The significance of estrogen receptor β in 301 surgically treated non-small cell lung cancers

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Objective: Estrogen receptor expression in lung cancer has been understudied, particularly in light of its potential biologic importance in the epidemic of lung cancer in women. The expression of estrogen receptors was investigated to better understand the possible role of sex hormones in lung cancer.

Methods: A total of 301 patients with surgically resected non-small cell lung cancers of stages I to III were explored. Sections of paraffin-embedded tumor samples were stained with estrogen receptor α and estrogen receptor β antibodies. Tumors with moderate-to-strong nuclear staining in at least 50% of the tumor cells were scored as positive for overexpression.

Results: The overall frequency of overexpression for estrogen receptor β was 45.8% (138/301). It was detected most frequently in female patients (in 54.3% of 127 tumors vs 39.7% of 174 tumors in men, $P = .012$). However, there was no estrogen receptor α nuclear staining detectable in non-small cell lung cancers. Interestingly, a significant correlation between estrogen receptor β expression, stage of disease, grade of differentiation, smoking status, vascular invasion, and survival in patients with stage II and III disease was found. By using multivariate analysis of survival among patients with stage II and III disease, estrogen receptor β overexpression, stage II tumor, well differentiation, nonsmoking status, and lack of vascular invasion were significantly favorable prognostic factors.

Conclusions: The results presented here show for the first time that immunohistochemical expression of estrogen receptor β can be used as a prognostic indicator in patients with surgically resected stage II and III non-small cell lung cancers. These observations might offer a possibility for hormonal therapy in patients with lung cancer.

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Lung cancer is one of the leading causes of cancer death in Taiwan¹ and the most common cause of cancer death in Western countries.² With the 5-year survival rate at only about 10%, lung cancer causes more than 6000 deaths per year in Taiwan. The majority of these tumors are non-small cell lung carcinomas (NSCLCs), of which adenocarcinoma is the most common histologic type.^{3,4}

Although cigarette smoking is the major risk factor, most people who smoke do not have lung cancer. Only approximately 3% of lung cancers occurred in nonsmokers⁵ in the Western world, and the majority of female patients with lung cancer were nonsmokers in Taiwan.⁶ Therefore factors other than cigarette smoking must also have a role in the pathogenesis of lung cancer. Such factors might be environmental (water and air pollution or occupational exposure) or host related (genetic or nutritional).

Sex hormones might be a host-related factor that influences the development of lung cancer in men and women. Estrogen receptors (ERs) are normally found in the breast and tissues of the female reproductive tract. Additionally, they are sometimes found in the neoplasms of these tissues.⁷ The existence of ERs has also been

demonstrated in a wide array of neoplasms, including meningiomas and primary brain tumors, malignant melanomas, sarcomas, carcinomas of the head and neck, thyroid gland, lungs, liver, pancreas, stomach, colon, and prostate gland.⁸

The human ER, now termed ER α , was first cloned in 1986. For the next 10 years, research on its ligand-dependent effects focused on the binding of estrogen to one cognate receptor.⁹ In 1996, a second isoform of ER, referred to as ER β , was discovered, which redefined concepts of the estrogen signaling pathway.¹⁰ The ER β protein has similarities to the classical ER α in terms of structure and function. The tissue distribution of these 2 receptors is not identical, but it appears to overlap in some cases.¹¹ ER α and ER β also differ in their effect on transcription at activator protein-1 (AP-1) sites.¹² Whereas ER α as a ligand to estradiol activates transcription at AP-1 sites, ER β as a ligand to estradiol inhibits transcription at AP-1 sites. This evidence suggests that ER α and ER β might be regulated by distinct mechanisms and play different roles in gene regulation, although sharing functional characteristics. Thus far, only limited data are available on the activity and expression of ERs in human lung neoplasms. Because there have been conflicting results in the literature, possibly as a result of the small number of tumors studied, the role of ERs has remained obscure.^{8,13-19}

The evolution from nonselective methods of detection of ERs (biochemical assay) to a more reliable method making use of monoclonal antibodies (immunohistochemical assay) has allowed the identification of the receptors in their functional site, the nucleus. The immunohistochemical assay is more selective and specific because of the use of monoclonal antibodies reacting with epitopes that offer only minimal variability. In the present study, the expression of ER α and ER β , as determined by means of immunohistochemistry, in 301 surgically resected NSCLCs was investigated. These findings were correlated with the clinicopathologic features, including sex, smoking history, histologic type and differentiation, tumor size, stage, vascular invasion, lymph node metastasis, and survival.

