

ORIGINAL ARTICLE

Specificities of Lung Adenocarcinoma in Women Who Have Never Smoked

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Introduction: No clear data are available on the high rate of tobacco-independent lung cancer in women. We hypothesize that genetic events or hormonal factors may be partly involved.

Methods: We aimed to compare clinical, pathological, and biological characteristics of lung cancer in two cohorts of women: smokers and never-smokers. A total of 140 women (63 never-smokers and 77 former/current smokers) with adenocarcinoma, were included in this study.

Results: The never-smokers were characterized by a higher age (67 versus 58.7 years; $p < 0.0001$) and a higher frequency of lepidic features (60.3% versus 37.7%; $p = 0.008$) compared with smokers. We observed differential genetic alteration repartition in women according to their tobacco status: 50.8% of never-smokers displayed an epidermal growth factor receptor (*EGFR*) mutation versus 10.4% of smokers ($p < 0.001$). In contrast, *K-Ras* was more frequently mutated in smokers (33.8%) than in never-smokers (9.5%; $p = 0.001$). We also observed a higher percentage of estrogen receptors (ER) α expression ($p = 0.03$; and $p = 0.008$ with two different antibodies) in patients who never smoked when compared with smokers. There was no significant difference in ER β and progesterone receptors between the groups. Finally, ER α expression was correlated with the presence of an *EGFR* mutation.

Conclusions: This study suggests that when lung cancer occurs in women who have never smoked, it is more frequently associated with an *EGFR* mutation and ER α expression, with a correlation between both markers. These findings underline the possibility of treating women who have never smoked by targeting both hormonal factors and genetic abnormalities.

Key Words: Epidermal growth factor receptor, Estrogen receptor, K-Ras, Non-small-cell-lung-cancer, Tobacco.

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The incidence of lung cancer in women affects an estimated 516,000 women worldwide, of which 100,000 are in the United States and 70,000 in Europe.¹ Lung cancer is now the fourth most frequent cause of death from cancer worldwide, and the first cause in the United States and some European countries. These epidemiological data underline that cancer in women deserves specific attention.

Until now, lung cancers occurring in women have been treated similarly to lung cancers in men. However, numerous studies have highlighted different characteristics of lung cancer in women. We along with others have described the specificities of clinical and radiological presentations, pathology types, the response-to-cancer treatments, and patient outcomes in women.^{2,3} Besides these clinical observations, other research has added new data that reinforce the specificities of lung cancer in women.

Two main mechanisms have emerged from recent findings on lung carcinogenesis in women: the high prevalence of genetic alterations, such as epidermal growth factor receptor (*EGFR*) mutations,^{4,5} and the potential involvement of hormonal factors.⁶ *EGFR* seems to be more frequently mutated in women than in men, leading to a better response rate to *EGFR*-tyrosine kinase inhibitor (TKI) therapy.⁷ Recent epidemiological and clinical studies have provided evidence of a role for estrogens in the genesis and progression of lung cancer, especially non-small-cell lung tumors.^{6,8}

Preclinical studies have shown a solid rationale for the crucial involvement of hormones in lung carcinogenesis. Many hormonal receptors, such as ER α , ER β (estrogen receptors α and β), and PR (progesterone receptors) have been isolated from lung cancer tissues. We have recently reported a higher expression of ERs in women operated on for lung cancer compared with men.⁹ Moreover, ERs have been shown to be involved in the onset of lung cancer in cells and animal models.¹⁰ Last, the interaction of ERs with growth factor receptor signaling, including *EGFR*, has been demonstrated.¹¹ A direct correlation between both pathways has been also suggested by two recent studies,^{12,13} indicating that lung cancer

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treatment could include both EGFR- and hormone-targeting drugs.¹⁴

Tobacco smoking is the main cause of lung cancer. However, lung cancer also occurs in people who have never smoked, and it ranks as the seventh most common cause of cancer death worldwide.^{15,16} Lung cancer in never-smokers is more frequently observed in women, representing 20% to 70% of these cases, according to geographical origin and patient selection.¹⁷ Lung cancer in never-smokers is also characterized by a higher rate of gene mutations, involving *EGFR*, *HER2*, or *PI3K*, and of *EML4-ALK* translocations. We have recently identified, in a national collaborative study, other genetic abnormalities in this population.¹⁸ Nevertheless, no clear data are available to improve our understanding of the higher rate of tobacco-independent lung cancer in women.

