receptor β 14C8 (estrogen receptor β-2), which was incubated overnight at 4°C, washed with PBS, and incubated with biotin-labeled secondary antibody (Envision Dual Link+, DAKO) for 30 min. Staining for the slides was developed with 0.05% 3,3-diaminobenzidine tetrahydrochloride, which had been freshly prepared in 0.05 mol/L Tris buffer (pH 7.6) containing 0.02% H2O2, and then, the slides were counterstained with hematoxylin, dehydrated, and mounted. Formalin-fixed, paraffin-embedded normal breast tissue was used as the positive control. For the negative control, we used the same specimens used for the positive controls but
replaced the primary antibody with PBS. For each antibody, we did titration experiments using a relatively wide range of antibody concentrations (1:50, 1:100, 1:200, and 1:500), including the concentration suggested by the manufacturer. The selection of the antibody dilution was based on the consistency in the expression in the breast cancer and normal tissues with known estrogen receptor and progesterone receptor expression used as control.

In addition, to study the correlation between the immunohistochemical expression of estrogen receptors and their ability to detected different isoforms of the proteins by Western blot, we examine by immunohistochemistry, formalin-fixed, paraffin-embedded cell lines and by Western blot protein obtained from seven NSCLC cell lines (H157, H3255, HCC778, H1359, H1666, and H1174). For both techniques, we used the same antibodies that we used to examine the tissue specimens, except estrogen receptor α-2, which recently was replaced by the new manufacturer by a new clone (clone E115, Millipore; Supplementary Table S2). For Western blot analysis, cells were lysed with radioimmunoprecipitation assay buffer (50 mmol/L Tris-HCl, pH 7.4; 150 mmol/L NaCl; 1 mmol/L EDTA; 1% Triton X-100; 0.1% SDS; 1 mmol/L phenylmethylsulfonylfluoride; 1 mmol/L Na3VO4; and protease inhibitor tablet; Roche Diagnostics), and 75 μg of protein was separated by SDS-PAGE. A new blot was run for each antibody to eliminate any potential cross-reactivity due to incomplete stripping of previous antibodies. Antibodies were diluted in 4% milk in Tris-buffered saline tween-20 (TBST) and incubated overnight at 4°C. Two observers (M.G. Raso and I.I. Wistuba) jointly quantified the immunohistochemical expression of estrogen receptors and progesterone receptor using light microscopy (original magnification, ×20). Both nuclear and cytoplasmic expressions were quantified using a four-value scale (0, no appreciable staining; 1+, barely detectable staining in epithelial cells compared with the stromal cells; 2+, readily appreciable staining; 3+, dark brown staining of cells). Next, an expression score was obtained by multiplying the intensity and reactivity extension values (range, 0-300).

**EGFR mutation analysis.** Exons 18 to 21 of EGFR were PCR-amplified using intron-based primers, as previously described (24, 25). Approximately 200 microdissected, formalin-fixed, paraffin-embedded cells were used for each PCR amplification. All PCR products were directly sequenced using the Applied Biosystems PRISM dye terminator cycle sequencing method. All sequence variants were confirmed by independent PCR amplifications from at least two independent microdissections and DNA extraction, and the variants were sequenced in both directions, as previously reported (24, 25).

**Statistical analysis.** The immunohistochemical expression and clinicopathologic data were summarized using standard descriptive statistics and frequency tabulations. BLIP plots were generated to summarize the distribution of estrogen receptor and progesterone receptor expressions. Associations between the marker expression and patients’ clinical and demographical variables (including age, sex, smoking history, histology type, and pathologic stage) were assessed using appropriate methods, including the χ2 or Fisher’s exact test for categorical variables, and Wilcoxon rank sum or Kruskal-Wallis test for continuous variables. The Spearman rank ρ was used to estimate the correlation between immunohistochemistry markers. Kaplan-Meier survival curves for patient overall survival and recurrence-free survival were also generated. The log-rank test was used to identify the difference between the patient groups for overall and recurrence-free survival. For univariate and multivariate analyses for immunohistochemical expressions, the Cox proportional hazard model was used. Two-sided Ps < 0.05 were considered statistically significant.

