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p53 expression in tumor-stromal fibroblasts forming and not forming fibrotic foci in invasive ductal carcinoma of the breast

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The purpose of this study was to determine whether p53 protein expression in tumor-stromal fibroblasts forming fibrotic foci is a significant outcome predictor, similar to p53 protein expression in tumor-stromal fibroblasts not forming fibrotic foci, and whether the combined assessment of p53 expression in tumor-stromal fibroblasts forming and not forming fibrotic foci served as an important outcome predictor among 1039 patients with invasive ductal carcinoma of the breast. We analyzed the outcome predictive power of the Allred score risk classification for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci using multivariate analyses with well-known clinicopathological factors. The Allred score risk classifications for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci were superior to the Allred scores for p53 in tumor-stromal fibroblasts not forming fibrotic foci alone for accurately predicting the tumor-related death of patients with invasive ductal carcinoma when examined using multivariate analyses. The Allred score risk classification for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci significantly increased the hazard rates for tumor recurrence and tumor-related death independent of the UICC pTNM stage in the multivariate analyses. These results indicated that the Allred score risk classification based on the combined assessment of p53 expression in tumor-stromal fibroblasts forming and not forming fibrotic foci is a very useful outcome predictor among patients with invasive ductal carcinoma.
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Keywords: fibroblast; fibrotic focus; p53; tumor cell–stromal cell interaction; breast

Along with others, we have already reported that a fibrotic focus, a characteristic histological feature of tumor stroma, is a very useful histological tumor-stromal indicator for accurately predicting the outcome of patients with invasive ductal carcinoma (IDC),[1–5] and the proliferative activity of tumor-stromal fibroblasts forming and not forming fibrotic foci has a very important function in nodal metastasis and distant organ metastasis by IDCs.[6,7] Because it has recently been reported that the gene expression profile and protein expression profile of the tumor stroma have a very important function in tumor progression in carcinoma[8,9] and that the interactions between tumor cells and stromal cells also are very important in tumor progression in carcinomas,[10,11] these findings strongly suggest that the tumor stroma has a significant function in tumor progression in IDCs. Mutations of the p53 tumor suppressor gene have been described in the stromal fibroblasts of breast and prostate carcinomas in

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humans and experimental animals,[12–14] and p53 mutations in breast cancer stromal cells have been reported to be closely associated with nodal metastasis.[15] However, some studies have reported that p53 mutations are not observed in the tumor stroma of breast cancer,[16,17] and the possibility of technical problem, eg polymerase chain reaction artifacts for the p53 gene abnormality, has been suggested by Campbell *et al*.[18] We recently showed that p53 expression in tumor-stromal fibroblasts not forming fibrotic foci was a very important outcome predictor for IDC patients who had or had not received neoadjuvant therapy.[19,20] On the basis of the above findings, the p53 status of tumor-stromal fibroblasts not forming fibrotic foci probably has a very important function in tumor progression in IDCs.

We also previously reported that our newly devised grading system for lymph vessel tumor emboli is a very useful histological grading system for accurately predicting the outcome of patients with IDC who have not received neoadjuvant therapy; furthermore, this grading system can be used to classify the prognosis of IDC patients with lymph vessel invasion into low-risk, intermediate-risk, and high-risk groups.[21] In addition, we recently confirmed that this grading system for lymph vessel tumor emboli was a very important outcome predictor for patients with IDC in a different patient group.[22]

The purpose of this study was to determine whether the combined assessment of p53 expression in tumor-stromal fibroblasts forming and not forming fibrotic foci served as an important outcome predictor among patients with IDC of the breast using multivariate analyses with well-known prognostic factors and our grading system for lymph vessel tumor emboli. The results indicated that a score classification based on the combined assessment of p53 expression in tumor-stromal fibroblasts forming and not forming fibrotic foci was a very useful outcome predictor among patients with IDC of the breast.

Materials and methods

Cases

The subjects of this study were 1039 consecutive patients with IDC of the breast who did not receive neoadjuvant therapy and who were surgically treated at the National Cancer Center Hospital between January 2000 and December 2005 (almost the same case series as that used in our previous study).[19,22] The IDCs were diagnosed preoperatively using needle biopsy, aspiration cytology, a mammography, or ultrasonography. All the patients were Japanese women, ranging in age from 23 to 72 years (median, 55 years). All had a solitary lesion; 497 patients were premenopausal and 542 were postmenopausal. A partial mastectomy had been performed in 455 patients, and a modified radical mastectomy had been performed in 584. A level I and level II axillary lymph node dissection had been performed in all the patients, and a level III axillary lymph node dissection had been performed in some of the patients with IDC.

Of the 1039 patients, 873 received adjuvant therapy, consisting of chemotherapy in 218 patients, endocrine therapy in 281 patients, and chemoendocrine therapy in 374 patients. The chemotherapy regimens used were anthracycline-based with or without taxane and non-anthracycline-based, and the endocrine therapy regimens consisted of tamoxifen with or without a gonadotropin-releasing hormone agonist, tamoxifen, with or without an aromatase inhibitor, an aromatase inhibitor alone, or a gonadotropin-releasing hormone agonist alone. No cases of inflammatory breast cancer were included in this series. All the tumors were classified according to the pathological UICC-TNM (pTNM) classification.[23] The protocol of this study (20-112) was reviewed by the institutional review board of the National Cancer Center.