We hypothesize that genetic events or hormonal factors may be part of this observation. Herein, we have aimed to compare clinical and pathological characteristics of lung cancer in two cohorts of women smokers and women never-smokers, with a specific focus on driver oncogenes and hormonal receptors.

PATIENTS AND METHODS

Patients and Tissues

Tumor specimens were collected from 50 women who underwent surgery at the Thoracic Oncology Department (Toulouse University Hospital, France; this comprised the Ligue collection). A second collection of 90 tumor samples was collected from the Lung Genes (LG-collection) study, which involved 13 centers in France.¹⁸ Only women were included in this study, and all patients had been treated with surgery.

We collected specific tobacco-exposure status from all women by checking each patient's file and subsequently performing a telephone- or mail-based survey. The 63 patients who had never smoked were defined according to current guidelines¹⁹ as persons with lifetime exposure of less than 100 cigarettes.

A lung cancer pathologist assessed the diagnoses by applying the latest World Health Organization classification,²⁰ and the clinicopathological stage was assigned according to the tumor, node, metastasis (TNM) classification.²¹ Patients who were included before the last classification were reclassified, especially for lepidic components. All patients signed an informed consent permitting analysis of tissues. Patients were treated and follow-up was done at our institutions, to ensure collection of clinical data.

Immunohistochemistry

We selected the most relevant antibodies^{8,10,13,22,23} from the literature (Fig. 1 and Table 1). We chose the N-terminal ER α (1D5 clone DAKO) and the PR (PgR 636 clone DAKO) used in breast carcinoma because of their therapeutic implications. A C-terminal α -ER monoclonal antibody (clone sc-8002 F10; Santa-Cruz Biotechnology, Dallas, TX) and an ER β polyclonal rabbit antibody (Biogenex, Fremont, CA) were also chosen because of their supposed prognostic implications.⁹

