Combined analysis of estrogen receptor β−1 and progesterone receptor expression identifies lung cancer patients with poor outcome

Running title: ERβ−1 and PR together predict survival in lung cancer

Key words: estrogen receptor, progesterone receptor, aromatase, epidermal growth factor receptor, lung cancer

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Grant support: NCI P50 CA090440 SPORE in Lung Cancer (JMS) and Flight Attendant Medical Research Institute (LPS)

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Abbreviations Used: estrogen receptor alpha, ERα; estrogen receptor beta, ERβ; progesterone receptor, PR; epidermal growth factor receptor, EGFR; overall survival, OS; time to progression, TTP; hazard ratio, HR; non-small cell lung cancer, NSCLC; tissue microarrays, TMAs; immunohistochemistry, IHC; cytoplasmic, cyto.
Statement of Translational Relevance

A better understanding of the role and interaction of hormone and growth factor pathways in the lung is necessary to elucidate novel effective preventative and treatment strategies for lung cancer. This work examines the combined effect of expression of estrogen receptor β−1 and progesterone receptor in tissues from lung cancer patients and the influence of expression of other markers including the epidermal growth factor receptor and aromatase on patient survival. Antibody staining detected in both the cytoplasmic and nuclear compartments was taken into account. The correlations between expression of these markers as well as their combined influence on patient survival suggest that phenotyping these interacting markers together in lung cancer patients may better predict patient survival as well as suggest which patients could be candidates for hormonal therapy for lung cancer treatment. Interacting hormonal factors appear important in both men and women with lung cancer and a validation study will be necessary to confirm these findings.
Abstract

Purpose: Steroid hormones and growth factors affect lung cancer, and it is possible they act in concert to influence patient outcome.

Experimental Design: Primary lung tumors and normal lung tissue were analyzed for expression and localization of estrogen receptor α and β−1 (ERα and ERβ), aromatase, progesterone receptor (PR), and epidermal growth factor receptor (EGFR).

Results: Tumors expressed higher levels of ERβ compared to matched normal lung, while the reverse was true of PR. High cytoplasmic ERβ expression was identified as an independent negative prognostic predictor of overall survival (OS) (HR=1.67), and low total PR was identified as an independent negative predictor of time to progression (TTP) (HR=1.59). After adjusting for stage, age, sex and smoking, combined high cytoplasmic ERβ and low total PR showed enhanced effects on OS (HR=2.64) and on TTP (HR=6.02). Further effects on OS were observed when EGFR expression was included (HR=5.32). Patients with low cytoplasmic ERβ, low aromatase, low EGFR and high total PR had shorter OS than patients with the opposite pattern (HR= 6.60). Contribution of these markers to survival showed no significant sex differences in a multivariable model. ERα was elevated in tumors but was not predictive of survival, and appears to represent a variant ERα protein that is only recognized by a C-terminal antibody.

Conclusions: Hormonal and EGFR pathways together may contribute to lung cancer prognosis. Lung tumors with high ERβ−1/low PR may define patients with aggressive biology. A validation study is necessary to fully assess the predictive value of these markers.
Introduction

Estrogens are known to stimulate non-small cell lung cancer (NSCLC) cell proliferation, while the antiestrogen fulvestrant inhibits this effect (1). Cell lines derived from lung tumors of both men and women express ERs and respond to estrogens. Numerous studies have linked estrogen status to lung cancer outcome. Postmenopausal women diagnosed with lung cancer had significantly enhanced survival compared to premenopausal women (2). Albain et al. reported that women over age 60 with advanced NSCLC had a survival advantage over men and younger women (3). Men with advanced NSCLC and high free $\beta$-estradiol serum levels had significantly worse survival compared to men with lower $\beta$-estradiol levels (4). Furthermore, it has been recently shown that anti-estrogens may reduce risk of death from lung cancer (5) while hormone replacement therapy (HRT) may increase this risk (6).

Cellular responses to estrogens are mediated by two distinct receptors, ER$\alpha$ and ER$\beta$. In lung cancer cells, ER$\beta$ is sufficient to induce the full range of estrogenic responses when no detectable full-length ER$\alpha$ protein is present (7). Nuclear ER$\beta$ has been reported as a favorable prognostic factor for lung cancer (8-12), although sometimes only in sub-groups such as males (9,11) or patients with EGFR mutations (12). Past results are summarized in Supplemental Table 1. Cytoplasmic ER$\beta$ expression was usually not considered, or cytoplasmic and nuclear ER$\beta$ staining were scored together. ER$\alpha$ expression results are also variable with either no evidence of expression using the monoclonal ID5 (epitope= N-terminus) or 6F11 (epitope= full length ER$\alpha$) antibodies (8-
10,13,14) or mainly cytoplasmic localization using the HC-20 (epitope= C-terminus) or ID5 antibodies (10-12). In one report, cytoplasmic ERα expression was associated with poor survival (10). Raso et al. observed both nuclear and cytoplasmic ERα expression to different extents using all three of the above antibodies in lung tumors; however cytoplasmic and not nuclear ERα expression was associated with worse survival and correlated with epidermal growth factor (EGFR) mutation (15).

