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Original Article

Prognostic significance of aromatase and estrogen receptor beta expression in *EGFR* wild-type lung adenocarcinoma

Kazumi Tanaka[1], Kimihiro Shimizu[1], Seiichi Kakegawa[1], Yoichi Ohtaki[1], Toshiteru Nagashima[1], Kyoichi Kaira[2], Jun Horiguchi[1], Tetsunari Oyama[3], Izumi Takeyoshi[1]

Departments of [1]Thoracic and Visceral Organ Surgery, [2]Medicine and Molecular Science, [3]Division of Diagnostic Pathology, Graduate School of Medicine, Gunma University, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan

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Abstract: Objectives: Based on recent findings of aromatase and estrogen receptor beta (ERβ) expression in non-small-cell lung cancer, we assessed the clinicopathological and prognostic significance of aromatase and ERβ expression and their relationship to epidermal growth factor receptor (*EGFR*) mutation in lung adenocarcinoma. Materials and methods: We evaluated 150 resected primary lung adenocarcinoma specimens. Expression of aromatase, ERα, ERβ, progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) was evaluated by immunostaining, and *EGFR* and *KRAS* mutations were analyzed. Overall survival (OS) and recurrence-free survival (RFS) were calculated using the Kaplan-Meier method. Results: Expression of aromatase, ERα, ERβ, PR, and HER2 was detected in 88.0%, 1.3%, 79.3%, 2.7%, and 39.3% of specimens, respectively. In patients with *EGFR* wild-type lung adenocarcinoma, high aromatase expression was an independent predictor of poor OS (hazard ratio [HR]=2.638; 95% confidence interval [CI], 1.173-5.936; *P*=.019) and RFS (HR=2.505; 95% CI, 1.154-5.434; *P*=.020). Positive ERβ expression was also an independent predictor of poor RFS (HR=4.013; 95% CI, 1.219-13.207; *P*=.022). Furthermore, high aromatase expression was a significant predictor of poor survival only in females (OS, *P*=.010; RFS, *P*=.007), whereas positive ERβ expression was an important predictor of poor survival only in males (OS, *P*=.073; RFS, *P*=.051). No prognostic significance was observed in patients with *EGFR* mutations. Conclusions: Our findings suggest that *EGFR* wild-type lung adenocarcinoma is an estrogen-dependent carcinoma, and aromatase expression and ERβ expression are potent prognostic markers for *EGFR* wild-type lung adenocarcinoma.

Keywords: Aromatase, estrogen receptor beta (ERβ), estrogen signaling pathway, *EGFR* mutation, lung adenocarcinoma

Introduction

Lung cancer is one of the most common cancers globally and is currently the leading cause of death in both females and males [1]. Smoking remains the major cause of lung cancer, but ~53% of all females with lung cancer are non-smokers [2]. Interestingly, a gradual increase in the adenocarcinoma subtype of lung cancer has been reported, despite a decline in the smoking population [3-4]. Therefore, etiologic factors other than tobacco may also play a role in the development of lung adenocarcinoma.

Epidermal growth factor receptor (*EGFR*) is the most frequently mutated proto-oncogene, particularly in lung adenocarcinoma of non-smoker females, and its mutations are thought to play an important role in carcinogenesis [5]. However, recent evidence has suggested that estrogen may also play an important role in the development of non-small cell lung cancer (NSCLC), particularly adenocarcinoma [6]. Several studies have reported that estrogen stimulates the proliferation and progression of lung carcinoma cells, functions that were shown to be significantly suppressed by antiestrogenic agents both in vitro and in vivo [7-10].

Estrogen is converted from androgen by aromatase, a key enzyme in estrogen biosynthesis. In addition to its expression in the ovary and pla-

Table 1. Clinicopathological factors of the patients

Characteristics	N	%
No. of patients	150	100.0
Age (year)		
Mean	66	
Range	36-84	
Sex		
Male	63	42.0
Female	87	58.0
Menopause		
Premenopausal	4	4.6
Postmenopausal	81	93.1
Unknown	2	2.3
Smoking status		
Ever-smoker	68	45.3
Non-smoker	82	54.7
Tumor diameter (mm)		
Mean	24	
Range	6-70	
Pathologic stage		
IA	85	56.7
IB	26	17.3
IIA	5	3.3
IIB	6	4.0
IIIA	22	14.7
IIIB	6	4.0
IV	0	0.0
Pleural invasion		
Absent	102	68.0
Present	48	32.0
Lymphatic invasion		
Absent	101	67.3
Present	49	32.7
Vascular invasion		
Absent	104	69.3
Present	46	30.7

centa, aromatase is present in male and female extragonadal tissues, including breast and lung [11]. Aromatase expression is elevated in certain malignancies, such as breast carcinomas, suggesting that tumor progression caused by stimulation of estrogen signaling pathway could be enhanced by circulating estrogen as well as by localized autocrine or paracrine production of estrogen by aromatase. Recently, aromatase expression in NSCLC has also been reported [12-14]. Weinberg et al. demonstrated that aromatase was expressed in NSCLC cell lines, and aromatase inhibitor (AI) suppressed tumor growth in vitro and in vivo [12]. Mah et al. reported that low aromatase expression was associated with favorable survival in female NSCLC patients, particularly those older than 65 years [13].

In estrogen signaling pathway, estrogens exert their effects mainly via estrogen receptor (ER) [6]. ER is a hormone receptor, as is progesterone receptor (PR). ER has two isoforms, ERα and ERβ, which are encoded by distinct genes and are expressed in various tissues or at various levels in the same tissue [15]. In the normal lung, ERβ has been reported to be expressed at a higher level than ERα [9]. Although the expression patterns of ERα and ERβ in NSCLC were highly inconsistent among these reports [6], most of the results showed that there were no or a low (under 10%) rate of ERα-positive cases and a higher rate (over 50%) of ERβ-positive cases [16]. Previously, ERα was considered a tumor promoter, whereas ERβ was believed to inhibit tumorigenesis [17]. However, recent studies have demonstrated that ERβ can function as a tumor promoter in the absence of ERα expression [18-22]. The association between ERβ expression and the prognosis of lung cancer patients remains controversial [23-27]. Wu et al. reported that ERβ expression was associated with favorable prognosis in NSCLC [23]. However, Stabile et al. reported that ERβ expression was associated with a poor prognosis in lung cancer [26].

Recent reports have suggested an interaction between *EGFR* pathway and estrogen signaling pathway in the development of breast and lung cancer; additionally, estrogen signaling pathway is regulated by membrane receptor tyrosine kinases, including EGFR and human epidermal growth factor receptor 2 (HER2) [26, 28-32]. The *EGFR* pathway becomes activated when estrogen is depleted, and ERβ expression is increased following treatment with *EGFR* tyrosin kinase inhibitors (EGFR-TKIs) in NSCLC cells [33-35]. Nose et al. reported that strong nuclear expression of ERβ is correlated with *EGFR* mutations in lung adenocarcinoma [31]. However, the role of aromatase and ERβ in estrogen signaling pathway and the association between the expression of these proteins and clinicopathological factors, including *EGFR*

Table 2. Primary antibodies used in the present study

Antigen	Clone	Dilution	Source
ERα	1D5	1:50	DakoCytomation, Glostrup, Denmark
ERβ	14C8	1:200	GeneTex, CA, USA
PR	PGR636	1:800	DakoCytomation, Glostrup, Denmark
Aromatase	#677/H7	1:1000	Contributed by Dr. Evans DB, Novartis, Basel, Switzerland
Ki-67	MIB1	1:150	DakoCytomation, Glostrup, Denmark
HER2	4B5	1:1	Ventana, Tucson, AZ, USA

ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

mutation, in lung adenocarcinoma, are not well understood.