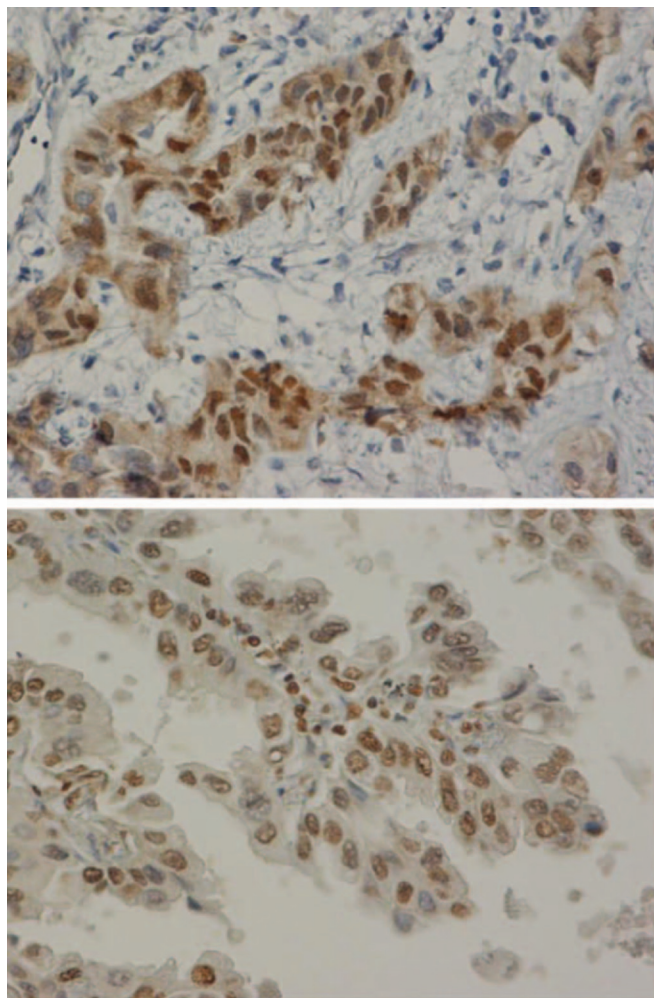


FIGURE 1. Immunohistochemistry of hormonal tumor staining (scale magnitude $\times 400$). A, ER α staining of lung tumor tissue ($\times 400$) (F10). Nucleus score is +++, cytoplasm score is ++; (B), ER β staining of lung tumor tissue ($\times 400$). ER β score is 6 (A). ER, estrogen receptor.

Samples (5- μ m sections) of lung tumor tissue were mounted on positively charged slides and dried for 1 hour at 60°C before they underwent immunohistochemical assays for ER α , ER β , and PR expression. We used the PT link pretreatment module (DAKO, Carpinteria, CA), which allows the entire pretreatment process of deparaffinization, rehydration, and epitope retrieval to be combined in a three-in-one specimen-preparation procedure.

The slides were treated with 3% hydrogen peroxide to block endogenous peroxidase activity. Nonimmune serum was used to block nonspecific staining. Antigen retrieval was achieved by proteinase K digestion for 10 minutes, and the primary antibody was applied at a dilution of 1:50, and then incubated for 2 hours at room temperature. Biotinylated secondary antimouse antibodies (1:100) were applied to the sections for 20 minutes. Visualization was obtained, using the labeled streptavidin-biotin staining method (LSAB kit; DAKO). We then evaluated staining intensity, its nuclear or

TABLE 1. Antibody Staining and Scores

ERα 1D5 and F10, Breast Cancer Score	0	0%–10% of Tumoral Cells Stained	
	1	>10% of tumoral cells stained	
ERα 1D5 nucleus intensity	0	No staining	
	+	Low staining	
	++	Medium staining	
	+++	High staining	
ERα 1D5 cytoplasm score	0	0%–10% of tumoral cells stained	
	1	10–50% of tumoral cells stained	
	2	>50% of tumoral cells stained	
ERα 1D5 cytoplasm intensity	0	No staining	
	+	Low staining	
	++	Medium staining	
	+++	High staining	
ERα F10 nucleus score	0	0%–10% of tumoral cells stained	
	1	>10% of tumoral cells stained	
ERα F10 nucleus intensity	0	No staining	
	+	Low staining	
	++	Medium staining	
	+++	High staining	
ERα F10 cytoplasm intensity	0	No staining	
	+	Low staining	
	++	Medium staining	
	+++	High staining	
ERβ score % of stained nucleus	1	0%–1% of nucleus staining in tumoral cells	
	2	1%–10%	
	3	10%–30%	
	4	30%–70%	
	5	70%–100%	
ERβ score intensity staining	0	No staining	
	1	Low staining	
	2	Medium staining	
	3	High staining	
Score of 0–8 = Score % of Stained Nucleus + Score Intensity Staining	0	C	Low expression
ERβ score	1	C	Low expression
	2	C	Low expression
	3	C	Low expression
	4	B	Medium expression
	5	B	Medium expression
	6	A	High expression
	7	A	High expression
	8	A	High expression

ER, estrogen receptor.

cytoplasmic localization, and the proportion of positively stained cells. We used semiquantitative scores for nuclear and cytoplasmic staining: that is, mild (+), moderate (++), and high (+++).

For all hormonal receptors, we applied the same score as used in breast cancer, with a 10% positive-cell threshold.²⁴ However, for estrogen,β receptors there is no currently validated score, so we applied a published *proportion score*.²³

For this score, we used a high-score class (6–8) and a low-score class (0–4) by adding the proportion of positively stained cells (five scores from 1–5) and one of the three intensity scores (1–3).