Effects of progesterone are mediated by PR, and reported PR expression in lung tumors is variable, with three studies reporting a high expression frequency (39-63%; 15-17) and others showing little or no expression (11,13,14). Ishibashi et al. demonstrated that PR expression is a strong prognostic factor for NSCLC (16) while other survival studies with PR were negative (15). Progesterone supplementation has been shown to inhibit the growth of PR positive lung tumors in mice (16).

Estrogen can be synthesized in the lung by the enzyme aromatase (CYP19A1) (18). Aromatase is present in NSCLC cells and tumor tissues and is functional. High aromatase expression has been correlated with poor prognosis in post-menopausal women with early lung cancer (19), and aromatase inhibitors inhibited lung tumors in mice (18). A decreased incidence of lung cancer was observed in breast cancer patients treated with an aromatase inhibitor after tamoxifen therapy compared with continued tamoxifen therapy (20).

Non-nuclear ER signaling has been reported to interact with growth factor pathways such as EGFR in the lung (21-22). The EGFR pathway is frequently involved in NSCLC and is a target for therapy. However, the clinical significance of EGFR expression in lung tumors remains unclear. Combining fulvestrant with an EGFR
inhibitor had maximum inhibitory effects on lung tumorigenesis (21), and a Phase II trial is underway evaluating combination treatment of erlotinib and fulvestrant in lung cancer (23). Recent lung cancer studies linking ER expression status with EGFR mutation (12,15,24) and with aromatase (14) suggest that considering these signaling pathways together may provide important insight into lung cancer biology.

This study addresses the hypothesis that ERs, PR, and EGFR act together to influence lung cancer biology, and that effect of ER status on outcome will be modulated by expression of these other proteins. In addition, steroid receptors may affect biology of lung tumors through both nuclear and cytoplasmic compartments. We examined the relationship between survival and expression/localization of ERα, ERβ-1 (the main isoform), PR, and EGFR, as well as aromatase, in tumors from 183 lung cancer patients, 98% NSCLC. The results from this study provide novel insight into combined hormonal phenotypes that influence lung cancer outcome.
Materials and Methods

**Human tissue samples.** Lung cancer patients who underwent thoracic surgical procedures at the University of Pittsburgh Cancer Institute were prospectively recruited to a tissue bank protocol approved by the University of Pittsburgh IRB. Tissue microarrays (TMAs) were constructed using randomly selected archival tumor specimens (date range 1992-2006) from 121 of these lung cancer patients. Tissues included primary lung tumor, and where possible, matched non-neoplastic lung from patients with outcome data. At least three cores were extracted from formalin-fixed, paraffin-embedded tissue blocks from each specimen and arrayed on recipient blocks. For TMA quality assessment, one H&E slide was evaluated per ten tissue sections. Slides from 62 patient tumor blocks were also included, which were not incorporated onto arrays, yielding 183 patients.

**Immunohistochemistry (IHC) evaluation.** IHC analyses were conducted by a single Board-certified pathologist (SD) blinded to clinical outcome. Tissue expression was measured by staining TMAs and individual blocks with anti-ERα (HC-20, Santa Cruz Biotechnology, Santa Cruz, CA), anti-ERβ (MCA1974ST, AbD Serotec, Raleigh, NC), anti-PR (MAB429, Millipore, Billerica, MA), CYP19A1 aromatase (MCA2077, AbD Serotec), and anti-EGFR (E3138, Sigma Diagnostics). The ERβ antibody is raised against the carboxy-terminus of the ERβ-1 isoform and was chosen for its specificity in Western and IHC analyses. Heat induced antigen retrieval was performed using 10mM citrate buffer, pH 6 using a pressure cooker. Endogenous peroxidase was quenched with 3% hydrogen peroxide for 5 min at room temperature (RT). Blocking was performed
with non-immune normal serum. Antibodies were diluted in PBS: ERα, 1:200 for 30 min at RT; ERβ, 1:20 overnight at 4°C; PR, 1:80 for 1 hour at RT; aromatase, 1:50 overnight at 4°C; EGFR, 1:7,500 for 30 min at RT. ERα, ERβ and EGFR staining was performed using the EnVision method (DAKO Corp., Carpinteria, CA) while PR and aromatase staining utilized the Vector ABC method (Vector Labs, Burlingame, CA). Two additional monoclonal ERα antibodies, ID5 (DAKO) and NCL-ER6F11 (Novacastra Labs) were tested on a subset of samples (n=20).

Immunoreactive cells were visualized following incubation with diaminobenzidine chromogenic substrate and counterstained with hematoxylin. Each individual core or section was scored semi-quantitatively. The percentage of immunoreactive cells was rounded to the nearest 5th percentile. To obtain a proportion score, the following cut-offs for positive staining were utilized: 0 (no positive cells), 1 (0-1%), 2 (2-10%), 3 (11-33%), 4 (34-66%), 5 (67-100%). Intensity was scored on a scale of 0-3. The Allred scoring system was used which generates values from 0-8 based on the sum of the proportion score and the intensity score (25). Scores were averaged over multiple cores from each patient. A nuclear score, a cytoplasmic (cyto) score, and a total score for ERα, ERβ and PR took into account cellular localization. The total score is the sum of the nuclear and cytoplasmic scores (ranging from 0-16). Fewer than 10% of tumors were positive for nuclear aromatase, so only cytoplasmic aromatase score was analyzed. EGFR membranous staining only was scored on a scale of 0-3: 0 (staining in <10% cells), 1 (light staining in >10% cells), 2 (moderate staining in >10% cells), 3 (strong staining in >10% cells). For normal tissue, type I pneumocytes and bronchial epithelium were scored.
For each experimental run the following controls were performed and representative images are shown in Supplemental Figure 1: positive control for ERα, ERβ, and PR was breast cancer tissue; placenta was the positive control for aromatase, and laryngeal squamous cell carcinoma was the EGFR positive control. Negative controls were performed for all markers by eliminating the primary antibody. No significant differences were observed between the median expression scores on individual blocks compared to the TMA samples.