**Results**

**Correlation of expression by estrogen receptor antibodies.** We examined four commercially available antibodies against estrogen receptor α: two using the same clone (6F11) and two antibodies against estrogen receptor β (Supplementary Table S2). Using the scores of expression generated from all NSCLCs, we analyzed the correlation of the expression in the malignant cells for the four estrogen receptor α and the two estrogen receptor β antibodies tested. All four of the estrogen receptor α antibodies showed nuclear staining, and two of the four antibodies also detected expression in the cytoplasm of malignant cells (estrogen receptor α-3, clone HC20, and estrogen receptor α-4, clone 1D5). Both estrogen receptor α clones 6F11 antibodies (estrogen receptors α-1 and α-2) against the full length of the protein, obtained from two different companies, showed only nuclear staining. Using the scores of expression, all four of the estrogen receptor α antibodies significantly correlated with each other at nuclear expression (Spearman rank correlation, r = 0.32-0.48; P < 0.0001; Supplementary Table S3). However, when only tumors expressing nuclear estrogen receptor α using any antibody were examined, no correlation in the expression using these four antibodies was observed (Supplementary Table S4). Similarly, although significant correlation was detected in the staining of the two estrogen receptor α antibodies (estrogen receptor α-3 and -4) showing cytoplasmic expression (r = 0.43; P < 0.0001) using the scores, no correlation was detected when only tumors showing cytoplasmic estrogen receptor α expression were examined. There was no statistically significant correlation between both of the estrogen receptor β antibodies examined in their nuclear expression; although they significantly correlated at their cytoplasmic expression, the r was very low (r = 0.17; P = 0.005).

To assess the ability of the antibodies to detect full length and isoforms of the estrogen receptor proteins, we examined their expression by immunohistochemistry (cell lines pellets) and Western blot in a panel of seven NSCLC cell lines. We identified that, by Western blot, all four estrogen receptor α and both estrogen receptor β antibodies used detect their full-length proteins of 66 and 60 kDa, respectively (data not shown). In addition, antibodies estrogen receptor α-1 and α-3 detect other isoforms of 46 kDa in NSCLC cell lines. We did not find correlation between the immunohistochemical nuclear and cytoplasmic expression and the patterns of isoforms detected by Western blot.

**Frequency of estrogen receptor and progesterone receptor expression in NSCLC specimens by histology.** We analyzed the frequency of any estrogen receptor and progesterone receptor immunohistochemical expression (positive cases, score > 0) for each antibody tested by NSCLC tumor histology, and the data are summarized in Table 1. Representative microphotographs of the expression of estrogen receptor and progesterone receptor with some of the antibodies tested are shown in Fig. 1. Estrogen receptors and progesterone receptor were detected in the nucleus of malignant cells by all of the corresponding antibodies tested. However, when expressed, the percentage of malignant cells showing staining was low in general, with an average percentage of positive expression of 19% (range, 2-90%) for estrogen receptor α-1 nuclear; 13% (range, 2-93%) for estrogen receptor α-2 nuclear; 21% (range, 1-60%) and 19% (range, 3-73%) for estrogen receptor α-3 nuclear and cytoplasmic, respectively; and 11% (range, 3-97%) and 7% (range, 3-30%) for estrogen receptor α-4 nuclear and cytoplasmic, respectively. The average percentages of positive cells expressing estrogen receptor β were 37% (range, 3-90%) and
37% (range, 3-97%) for estrogen receptor β-1 nuclear and cytoplasmic, respectively, and 13% (range, 1-77%) and 24% (range, 3-67%) for estrogen receptor β-2 nuclear and cytoplasmic, respectively.

Although there are important variations in the frequency of expression between the nuclear estrogen receptor α antibodies tested, adenocarcinoma histology showed significantly higher frequency of expression than squamous cell carcinomas for all estrogen receptor α antibodies (P < 0.0001-0.048; Table 1). For nuclear expression of estrogen receptor β, the data obtained with both antibodies tested were relatively consistent, and the adenocarcinoma histology showed a significantly higher frequency of expression than the squamous cell carcinoma did with the estrogen receptor β-2 antibody (P = 0.0069). Two of the estrogen receptor α (estrogen receptor α-3 and α-4) and both estrogen receptor β antibodies also detected estrogen receptor expression in the cytoplasm of NSCLC cells (Table 1). Although the estrogen receptor β-2 antibody detected protein expressed in the cytoplasm of a subset of NSCLCs, the estrogen receptor β-1 antibody detected expression in nearly all of the tumors. Cytoplasmic expression, only for the estrogen receptor α-3 antibody, was significantly higher in adenocarcinomas when compared with squamous cell carcinomas (P = 0.0064).

