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Molecular Epidemiological Study on Passive Smoking and Estrogen Receptor Expression in Never-smokers with Non-small Cell Lung Cancer

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Abstract

Background

Although sex hormones are thought to play an important role in the carcinogenesis of non-small cell lung cancer (NSCLC) in never-smokers, the causative mechanism remains unknown. Passive smoking (PS) is common among East Asian women and has been suggested to be a potential cause of the disease.

Methods

We systematically evaluated the expression of estrogen receptor (ER), the prevalence of PS, and genetic mutations using tumor samples from a prospectively registered cohort of never-smokers with lung cancer. The study enrolled 92 never-smokers with NSCLC. Expression of ERα, ERβ, and progesterone receptor (PR) was examined via immunohistochemical staining (IHC). Detailed PS information was obtained through a standardized questionnaire. The cumulative dose of PS (CPS) was evaluated as a sum of the number of exposure years at home and/or in the work place.

Results

Nuclear expression of ERα, ERβ, and PR was detected in 0, 14, and 3 cases, respectively. ERβ was more frequently overexpressed in earlier stage cancer ($p=0.043$). Ninety patients (97.9%) had a PS history, and the median CPS was 47.5 years (range, 0-103 years). There was no significant correlation between the amount of PS and ERβ expression ($p=0.101$). Twelve patients (85.7%) had Epidermal growth factor receptor (*EGFR*) mutations in 14 tumors expressing ERβ, and a trend towards an association between ERβ expression and *EGFR* mutations ($p=0.067$) was observed.

Conclusions

Nuclear expression of ERβ was more frequently observed in early stage NSCLC in never-smokers.

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ERβ expression tended to correlate with *EGFR* mutations, but not PS.

Key Words: Passive smoking; Estrogen; Lung cancer; Never-smokers; Epidermal growth factor receptor mutations

Introduction

Lung cancer is the leading cause of cancer-related death. Although the disease is predominantly caused by tobacco smoke, approximately 25% of all lung cancer cases worldwide are not attributable to such a cause. In fact, about 30% of all participants in a Japanese cohort study of more than 20000 non-small cell lung cancer (NSCLC) patients were never-smokers[1,2]. Although the cause of lung cancer in never-smokers remains unknown, the disease presents unique clinical characteristics and is thought to be a distinct entity, which has emerged as a global public health concern[3].

One of its characteristics is its predominance in women[4], and estrogen has been suggested to play an important role in carcinogenesis. Estrogen receptors (ERs) are expressed in both normal and neoplastic tissues and mediate the growth and maturation of normal tissue[5]. A previous randomized study showed that estrogen plus progestin therapy was associated with an increased risk of lung cancer. The prospective Vitamins and Lifestyle Study followed a cohort of more than 36000 peri- and post-menopausal women for six years[6]. After adjusting for smoking and other confounding factors, the investigators reported that the incidence of lung cancer was higher among those receiving estrogen plus progestin. The increased risk was proportional to the duration of hormone exposure (hazard ratio [HR]=1.48, 95% confidence interval [CI]=1.03-2.12 for those with ≧10 years of exposure to estrogen plus progestin).

Passive smoking (PS) is common among East Asian women and is thought to be a potential cause of lung cancer in never-smokers. A prospective study by the Japan Public Health Center showed that second-hand smoke exposure was clearly related to the development of lung adenocarcinoma in never-smokers[7]. The study identified a statistically significant dose-response relationship between the quantity and intensity of husbands' smoking and their wives' incidence of lung cancer. Furthermore, a preclinical study demonstrated that second-hand smoke contained a higher concentration of nicotine than mainstream smoke and that nicotine had a strong synergistic effect on the ER pathway in carcinogenesis[8].

Based on the above-mentioned findings, we hypothesized that estrogen contributes to the development of lung cancer and that the expression of ERs is associated with PS among never-smokers. In the present study, we systematically evaluated ER expression, PS, and genetic mutations using tumor samples from a prospectively registered cohort of never-smokers with lung cancer.