The purpose of the present study was to examine the correlation between aromatase/ERβ expression and clinicopathological prognostic factors, including *EGFR* mutations, and to evaluate the prognostic significance in lung adenocarcinoma.

Materials and methods

Patients and tissue specimens

One hundred and fifty lung adenocarcinoma specimens were obtained from patients who underwent complete surgical resection consecutively from 2004 to 2008 at Gunma University Hospital. The clinicopathological factors of the patients are shown in **Table 1**. The disease stage was determined according to the seventh edition of the TNM classification for lung and pleural tumors [36]. All of the procedures were approved by the Ethics Committee on Human Research of Gunma University Graduate School of Medicine, and written informed consent was obtained from all of the patients before surgery.

Immunohistochemistry

Serial tissue sections of 4-μm thickness sliced from paraffin-embedded specimens were used for immunohistochemistry using the labeled streptavidin-biotin method. Immunostaining for ERα, ERβ, PR, aromatase, Ki-67, and HER2 was performed with the antibodies listed in **Table 2**. The slides were deparaffinized with xylene and rehydrated with ethanol. For ERα, PR, and Ki-67 analysis, antigen retrieval was performed according to the manufacturer's instructions. For ERβ, antigen retrieval was carried out by autoclaving the slides in citrate buffer (0.01 mol/L) at 121°C for 5 min. For HER2, immunohistochemical staining was performed using BenchMark XT (Ventana, Tucson, AZ, USA), an automatic immunohistochemical staining system. Nuclear positive immunoreactivity for ERα, ERβ, and PR was counted among 1000 cells per case and was recorded as "positive" for positive results of more than 10% [14]. Immunoreactive intensity of ERβ was scored into four phases (0, negative; 1+, weak; 2+, moderate; and 3+, strong). For Ki-67, 1000 cells were counted per case, and the proliferative activity was assessed as the percentage of Ki-67-stained nuclei (Ki-67 labeling index; LI) in each sample. Cytoplasmic staining for aromatase in over 10% of the cancer lesion was recorded as "positive", and immunoreactive intensity was scored into four phases (0, negative; 1+, weak; 2+, moderate; and 3+, strong) [6, 13]. HER2 immunoreactivity was evaluated using the DAKO HercepTest scoring system (DakoCytomation), and over 2+ was considered "positive". Two observers (K.T. and T.O.) who were unaware of the clinical data independently reviewed all pathological slides.

Gene mutation analysis

We examined *EGFR* and *KRAS* mutations in the present study. Genomic DNA was extracted from a 3- to 5-mm cube of tumor tissue using a DNA Mini Kit (Qiagen, Hilden, Germany) and subsequently diluted to 20 ng/μL. *KRAS* and *EGFR* mutations were analyzed by sequencing as described previously [37, 38].

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics 21.0 (IBM Co., Armonk, NY, USA). Student's *t*-test and chi-squared test were used to compare percentages and mean values, respectively. Survival was calculated using the Kaplan-Meier method and confirmed using the log-rank test. Overall survival (OS) was

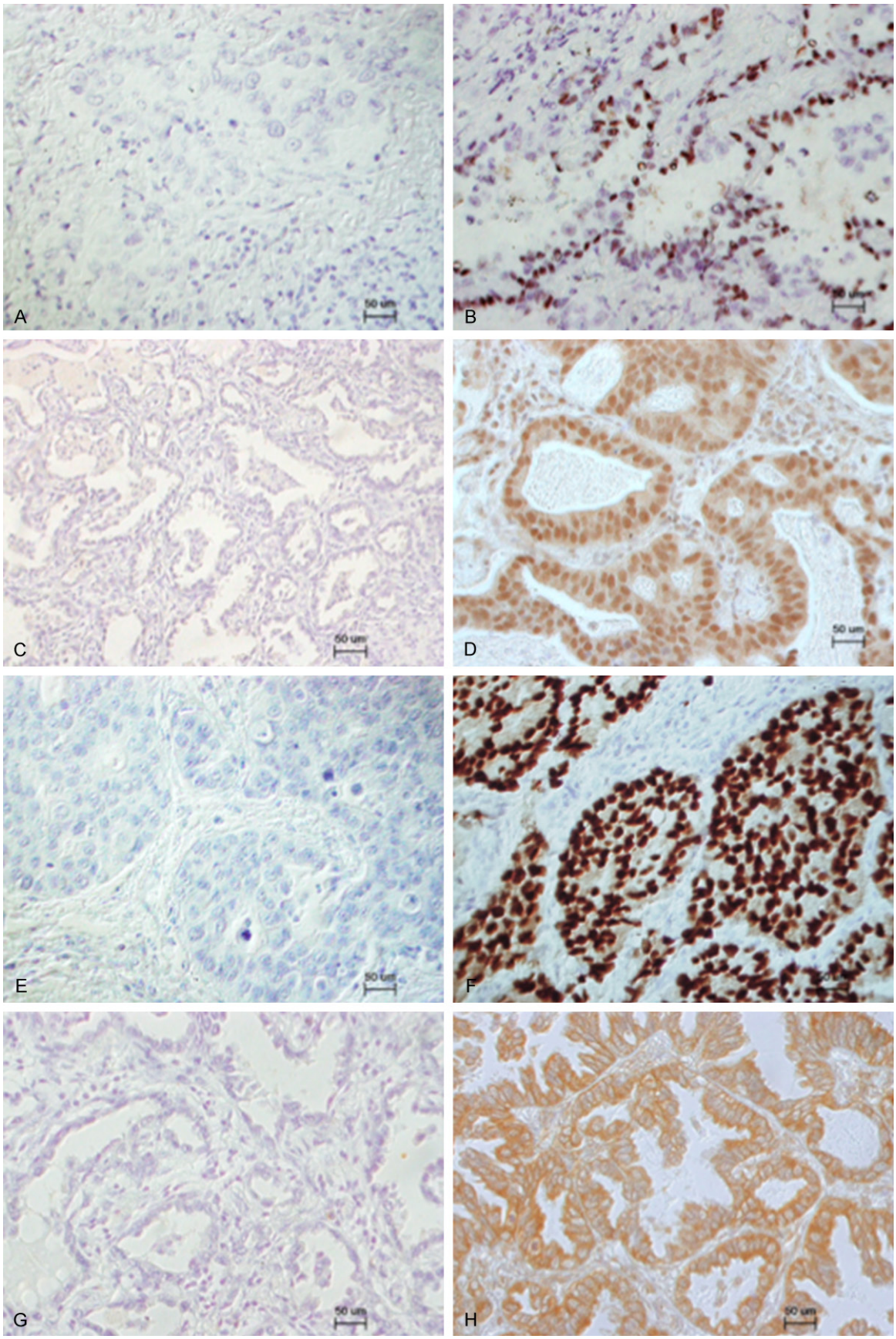
A
B
C
D
E
F
G
H

Figure 1. Representative immunohistochemical staining of ERα, ERβ, PR, and aromatase in lung adenocarcinoma. A. Negative staining of ERα; B. Positive staining of ERα; C. Negative staining of ERβ; D. Positive staining of ERβ; E. Negative staining of PR; F. Positive staining of PR; G. Negative staining of aromatase; H. Positive staining of aromatase.