In the NSCLC tissues, progesterone receptor expression was frequently detected in the nuclei of malignant cells only. Squamous cell carcinoma histology showed a marginally significant higher frequency of expression than that of the adenocarcinomas (P = 0.05; Table 1).

**Correlation between estrogen receptor and progesterone receptor expression in NSCLC and patients’ clinicopathologic features.** We correlated expression of estrogen receptors and progesterone receptor for each antibody tested with the patients’ clinicopathologic characteristics, including histology, gender, tobacco history, and tumor-node-metastasis pathologic stage using the expression score as a continuous variable. Using this type of analysis, adenocarcinoma histology also showed a statistically significant higher nuclear expression for all estrogen receptor α antibodies and for the estrogen receptor β-2 antibody than squamous histology (Table 2). Of great interest was the fact that the NSCLC tissues obtained from females and never smokers showed statistically significant higher expression of nuclear estrogen receptor α and β for several of the antibodies used (Table 2). No correlations between the expression of progesterone receptor and the clinicopathologic characteristics were found.

We did overall survival and recurrence-free survival analyses to determine the expression of estrogen receptors and progesterone receptor for each antibody tested by using specimens from 317 patients with NSCLC with a median follow-up of 6.1 years for overall survival and 4.2 years for recurrence-free survival. No association was detected between the expression of estrogen receptor and progesterone receptor and overall survival. Of interest, any expression of cytoplasmic estrogen receptor α, using estrogen receptor α-4 antibody, and nuclear estrogen receptor β, using the estrogen receptor β-1 antibody, conferred to patients a significantly worse recurrence-free survival in the univariate and multivariate analysis (Fig. 2; Table 3). However, only the cytoplasmic expression of estrogen receptor α-4 correlated with worse recurrence-free survival when dichotomized score was being used (hazard ratio, 1.77; 95% confidence interval, 1.11-2.81; P = 0.0156; Table 3).

**Correlation between estrogen receptor and progesterone receptor expression in NSCLC and tumor EGFR mutation status.** Among 182 adenocarcinoma cases, EGFR mutations of the tyrosine kinase domain (exons 18-21) were detected in 31 (17%) cases. Most (88%) EGFR mutations were detected in the exons 19 and 21, and we did not find correlation between the location of the mutation and estrogen receptor α and β expression. We correlated the estrogen receptor and progesterone receptor scores and any expression (positive cases, score > 0) with EGFR mutation status. Interestingly, EGFR mutant adenocarcinomas showed statistically significant higher expression than wild-type tumors of nuclear estrogen receptor α, cytoplasmic estrogen receptor α, and nuclear estrogen receptor β when tested with

<table>
<thead>
<tr>
<th>Marker</th>
<th>Location</th>
<th>ADCA</th>
<th>SCC</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cases</td>
<td>Positive, n (%)</td>
<td>No. of cases</td>
<td>Positive, n (%)</td>
<td></td>
</tr>
<tr>
<td>ERα-1</td>
<td>Nucleus</td>
<td>187</td>
<td>20 (11)</td>
<td>109</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>187</td>
<td>0</td>
<td>108</td>
<td>0</td>
</tr>
<tr>
<td>ERα-2</td>
<td>Nucleus</td>
<td>185</td>
<td>84 (45)</td>
<td>110</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>185</td>
<td>1 (&lt;1)</td>
<td>111</td>
<td>0</td>
</tr>
<tr>
<td>ERα-3</td>
<td>Nucleus</td>
<td>191</td>
<td>16 (8)</td>
<td>114</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>190</td>
<td>92 (48)</td>
<td>114</td>
<td>37 (33)</td>
</tr>
<tr>
<td>ERα-4</td>
<td>Nucleus</td>
<td>185</td>
<td>74 (40)</td>
<td>109</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>185</td>
<td>35 (19)</td>
<td>109</td>
<td>18 (17)</td>
</tr>
<tr>
<td>ERβ-1</td>
<td>Nucleus</td>
<td>189</td>
<td>102 (54)</td>
<td>112</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>189</td>
<td>185 (98)</td>
<td>112</td>
<td>110 (98)</td>
</tr>
<tr>
<td>ERβ-2</td>
<td>Nucleus</td>
<td>174</td>
<td>83 (48)</td>
<td>100</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>172</td>
<td>37 (22)</td>
<td>100</td>
<td>16 (16)</td>
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<td>PR</td>
<td>Nucleus</td>
<td>177</td>
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<tr>
<td>Cytoplasm</td>
<td>176</td>
<td>0</td>
<td>112</td>
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</tr>
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</table>

NOTE: Any expression score > 0 is considered positive. Abbreviations: ADCA, adenocarcinomas; SCC, squamous cell carcinoma; ER, estrogen receptor; PR, progesterone receptor.