**Statistical analysis.** Kruskal Wallis tests and Wilcoxon-Mann-Whitney tests were performed to examine differences by sex, histology, and smoking status. Spearman correlations between markers were computed. Paired t-tests were used to determine differences between normal lung tissue and lung tumors from the same patient. To adjust for multiple comparisons where appropriate, we controlled the family-wise error rate using the Hochberg method. In this method, the largest p-value is compared with 0.05, the second with .05/2, and so on, with the smallest p-value being compared against .05/11. Because several markers were investigated in this study, we provided both conventional p-values (p) and conservative p-values (p_{adj}) that are adjusted for the number of comparisons. Results for which p_{adj}<.05 may be viewed as confirmatory evidence. Log-rank tests were used to compare overall survival (OS) and time to progression (TTP) between males and females and between high and low marker expression. TTP was defined as the time between surgery date and date of a clearly documented progression or recurrence. Second primaries were not counted as events therefore completely new lung malignancies were not included in tumor progression analysis. OS was defined as the time between surgery date and the date of death or date
of last contact. Time was censored at the date of last follow-up or death without progression. The cut-off for ERα and aromatase for high versus low expression, selected a priori based on past literature (10), was 0-4 versus > 4. It was necessary to dichotomize ERβ and PR as 0-7 versus >7, and EGFR as 0 versus >0, because distribution of those markers was highly skewed (Supplemental Table 2). These cut-offs were selected, without considering clinical outcomes, to be as consistent as possible across biomarkers while balancing sample sizes between high and low groups and by being as close to the median as possible; the same cut-off was used for nuclear and cytoplasmic compartments. Cox proportional hazards regressions were used to model OS and TTP while controlling for age at diagnosis, stage, sex, and smoking status. Smoking status was not controlled for in TTP models since it was not significant in preliminary analyses. Hazard ratios (HR) were estimated from Cox regression models. Analyses were performed using SAS ver. 9.2 (Cary, NC).

**RNA isolation and RT-PCR.** RNA was isolated from cell lines as described (1). One microgram of RNA was used to generate cDNA using reverse transcription. PCR conditions are in Supplemental Table 5. PCR products were isolated and sequenced for confirmation. cDNA from MCF7 breast cancer cells were used as a positive control for each reaction. A negative control was performed for each reaction without the addition of cDNA.
Results

**Biomarker expression in the cohort.** The cohort analyzed is described in Table 1. Demographic and outcome data from 183 patients were obtained, with a median follow-up time of 3.8 years (range= 2.0 days-14.3 years). The cohort represented a range of lung cancer histologies and stages. Median TTP was 1.62 years; median OS was 2.67 years. Number of patients with data for each marker and the mean, median and score ranges are in Supplemental Table 2. Supplemental Table 3 shows the percent of positive tumors for each marker. Only 1% of cases were negative for all markers. Localization of ERα, ERβ, and PR in both the cytoplasm and nucleus was seen in over 50% of cases, so both locations may be biologically significant, and non-nuclear steroid hormone signaling is known to occur (21,26). No difference in expression of any marker was observed by sex or histology (p>0.05 in all cases). Figure 1 shows representative immunostaining.

ERα expression has been reported to be increased in never smokers (15), so we examined impact of smoking on these markers. Non-active smokers (ex-smokers and never smokers) had a significantly higher expression than active smokers for ERα nuclear (p=0.016, p_adj=0.140), PR cyto (p=0.012, p_adj=0.115) and PR total (p=0.002, p_adj=0.019). Ever smokers (ex-smokers and active smokers) had lower total PR expression than never smokers (p=0.030, p_adj=.334). No significant correlations with smoking were observed for other markers.

**Correlation of expression among markers.** Expression of several markers was correlated (p<0.001; rho>.5; Supplemental Table 4). For example, cytoplasmic and nuclear compartments of ERα and ERβ were moderately to highly correlated with each
other and to their total scores. Other significant correlations were: ER\(\alpha\) nuclear with both PR cyto and PR total, and ER\(\alpha\) cyto with all ER\(\beta\) results. Other correlations were weak: ER\(\beta\) cyto and PR total were not strongly correlated (rho=.210), as well as ER\(\beta\) cyto and EGFR (rho=.428) and PR total and EGFR (rho=.172). Correlations did not differ by sex. These correlations suggest that ER\(\alpha\), ER\(\beta\), and PR are often expressed in both the cytoplasmic and the nuclear compartments, and may occur together.