Methods

Patients

Patients' information and tissue specimens were obtained from a prospective registry of never-smokers with NSCLC, which was initiated at the Kinki-Chuo Chest Medical Center, Japan, in 2008. Patients who were never-smokers (<100 cigarettes lifetime) and whose tumor tissue samples were available for analysis were eligible for the study. We consecutively enrolled all never-smokers after obtaining written informed consent, and collected their PS information via a questionnaire. As of 2011, there were 202 cases enrolled in the registry, and 92 with sufficient tumor specimens of optimal quality available were selected for this study. The materials were obtained from surgery (77.0%),

bronchoscopy (22.0%), or other procedures (1.0%). Fifty-seven patients included in this study were also participants in our previous study on PS and Epidermal growth factor receptor (*EGFR*) mutations[9]. The studied patients included 80 women (87.0%) and 12 men (13.0%), with a median age of 67 years (range, 29-84 years). Almost all cases were of the adenocarcinoma histological subtype (92.4%).

PS information

PS was defined as regular exposure to tobacco smoke produced by an active smoker within a confined space for at least one year. Detailed PS information was obtained through a standard questionnaire, which was carefully supported by interview with trained personnel. The questionnaire included items for years of PS from parents and other relatives as a child, years of PS from spouse/partner and/or children at home, and years of PS from co-workers at workplace. The cumulative dose of PS was assessed in terms of total smoker-years[9]. This assessment was constructed to add years of PS from three different parts in the questionnaire: (1) from parents and/or other relatives during the participants' childhood; (2) from spouse/partner and/or children at home; and (3) from co-workers at a workplace.

Immunohistochemical staining for sex hormone receptor expression

Immunohistochemical analysis of ERα, ERβ, and PR expression was performed on all 92 formalin-fixed paraffin-embedded (FFPE) samples. Sections of 4-μm thickness were dewaxed and incubated in the target retrieval solution (Target Retrieval Solution, DAKO, Japan) for 40 minutes at 95℃. The primary antibodies used included a mouse monoclonal anti-ERα antibody (ID5, DAKO, Japan; 1:50 dilution), a mouse monoclonal anti- ERβ antibody (PPR5/10, DAKO, Japan; 1:20 dilution), and a mouse monoclonal anti-PR (PgR 636, DAKO, Japan; 1:800 dilution).

We used the Allred scoring method to categorize nuclear expression in the stained specimens[10]. Four scoring categories each were assigned according to staining proportion and staining intensity. The proportion and intensity scores were summed to achieve a final score, ranging from 0 to 8. A tumor was considered positive for expression when the final score was more than 3. All stained slides were independently examined by a pathologist (S.S.) who was blinded to the clinicopathological data.

EGFR mutational analysis

DNA was successfully extracted from FFPE samples, and a genetic analysis was performed to detect *EGFR* mutations in exons 18 to 21. The nucleotide sequence of the kinase domain of the *EGFR* gene was determined using a PCR-INVADER assay for the individual exons[11].

Statistical analysis

The Pearson's chi-squared test, Fisher's exact test, Mann-Whitney U test, or Mantel test was used to determine statistical differences of variables as appropriate.

This study was approved by the institutional review board of the National Hospital Organization Kinki-Chuo Chest Medical Center, Osaka, Japan. All patients provided written informed consent before enrollment.

Results

Sex hormone receptor expression and clinicopathological factors

The proportion score for ERα, ERβ, and PR was more than 1 in 0 (0%), 12 (13.0%), and 2 (2.2%) cases, respectively, and the intensity score was not less than 1 in 0 (0%), 21 (22.8%), and 4 (4.3%) cases, respectively (Fig. 1). On the basis of the combined score, ERα expression was negative in all

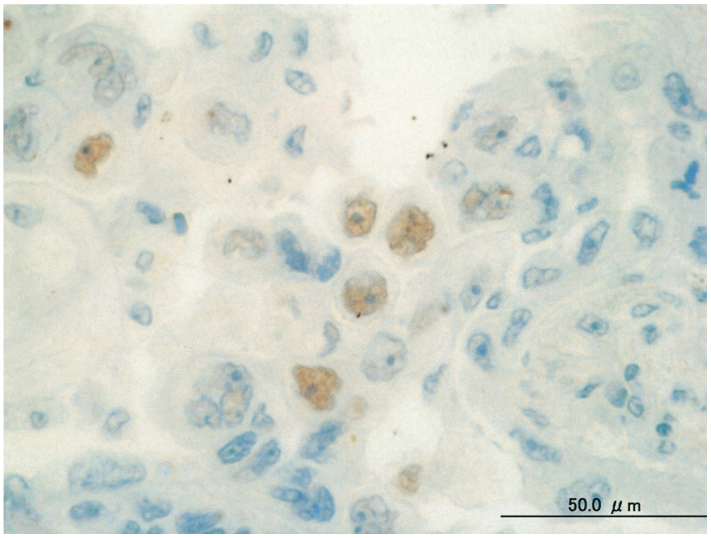

Figure 1. Representative immunohistochemical staining for estrogen receptor β (ERβ). The primary antibody is rabbit polyclonal anti-ERβ (PPR5/10; DAKO, Japan; 1:20 dilution). Several positive cells are observed (proportion score=2, intensity score=2) in the tumor.