Materials and Methods

Patients and Tumor Specimen

Three hundred one NSCLC specimens were obtained from patients who underwent complete surgical resection at National Taiwan University hospital between January 1990 and December 2001. The study was approved by the Research Ethics Committee of this hospital. These patients were not treated with neoadjuvant chemotherapy and irradiation therapy. All specimens were formalin fixed and sectioned for microscopic examination after applying hematoxylin and eosin stain. Histologic diagnosis and pathologic features were obtained, including tumor cell type, degree of differentiation, vascular invasion, and regional lymph node metastasis. Pathologic staging was performed according to the international staging system for lung cancer,²⁰ which was based on tumor size, location and involvement, and the presence of lymph node metastasis. Adenosquamous carcinomas were not included in the clas-

sification of histologic type and grade of differentiation because of the discrepancy in each tumor. The patient numbers in some parameters might be less because the information was not available in a few cases. The women were grouped into 2 categories on the basis of the average US menopausal age of 51 years, as defined by the American College of Obstetricians and Gynecologists. Premenopausal was defined as women aged less than 51 years and postmenopausal was defined as women aged at least 51 years.²¹ The follow-up period ranged from 2 to 168 months (median survival, 41 months).

Immunohistochemistry

For immunohistochemical demonstration of ER β or ER α expression in the tumor tissue, 4- μ m-thick sections from each formalin-fixed, paraffin-embedded tissue block were dewaxed with xylene and rehydrated through a graded series of ethanol.

The sections for immunohistochemistry of the ER β and ER α expression were autoclaved in 0.01 mol/L citrate buffer (pH 6.0) at 121°C for 10 minutes (ER β) and in EDTA buffer (pH 8.0) for 8 minutes (ER α). They were treated with 3% H₂O₂-methanol solution to reduce endogenous peroxidase activity. These were then incubated with normal goat serum to reduce nonspecific antibody binding and were subsequently subjected to the primary antibody reaction. The antibodies for ER β (1:100, BioGenex) and ER α (1:30, BioGenex) were left to react with the sections overnight (ER β) and for 2 hours (ER α) at room temperature. Detection of the immunoreactive staining was carried out by using the avidin-biotin-peroxidase complex method, according to the manufacturer's instructions (Dako Corp). The primary antibody was replaced with bovine serum albumin to check for nonspecific staining by the avidin-biotin-peroxidase complex detection system. The sections were then subjected to a color reaction with 0.05% 3,3-diaminobenzidine in 0.05 mol/L Tris base (pH 7.6) containing 0.01% H₂O₂ and were lightly counterstained with hematoxylin.

Assessment of ER Expression

By using an infiltrating ductal carcinoma of the breast with a high content of ER β and ER α as a positive control, the expression of ER β and ER α for each tumor was assessed. Tumors were considered to be positive when there was moderate-to-strong nuclear staining of more than 50% of the neoplastic cells. Two independent pathologists (C-TW and Y-LC) were involved in the assessment of expression.

Statistical Analysis

The correlation between various clinical or pathologic parameters and the expression of ER β was analyzed by using the Pearson χ^2 test. Survival curves were estimated by using the Kaplan-Meier method. The log-rank test was used to compare survival curves. Cox proportional hazards regression was used to model survival with ER β overexpression and clinicopathologic variables typically associated with prognosis. The following factors were modeled in a univariate fashion: sex (male or female); histologic type (squamous cell carcinomas or bronchioloalveolar carcinomas and adenocarcinomas); tumor size (>3 cm or $\leq$ 3 cm); stage (I, II, and III); grade of differentiation (well, moderately, or poorly differentiated); smoking status (smokers or nonsmokers); vascular invasion (absent or present); and ER β overexpression (positive or negative).