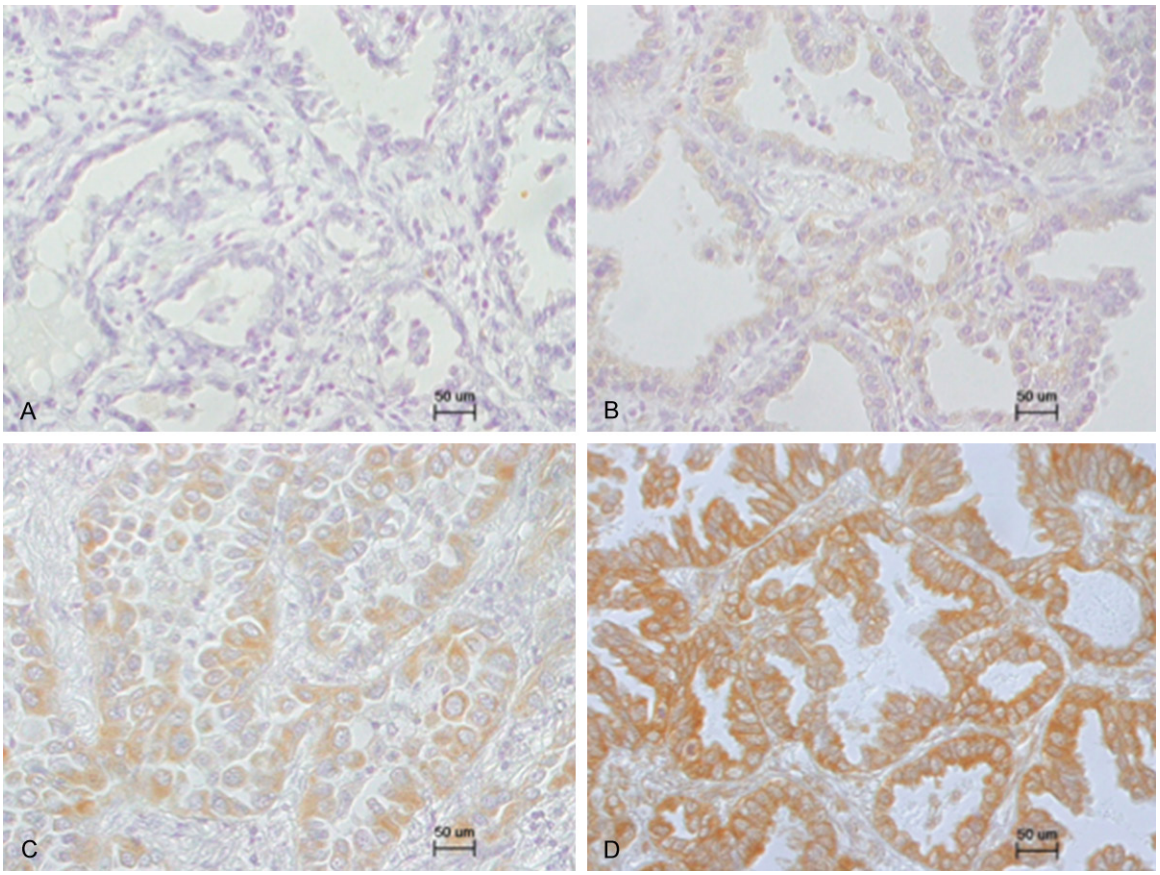

Figure 2. Representative immunohistochemical staining pattern of aromatase in lung adenocarcinoma. Specimens were assigned one of four scores (0, negative; 1+, weak; 2+, moderate; and 3+, strong) according to the intensity of immunoreactivity. A, 0; B, 1+; C, 2+; D, 3+.

determined as the time from tumor resection to death from any cause. Recurrence-free survival (RFS) was defined as the time between tumor resection and first disease recurrence or death. The median follow up for survivors was 65.5 months (average, 63.7 months; range, 1-117 months). Variables with *P* value less than .05 after univariate analysis were entered into multivariate analysis using the Cox proportional hazards model. $P < .05$ was deemed to indicate statistical significance. The midpoint and median intensity (1.5) was used to define low and high aromatase expression as previously described [13]. For univariate and multivariate analyses, each continuous variable (age and Ki-67 LI) was dichotomized at the median value.

Results

Immunohistochemical analysis

Among the 150 cases, ERα, ERβ, PR, aromatase, and HER2 were detected in 2 (1.3%), 119 (79.3%), 4 (2.7%), 132 (88.0%), and 7 (4.7%) cases, respectively. **Figure 1** shows representative staining for ERα, ERβ, PR, and aromatase. Regarding the immunoreactive intensity of aromatase, 18 (12.0%) cases were scored as 0, 60 (40.0%) cases were scored as 1+, 51 (34.0%) cases were scored as 2+, and 21 (14.0%) cases were scored as 3+ (**Figure 2**). Therefore, low expression group comprised 78 (52.0%) cases, and high expression group comprised 72 (48.0%) cases. ERβ staining was

Table 3. Univariate and multivariate analyses of prognostic factors in all patients

Variable	No. of patients (%)		Univariate analysis				Multivariate analysis					
							OS			RFS		
			5-year OS (%)	*P* value	5-year RFS (%)	*P* value	HR	95% CI	*P* value	HR	95% CI	*P* value
All cases	150	(100.0)	73.6		63.8							
Age (years: median 69)												
<69	72	(48.0)	81.6		72.1		1.000	-	-	1.000	-	-
≥69	78	(52.0)	66.0	0.001	56.1	0.011	3.178	1.527-6.616	0.002	1.861	1.035-3.348	0.038
Sex												
Male	63	(42.0)	69.5		60.2							
Female	87	(58.0)	76.6	0.106	66.3	0.150						
Smoking status												
Non-smoker	82	(54.7)	81.5		71.9		1.000	-	-	1.000	-	-
Ever-smoker	68	(45.3)	63.8	0.004	54.0	0.005	0.770	0.343-1.729	0. 526	0.989	0.549-1.779	0.969
Pathologic stage												
I, II	122	(81.3)	84.2		76.1		1.000	-	-	1.000	-	-
III	28	(18.7)	28.6	<0.001	10.7	<0.001	2.024	1.358-3.016	0.001	1.697	1.223-2.353	0.002
Pleural invasion												
Absent	102	(68.0)	85.0		75.2		1.000	-	-	1.000	-	-
Present	48	(32.0)	50.0	<0.001	39.6	<0.001	0.985	0.481-2.019	0.968	1.201	0.676-2.132	0.533
Lymphatic invasion												
Absent	101	(67.3)	88.9		82.0		1.000	-	-	1.000	-	-
Present	49	(32.7)	41.9	<0.001	26.5	<0.001	1.609	0.664-3.900	0.292	2.418	1.101-5.309	0.028
Vascular invasion												
Absent	104	(69.3)	86.4		79.6		1.000	-	-	1.000	-	-
Present	46	(30.7)	44.6	<0.001	28.3	<0.001	1.727	0.735-4.059	0.210	1.362	0.661-2.808	0.402
EGFR mutation												
Mutant	62	(44.3)	83.8		69.3		1.000	-	-			
Wild type	78	(55.7)	65.1	0.001	58.8	0.079	2.954	1.245-7.009	0.014			
KRAS mutation												
Mutant	23	(16.4)	52.2		47.8		1.205	0.548-2.651	0.643			
Wild type	117	(83.6)	77.6	0.035	66.6	0.126	1.000	-	-			
Aromatase												
Low expression	78	(52.0)	81.7		70.3		1.000	-	-			
High expression	72	(48.0)	65.1	0.039	56.9	0.101	2.235	1.142-4.374	0.013			