Table 1. Patient characteristics

Characteristic	ERβ positive		ERβ negative		p
	No. of Patients	%	No. of Patients	%	
Age, years					0.624
≦65	6	42.9	36	46.2	
65<	8	57.1	42	53.3	
Median	66.5 (51-82)		67 (29-84)		
Sex					0.201
male	0	0	12	15.4	
female	14	100	66	84.6	
Pathological Stage					0.043
Ⅰ	11	78.6	42	53.9	
Ⅱ	1	7.1	4	5.1	
Ⅲ	2	14.3	17	21.8	
Ⅳ	0	0	15	19.2	
Histology					0.446
Adenocarcinoma	11	7.1	74	94.9	
Squamous cell carcinoma	1	78.6	3	3.8	
Others	2	14.3	1	1.3	
EGFR mutation status					0.340
Positive	12	85.7	47	60.2	
exon 19	5	41.7	19	40.4	
L858R	5	41.7	20	42.6	
Others	2	16.6	8	17.0	
Negative	2	14.3	31	39.8	
ERα					1
Positive	0	0	0	0	
Negative	14	100	78	100	
PR					0.0029
Positive	3	21.4	0	0	
Negative	11	78.6	78	100	
Total	14		78		

No., number; ER, estrogen receptor; EGFR, epidermal growth factor receptor; and PR, progesterone receptor.

cases, ERβ expression was positive in 14 cases (15.2%), and PR expression was positive in three cases (3.3%). Clinical characteristics according to the tumor ERβ status of the 92 cases are summarized in Table 1. ERβ positivity was significantly associated with PR positivity (p=0.003). ERβ was more frequently overexpressed in earlier stage cancers (p=0.043).

Sex hormone receptor expression and amount of PS

Ninety patients (97.9%) had a PS history, and the median cumulative dose of PS was 47.5 years (range, 0-103 years). No PS history was reported in two patients; both of those had ERβ-expressing tumors and one had a PR-expressing tumor. As shown in Table 2, there was no significant association between sex hormone receptor expression and the amount of PS.

Relationship between sex hormone receptor expression and EGFR mutations

EGFR mutations were detected in 59 of 92 patients (64.1%). Thirty cases (32.6%) had in-frame deletions in exon 19, 26 (28.3%) had L858R point mutations in exon 21, two had G719C in exon 18, and one had in-frame deletions in exon 18. Three *EGFR* mutations were detected in all of the PR-expressing tumors, and 56 (62.9%) mutations were detected in 89 tumors without PR expression. There was no significant difference in the occurrence of *EGFR* mutations between PR-positive and PR-negative tumors (p=0.576). In contrast, 12 patients (85.7%) had *EGFR* mutations in 14 ERβ-expressing tumors and 47 (60.2%) had mutations in 78 non-ERβ-expressing tumors. There was a tendency toward an association between *EGFR* mutations and ERβ expression (odds ratio=3.957; 95% CI: 0.915-16.751, p=0.067, Table 3).

Table 2. PS and sex hormone status in tumor

PS	ERβ positive			PR positive		
	No. of Patients	%	p	No. of Patients	%	p
0-25	3	20	0.307	1	6.7	0.462
26-50	7	18.9		1	2.7	
51-75	3	9.4		1	3.1	
76-103	1	12.5		0	0	
Total	14	15.2		3	3.3	

PS, Passive smoking; No., number; ER, estrogen receptor; and PR, progesterone receptor.

Table 3. Summary of results from previous relevant studies and the present investigation

Author/ Country	n	Smoking history			ERβ		ERβ positive*	ERβ negative*	p
		Ever	Never	Unknown	Antibody	Positive* %	EGFR mutation positive/total n (%)	EGFR mutation positive/total n (%)	
Nose N. /Japan	447	271	164	12	Clone H150	48.5	113/217 (52.1)	56/230 (24.3)	<0.001
Raso G.M. /U.S.A.	317	262	54	1	Clone H150 Clone 14C8	55.8 41.6	22/92 (23.9) N.D.	5/80 (6.3) N.D.	<0.01 -
Toh C.K. /Singapore	109	47	55	7	Clone PPG5/10	9.4	6/10 (60.0)	33/87 (37.1)	0.157
Current study /Japan	92	0	92	0	Clone PPG5/10	15.2	12/14 (85.7)	47/72 (60.3)	0.067

*nuclear staining. n, number of cases; N.D., not described; ER, estrogen receptor; EGFR, and epidermal growth factor receptor.