*Not tested.
antibodies estrogen receptor α-3, estrogen receptor α-4, and estrogen receptor β-1, respectively (Table 4; Fig. 3). Because there was a higher incidence of EGFR mutation in lung adenocarcinoma cases from patients with a history of never smoking, Asian ethnicity, or female characteristics (data not shown), we adjusted the effects of age, gender, smoking history, ethnicity, and pathologic stage in the correlation of estrogen receptor α and β with EGFR mutation status. After linear regression analysis,

**Table 2.** Significant correlations between immunohistochemical expression of estrogen receptor and progesterone receptor and NSCLC patients’ clinicopathologic features

<table>
<thead>
<tr>
<th>Estrogen receptor</th>
<th>Histology</th>
<th>Gender</th>
<th>Tobacco history</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ADCA (n = 201) &gt;</td>
<td>Female (n = 167) &gt;</td>
<td>Never (n = 54) &gt;</td>
</tr>
<tr>
<td></td>
<td>SCC (n = 116)</td>
<td>Male (n = 150)</td>
<td>Ever (n = 262)</td>
</tr>
<tr>
<td>ERα-1 nucleus</td>
<td>0.0048</td>
<td>0.0051</td>
<td>NS</td>
</tr>
<tr>
<td>ERα-2 nucleus</td>
<td>&lt;0.0001</td>
<td>0.0109</td>
<td>0.0006</td>
</tr>
<tr>
<td>ERα-3 nucleus</td>
<td>0.0015</td>
<td>NS</td>
<td>0.0242</td>
</tr>
<tr>
<td>ERα-4 nucleus</td>
<td>0.0004</td>
<td>0.0148</td>
<td>0.0044</td>
</tr>
<tr>
<td>ERβ-1 nucleus</td>
<td>NS</td>
<td>NS</td>
<td>0.0290</td>
</tr>
<tr>
<td>ERβ-2 nucleus</td>
<td>0.0016</td>
<td>0.044</td>
<td>NS</td>
</tr>
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NOTE: Estrogen receptor and progesterone receptor were tested using expression score. Abbreviation: NS, not significant.
Table 3. Multivariate recurrence-free survival analysis using Cox regression model in NSCLC patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR</th>
<th>95% CI of HR</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER as continuous variable</td>
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<td></td>
</tr>
<tr>
<td>ERα-4 cytoplasm</td>
<td>1.05</td>
<td>1.01 1.08</td>
<td>0.0068</td>
</tr>
<tr>
<td>ERβ-1 nucleus</td>
<td>1.01</td>
<td>1.00 1.02</td>
<td>0.0034</td>
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<tr>
<td>Stage II vs I</td>
<td>1.90</td>
<td>1.14 3.18</td>
<td>0.0145</td>
</tr>
<tr>
<td>Stage III/IV vs I</td>
<td>3.17</td>
<td>1.98 5.08</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ER dichotomized</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERα-4 cytoplasm: &gt;0 vs 0</td>
<td>1.77</td>
<td>1.11 2.81</td>
<td>0.0156</td>
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<tr>
<td>ERβ-1 nucleus: &gt;0 vs 0</td>
<td>1.36</td>
<td>0.91 2.05</td>
<td>0.1388</td>
</tr>
<tr>
<td>Stage II vs I</td>
<td>1.79</td>
<td>1.08 2.99</td>
<td>0.0250</td>
</tr>
<tr>
<td>Stage III/IV vs I</td>
<td>3.13</td>
<td>1.97 4.99</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

NOTE: With only significant covariates.
Abbreviations: 95% CI, 95% confidence interval; HR, hazard ratio.