ERα												
Negative	148	(98.7)	73.9		64.0							
Positive	2	(1.3)	50.0	0.405	50.0	0.568						
ERβ												
Negative	31	(20.7)	76.9		74.2							
Positive	119	(79.3)	72.7	0.447	61.0	0.145						
PR												
Negative	146	(97.3)	73.6		63.5							
Positive	4	(2.7)	75.0	0.724	75.0	0.573						
HER2												
Negative	143	(95.3)	74.5		64.9					1.000	-	-
Positive	7	(4.7)	57.1	0.091	42.9	0.015				3.093	1.151-8.311	0.025
Ki-67 LI (%: median 11.5)												
Low score	75	(50.0)	87.7		84.0		1.000	-	-	1.000	-	-
High score	75	(50.0)	59.3	<0.001	43.3	<0.001	2.591	1.115-6.021	0.027	2.620	1.299-5.284	0.007

OS, overall survival; RFS, recurrence-free survival; HR, hazard ratio; CI, confidence interval; *EGFR*, epidermal growth factor receptor; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; LI, labeling index.

Table 4. Univariate and multivariate analyses of prognostic factors in patients with *EGFR* wild-type lung adenocarcinoma

Variable	No. of patients (%)		Univariate analysis				Multivariate analysis					
			5-year OS (%)	*P* value	5-year RFS (%)	*P* value	OS			RFS		
							HR	95% CI	*P* value	HR	95% CI	*P* value
All cases	78	(100.0)	65.1		58.8							
Age (years: median 69)												
<69	33	(42.3)	78.0		75.5		1.000	-	-	1.000	-	-
≥69	45	(57.7)	55.6	0.003	46.7	0.003	3.497	1.436-8.514	0.006	2.775	1.193-6.455	0.018
Sex												
Male	40	(51.3)	59.6		54.8							
Female	38	(48.7)	70.7	0.105	63.2	0.180						
Smoking status												
Non-smoker	34	(43.6)	79.1		70.6		1.000	-	-	1.000	-	-
Ever-smoker	44	(56.4)	54.1	0.009	54.8	0.021	0.788	0.248-2.505	0.687	0.453	0.147-1.395	0.168
Pathologic stage												
I, II	62	(79.5)	77.2		71.0		1.000	-	-	1.000	-	-
III	16	(20.5)	18.8	<0.001	12.5	<0.001	1.917	1.158-3.175	0.011	1.715	1.037-2.836	0.036
Pleural invasion												
Absent	47	(60.3)	82.9		76.4		1.000	-	-	1.000	-	-
Present	31	(39.7)	38.7	<0.001	32.3	0.001	1.034	0.424-2.524	0.941	1.247	0.527-2.955	0.615
Lymphatic invasion												
Absent	47	(60.3)	86.9		80.7		1.000	-	-	1.000	-	-
Present	31	(39.7)	32.3	<0.001	25.8	<0.001	1.454	0.521-4.056	0.474	3.275	1.079-9.941	0.036
Vascular invasion												
Absent	49	(62.8)	85.6		79.5		1.000	-	-	1.000	-	-
Present	29	(37.2)	30.7	<0.001	24.1	<0.001	2.893	1.076-7.775	0.035	1.645	0.572-4.734	0.356
KRAS mutation												
Mutant	23	(29.5)	52.2		47.8							
Wild type	55	(70.5)	70.6	0.473	63.4	0.430						
Aromatase												
Low expression	43	(55.1)	78.9		72.1		1.000	-	-	1.000	-	-
High expression	35	(44.9)	48.2	0.005	42.9	0.010	2.638	1.173-5.936	0.019	2.505	1.154-5.434	0.020
ERα												
Negative	76	(97.4)	65.5		59.1							
Positive	2	(2.6)	50.0	0.663	50.0	0.687						

ERβ												
Negative	19	(24.4)	78.9		78.9					1.000	-	-
Positive	59	(75.6)	60.5	0.079	52.3	0.031				4.013	1.219-13.207	0.022
PR												
Negative	75	(96.2)	65.0		58.5							
Positive	3	(3.8)	66.7	0.638	66.7	0.637						
HER2												
Negative	74	(94.9)	66.1		59.5							
Positive	4	(5.1)	50.0	0.060	50.0	0.133						
Ki-67 LI (%: median 11.5)												
Low score	36	(46.2)	83.0		80.6		1.000	-	-	1.000	-	-
High score	42	(53.8)	49.5	<0.001	40.1	<0.001	1.634	0.501-5.334	0.416	2.239	0.752-6.666	0.148

OS, overall survival; RFS, recurrence-free survival; HR, hazard ratio; CI, confidence interval; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; LI, labeling index.