TABLE 1. Relationship between patient demographics and smoking history

Parameters	No. of patients	No. of patients with smoking history	Smokers (%)	Nonsmokers (%)	P value
Total patients	301				
Sex					
Male	174	164	120 (73.2)	44 (26.8)	<.001
Female	127	126	11 (8.7)	115 (91.3)	
Histologic type					
Squamous cell carcinoma	90				
Male	77	72	65 (90.3)	7 (9.7)	<.001
Female	13	13	4 (30.8)	9 (69.2)	
Adenocarcinoma	194				
Male	85	80	49 (61.3)	31 (38.8)	<.001
Female	109	108	7 (6.5)	101 (93.5)	
Adenosquamous carcinoma	17				
Male	12	12	6 (50)	6 (50)	.049
Female	5	5	0 (0)	5 (100)	
Age (y)					
≤50	41	38	12 (31.6)	26 (68.4)	.071
>50	260	252	119 (47.2)	133 (52.8)	

A multivariate Cox proportional hazards regression model was fit with those variables that demonstrated statistical significance ($P < .050$) in the univariate models. SPSS version 10.0 software (SPSS Inc) was used for all analyses.

Results

Patient Demographics

Of the 301 patients in this study, 174 were men, and 127 were women. Their ages ranged from 30 to 83 years, with a mean age of 63 years. All patients underwent complete surgical resection with regional lymph node dissection.

The majority of the male patients were smokers (120/164 [73.2%]), whereas the majority of the female patients were nonsmokers (115/126 [91.3%], $P < .001$). There were 90 squamous cell carcinomas, 194 adenocarcinomas, and 17 adenosquamous carcinomas. Among patients with squamous cell carcinomas, most of the male patients were smokers (65/72 [90.3%]), whereas the female patients were predominantly nonsmokers (9/13 [69.2%], $P < .001$). Among patients with adenocarcinomas, most of the male patients were smokers (49/80 [61.3%]), and 101 (93.5%) of 108 female patients were nonsmokers. This difference was also statistically significant ($P < .001$, Table 1).

Correlations Between ERβ Expression and Clinicopathologic Features

In the current study, ERβ nuclear reactivity was detected in 138 (45.8%) of the 301 lung cancers, usually of moderate-to-strong intensity. The remaining 163 (54.2%) lung cancers were characterized by complete absence of nuclear staining or 50% or less immunoreactivity. The relationship between clinical parameters or pathologic characteristics and the

frequency of ERβ reactivity is shown in Table 2. Higher ERβ expression was noted in female patients, nonsmokers, patients with tumors of 3 cm or smaller, patients without lymph node metastasis, and patients with tumors with a lower grade of differentiation.

Overexpression of ERβ was significantly more common in tumors occurring in nonsmokers (53.5%) than in those of smokers (36.6%, $P = .004$). Among the nonsmokers, higher ERβ expression was observed more frequently in female patients (58.3%) than in male patients (40.9%), and such a difference was also statistically significant ($P = .050$). In both squamous cell carcinoma and adenocarcinoma (including bronchioloalveolar carcinoma), the overexpression of ERβ was observed preferentially in well-differentiated subtypes ($P < .001$).

Differences in ERβ expression were not statistically significant ($P = .579$) between the premenopausal and postmenopausal groups of female patients.

Correlations Between ERβ Expression, Clinicopathologic Features, and Survival

The influence of various clinicopathologic parameters on overall patient survival was analyzed (Table 3). By using univariate analysis of survival, significantly better prognostic indicators included tumors of 3 cm or smaller, early-stage tumors, tumors with a lower grade of differentiation, tumors without vascular invasion, and increased levels of ERβ expression. By using multivariate analysis, early-stage tumors, tumors with a lower grade of differentiation, and tumors without vascular invasion remained as statistically significant prognostic factors indicating better survival.