Discussion

Estrogen receptors α and β frequently expressed in our NSCLC cases, and estrogen receptor α expression distinguished a subset of NSCLC that has defined clinicopathologic and genetic features. Although the immunohistochemical expression of estrogen receptors α and β has been reported in tumor tissue specimens from surgically resected NSCLCs, the data on the fraction of tumors expressing estrogen receptor are still controversial. Previous studies on estrogen receptor α immunohistochemical expression in formalin-fixed and paraffin-embedded NSCLC specimens using six different antibodies identified nuclear expression in malignant cells in frequencies that ranged from none (10, 14) to 18% (26) and 38% (12). Similarly, in other studies, the frequency of estrogen receptor α cytoplasmic expression in NSCLC ranged from 0% to 3% (12, 26) to 35% (11) and 73% (27). In the current study, using four different commercially available estrogen receptor α antibodies, we also identified a wide range of percentages in the frequency of NSCLCs exhibiting any expression of estrogen receptor α in the nucleus (7-54%) and in the cytoplasm (0-42%) of tumor cells. However, in our study, when the scores of immunohistochemical expression were analyzed as continuous variables, all of the estrogen receptor α antibodies significantly correlated with each other at nuclear and cytoplasmic locations.
A similar situation is observed when the estrogen receptor β immunohistochemical expression data are examined in NSCLC. Several previous studies, using six different antibodies, have reported frequencies of estrogen receptor β expression in tumors with a wide range of percentages at the nuclear location, 0% (9), 34% to 47% (10, 12, 14), and 61% to 84% (9, 11), but not in the cytoplasm of malignant cells, wherein most of the studies have shown no reactivity (9, 10, 12, 14); some expression was seen in a small number of cases (6) or low frequency of expression in a large number of cases (10%; ref. 11). In the present study, using two antibodies, any estrogen receptor β nuclear expression was detected in about half (56% and 42%) of the NSCLCs, and cytoplasmic expression was found in a wider range (20-98%) of our cases. We do not have a definitive explanation to the high levels of expression of estrogen receptor β in NSCLC cells in our study and the discordance with previous reports. However, immunohistochemical analysis has shown the distribution of estrogen receptor β to be much more widespread than estrogen receptor α (28–30). Several studies have reported that estrogen receptor β immunohistochemical expression is frequently detected in the nucleus and cytoplasm of normal respiratory cells (28). Although expression has been questioned by suggestions that this observation is based on nonspecific binding produced by unpurified antibodies (31), multiple reports have shown the presence of a nonnuclear pool of estrogen receptors in normal and malignant cells (32–35). Yang et al. (35) used one of the same estrogen receptor β antibodies that we used (estrogen receptor β-1) and showed mitochondrial localization of this receptor in several normal human and murine cells, suggesting a role for estrogen receptor β receptor in the cytoplasm of cells. Our finding of high frequency of estrogen receptor β expression, using estrogen receptor β-1 antibody, in the cytoplasm of normal respiratory cells from our lung adenocarcinoma patients are consistent with these findings.

### Table 4. Significant correlations between immunohistochemical expression of estrogen receptor and EGFR mutation status in adenocarcinoma

<table>
<thead>
<tr>
<th>ER expression by antibody</th>
<th>EGFR mutation status</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type, n positive/total (%)</td>
<td>Mutant, n positive/total (%)</td>
</tr>
<tr>
<td>ERα-3 nucleus</td>
<td>9/146 (6)</td>
<td>7/28 (25)</td>
</tr>
<tr>
<td>ERα-3 cytoplasm</td>
<td>68/146 (47)</td>
<td>21/27 (78)</td>
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<tr>
<td>ERα-4 nucleus</td>
<td>50/143 (35)</td>
<td>18/27 (67)</td>
</tr>
<tr>
<td>ERα-4 cytoplasm</td>
<td>20/143 (14)</td>
<td>13/27 (48)</td>
</tr>
<tr>
<td>ERβ-1 nucleus</td>
<td>70/145 (48)</td>
<td>22/27 (82)</td>
</tr>
</tbody>
</table>

NOTE: Estrogen receptor and progesterone receptor were tested using expression score.