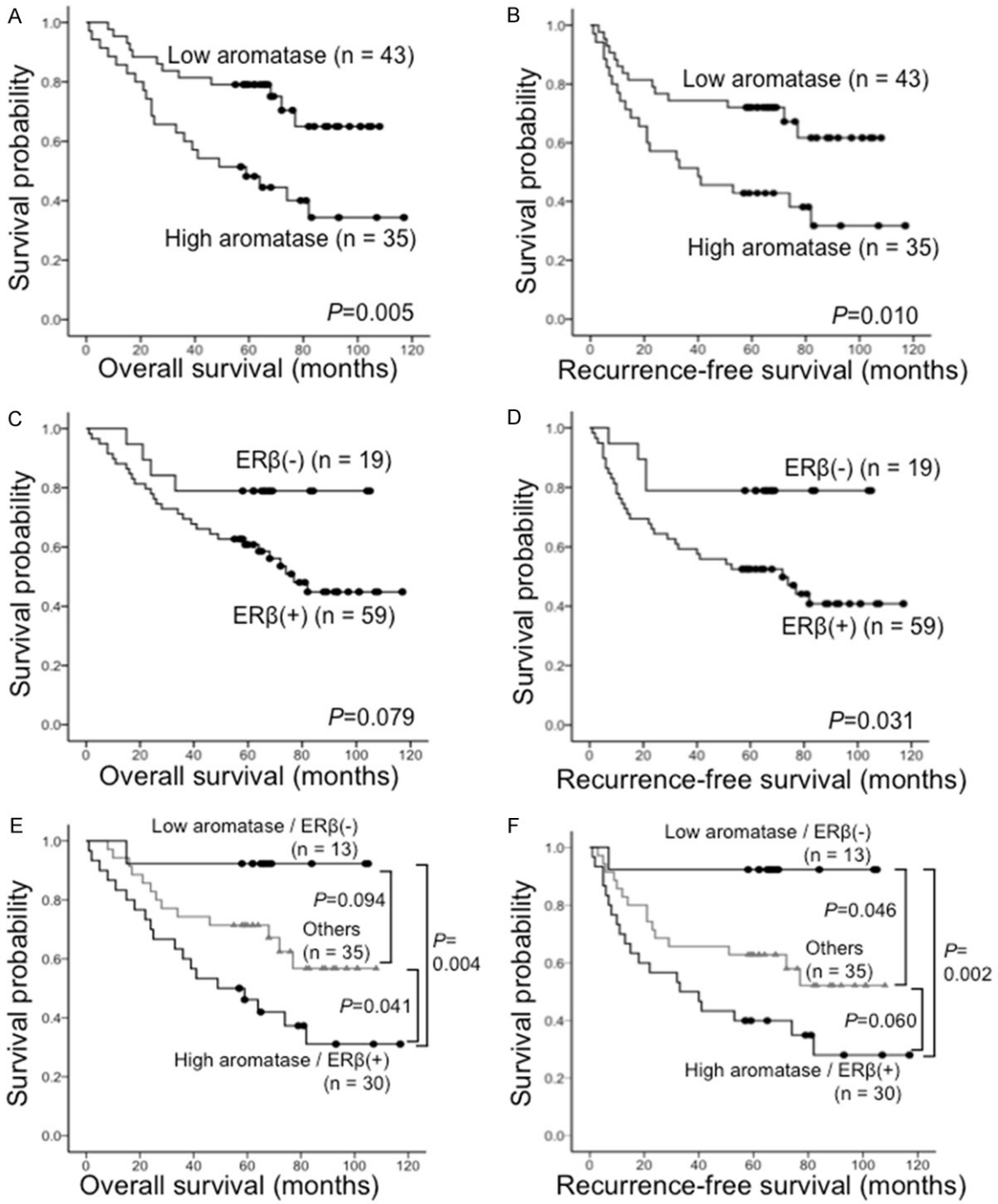

Figure 3. Kaplan-Meier survival curves of 78 patients with *EGFR* wild-type lung adenocarcinoma according to the immunoreactivity results for aromatase and ERβ. A. Overall survival (OS) stratified by high versus low expression of aromatase. B. Recurrence-free survival (RFS) stratified by high versus low expression of aromatase. C. OS stratified by positive versus negative expression of ERβ. D. RFS stratified by positive versus negative expression of ERβ. E. OS stratified by combined high expression of aromatase/positive expression of ERβ versus combined low expression of aromatase/negative expression of ERβ versus others. F. RFS stratified by combined high expression of aromatase/positive expression of ERβ versus combined low expression of aromatase/negative expression of ERβ versus others.

seen in both the cytoplasm and nucleus. Regarding the immunoreactive intensity of ERβ, 31 cases were scored as 0 (20.7%), 98 as 1+ (65.3%), 18 as 2+ (12.0%), and 3 as 3+ (2.0%). Additionally, among the tumors that stained positive for ERβ, almost all of the tumor tissue

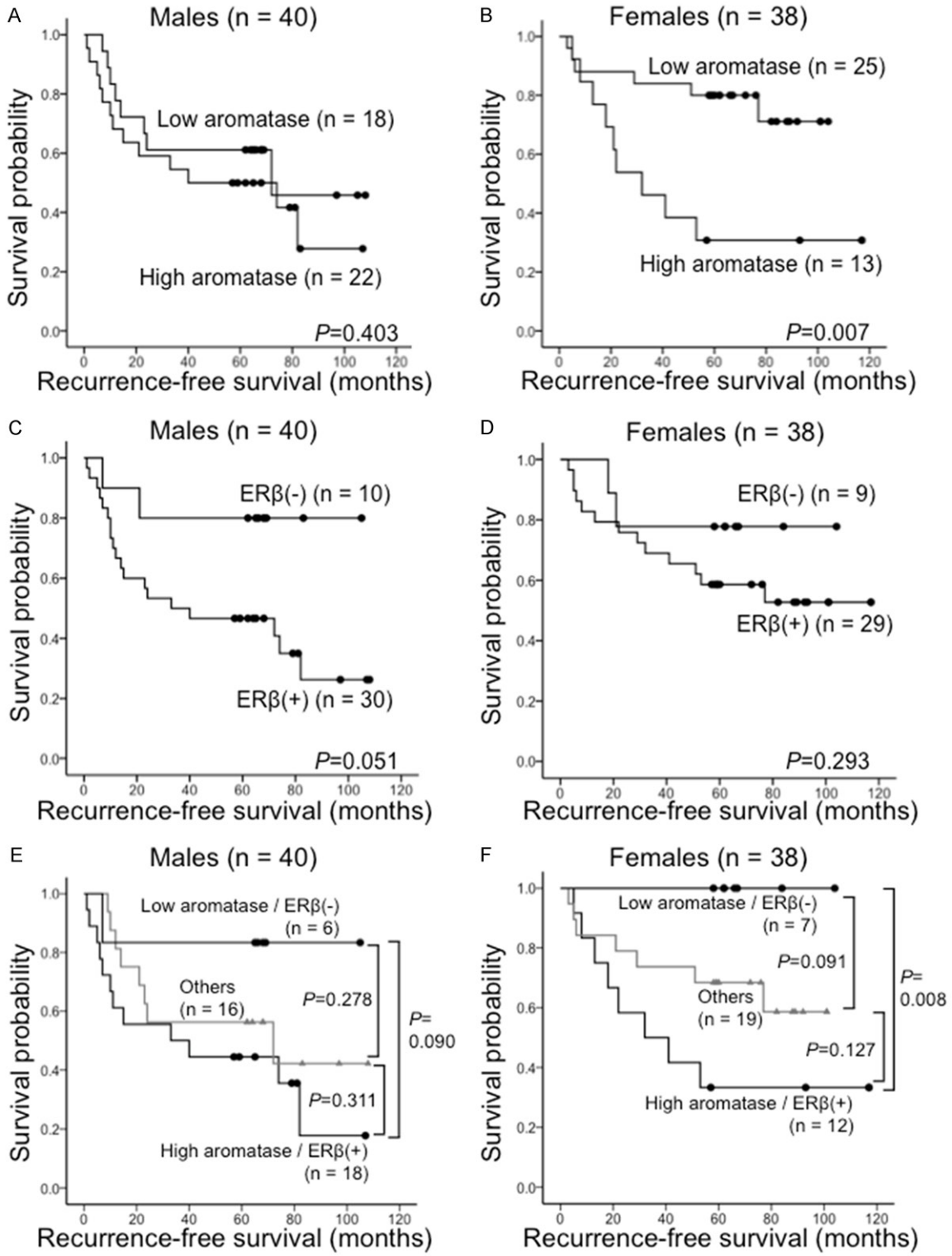

Figure 4. Kaplan-Meier survival curves of male and female patients with *EGFR* wild-type lung adenocarcinoma according to the immunoreactivity results for aromatase and ERβ. A. Recurrence-free survival (RFS) of males stratified by high versus low expression of aromatase. B. RFS of females stratified by high versus low expression of aromatase. C. RFS of males stratified by positive versus negative expression of ERβ. D. RFS of females stratified by positive versus negative expression of ERβ. E. RFS of males stratified by combined high expression of aromatase/positive expression of ERβ versus combined low expression of aromatase/negative expression of ERβ versus others. F. RFS of females stratified by combined high expression of aromatase/positive expression of ERβ versus combined low expression of aromatase/negative expression of ERβ versus others.

stained positive. Therefore, ERβ immunoreactivity was not scored using scoring system such as the Allred score [39].