TABLE 2. Frequency of ER β expression with relation to clinical parameters and pathologic characteristics

Parameters	No. of patients*	ER β >50% (%)	ER β $\leq$ 50% (%)	P value
Patient no.	301	138 (45.8)	163 (54.2)	
Menopausal age (y)				
$\leq$ 50 (premenopausal)	20	12 (60.0)	8 (40.0)	.579
>50 (postmenopausal)	107	57 (53.3)	50 (46.7)	
Sex				
Male	174	69 (39.7)	105 (60.3)	.012
Female	127	69 (54.3)	58 (45.7)	
Smoking history				
Positive	131	48 (36.6)	83 (63.4)	.004
Negative	159	85 (53.5)	74 (46.5)	
Histologic type†				
Squamous cell carcinoma	90	37 (41.1)	53 (58.9)	.163
Adenocarcinoma	194	97 (50.0)	97 (50.0)	
Tumor size				
$\leq$ 3 cm	127	68 (53.5)	59 (46.5)	.022
>3 cm	174	70 (40.2)	104 (59.8)	
Tumor stage				
I	114	61 (53.5)	53 (46.5)	.112
II	67	27 (40.3)	40 (59.7)	
III	120	50 (41.7)	70 (58.3)	
Vascular invasion				
Positive	70	34 (48.6)	36 (51.4)	.602
Negative	231	104 (45.0)	127 (55.0)	
Lymph node metastasis				
Positive	168	66 (39.3)	102 (60.7)	.010
Negative	133	72 (54.1)	61 (45.9)	
Grade of differentiation†				
Well	106	66 (62.3)	40 (37.7)	<.001
Moderate	149	59 (39.6)	90 (60.4)	
Poor	29	9 (31.0)	20 (69.0)	

*Some of the patients lack information. †Adenosquamous carcinomas are not included.

These patients were then divided according to their tumor stage. In patients with stage I disease, there were no significant prognostic indicators, including ER β overexpression, determined by using univariate analysis of survival. A total of 187 patients had stage II and III tumors. An analysis of the effect of various clinicopathologic factors on patient survival was performed for these patients with stage II and III disease (Table 4). By using univariate analysis, stage II tumors, tumors with a lower grade of differentiation, non-smoking status, tumors without vascular invasion, and increased expression of ER β were found to be significantly better prognostic indicators. By using multivariate analysis, all 5 factors remained statistically significant favorable prognostic indicators (Figure 1).

ER α Expression

In contrast, there was no ER α nuclear staining in any NSCLCs. Only 8 cases demonstrated cytoplasmic staining. The staining intensity of most cases was weak.

Discussion

The presence of ERs in human lung cancers has been controversial for many years. Some studies have been performed to assess the expression of ERs in human lung cancers to explain sex differences in incidence, histology, and prognosis of human NSCLCs between female and male patients.^{5,8,13,14,22} However, reported values have varied widely, and the involvement of this receptor in lung physiology, pathology, or both remains unclear.

ERs, members of the nuclear steroid receptor superfamily, mediate cellular response to estrogen. Two ERs have been identified, ER α and ER β , which are encoded by separate genes. ER β is localized on human chromosome 14, whereas ER α is located on chromosome 6.²³ These proteins function as sequence-specific, ligand-activated transcription factors. Both receptors possess several distinct functional domains, including a central DNA-binding domain that is highly conserved between ER α and ER β .²⁴ These functional differences could be due to the dissimilarities in

TABLE 3. Correlations between ER β expression, clinicopathologic features, and survival in 301 patients with stage I to III disease