Molecular Analysis of *EGFR* and *K-Ras*

For *EGFR* and *K-Ras*, direct sequencing was performed after polymerase chain reaction amplification of

EGFR exons 18, 19, 20, 21 (NM_005228.3), and *K-Ras* exons 2 and 3 (NM_033360.2). Purified DNA was sequenced using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Sequencing reactions were analyzed on a 16-capillary ABI3130 or a 48-capillary 3730 DNA analyzer in both sense and antisense directions, from at least two independent amplifications. Sequence reading and alignment were performed with SeqScape software (Applied Biosystems).

Statistics

Continuous variables are presented by their means \pm standard deviations. We first described and compared the patients' characteristics between our local series and the national collection. Second, we tested whether the association between smoking status and the patients' characteristics depended on where the data were collected from; we did not observe any significant interaction between the patients' characteristics and the place of collection. We subsequently decided to pool data from both these series for the final analysis. The patients' characteristics were compared between smokers and nonsmokers. The χ^2 or Fisher's exact test was used to compare categorical variables, and Student's *t* test or a nonparametric Wilcoxon test was used for continuous variables. We estimated Spearman's correlation coefficients (\tilde{r}) between each biological characteristic and tested the null hypothesis ($\tilde{r} = 0$) using a Benjamini–Yekutieli correction for multiple tests. All analyses were conducted using Stata SE version 11.2.

RESULTS

Clinical and Pathological Characteristics of Lung Cancer in Women Never-Smokers

A total of 140 women with adenocarcinoma (AC) were included in this study. Of these, 63 were never-smokers and 77 were former or current smokers. We double-checked this status by collecting data from the patients' files and by sending a specific survey to each patient to ensure that she fulfilled the abovementioned criteria. We compared both groups (Table 2) and did not observe any significant differences regarding either TNM status or tumor stage. In contrast, we observed a higher average age at disease appearance for women who never smoked (67 years) compared with smokers (58.7 years; $p < 0.0001$). In addition, lepidic features were more frequent in the never-smokers (60.3% versus 37.7%; $p = 0.008$).

Genetic Characteristics of Lung Cancer in Women Never-Smokers

For all patients, we assessed the mutational status of *EGFR* (exon 19 and 21) and *K-Ras* (exon 2). Patients with a mutated *EGFR* represented 28.6% of the overall population in this study. More precisely, 50.8% of never-smokers displayed the mutation compared with 10.4% of former or current smokers ($p < 0.001$; Table 3). In contrast, the *K-Ras* mutation was more frequent in smokers (33.8%) compared with never-smokers (9.5%; $p = 0.001$) (Fig. 2).

Hormone-Receptor Expression in Women Never-Smokers

We analyzed the ER α , ER β , and the PR for all patients using immunohistochemistry. For all markers, we analyzed the percentage of positive cells and the intensity of staining in both cytoplasm and the nucleus.

We analyzed ER α expression according to the breast cancer testing procedure, as previously described,⁹ but we also extended our analysis to include other parameters, such as cytoplasm expression and intensity staining. Moreover, we used two different antibodies directed against ER α , as described in the literature⁹: ER α 1D5 (epitopes NH2 terminal) used in breast cancer diagnosis and ER α F10sc8002 (epitopes COOH terminal)^{9,13} (Table 1).

For the breast cancer–derived procedure (threshold of 10% nucleus staining with 1D5 antibody), 11.1% of the never-smokers and 3.9% of smokers ($p = 0.11$) had a positive score (Table 3). We hypothesize that this score might be inappropriate for lung cancer, so we also looked for other parameters. We found that both the 1D5 nucleus and the intensity of cytoplasm staining were significantly higher in never-smokers versus smokers ($p = 0.008$ and $p = 0.03$, respectively). Using the ER α F10 antibody, we observed a higher percentage of positive tumor samples (as for the breast cancer score) for never-smokers compared with smokers, 23.8% versus 10.4%, respectively ($p = 0.03$). In summary, we observed significant differences in nucleus-intensity staining with both antibodies for ER α according to the tobacco status of women with NSCLC (Table 3 and Fig. 2).