Association between aromatase/ERβ expression and clinicopathological factors, including EGFR and KRAS mutation

In 10 of the 150 cases, DNA could not be extracted because the specimen was too small. Among the remaining 140 cases, *EGFR* and *KRAS* mutations were found in 62 (44.3%) and 23 (16.4%) cases, respectively. The occurrences of these mutations were mutually exclusive. Aromatase expression status was significantly associated with pleural invasion (*P*=.037), and ERβ expression was not significantly associated with clinicopathological factors (Table S1).

Survival analysis

On univariate analysis, 10 variables were found to be significantly associated with poor OS. For RFS, eight variables were identified as statistically significant factors (**Table 3**). Multivariate analysis demonstrated that older age, advanced pathological stage, *EGFR* wild-type status, high aromatase expression, and high Ki-67 LI score were significant independent predictors of poor OS; additionally, older age, advanced pathological stage, lymphatic invasion, HER2 expression, and high Ki-67 LI score were significant independent predictors of poor RFS (**Table 3**).

Survival analysis according to EGFR/KRAS mutation status

To clarify the prognostic significance of aromatase/ERβ expression according to *EGFR/KRAS* mutation status, we examined survival analysis stratified by *EGFR/KRAS* mutation status. No relationship was found between survival and *KRAS* mutation status (data not shown); however, a significant relationship was found between survival and *EGFR* mutation status. On univariate analysis of the *EGFR* wild-type population, eight variables were significantly associated with poor OS. Regarding RFS, nine variables were identified as statistically significant factors (**Table 4**). Multivariate analysis showed that older age, advanced pathological stage, vascular invasion, and high aromatase expression were significant independent predictors of poor OS. Concerning RFS, older age, advanced pathological stage, lymphatic invasion, high aromatase expression, and ERβ positive status were significant independent predictors (**Table 4**).

Figure 3 shows the survival curves of 78 patients with *EGFR* wild-type lung adenocarcinoma according to aromatase and ERβ expression, respectively. Interestingly, differences in survival became clearer when patients were stratified by aromatase and ERβ expression. Patients with high aromatase expression had poor prognosis in both OS (**Figure 3A**; *P*=.005) and RFS (**Figure 3B**; *P*=.010). Patients with ERβ-positive also had poor prognosis in terms of RFS (**Figure 3D**; *P*=.031); an identical tendency was observed for OS (**Figure 3C**; *P*=.079). Furthermore, patients with high expression of aromatase and ERβ-positive had a poorer prognosis than patients with low expression of aromatase and ERβ-negative in terms of both OS (**Figure 3E**; *P* =.004) and RFS (**Figure 3F**; *P*=.002).

Conversely, no significant difference was noted in the survival of patients with *EGFR* mutant lung adenocarcinoma according to aromatase and ERβ expression (Table S2 and Figure S1).

Survival analysis according to sex in patients with EGFR wild-type lung adenocarcinoma

Next, we performed a survival analysis stratified by sex to clarify the prognostic impact of hormonal effect on sex in patients with *EGFR* wild-type lung adenocarcinoma. **Figure 4** shows the RFS curves of males and females with *EGFR* wild-type lung adenocarcinoma according to aromatase and ERβ expression, respectively. High aromatase expression was significantly associated with poor prognosis only in females (**Figure 4B**; *P*=.007), whereas ERβ-positive had a tendency for poor prognosis only in males (**Figure 4C**; *P*=.051). Furthermore, patients with high expression of aromatase and ERβ-positive had a poorer prognosis than patients with low expression of aromatase and ERβ-negative, but only among females (**Figure 4F**; *P*=.008).

Discussion

Understanding the role of estrogen and *EGFR* pathways in lung adenocarcinoma is necessary to develop new preventative and treatment strategies. We report here for the first time that aromatase and ERβ expression are indepen-

dent, unfavorable prognostic factors in *EGFR* wild-type lung adenocarcinoma. Furthermore, regarding *EGFR* wild-type lung adenocarcinoma, we showed that high aromatase expression was a significant predictor of poor survival only in females, whereas ERβ expression was an important predictor of poor survival only in males. These observations indicate that these pathways are important to a different extent in males and females with lung adenocarcinoma.

In our study, PR was detected only in 2.7%. The rate of PR expression in lung tumors varies among reports, ranging from no expression to marginal (22%-35%) or even high expression (39%-63%) [40]. This difference may be related to the antibody used in each study. The antibody we used is a representative PR antibody widely used for breast cancer research and to guide clinicians' choice of therapy. However, its use remains limited for lung tumors, and the most appropriate antibody for PR staining in lung tumors is yet to be identified.

Recent studies linking ER expression status with *EGFR* mutation have suggested that considering these signaling pathways together may provide important insight into lung cancer biology [31, 41-42]. Thus, we analyzed the association of aromatase/ERβ expression with *EGFR* mutation status. Interestingly, our subgroup analysis showed a significant difference in OS and RFS according to aromatase expression, and a significant difference in RFS according to ERβ expression, only in *EGFR* wild-type lung adenocarcinoma.

Mah et al. reported that low aromatase expression was found to be associated with favorable survival in female NSCLC patients in the United States [13]. However, in reports based on Asian populations, aromatase expression had no association with prognosis, although aromatase was expressed in more than 60% of lung cancer patients [14, 27]. The result of Mah's report [13] is consistent with that of ours, although those of later reports are not. However, those previous studies grouped adenocarcinoma and other histological types of NSCLC together. Furthermore, *EGFR* mutation status was not analyzed in those studies. The precise mechanism underlying the worse survival regarding the association of aromatase expression with *EGFR* mutation status in our study is unknown. However, the frequency of *EGFR* mutations in NSCLC varies among races: from 27 to 60% in Asians and from 8 to 16% in Europeans, Africans, and Caucasian Americans [43-44]. Based on these observations and our findings, we speculate that the prognostic discrepancy in relation to the aromatase expression level among previous reports might be attributable to the difference in *EGFR* mutation status in each study.

To further examine aromatase and ERβ as a predictor of survival, and to assess the importance of sex, we analyzed the association between aromatase/ERβ expression and sex in *EGFR* wild-type patients according to survival. As expected, our result showed that high aromatase expression was a significant predictor of poor survival only in females, and we found no predictive value for aromatase expression levels in males. By contrast, our result showed that ERβ-positive was a significant predictor of poor survival only in males, and we found no predictive value for ERβ expression levels in females.