A similar situation is observed when the estrogen receptor β immunohistochemical expression data are examined in NSCLC. Several previous studies, using six different antibodies, have reported frequencies of estrogen receptor β expression in tumors with a wide range of percentages at the nuclear location, 0% (9), 34% to 47% (10, 12, 14), and 61% to 84% (9, 11), but not in the cytoplasm of malignant cells, wherein most of the studies have shown no reactivity (9, 10, 12, 14); some expression was seen in a small number of cases (6) or low frequency of expression in a large number of cases (10%; ref. 11). In the present study, using two antibodies, any estrogen receptor β nuclear expression was detected in about half (56% and 42%) of the NSCLCs, and cytoplasmic expression was found in a wider range (20-98%) of our cases. We do not have a definitive explanation to the high levels of expression of estrogen receptor β in NSCLC cells in our study and the discordance with previous reports. However, immunohistochemical analysis has shown the distribution of estrogen receptor β to be much more widespread than estrogen receptor α (28–30). Several studies have reported that estrogen receptor β immunohistochemical expression is frequently detected in the nucleus and cytoplasm of normal respiratory cells (28). Although expression has been questioned by suggestions that this observation is based on nonspecific binding produced by unpurified antibodies (31), multiple reports have shown the presence of a nonnuclear pool of estrogen receptors in normal and malignant cells (32–35). Yang et al. (35) used one of the same estrogen receptor β antibodies that we used (estrogen receptor β-1) and showed mitochondrial localization of this receptor in several normal human and murine cells, suggesting a role for estrogen receptor β receptor in the cytoplasm of cells. Our finding of high frequency of estrogen receptor β expression, using estrogen receptor β-1 antibody, in the cytoplasm of normal respiratory cells from our lung adenocarcinoma patients are consistent with these findings.

**Fig. 3.** Representative examples of estrogen receptor α and β immunohistochemical expression (top figures) and EGFR mutations (bottom figures) in lung adenocarcinomas. A, estrogen receptor α (antibody estrogen receptor α-4) positive in the nucleus of malignant cells and sequencing chromatograms showing the presence of mutant form of EGFR (15-bp deletion in exon 19). Arrow, in-frame deletion mutation sequence. B, estrogen receptor β (antibody estrogen receptor β-1) positive in the nucleus of malignant cells and sequencing chromatograms showing the presence of mutant EGFR (L858R point mutation in exon 21). Arrow, CTG to CGG mutation. C, estrogen receptors α and β (same antibodies than as A and B) with negative expression in the malignant cells and sequencing chromatograms showing the presence of wild-type form of EGFR exon 19. Line, sequences 746 to 750.
Several discrepancies were observed when we compared our results with those published previously (6, 9, 10, 12) using the same antibodies, especially for estrogen receptor α. For example, our estrogen receptor α-3 antibody, raised against the COOH-terminus region of the protein, detected nuclear and cytoplasmic expressions in 54% and 42% of our NSCLC cases, respectively. Using this antibody, nuclear expression was reported in a small number of NSCLC tumors by Stabile et al. (6) and in none of the 130 tumors examined by Kawai et al. (10). At the cytoplasmic location of malignant cells, both studies reported positive immunostaining (6, 10), with up to 73% of cases in the study done by Kawai et al. (10).

Why these inconsistent results on the immunohistochemical expression of estrogen receptor α and β occur raises a very important question. Clearly, the reasons for the inconsistent results include the use of different antibodies manufactured from different clones and by different companies. Indeed, some of these antibodies have been made against different parts of the protein: full length, NH2-terminus, and COOH-terminus regions. It has been shown that several mRNA splicing variants of estrogen receptor α have been detected in lung cancer cell lines, and antibodies raised against epitopes in the deleted exons of estrogen receptor may give conflicting results (6). Although a number of estrogen receptor α mRNA variants have been reported, in most cases, wild-type mRNA estrogen receptor α is coexpressed along with splicing variants (36). In our study, we identified a high level of discordance in the frequency and location in the malignant cells of the expression of estrogen receptor α and estrogen receptor β antibodies examined. By examining NSCLC cell lines using immunohistochemistry and Western blot, we did not find correlation between the patterns of immunostaining (nuclear versus cytoplasmic) and the expression protein isoforms. In addition, it is important to note that there are multiple criteria reported to assess estrogen receptor α and β positivity in NSCLC tissues. Although most studies considered different expression intensity levels (usually a scale 0–3+) at nuclear and cytoplasmic locations combined with the percentage of malignant cells expressing a given intensity, the cutoff levels of expression vary significantly between studies (e.g., 1+ in >10% of cells; 1+ in 1–25% of cells; >50% of cells; score 0–8, etc.; refs. 6, 9–12, 14, 26).