For ER β we used a polyclonal rabbit antibody.²³ We observed 100% expression of ER β in never-smokers compared with 92% in smokers, but ER β nuclear expression was not significantly different ($p = 0.12$). Last, we analyzed PR expression and found a very low rate of expression in both populations, with no significant differences (Table 3).

Correlations between Biological Data

We found a negative correlation between the *K-Ras* and *EGFR* mutations in this female population. Both mutations

TABLE 2. Clinical and Pathological Characteristics of Women Who Have Never Smoked ($n = 63$) Versus Female Smokers ($n = 77$)

	Never Smoked	Smokers	<i>p</i>
% of total	45.0%	55.0%	—
Age (average) (yr)	67.0 \pm 9.7	58.7 \pm 9.8	<0.0001
Stage as % of total			
IA	23.8%	29.9%	0.35
IB	39.7%	22.1%	—
IIA	3.2%	2.6%	—
IIB	9.5%	10.4%	—
IIIA	19.0%	23.4%	—
IIIB	4.8%	10.4%	—
IV	0.0%	1.3%	—
Lepidic component (%)	60.3%	37.7%	0.008
Tumor size (average, mm)	33.9 \pm 15.4	36.1 \pm 22.2	0.85

TABLE 3. Biological Characteristics of Women Who Have Never Smoked ($n = 63$) Versus Female Smokers ($n = 77$)

	Never Smoked	Smokers	<i>p</i>
<i>EGFR</i> mutation as % of each subgroup	50.8%	10.4%	<0.001
<i>K-Ras</i> mutation as % of each subgroup	9.5%	33.8%	0.001
ER α nucleus (1D5), % positive/ breast score	11.1%	3.9%	0.11
ER α nucleus (1D5), staining intensity,			
0	69.8%	89.6%	0.008
+	20.6%	9.1%	—
++	9.5%	1.3%	—
+++	0.0%	0.0%	—
ER α nucleus (F10), % positive/ breast score	23.8%	10.4%	0.03
ER α nucleus (F10), staining intensity,			
0	54.0%	75.3%	0.04
+	28.6%	18.2%	—
++	14.3%	5.2%	—
+++	3.2%	1.3%	—
ER β score			
0	0.0%	7.8%	0.02
1–8	100%	92.2 %	
ER β nucleus staining intensity			
0	50.8%	38.2%	0.12
+	38.1%	38.2%	—
++	11.1%	23.7%	—
+++	0.0%	0.0%	—
PR nucleus % positive/score	1.6%	0.0%	0.45
PR nucleus staining intensity			
0	85.7%	92.2%	0.25
+	7.9%	5.2%	—
++	0.0%	1.3%	—
+++	0.0%	0.0%	—

EGFR, epidermal growth factor receptor; ER, estrogen receptor; PR, progesterone

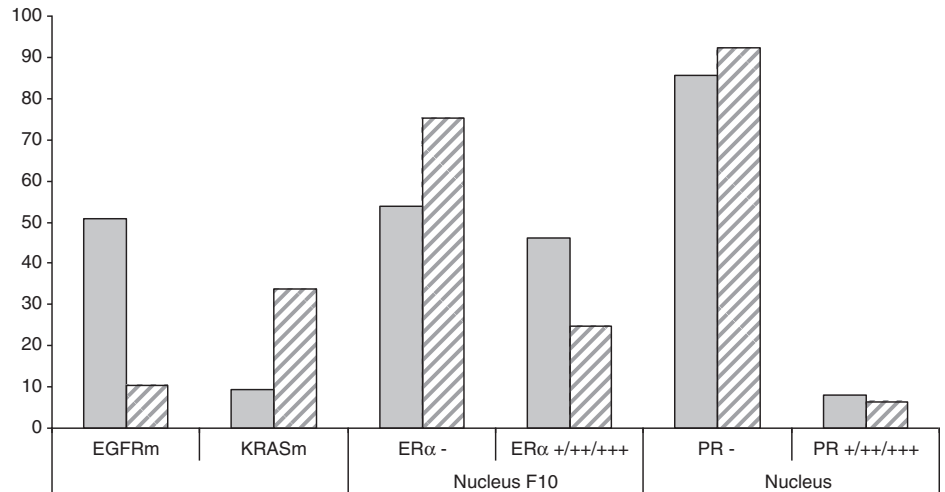
remain mutually exclusive for all patients. Interestingly, ER α nucleus-intensity staining (of both antibodies) correlated significantly with the *EGFR* mutation status of all the women in the study (Table 4). We did not find any correlation between biomarkers according to smoking status (data not shown).