**Tumors express higher ER\(\alpha\), ER\(\beta\), and lower PR compared to matched normal lung tissue.** Biomarker expression was significantly higher in lung tumor compared to normal lung for ER\(\alpha\) cyto (p=0.004, \(p_{adj}=0.035\)), ER\(\beta\) cyto (p<0.001, \(p_{adj}=0.001\)), and ER\(\beta\) nuclear (p<0.001, \(p_{adj}=0.003\)) (Fig. 2A-B). ER\(\alpha\) total (p=0.041, \(p_{adj}=0.247\)) and ER\(\beta\) total (p<0.001, \(p_{adj}=0.001\)) also showed significant differences (not shown). Nuclear PR expression was significantly higher in normal lung compared to lung tumor (p=0.007, \(p_{adj}=0.051\)) (Fig. 2C). No other significant differences were observed between tumor and normal tissue.

**Survival analyses.** Consistent with other reports (27), we observed that women had significantly better OS (p=0.014; median 3.50 years) and longer TTP (p=0.009, median 2.66 years) compared to men (median OS= 1.57 years, median TTP= 1.02 years). Among women, there was no significant difference in OS or TTP between pre-menopausal and peri- or post-menopausal women (age>51). Age at diagnosis, stage, sex and smoking status were significant predictors of OS while age, stage, and sex were significant in TTP.

In initial analyses, the total score for each marker was included as a continuous variable in separate Cox proportional hazards models adjusted for age at diagnosis, stage,
sex, histology and smoking status. ERβ was a significant predictor of worse OS (p=0.039; HR=1.05; 95% CI=1.00, 1.10) and EGFR approached significance as a predictor for worse OS (p=0.060; HR= 1.29; 95% CI=0.99,1.68). PR approached significance as a predictor for longer TTP (p=0.066; HR= 0.96; 95% CI=0.91, 1.00). ERα and aromatase as a single continuous variable showed no effects on survival so no further analyses were performed on these markers alone.

To further examine ERβ as a predictor of survival, and to assess whether cellular compartment is important, we stratified all patients by high (>7) versus low (≤ 7) ERβ score in the cytoplasm (ERβ cyto) and the nucleus, as well as total ERβ (Supplemental Table 2). There was no significant difference in OS between patients with high and low ERβ total scores (p=0.161) or between patients with high and low ERβ nuclear scores (p=0.317). However, there was a significant difference in OS between patients with high (median OS= 1.87 years) and low (median OS= 3.50 years) ERβ cyto scores (p=0.021, Fig. 3A). Sub-group analysis showed that OS by ERβ cyto high/low stratification without other adjustments may differ in men (median OS= 1.34 years versus 2.13 years; p=0.057), but not in women (p=0.251). There was also a significant difference in TTP among those with high (median TTP= 0.90 years) and low ERβ cyto scores (median TTP =1.96 years; p=0.010), but not using ERβ nuclear or ERβ total scores. When tested in sub-group analysis this difference remained significant in men (median TTP= 0.58 years versus 1.42 years; p=0.026), but not in women (p=0.103).

PR was also stratified with a cut-off of 7 and examined as total staining and by cellular compartment (Supplemental Table 2). There were no significant differences in
OS between patients with high versus low PR cyto, nuclear, or total expression. However, TTP was significantly longer in patients with high (median TTP= 1.93 years) versus low (median TTP=1.03 years) PR total scores (p=0.038, Fig. 3B), while a difference in TTP was not observed between patients for PR cyto or PR nuclear scores. This suggests that a combined effect of PR from both cellular compartments may contribute to survival. In a sub-group analysis by sex, PR total was not significant.

Figures 3C-D illustrate the additional prognostic significance of ERβ cyto and PR total expression when considered in tandem, without other adjustments. Median OS was 1.48 years for high ERβ cyto/low PR total versus 2.94 years for low ERβ cyto/high PR total (Fig. 3C), suggesting increased prediction for the combination. Patients with high ERβ cyto/low PR total also had a shorter TTP (median TTP= 0.67 years vs. 2.75 years; p=0.004; Fig. 3D). Examining survival differences by sex using log-rank test showed a significant difference for men in OS (p=0.001) and TTP (p<0.001) comparing high ERβ cyto/low PR total (median OS= 0.72 years; median TTP= 0.23 years) with the opposite (median OS= 2.67; median TTP= 1.93 years). There was also a significant difference in TTP (p=0.041) among women with high ERβ cyto/low PR total (median TTP= 0.85 years) compared with the opposite (median TTP= not reached), but no significant difference in OS.