Because there were different levels of estrogen receptor α and β immunohistochemical expression detected using different antibodies in ours and the previous studies (6, 9–12, 14, 26), we correlated the expression of estrogen receptor using all of the antibodies we tested with the patients’ clinicopathologic features and the tumors’ EGFR mutation status. The evaluation of multiple antibodies for estrogen receptor expression adds strength to our findings. In our study, we analyzed the immunohistochemical scores as continuous and dichotomized variables, and a significantly higher expression of nuclear estrogen receptor α was detected with all four antibodies tested in adenocarcinoma than squamous cell carcinoma histology, three of the four antibodies tested in tumors obtained from females compared with males and from people who had never smoked compared with smokers. The biological implication of the large range of positive lung cancer cells for estrogen receptor and progesterone receptor is unknown. The cutoff that we selected (positive, >0) to study correlation with clinicopathologic features was based in the fact that a large number of tumors showed low percentage of malignant cells expressing these receptors. The two previous studies reporting estrogen receptor α nuclear expression in NSCLC, which examined a relatively large series of cases, did not address differences of expression based on histology types or patients’ clinicopathologic features (12, 26). In the NSCLC tissues that we reviewed, higher expression of estrogen receptor β correlated significantly with tumor adenocarcinoma histology and the patients’ female sex for estrogen receptor β-1 antibody and correlated with the patients’ history of never smoking with the estrogen receptor β-2 antibody.

Few studies have shown inconsistent results on whether estrogen receptor expression is biased to any sex using different types of specimens and assays (6–9). Schwartz et al. (9), using a different antibody than ours, reported that NSCLCs obtained from females were 46% less likely to have estrogen receptor β–positive tumors than males in a multivariate analysis. In addition, mRNA expression of estrogen receptor α has been reported to be significantly higher in lung tumors from women than from men (8). In a small number of NSCLC tumor tissue specimens, estrogen receptor α and β gene transcripts have been found to be expressed in similar levels when comparing samples obtained from females and males (7). Adenocarcinoma of the lung, which shows a weaker association with tobacco smoking than with other types of lung cancer, is also found predominantly in women, suggesting a possible role for female hormones in the pathogenesis of this type of lung cancer (5).

In previous studies, estrogen receptor β expression in NSCLC tumors has been associated with improved survival (9–11), whereas the immunohistochemical expression of estrogen receptor α has been shown to be a poor prognostic factor (9). Thus, both estrogen receptors have been proposed to play opposite roles in cell proliferation, with estrogen receptor α promoting proliferation and estrogen receptor β having an antiproliferative effect (37, 38). In our study, we did not find a correlation between overall survival and recurrence-free survival and estrogen receptor β expression, but we did find that only the expression of cytoplasmic estrogen receptor α (using one antibody) conferred to patients a significantly worse recurrence-free survival, but not overall survival, in multivariate analysis.

Several studies have shown that estrogen signaling plays a role in the development of the epithelium in the lung and that estrogen could potentially promote lung cancer (6, 7, 13, 16, 17). In addition, antiestrogen drugs have been suggested to have a role in the therapy of lung cancer (6, 19). NSCLC cell lines and in vivo tumor xenografts have been shown to respond to estrogens, and tumor growth can be inhibited up to 40% by the antiestrogen fulvestrant (6). In the past few years, significant advances have been made in the development of new molecularly targeted agents for lung cancer (39). The identification of the subset of patients with NSCLC who will benefit with targeted therapy is a key element in the development of personalized treatment approaches in this disease. A pilot study on combined therapy using fulvestrant and gefitinib in advanced NSCLC has shown to be well tolerated and has shown some tumor responses (40). Our study results strongly suggest that NSCLC tumors obtained from patients with adenocarcinoma histology, female gender, and history of never smoking have a higher chance of expressing estrogen receptors and have the potential to respond positively to antiestrogen therapy.
While our manuscript was under review, Nose et al. (41), using one antibody against estrogen receptor \( \alpha \) (same antibody as our estrogen receptor \( \alpha \)-3) and estrogen receptor \( \beta \) (same antibody as our estrogen receptor \( \beta \)-1), reported in 447 surgically resected lung adenocarcinomas (stages I-IV) from Japanese patients that estrogen receptor \( \beta \) nuclear expression was significantly \((P < 0.001)\) higher in tumors with \( EGF R \) mutation. They also reported that estrogen receptor \( \beta \) nuclear expression correlated with increasing disease-free survival (hazard ratio, 2.18; 95\% confidence interval, 1.18-4.06; \( P = 0.014 \)) in the patients with \( EGF R \) mutant tumors. Although, in this study, a different scoring system was used to assess estrogen receptor immunohistochemical expression and the expression of estrogen receptors \( \alpha \) and \( \beta \) in the malignant cells was reported at cytoplasmic and nuclear levels only, respectively, the finding of significant correlation of estrogen receptor \( \beta \) nuclear expression with \( EGF R \) mutation in lung adenocarcinoma agrees with our findings. Thus, our study is the first to report an association between \( EGF R \) mutation and estrogen receptor \( \alpha \) expression in lung adenocarcinomas. Importantly, we have shown that the correlation between estrogen receptor expression and \( EGF R \) mutation is independent of the clinicopathologic features associated with both abnormalities, such as adenocarcinoma histology, female gender, and history of never smoking (42). Based on the interactions between estrogen receptor and \( EGF R \)-signaling pathways, there is evidence showing that targeting both pathways by using antiestrogens (fulvestrant) and \( EGF R \) tyrosine kinase inhibitors (gefitinib), the antitumor effect in lung cancer models of the drug combination is higher than in treatment with each drug alone (19). Thus, our findings of an association between the activation of both pathways further strengthen the concept of combined antiestrogen and \( EGF R \) inhibitor therapy for a selected group of patients with lung adenocarcinoma.