Discussion

In this study, nuclear ERβ and PR expression was observed in 15.2% and 3.3% of the never-smokers with NSCLC. ERβ expression was more frequently observed in earlier stage cancers. Although the pattern of hormone receptor expression was not associated with the amount of PS, the expression of ERβ tended to correlate with *EGFR* mutations. To our knowledge, this is the first report to focus on ER expression and PS in never-smokers with NSCLC.

Regarding sex hormone receptor expression, we did not detect any nuclear ERα expression in NSCLC. The antibody we used recognizes the NH2 domain of ER, and no positive staining was noted when using the same antibody against the N-terminus in a previous study[12]. Furthermore, ERα staining in lung tumor tissues and cell lines has been reported to be primarily cytoplasmic and membranous, with rare expression in the nucleus[13]. The frequency of ERβ expression varies according to the type of antibody and the dilution used in different studies. We used the Clone PPR5/10 antibody, which is routinely employed for immunohistochemical staining of breast cancer specimens in the clinical setting. Expression detected with this clone is generally lower than that detected by Clone H150.

Previous studies[10,13] using the same antibody as we did reported a 69% ERβ expression rate at a 1:10 dilution and a 9% expression rate at a 1:100 dilution. Different conditions in smoking status and clinical stage in the study population might also contribute to the different results. In contrast, our PR expression results were similar to those of other studies, which mostly reported relatively a low percentage of PR positivity in lung cancer using different assessment techniques[10,13].

Several studies have suggested that ERβ might play a functional role in the lungs, and its nuclear expression was observed in the majority of lung cancer cases[5]. We showed here that ERβ expression was more frequently observed in earlier stage NSCLC, which supports a role for ERβ in tumor development. Even in the advanced stage, a certain number of cells express the receptor, and ERβ could be a potential therapeutic target for NSCLC treatment, as is the case for breast cancer.

In a preclinical study, fulvestrant, an ER antagonist, inhibited the proliferation of NSCLC cells in vitro and in lung tumor xenografts in immunocompromised mice[14]. Recently, a phase Ⅱ trial has compared the combination of erlotinib and fulvestrant versus erlotinib alone[15]. Among patients harboring tumors with wild-type *EGFR*, the clinical benefit rate was significantly higher in those treated with the combination than in those receiving erlotinib alone, with trends towards improved survival. Such a result suggested that targeting the ER pathway in conjunction with the *EGFR* pathway might offer beneficial anti-tumor effects in NSCLC.

In this study, we failed to demonstrate the association between ER expression and PS. A recent large-scale prospective study indicated that PS in adults for >30 years tended to be associated with lung cancer development[16]. As their PS exposure categories differed according to the methods used in different studies, we examined whether any other categories of PS were associated with ER expression in our samples. However, we could not detect any significance for the association (data not shown). Other environmental factors and/or ethnicity can be associated with ER expression, and further studies are required to elucidate the mechanism of estrogen regulation in lung cancer.

There were several studies on ERβ and *EGFR* mutations[10,17,18]. As shown in Table 3, most of these, including ours, indicated that ERβ was associated with *EGFR* mutations. The Japanese and US studies of surgically resected adenocarcinoma specimens reported strong nuclear expression of ERβ being associated with *EGFR* mutations[17]. This study showed a trend of association between *EGFR*

mutations and ERβ positivity in the tumors. The statistical insignificance observed was probably due to the small numbers of cases. *EGFR* mutations are significantly associated with smoking status, and distinguishing between smokers or never-smokers is critical for the investigation of these mutations. Therefore, enrollment of only never-smokers could be an advantage in our study.

The main limitations of our study were its small sample size and the lack of validation for the questionnaire. No definite biomarker has been established to date for the evaluation of PS, and a detailed questionnaire remains essential for further study. To overcome recall bias, questionnaire completion was supported by trained personnel to help obtain reproducible and accurate information.

In conclusion, the present study demonstrated nuclear expression of ERβ and PR in NSCLC in never-smokers. ERβ was more frequently observed in the earlier stages of cancer. Although there was no correlation between sex hormone receptor expression and PS, ERβ expression tended to correlate with *EGFR* mutations.

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