To examine the contribution of the these biomarkers while accounting for other survival factors, Cox proportional hazards models that adjusted for age, stage, sex, (and smoking status for OS) were performed. The models showed that individuals with high ERβ cyto expression had a higher risk of death than those with low ERβ cyto expression (HR=1.67, p=0.009; Table 2). Individuals with low PR total expression were at
increased risk for disease progression relative to those with high PR total expression (HR= 1.58, p=0.028; Table 2). Examining both biomarkers together resulted in a HR of 2.64 for patients with high ERβ cyto/low PR total expression relative to patients with the opposite for OS (p=0.002; Table 2) and a HR of 6.02 for TTP (p<0.001; Table 2). These models were adjusted for sex and therefore the HRs were equivalent in men and women. To examine possible sex differences, we included an interaction term between sex and the predictors. Sex alone was a significant covariate however none of the interactions between sex and the biomarkers of interest were significant, indicating that the effects of high ERβ cyto or low PR total, or the combination, did not differ by sex. We also tested the degree of significance for the HRs in a model combining ERβ cyto with PR compared to a model with ERβ cyto alone. The HR in the combined model was significantly different from ERβ cyto alone for both OS (p=0.018) and TTP (p<0.0001).

**Impact of EGFR.** Since EGFR approached significance in initial modeling as a continuous variable, we also analyzed OS by high (>0) versus low (0) EGFR expression score using log-rank tests, but found no significant difference. EGFR score was then examined together with ERβ cyto and PR total for both OS and TTP (Fig. 4). Patients with high ERβ cyto, high EGFR and low PR total (n = 12) had shorter OS (median OS= 1.66 years; p=0.026) and TTP (median TTP= 0.76 years; p=0.004) than those with the opposite (n = 44, median OS= 3.50 years, median TTP= 2.75 years). Six men and six women were in the high-risk group and the stage distribution was: stage IA/B (n=8), stage IIB (n=3), and stage IIIA (n=1). Besides the 44 low-risk patients, the remaining patients had intermediate scores for these markers and the survival curves fell between the two extremes (Fig. 4). When using a Cox model that controlled for age, stage, sex,
and smoking status, including EGFR expression increased the HR for OS, but did not increase the HR for TTP (Table 2). The OS and TTP HR for high ERβ cyto/low PR/high EGFR relative to the opposite group was 5.32 (p=0.001) and 4.80 (p=0.002), respectively.

**Survival analysis with all four markers.** Controlling for age, stage, sex, (and smoking status for OS), patients with high expression for ERβ cyto, aromatase, and EGFR, with low PR total expression had a higher risk of death (HR= 6.6, p=0.006) and a higher risk of disease progression than patients with the opposite (HR=4.09, p=0.060; Table 2). Median OS and TTP were 1.71 years and 0.76 years, respectively, for high ERβ cyto/low PR total/high EGFR/high aromatase and 3.50 years and 2.75 years for the opposite. Since the number of subjects in these subset populations is extremely small, it will be important to validate this finding in a larger dataset to determine to what extent aromatase adds predictive value to the model.

**ERα variants.** ERα expression was observed only with the C-terminal HC-20 antibody that detects known ERα exon 2-7 splice variants as well as full-length ERα. The full length (6F11) or N-terminus (ID5) ERα antibodies, commonly used in breast cancer, were uniformly negative in these lung cancer and normal lung tissues, similar to other reports (8-10, 13, 14). In order to determine the reason for this discrepancy using different primary antibodies, we examined NSCLC and normal lung fibroblast cell lines for ERα mRNA full-length transcripts. All cell lines examined contained deletions in ERα exons 2, 3+4, 4 and 7 (Supplemental Table 6 and Supplemental Figure 2). Since we did not observe ERα full-length protein by immunoblot with the C-terminal antibody in
primary lung tumor extracts or in cell lines (1,7), the mRNA results suggest that most if not all of the ERα expression observed by IHC in patient specimens represents protein products of mRNA splice variants. In contrast, full-length ERβ mRNA was observed with every primer pair tested (Supplemental Figure 2), in agreement with our previous reports showing full-length ERβ by immunoblot in lung tumor cell lines and extracts of primary lung tumors (1,7). Other investigators (15, 22, 28) have also reported expression of ERα and ERβ mRNA and/or protein in lung tumor cell lines and in some cases full-length ERα has been reported.
Discussion