Although progesterone receptor expression has been reported to be present in NSCLC cell lines and tumor specimens, the data are controversial like those for estrogen receptors (11, 12, 15, 43, 44). Of four studies reporting on immunohistochemical expression of progesterone receptor in surgical resected and formalin-fixed NSCLC tissue specimens using different antibodies, there were two studies that reported a relatively high frequency of progesterone receptor expression in tumors (39\% and 47\%; refs. 12, 15); the remaining two reports showed no expression (11, 44). In the present study, progesterone receptor was frequently (63\%) detected in the nuclei of malignant NSCLC, with a trend to higher expression in squamous cell carcinoma histology. We did not find a correlation between progesterone receptor and any of the clinicopathologic characteristics we studied, including survival. In contrast, Ishibashi et al. (12) reported that progesterone receptor immunohistochemical expression was higher in NSCLCs obtained from females and correlated with better overall survival in stages I to III tumors. In breast cancer, transcription of the progesterone receptor gene is well known to be regulated by estrogenic actions through estrogen receptors, and a positive progesterone receptor status is generally regarded as one of the markers of functional estrogenic pathways. In our study, we found no statistical correlation between progesterone receptor and any of the estrogen receptor antibodies studied. In vivo and in vitro studies have shown that administration of progesterone inhibits the growth of progesterone receptor-positive NSCLC cell lines, which is similar to what has been shown to happen in breast and endometrial carcinomas (12).

Lung cancer is believed to develop from a series of preneoplastic lesions in the respiratory mucosa, and these abnormalities are frequently extensive and multifocal throughout the respiratory epithelium, indicating a field-effect or field-cancerization phenomenon (45). Our findings of relatively frequent expression of nuclear progesterone receptor and lack of expression of estrogen receptor \( \alpha \) in the normal epithelium adjacent to adenocarcinomas expressing these receptors suggest that progesterone receptor, but not estrogen receptor \( \alpha \) expression, may represent a field-effect phenomenon. Of interest, all but one case with normal epithelium expression of progesterone receptor showed expression of this receptor in the corresponding tumor. The frequent finding of cytoplasmic estrogen receptor \( \beta \) in normal epithelium may represent a constitutive expression in normal respiratory cells and is probably not related to the carcinogenesis process (35).

In summary, our findings show that estrogen receptors \( \alpha \) and \( \beta \) and progesterone receptor are frequently expressed in NSCLC, and estrogen receptor expression distinguishes a subset of NSCLC that has defined clinicopathologic and genetic features. In our study, there is a bias toward early stages of lung cancer because we examined surgically resected tumors, being most (98\%) of them stages I to III. The frequency of estrogen receptor and progesterone receptor expression in advanced metastatic tumors need to be further examined. The correlation between estrogen receptor and \( EGF R \) mutation in lung adenocarcinoma suggests that it might be important to target both pathways simultaneously for lung cancer chemoprevention and therapy.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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