Variables	No. of patients*	No. of deaths	Univariate			Multivariate		
			Hazard ratio	95% CI	P value	Hazard ratio	95% CI	P value
ER β overexpression								
Negative	163	84	1.000			1.000		
Positive	138	55	0.672	0.478-0.945	.022	0.719	0.504-1.026	.069
Size								
≤3 cm	127	48	1.000					
>3 cm	174	91	1.776	1.248-2.526	.001			
Stage								
I	114	21	1.000			1.000		
II	67	33	3.635	2.082-6.349	<.001	3.084	1.732-5.489	<.001
III	120	85	6.870	4.201-11.236	<.001	6.021	3.577-10.136	<.001
Differentiation†								
Well	106	35	1.000			1.000		
Moderate	149	75	1.984	1.322-2.977	.001	1.372	0.906-2.079	.136
Poor	29	19	3.229	1.837-5.676	<.001	2.895	1.630-5.142	<.001
Vascular invasion								
Negative	231	93	1.000			1.000		
Positive	70	46	2.355	1.644-3.373	<.001	1.749	1.186-2.580	.005
Sex								
Female	127	52	1.000					
Male	174	87	1.387	0.983-1.958	.063			
Smoking history								
Negative	159	66	1.000					
Positive	131	66	1.403	0.997-1.974	.052			
Histologic type†								
Squamous cell carcinoma	90	45	1.000					
Adenocarcinoma	194	84	0.778	0.539-1.121	.177			

*Some of the patients lack information. †Adenosquamous carcinomas are not included.

the N-terminal domains, suggesting that these 2 receptors interact with different sets of proteins. The tissue distribution of ER α is also different from that of ER β , suggesting that these proteins function in a tissue-specific manner.¹¹

ER β has been shown to be expressed and functional in most human NSCLC cell lines and cells derived from normal lung and tissues.^{2,18,19} Most investigations have been performed at the mRNA expression level with reverse transcriptase–polymerase chain reaction analysis; however, very few studies have applied immunohistochemistry.²⁵ In the present study a positive correlation between ER β protein overexpression and female sex, nonsmoking status, tumors of 3 cm or smaller, absence of lymph node metastasis, and tumors with a lower grade of differentiation was noted. We found positive ER β expression in squamous cell carcinomas and adenocarcinomas. Although it has been recognized that ER β expression is found to be higher in tumors from women¹⁵ and adenocarcinomas,¹⁸ other authors have also reported positive ER expressions in both squamous cell carcinomas and adenocarcinomas.^{22,26,27}

Bronchogenic carcinomas of all major cell types are thought to arise from a common precursor cell and share certain genetic abnormalities (ie, deletions or rearrangements of the short arm of chromosome 3).²⁸ Therefore it is not surprising that lung cancers of different cell types share a property like positive sex hormone receptor status.

The correlation between ER β and various clinicopathologic factors has revealed that ER β is expressed predominantly in well-differentiated and small-sized lung cancers. This result indicates that the expression of ER β is lost during dedifferentiation of the tumor cells. It is also noted that ER β is tightly associated with lymph node status. This suggests that the loss of ER β expression might be an indicator of a tumor phenotype with high metastatic potential. Such a role is in agreement with the findings of metastatic tendency in the axillary lymph nodes of breast cancers.²⁹ This implies that ER β might play a gatekeeper role by inhibiting invasion and proliferation and maintaining the low biologic aggressiveness and might be related to suppression of tumor growth.³⁰

TABLE 4. Correlations between ER β expression, clinicopathologic features, and survival in 187 patients with stage II and III disease

Variables	No. of patients*	No. of deaths	Univariate			Multivariate		
			Hazard ratio	95% CI	P value	Hazard ratio	95% CI	P value
ER β overexpression								
Negative	110	77	1.000			1.000		
Positive	77	41	0.581	0.397-0.849	.005	0.662	0.439-0.999	.049
Stage								
II	67	33	1.000			1.000		
III	120	85	1.880	1.251-2.823	.002	2.081	1.304-3.321	.002
Differentiation†								
Well	53	28	1.000			1.000		
Moderate	102	65	1.523	0.977-2.374	.063	1.183	0.745-1.878	.476
Poor	21	16	2.697	1.452-5.007	.002	2.233	1.158-4.305	.016
Smoking status								
Nonsmokers	98	57	1.000			1.000		
Smokers	82	57	1.651	1.142-2.387	.008	1.762	1.168-2.659	.007
Vascular invasion								
Negative	131	75	1.000			1.000		
Positive	56	43	1.830	1.248-2.684	.002	1.639	1.079-2.490	.021
Sex								
Female	76	45	1.000					
Male	111	73	1.384	0.954-2.007	.087			
Histologic type†								
Squamous cell carcinoma	58	36	1.000					
Adenocarcinoma	118	73	0.876	0.587-1.308	.518			
Size								
≤3 cm	53	32	1.000					
>3 cm	134	86	1.307	0.870-1.964	.197			