There is increasing evidence for steroid hormone effects in lung cancer. We report here for the first time that cytoplasmic ERβ (specifically ERβ-1) is an independent negative prognostic factor for lung cancer and confirm a previous report that PR is an independent positive prognostic factor. We further show that the combined analysis of these two receptors significantly increases calculated risk of progression and death from lung cancer. Nuclear ERβ or nuclear and cytoplasmic ERβ considered together showed no significant effect on survival in our analysis, suggesting that cytoplasmic ERβ signaling is more important for patient survival than its nuclear signaling. In the case of PR, impact on survival was only significant when considering the sum of cytoplasmic and nuclear PR staining, suggesting both cellular compartments contribute to PR effects in lung cancer. We observed few sex differences in the impact of cytoplasmic ERβ and PR on survival when the markers were considered together. It is possible that ERβ cytoplasmic expression has more impact on survival in men than women when considered as a single variable, but much less so when considered together with PR. Further, there were no significant interactions between sex and any of the biomarker(s) of interest when controlling for other variables. It does not appear that differences in these steroid hormone receptors can account for the large survival difference observed in general between men and women with lung cancer. We further found in both men and women that ERβ is upregulated in lung tumor tissues compared to normal lung tissue, while PR is downregulated in lung tumors compared to normal lung. We observed that these pathways are dysfunctional to the same extent in male and female lung cancer.
The reported expression frequency and localization of hormone receptors in lung cancer has been variable. These differences could be due to lack of standardization in multiple factors including 1) interpretation of the staining 2) antibody and dilution used 3) variability in the scoring assessment or 4) differences in patient cohort characteristics. These discrepancies probably contribute to variable results regarding prognostic significance. There are reports of nuclear ER$\beta$ as a positive prognostic factor for lung cancer (8-12), but in a recent report, survival effect of nuclear ER$\beta$ was limited to patients with EGFR mutations. Recently an association between high ER expression and EGFR mutant lung tumors was found (12, 15), and strong ER$\beta$ expression predicted a good clinical response and longer progression-free survival after EGFR TKI treatment for lung adenocarcinoma patients (29). In our cohort, EGFR mutation status was not available on all patients; EGFR mutations occur mainly in never smokers (30) and our cohort consisted of only 13 never smokers. We found 4 EGFR mutant tumors in these 13 patients. No statistical differences were observed in any of the biomarker expression levels between the 4 EGFR mutant tumors and the 9 EGFR wild-type tumors. Several EGFR mutations may have been missed, but are unlikely to contribute to survival effects we observed. We found no difference in ER$\alpha$ or ER$\beta$ cytoplasmic or nuclear expression between never smokers and all other patients, and no prognostic significance of ER$\beta$ when the never smokers were examined separately. PR expression however was significantly higher in never smokers, and could be an important survival variable for patients without tobacco exposure. Determining a selective effect of tobacco history or EGFR mutation on the variables we examined would require a much larger population of never smokers.
The significance of ERβ cytoplasmic staining as an independent negative prognostic factor for lung cancer has not been reported previously. In part, this could be because cytoplasmic ERβ staining has been largely ignored during staining interpretation or alternatively could be due to which antibody was used. We used an antibody that specifically detects the ERβ-1 isoform. Other commercial antibodies directed towards the amino-terminus of ERβ, such as H-150, may also recognize other isoforms of ERβ which may confound the results. A recent report shows that ERβ-1, but not ERβ-2 is linked to worse prognosis in Stage I lung adenocarcinoma in women (31). We have previously shown that full-length ERβ protein was detected by Western blot in both the nuclear and cytosolic fractions of NSCLC cells, demonstrating that cytoplasmic staining for ERβ is biologically meaningful (7). Hormone receptors do mediate effects through non-genomic pathways, which usually occur in the cytoplasm and involve bidirectional cross-talk with growth factor receptor pathways. We and others have reported rapid activation of MAPK by β-estradiol in NSCLC cells, which is dependent on immediate release of EGFR ligands (21-22). Our survival results suggest that non-genomic ERβ signaling in the lung has important clinical implications. We also observed that ERβ cytoplasmic and nuclear expression values are moderately correlated with each other but in our study nuclear expression did not predict survival. It is unknown how cellular compartmentalization of ERβ is controlled. We were unable to analyze the combined effect of high ERβ cyto/low ERβ nuclear versus the opposite due to the small number of patients whose tumors exhibited this combination. However, there may be sub-groups in which amount of nuclear ERβ protein is prognostic. Supplemental Table 1 summarizes
the results of our study and others on the prognostic significance of ERβ. Either an antibody that specifically recognizes ERβ-1 or an antibody to the N-terminus which recognizes all ERβ isoforms was used. Our study lies in the middle of the extremes of the staging reported in other studies and most likely does not contribute to differences in ERβ prognostic value observed. The main difference in these studies is in the scoring and interpretation. Whether or not nuclear staining only or nuclear and cytoplasmic staining were scored separately or together is a major variable as well as the cut-off points used for statistical survival analyses. Our study compared patients with the highest ERβ expression versus all others, unlike all other studies which compared negative versus positive or negative/weak versus strong.

We could not demonstrate a predictive value for extent of ERα expression, but did demonstrate that expression is dependent upon which antibody is used. Antibody reactivity is a function of presence or absence of the epitope against which the antibody is raised. Results from mRNA analysis of NSCLC cell lines suggest that a series of ERα mRNA splice variants are present with little or no full-length mRNA in the lung cancer cell lines that we examined. By IHC in lung tumors, an antibody to the ERα C-terminus, which is predicted to be present in proteins translated from the splice variants we detected, was the most reactive. Raso et al. recently tested a panel of antibodies for ERα IHC expression and reported varying expression results based on the antibody (15). Either ERα expression was not observed at all or high cytoplasmic expression was observed, in line with our findings. Whether or not ERα variants have any functional role is not known. We have previously shown that an ERα selective agonist does not activate
transcription from an estrogen response element in NSCLC cells, does not cooperate with EGF to promote lung tumor growth, and does not induce lung tumor xenograft growth (7), while ERβ selective agonists are active in these assays.

We found no general association between aromatase and survival in our cohort whereas Mah et al. have shown that aromatase was predictive, but only in women age 65 and older with early stage disease (19). In our patient population, only 41 women met these criteria, compared to 103 in the study by Mah et al. There was no difference in OS or TTP among high versus low aromatase for these 41 patients. Small sample size most likely limits our ability to observe this effect.