The differences in these sex-related results seem to depend on the difference in the status of, and sensitivity to, reproductive hormones according to sex. In postmenopausal females, circulating estrogen levels are decreased due to the decline in estrogen production by the ovaries [45]. Under these conditions, local estrogen production through aromatase might be an important determinant of estrogen levels. In fact, Niikawa et al. demonstrated that the intratumoral estradiol concentration was significantly higher in NSCLC than in nonneoplastic lung tissue and was positively correlated with intratumoral aromatase expression [46]. In our study, we did not distinguish females according to menopause status because only one of the females with *EGFR* wild-type lung adenocarcinoma was premenopausal. In other words, our result indicated that aromatase expression level is prognostic factor in postmenopausal females with *EGFR* wild-type lung adenocarcinoma. Therefore, intratumoral aromatase expression is associated with tumor progression via estrogen signaling pathway in postmenopausal females, particularly in *EGFR* wild-type lung adenocarcinoma.

In contrast to females, circulating estrogen levels in males are almost the same as those in postmenopausal females, and this condition

remains relatively constant with age [47]. Martin et al. demonstrated that ER-positive breast cancer cells are hypersensitive to low doses of estrogen with long-term estrogen deprivation [48]. Furthermore, in males, a higher level of androgen, which is a substrate of estrogen synthesis, is present than in females [47]. Based on these observations, at low expression levels, aromatase may produce an amount of estrogen sufficient for proliferation of ER-positive cells. In the present study, 92.1% of lung adenocarcinoma expressed aromatase at lower levels in males. Therefore, these findings support our hypothesis that lung adenocarcinoma in males supplies sufficient levels of estrogen to activate ER for tumor cell maintenance, and that the level of tumor proliferation activity-mediated via estrogen signaling pathway-is more dependent on ERβ than aromatase expression in males.

The role of AIs in lung adenocarcinoma is unclear. However, many studies have reported that AIs demonstrated significant anti-tumor effects in NSCLC expressing aromatase both in vitro and in vivo [12, 16, 33, 46, 49]. These observations and our findings revealed that *EGFR* wild-type lung adenocarcinoma patients with high aromatase expression are a suitable subset for AI treatment, particularly in postmenopausal females. Currently, a phase I clinical trial of the irreversible steroidal AI exemestane in combination with chemotherapy for late-stage lung cancer in postmenopausal females is underway (NCT01664754). We are awaiting the results of this phase I study, which may reveal that AI treatment is effective for lung cancer in postmenopausal females.

Stabile et al. reported that increased *EGFR* signaling might be caused by depletion of estrogen signals induced by endocrine therapy, and targeting both pathways could be beneficial for therapy [34]. A phase II trial of erlotinib (EGFR-TKI) or erlotinib + fulvestrant in previously treated male and female advanced NSCLC has been completed [Garon EB, Siegfried JM, Dubinett SM, Elashoff RM, Park DJ, Parikh RJ, Patel R, Hu EH, Reckamp KL, Adams B, Martinez D, Wang HJ, Kabbinavar F, Dacic S, Brennan M, Laux I, Márquez-Garban DC, Stabile LP, Slamon DJ, Pietras RJ. Results of TORI-L-03, a randomized, multicenter phase II clinical trial of erlotinib (E) or E + fulvestrant (F) in previously treated advanced non-small cell lung cancer (NSCLC). Presented at the 104th Annual Meeting of the American Association for Cancer Research; Washington DC. PA, April 6-10, 2013. p. Abstract 4664; Unpublished results]. Interestingly, the clinical benefit rate was significantly higher among patients treated with the combination compared with erlotinib alone among patients with *EGFR* wild-type tumors, although the survival and response rates were similar between the two treatment arms in unselected patients. The latter finding supports our suggestion that estrogen signaling pathway plays an important role in the development of *EGFR* wild-type lung adenocarcinoma. Thus, endocrine therapy could also be beneficial for *EGFR* wild-type lung adenocarcinoma as a combination therapy with EGFR-TKI.

The limitations of present study include selection of antibodies, the retrospective design, and relatively small number of patients, all of whom were Japanese. Thus, large, population-based prospective studies with ethnically diverse populations are warranted to elucidate the role of growth factor pathways, including *EGFR* and/or estrogen signaling pathways, in lung adenocarcinoma.

In conclusion, we demonstrated that aromatase and ERβ expressions are independent negative prognostic factors in *EGFR* wild-type lung adenocarcinoma and that high aromatase expression is a significant predictor of poor survival only in females, whereas ERβ-positive is an important predictor of poor survival only in males. We suggest that *EGFR* wild-type lung adenocarcinoma is a hormone-related carcinoma.

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Disclosure of conflict of interest

None.

Address correspondence to: Kimihiro Shimizu, Department of Thoracic and Visceral Organ Surgery, Gunma University Graduate School of Medicine, 3-39-22 Showa-machi, Maebashi, Gunma 371-85-11, Japan. Tel: 81-27-220-8245; Fax: 81-27-220-8255; E-mail: kmshimizu@gmail.com

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Table S1. Association between clinicopathologicalfactors and immunoreactivity for aromatase in 150 lung adenocarcinomas

	Aromatase					ERβ				
	High expression		Low expression		*P* value	Positive		Negative		*P* value
	N = 72	% (range)	N = 78	% (range)		N = 119	% (range)	N = 31	% (range)	
Age (years: median 69)	68	(40–83)	65	(36–84)	0.077	67	(40–83)	63	(36–84)	0.066
Sex										
Male	33	22.0	30	20.0		48	32.0	15	10.0	
Female	39	26.0	48	32.0	0.361	71	47.3	16	10.7	0.272
Smoking status										
Ever-smoker	35	23.3	33	22.0		54	36.0	14	9.3	
Non-smoker	37	24.7	45	30.0	0.438	65	43.4	17	11.3	0.573
Tumor diameter (mm)	25	(7–53)	23	(6–70)	0.377	25	(7–70)	20	(6–60)	0.050
Pathologic stage										
IA	38	25.3	47	31.4		62	41.3	23	15.4	
IB	14	9.3	12	8.0		23	15.4	3	2.0	
IIA	2	1.3	3	2.0		5	3.3	0	0.0	
IIB	4	2.7	2	1.3		4	2.7	2	1.3	
IIIA	12	8.0	10	6.7		20	13.3	2	1.3	
IIIB	2	1.3	4	2.7	0.763	5	3.3	1	0.7	0.207
Pleural invasion										
Absent	43	28.7	59	39.3		79	52.6	23	15.4	
Present	29	19.3	19	12.7	0.037	40	26.7	8	5.3	0.589
Lymphatic invasion										
Absent	48	32.0	53	35.3		79	52.6	22	14.7	
Present	24	16.0	25	16.7	0.867	40	26.7	9	6.0	0.399
Vascular invasion										
Absent	47	31.3	57	38.0		80	53.3	24	16.0	
Present	25	16.7	21	14.0	0.301	39	26.0	7	4.7	0.191
EGFR mutation										
Mutant	33	23.6	29	20.7		53	37.9	9	6.4	
Wild type	35	25.0	43	30.7	0.326	59	42.1	19	13.6	0.108
KRAS mutation										
Mutant	10	7.1	13	9.3		19	13.6	4	2.9	
Wild type	58	41.4	59	42.2	0.593	93	66.4	24	17.1	0.493
Aromatase										
High expression						60	40.0	12	8.0	
Low expression						59	39.3	19	12.7	0.168
ERα										
Positive	1	0.7	1	0.7		2	1.3	0	0.0	
Negative	71	47.3	77	51.3	0.731	117	78.0	31	20.7	0.628
ERβ										
Positive	60	40.0	59	39.3						
Negative	12	8.0	19	12.7	0.168					
PR										
Positive	2	1.3	2	1.3		4	2.7	0	0.0	
Negative	70	46.7	76	50.7	0.659	115	76.7	31	20.6	0.392
HER2										
Positive	4	2.7	3	2.0		7	4.7	0	0.0	
Negative	68	45.3	75	50.0	0.455	112	74.7	31	20.6	0.190
Ki-67 LI (%)	18.9	(1.0–63.1)	17.3	(0.2–93.6)	0.582	18.2	(1.0–66.4)	17.5	(0.2–93.6)	0.865

EGFR, epidermal growth factor receptor; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; LI, labeling index.