DISCUSSION

Herein, we have aimed to identify the specificities of lung cancer in women. We first report that lung cancer was similar regarding TNM status at the time of diagnosis. We found that lung cancer appeared later in women who had never smoked than in women who smoked. These data are consistent with a recent study performed in France on primary lung cancer.²⁵ An explanation for this might be that women are more sensitive to tobacco than men, and that lung cancer occurs earlier in this population. The diagnostic age of 58.7 years for women smokers is lower than the median age observed for men smokers. We also observed in our former study that women displayed lung cancer at 58 years (compared with 61.2 years for men).⁹ In contrast, women not exposed to a carcinogen such as tobacco developed lung cancer later.

We looked at the genetic profiles of both our populations and found that lung cancer had mutated *EGFR* more frequently and mutated *K-Ras* less frequently in women who had never smoked. These results are in line with already published data. *EGFR* is known to be mutated in 14% of women smokers compared with 55% who had never smoked.²⁶ Moreover, *EGFR* is more frequently mutated in never-smokers.²⁷ Overall, women who had never smoked represented a population characterized by a high probability of *EGFR* mutation. This finding is associated with a better response to *EGFR-TKI* as reported in many recent trials.^{7,28} Interestingly, we observed that *EGFR* was mutated in 10% of female smokers, suggesting that screening for *EGFR* mutations should not be limited to never-smokers. *K-Ras* was mutated in more than 33% of female smokers (whereas only 9.5% women who had never smoked were affected). *K-Ras* is known to be mutated by tobacco carcinogens, and remains difficult to inhibit by targeted therapy, but drugs such as mitogen-activated ERK kinase inhibitors are currently being tested.²⁹ Overall, these results show that more than 50% of women displayed either *EGFR* or *K-Ras* mutations that can be targeted by dedicated drugs. It would be of interest to obtain information on other biomarkers, such as

FIGURE 2. Biomarker incidence in women who have never smoked (shaded) versus women smokers (lined). $p < 0.001$ for *EGFR* mutation, $p = 0.001$ for *K-Ras* mutation, and $p = 0.008$ for *ERα*. *EGFR*, epidermal growth factor receptor; *ER*, estrogen receptor; *PR*, progesterone receptors.



Her2, *BRaf*, *Pi3K*, and *EML4-ALK*, which are known to be deregulated in lung cancer. Unfortunately, we were not able to test these biomarkers at the time of this study.

We found higher expression of both *ERα* and *ERβ* in female never-smokers. *ERs α* and *β* are expressed in normal lung cells (cultured normal lung fibroblast cell lines and normal bronchial epithelial cells) and in the lung tumors of both men and women.¹⁰ Several in vitro and in vivo studies have provided evidence supporting a biological role for estrogens in lung carcinogenesis by directly promoting cell proliferation. Estrogens stimulate the proliferation of NSCLC through *ER*-mediated signaling, whereas antiestrogens inhibit the growth of NSCLC cells.^{10,14} We, along with others, have previously reported that *ERα*, and to a lesser extent, *ERβ*, was more frequently overexpressed in women than in men, and in never-smokers than in smokers. Nose et al.¹² reported a clear correlation between *ERβ* and the nonsmoking status ($p < 0.001$) in univariate analysis. Nevertheless, the multivariate analysis failed to show any statistical difference for *ERβ* expression according to the sex ($p = 0.378$) and the smoking status ($p = 0.294$). However, the difference in *ER*

expressions according to the smoking status of women has not been addressed by the authors. We report here for the first time the difference between female never-smokers and smokers.^{9,13} These data suggest that oncogenesis in never-smokers relies more frequently on hormonal pathways.