Our results confirm a previous report that PR is a strong protective factor for lung cancer (16), similar to what is observed in breast cancer (32). Recent results from the Women’s Health Initiative showed that HRT (estrogen plus progestin) increased the risk of lung cancer death (6). Furthermore, the Vitamins and Lifestyle (VITAL) study demonstrated that women who took combined estrogen and progestin had an increased risk of lung cancer while estrogen-only used showed no effect (33). Progesterone exerts its tumorigenic effects by increasing angiogenesis (34), however progesterone is normally only produced during the menstrual cycle and pregnancy. Progesterone levels found in post-menopausal women and men are below the 1ng/ml or higher serum range required to stimulate the PR (35). In breast cancer, PR is known to signal through ligand-independent mechanisms due to phosphorylation by kinases (36). One molecular mechanism for loss of PR expression in breast tumors is down-regulation of PR by increased growth factor signaling which portends a more aggressive biology (37). It is unknown how the PR signals in NSCLC, however ligand-independent mechanisms may
be involved since the populations most at risk are older men and women, groups that do not have a source of progesterone synthesis. Additionally, the antibody used in this study does not distinguish between the PR-A and PR-B isoforms, which could exert different functions.

It will be important to determine the role of multiple sex hormone-related proteins in predicting lung cancer survival and understanding how interactions between hormones and growth factors are involved in lung cancer. A recent report shows that nuclear ERβ and aromatase are often expressed together (14). ERβ and aromatase were only weakly correlated in our study. The significant markers in our study, ERβ cyto, PR total, and EGFR were only weakly correlated suggesting that results with more than one marker are not artifacts of using markers that are highly correlated. In regards to survival, the combined contribution of ERβ and PR held up in a multivariate regression model after adjusting for other variables such as sex and stage. The HRs found for combined cytoplasmic ERβ and total PR suggested a 2.6 to 6.0-fold increase in poor outcome for at-risk patients and the increased risk for the combination was significantly different from that of ERβ cyto high alone. Combining other markers singly with either ERβ or PR was not significant.

Expression of EGFR by IHC has been reported in a wide range (35-83%) of lung tumors in clinical studies (38). A meta-analysis of 2972 NSCLC patients concluded that EGFR expression considered as a single variable has no effect on survival (38). While EGFR expression was only marginally significant as a single variable, adding EGFR expression to combined ERβ and PR expression showed enhanced survival effects for OS, but not for TTP. Whether this is a true selective effect or due to sample size
constraints is not known. Because of cross-talk between EGFR and ER in lung cancer, degree of EGFR expression might modify ER survival contributions. EGFR gene copy number or mutations are known to contribute to survival and response to therapy. EGFR copy number or mutation might have additional predictive value in a model including expression of EGFR, PR, and ERβ.

Based on our findings and those previously reported, a standardized approach (same antibody epitope, dilutions, epitope retrieval method, scoring system and interpretation) should be developed and validated for screening of lung tumor ERα, ERβ-1, PR, EGFR and aromatase expression in patients. A larger cohort will be needed to examine and validate the predictive value of these markers on lung cancer survival. EGFR and aromatase expression contributed to survival effects only in context with steroid hormone receptors. Following validation, systematic examination of these markers could potentially be prognostic and may be useful to identify patients of both sexes who might respond to anti-estrogen therapy, growth factor TKI therapy, or a combination of both.

Acknowledgements

We gratefully acknowledge Ms. Marianne Notaro and Ms. Leigha Sentir for technical support, Ms. Autumn Gaither-Davis for assistance with clinical records and Dr. Hong Wang for statistical support.
**Figure Legends**

**Fig. 1.** Representative immunohistochemistry of lung tumors for ERα, ERβ, aromatase, EGFR and PR.

**Fig. 2.** Scatter plot and representative images of (A) ERα cytoplasmic expression in matched lung tumor versus normal lung tissue (B) ERβ cytoplasmic and nuclear expression in matched lung tumor versus normal lung tissue and (C) PR nuclear expression in matched lung tumor versus normal lung tissue. Large open circle represents the mean value for each marker. Points above the vertical indicate cases for which the tumor tissue had higher expression than the matching normal tissue.

**Fig. 3.** Kaplan-Meier curves. (A) Overall survival in years stratified by high (dashed line) versus low ERβ cyto score (solid line). (B) Time to progression in years stratified by high (dashed line) versus low PR total scores (solid line). (C) Overall survival in years stratified by combined high ERβ cyto score/low PR total score (solid line) versus low ERβ cyto score/high PR score (dashed line). Dotted line represents all other patients. (D) Time to progression in years stratified by combined high ERβ cyto/low PR (solid line) versus low ERβ cyto/high PR (dashed line). Dotted line represents all other patients.

**Fig. 4.** Kaplan-Meier curves. (A) Overall survival in years stratified by high ERβ cyto/high EGFR/low PR (solid line) versus low ERβ cyto/low EGFR/high PR (dashed line). Dotted line represents all other patients. (B) Time to progression in years stratified by high ERβ cyto/high EGFR/low PR (solid line) versus low ERβ cyto/low EGFR/high PR (dashed line). Dotted line represents all other patients.
References


31. Sethi S, Coti M, Lonardo F. Expression of estrogen receptor beta 1, but not estrogen receptor beta 2 or alpha is linked to worse prognosis in stage I adenocarcinoma, in women, in a large epidemiological cohort but not in a smaller, single hospital based series. United States and Canadian Academy of Pathology. 2010. Abstract 1843.