*Some of the patients lack information. †Adenosquamous carcinomas are not included.

Of particular interest is our finding that patients with stage II tumors, a lower grade of differentiation, nonsmoking status, tumors without vascular invasion, and overexpression of ER β have a better clinical prognosis among patients with stage II and III disease. The meaning of this association is not clear and has never been reported before. However, our unexpected findings suggest that estrogens might be involved in lung carcinogenesis, probably either by acting as ER ligands and activating cell-proliferation pathways or by metabolic activation to reactive intermediates that can produce DNA adducts or cause oxidation damage, as mentioned in a recent study.³¹

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 this form of disease.^{32,33} In our study the distribution of hormone receptors in NSCLCs also appeared in male patients. This phenomenon might be partly explained by the fact that estrogen could have a role in cancer in male patients. Because the enzyme aromatase (CYP19), which is expressed in many tissues outside the reproductive tract, is able to convert

androgen to estrogen,^{31,34} local tissue production of estrogen might also occur in male patients. These results indicate that blocking the effects of estrogen might be an important therapeutic strategy to halt lung cancer progression or prevent recurrence in patients of both sexes.

From an endocrinologic point of view, the expression of ER β has been noted as more common in premenopausal and perimenopausal patients with cancer than in postmenopausal patients with breast cancer.²⁹ In one recent study examining a large multi-institutional database, premenopausal women were found to have more advanced lung cancer and to undergo more extensive resection, yet these women had a survival advantage compared with postmenopausal women.²¹ However, ER status in these patients' tumors was not examined. In this study we did not find a concordance between menopause status and ER β overexpression in NSCLCs.

Our results unequivocally demonstrate that ER β can be routinely detected in archival, formalin-fixed, and paraffin-embedded lung cancers by using the monoclonal antibody. This could have profound clinical implications because the level of sex hormone receptors in cancer tissue often is

Figure 1. Survival curves in patients with stage II and III disease: surgically resected non-small cell lung carcinomas with positive (>50%) or negative (≤50%) estrogen receptor β expression ($P = .004$, log-rank test). ER, Estrogen receptor.

considered as an indicator for the selection of appropriate treatment. The finding of ER β in a wide array of nontarget neoplastic tissues might be indicative of their common role in the regulation of cancer growth, irrespective of the organ involved.

On the basis of immunohistochemistry results, the forms of ER α expressed in our lung cancer tissues were located primarily in the cytoplasm, and the cases were few. The reason might be that lung cancer cells have little or no full-length ER α and that the stained variant is mainly cytoplasmic.¹⁹ According to several recent reports, immunohistochemical study also failed to demonstrate ER α nuclear expression in lung cancers.^{17,18} The reasons for this finding remain unclear.

In conclusion, this study is the first to demonstrate the association between ER β overexpression and female sex, nonsmoking status, tumors of 3 cm or smaller, absence of lymph node metastasis, and tumors with a lower grade of differentiation in a large cohort of patients with NSCLCs by means of immunohistochemistry. Interestingly, the present results also have clinical implications because of the observation of a statistically significant correlation between survival and tumors with stage II diseases, a lower grade of differentiation, nonsmoking status, tumors without vascular invasion, and increased expression of ER β in stage II and III NSCLCs. Thus it is strongly indicated that ER β overexpression could be a useful and statistically significant prognostic marker for surgically resected stage II and III NSCLCs.

These studies might also provide the possibility for a new type of adjuvant therapy in selected patients with lung cancer.

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