There are many reports on the immunohistochemical expression of hormonal receptors. Most data are conflicting because of two *ER* isoforms (α and β), the presence of truncated forms of *ERα*, the range of antibodies used, and the absence of a validated threshold or score. Thus, we selected specific antibodies that targeted both COOH-terminal and NH₂-terminal *ERα* and *ERβ*. We also used previously published scores; for *ERα*, we applied the same scores used in breast cancer, with a 10% positive-cell threshold and distinguished *ER* expression in the cytoplasm and the nucleus. Last, we used two antibodies for *ERα* staining, 1D5, targeting N-terminal part of the receptor, and F10, targeting the COOH part of the receptor. We first applied the breast cancer score with both antibodies and found a statistical difference by using 1D5 antibody and not the F10 antibody. Looking at nuclear intensity staining, we observed a statistical difference for both antibodies. Overall, our data suggest that a different score should be used in lung cancer when comparing it with breast cancer and that an antibody targeting its COOH part is probably more potent in identifying *ERα* expression in lung cancer. For *ERβ*, there is currently no validated score, and we used a proportion score.²³ Establishment of a dedicated score for *ER* in lung cancer is warranted to ensure homogeneity between data and studies.

Stabile et al.¹² reported that *EGFR* protein expression was up-regulated in response to antiestrogens in vitro, and that *ERβ* expression was decreased in response to epidermal growth factor and increased in response to gefitinib. In addition, in line with our results, two previous studies also reported an association between *ERα* and *ERβ* expression and *EGFR* mutations. Raso et al.¹³ reported the correlation between *EGFR* mutation and *ERα* expression in a population of ACs, 27 (in both men and women) of which were found with mutations. Nose et al.¹² also reported the correlation between *ERβ* expression and *EGFR* mutation in a large population of ACs. To date, our work is the largest study showing this interaction

TABLE 4. Biomarker Correlations for all Women in the Trial

	Mutant <i>EGFR</i>	Mutant <i>K-Ras</i>
<i>EGFR</i> mutation % of total	1	—
<i>K-Ras</i> mutation	-0.35	1
<i>ERα</i> nucleus (1D5), % positive/breast score	0.01	-0.02
<i>ERα</i> nucleus (1D5), staining intensity	0.28	-0.13
<i>ERα</i> nucleus (F10), % positive/ breast score	0.23	-0.11
<i>ERα</i> nucleus (F10), staining intensity	0.30	-0.09
<i>ERβ</i> score	-0.13	-0.09
<i>ERβ</i> nucleus staining intensity	-0.17	-0.06
<i>PR</i> nucleus % positive/score	-0.05	0.16
<i>PR</i> nucleus staining intensity	0.10	0.03

Bold font shows a significant correlation, Benjamini–Yekutieli: $p < 0.001$.

EGFR, epidermal growth factor receptor; *ER*, estrogen receptor; *PR*, progesterone receptor.

in a specific population. Recently, Garon et al.³⁰ reported the potential synergy of EGFR inhibitors and ER antagonists on preclinical models. Traynor et al.³¹ reported the result of a pilot study where postmenopausal women diagnosed with NSCLC were treated with gefitinib and fulvestrant. An ongoing clinical trial (NCT01556191) in France is testing the combination of EGFR inhibitors and fulvestrant.

Overall, our study suggests that lung cancer in women who have never smoked is more frequently associated with EGFR mutations and ER α overexpression and that both biomarkers are associated. These findings underline the possibility of treating women who have never smoked with drugs to target hormonal factors, genetic abnormalities, or both.

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