Stable Figure 1

ERα

ERβ

aromatase

EGFR

PR
Stable Figure 2

A  ERα cytoplasmic (n=101)  
Normal: mean=3.23  
Tumor: mean=3.97

B  ERβ cytoplasmic (n=101)  
Normal: cytoplasmic mean= 3.34; nuclear mean= 6.70  
Tumor: cytoplasmic mean= 5.14; nuclear mean= 7.39

C  PR nuclear (n=101)  
Normal: mean=5.46  
Tumor: mean=4.87
Figure 3

A: Overall Survival (in years)

B: Time to Progression (in years)

C: Overall Survival (in years)

D: Time to Progression (in years)
Table 1: Patient Cohort Characteristics

<table>
<thead>
<tr>
<th></th>
<th>frequency</th>
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<tbody>
<tr>
<td><strong>Race</strong></td>
<td></td>
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<tr>
<td>African American</td>
<td>16</td>
<td>9%</td>
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<tr>
<td>White</td>
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<td>88%</td>
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<td>unknown</td>
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<td>3%</td>
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<tr>
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<tr>
<td>Female</td>
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<td>50</td>
</tr>
<tr>
<td>Male</td>
<td>91</td>
<td>50</td>
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<tr>
<td><strong>Histology</strong></td>
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<tr>
<td>Adenocarcinoma, adenosquamous</td>
<td>103</td>
<td>56%</td>
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<tr>
<td>Large cell carcinoma</td>
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<td>7%</td>
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<tr>
<td>Squamous cell carcinoma</td>
<td>62</td>
<td>34%</td>
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<tr>
<td>Small cell carcinoma</td>
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<tr>
<td>Unclassified NSCLC</td>
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<tr>
<td><strong>Stage</strong></td>
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<tr>
<td>IA</td>
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<td>IB</td>
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<tr>
<td>Ex-smoker</td>
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<tr>
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<tr>
<td><strong>Disease progression</strong></td>
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</tr>
<tr>
<td>No progression</td>
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<td>42%</td>
</tr>
<tr>
<td>Progression</td>
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<td>58%</td>
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<tr>
<td><strong>Vital Status</strong></td>
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<tr>
<td>Dead</td>
<td>125</td>
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<tr>
<td>Alive</td>
<td>58</td>
<td>32%</td>
</tr>
<tr>
<td><strong>Age at tissue collection</strong></td>
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<tr>
<td>N</td>
<td>183</td>
<td></td>
</tr>
<tr>
<td>(min, max)</td>
<td>(38,92)</td>
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<tr>
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</tr>
<tr>
<td>mean</td>
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<tr>
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<tr>
<td><strong>Follow up time</strong></td>
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</tr>
<tr>
<td>Mean</td>
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<tr>
<td>median</td>
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Table 2. Multivariable Cox proportional hazards regression of OS and TTP.

<table>
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<tr>
<th>Analysis</th>
<th>Hazard Ratio (HR)</th>
<th>95% Confidence Interval for HR</th>
<th>p-value</th>
</tr>
</thead>
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<tr>
<td>OS: High ERβ cyto (n=64) relative to low ERβ cyto (n=105)</td>
<td>1.67</td>
<td>(1.14, 2.44)</td>
<td>0.01</td>
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<tr>
<td>OS: High ERβ cyto/ low PR (n=20) relative to opposite (n=59)</td>
<td>2.64</td>
<td>(1.43, 4.87)</td>
<td>&lt;0.01</td>
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<tr>
<td>OS: High ERβ cyto/ low PR/ high EGFR (n=10) relative to opposite (n=43)</td>
<td>5.32</td>
<td>(1.93, 14.7)</td>
<td>&lt;0.01</td>
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<tr>
<td>OS: High ERβ cyto/ low PR/ high EGFR/ high Aromatase (n=6) relative to opposite (n=37)</td>
<td>6.60</td>
<td>(1.71, 25.22)</td>
<td>&lt;0.01</td>
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<tr>
<td>TTP: Low PR (n=70) relative to high PR (n=99)</td>
<td>1.58</td>
<td>(1.05, 2.37)</td>
<td>0.03</td>
</tr>
<tr>
<td>TTP: Low PR/ high ERβ cyto (n=22) relative to opposite (n=58)</td>
<td>6.02</td>
<td>(2.89, 12.54)</td>
<td>&lt;0.01</td>
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<tr>
<td>TTP: Low PR/ high ERβ cyto/ high EGFR (n=12) relative to opposite (n=42)</td>
<td>4.80</td>
<td>(1.82, 12.69)</td>
<td>&lt;0.01</td>
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<tr>
<td>TTP: Low PR/ high ERβ cyto/ high EGFR / high Aromatase (n=8) relative to opposite (n=36)</td>
<td>4.09</td>
<td>(0.94, 17.75)</td>
<td>0.06</td>
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</table>
Clinical Cancer Research

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Laura P. Stabile, Sanja Dacic, Stephanie R. Land, et al.

Clin Cancer Res  Published OnlineFirst November 9, 2010.

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