Table S2. Univariate analysis of prognostic factors using the log-rank test in *EGFR* mutant cases

Variable	No. of patients (%)		5-year OS (%)	Univariate *P* value	5-year RFS (%)	Univariate *P* value
All cases	62	(100.0)	83.9		69.3	
Age (years: median 69)						
<69	34	(54.8)	85.3		67.5	
≥69	28	(45.2)	82.1	0.487	71.4	0.854
Sex						
Male	19	(30.6)	89.5		73.7	
Female	43	(69.4)	81.4	0.393	67.4	0.678
Smoking status						
Ever-smoker	19	(30.6)	84.2		63.2	
Non-smoker	43	(69.4)	83.7	0.867	72.1	0.500
Pathologic stage						
I, II	33	(53.2)	92.2		82.4	
III	29	(46.8)	36.4	<0.001	9.1	<0.001
Pleural invasion						
Absent	48	(77.4)	87.5		74.9	
Present	14	(22.6)	71.4	0.198	50.0	0.039
Lymphatic invasion						
Absent	46	(74.2)	91.3		82.6	
Present	16	(25.8)	62.5	0.016	31.3	<0.001
Vascular invasion						
Absent	47	(75.8)	89.4		80.9	
Present	15	(24.2)	66.7	0.078	33.3	0.001
KRAS mutation						
Mutant	0	(0.0)	---		---	
Wild type	62	(100.0)	83.9	---	69.3	---
Aromatase						
High expression	33	(53.2)	81.8		69.7	
Low expression	29	(46.8)	86.2	0.861	69.0	0.915
ERα						
Positive	0	(0.0)	---		---	
Negative	62	(100.0)	83.9	---	69.3	---
ERβ						
Positive	53	(85.5)	84.9		67.9	
Negative	9	(14.5)	77.8	0.575	77.8	0.677
PR						
Positive	1	(1.6)	100.0		100.0	
Negative	61	(98.4)	83.6	0.649	68.8	0.542
HER2						
Positive	3	(4.8)	66.7		33.3	
Negative	59	(95.2)	84.7	0.501	71.1	0.034
Ki-67 LI (%: median 11.5)						
Low score	33	(53.2)	93.9		90.9	
High score	29	(46.8)	72.4	0.011	44.8	<0.001

OS, overall survival; RFS, recurrence-free survival;ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; LI, labeling index.

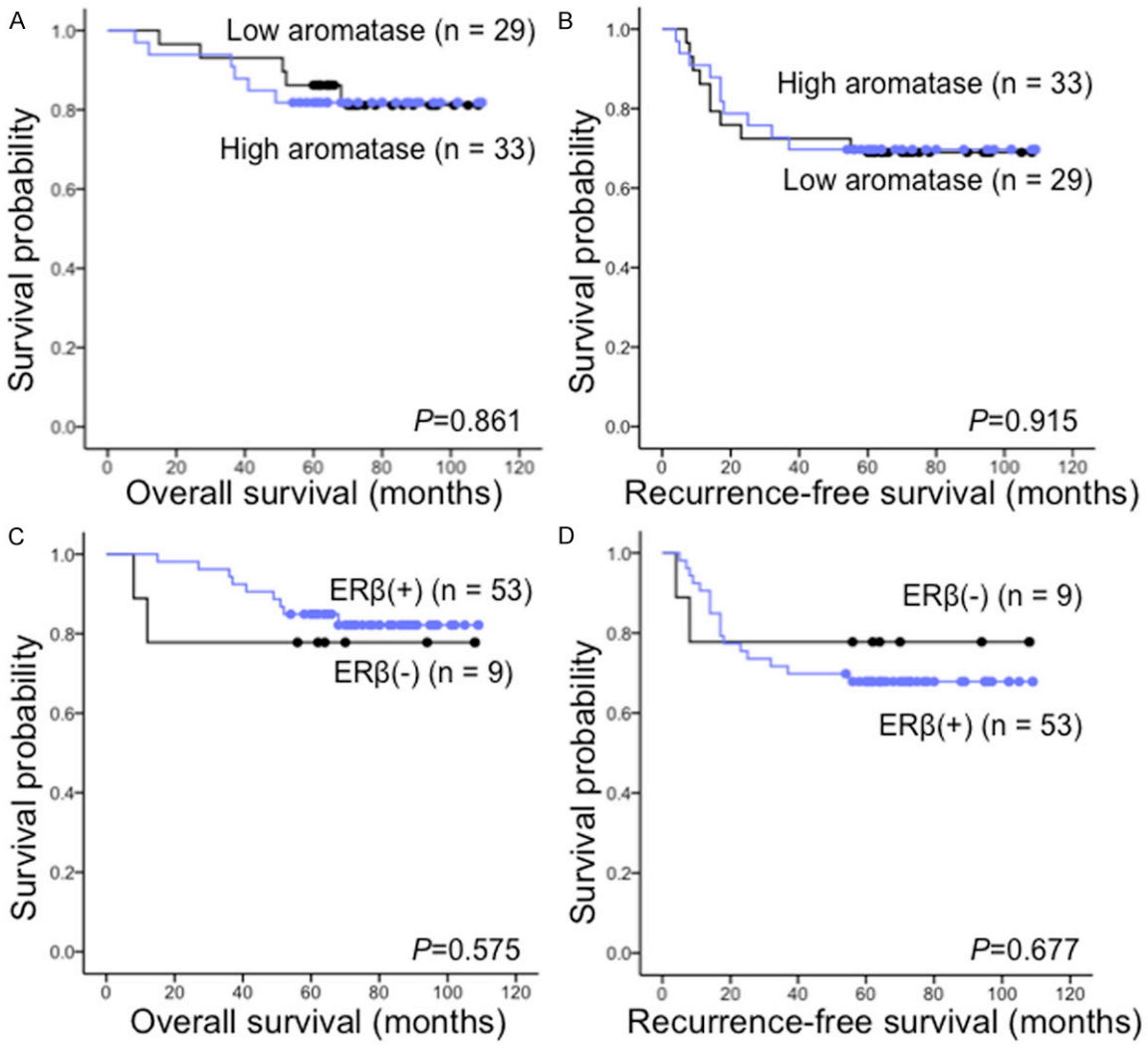

Figure S1. Kaplan-Meier survival curves of 62 lung adenocarcinoma patiens with EGFR mutations according to the immunoreactivity results for aromatase and ERβ. A. Overall (OS) stratified by high versus low expression of aromatase. B. Recurrence-free survival (RFS) stratified by high verus low expression of aromatase. C. OS stratified by positive versus negative expression of ERβ. D. RFS stratified by positive versus negative expression of ERβ.