For the pathological examination, we fixed the surgically resected specimens in 10% formalin, and the size and gross appearance of the tumors were recorded. The tumor size was confirmed by comparison with the tumor size on the histological slides; if more than one invasive focus was present, the size of the largest invasive focus was recorded as the invasive tumor size, based on a previously reported definition for determining the size of microinvasion in IDC with multiple microinvasive foci[23] in this study.

Histological Examination

Serial sections of each tumor area were cut from paraffin blocks. One section from each tumor was stained with hematoxylin and eosin and was examined histologically to confirm the diagnosis, and another section was subjected to immunohistochemistry. The following eight histological factors and the grading system for lymph vessel tumor emboli[21,22] were evaluated: (1) invasive tumor size (≤ 20, >20 to ≤ 50, >50 mm); (2) histological grade (1, 2, 3);[24] (3) tumor necrosis (absent, present);[25] (4) fibrotic focus (absent, fibrotic focus diameter ≤ 8 mm, fibrotic focus diameter >8 mm) (Figure 1);[1,2] (5) blood vessel invasion (absent, present); (6) adipose tissue invasion (absent, present); (7) skin invasion (absent, present); and (8) muscle invasion (absent, present).

Immunohistochemistry

Immunohistochemical staining for estrogen receptors, progesterone receptors, p53, and HER2 products was performed using an autoimmunostainer (Optimax Plus; BioGenex, San Ramon, CA, USA). The antigen retrieval device for Optimax Plus was

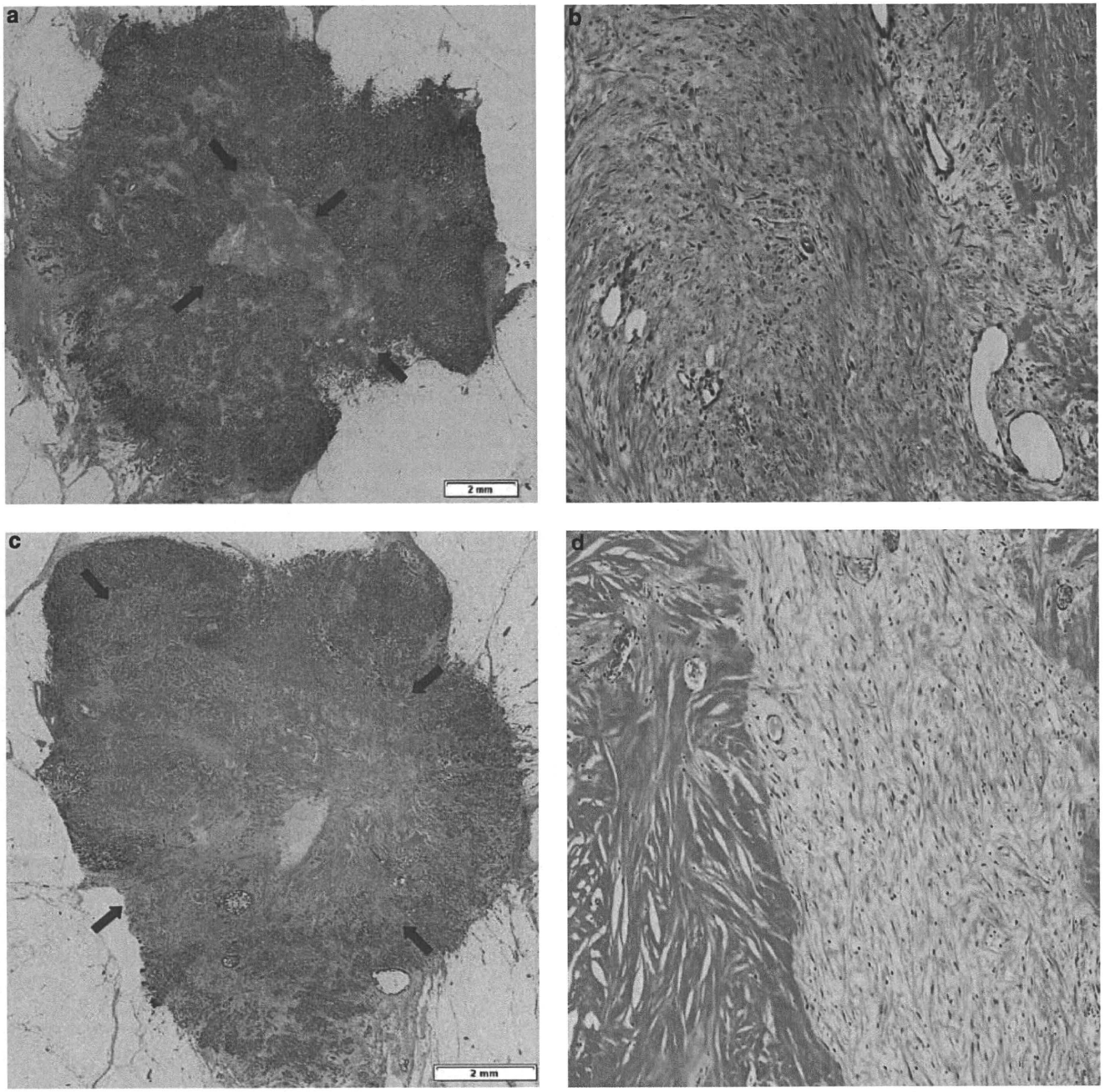


Figure 1 Invasive ductal carcinomas with fibrotic foci (**a–d**). (**a**) A fibrotic focus measuring 6.4 × 3.3 mm is visible within the tumor (panoramic view, arrows). The fibrotic focus shows a scar-like feature and is surrounded by invasive ductal carcinoma cells. (**b**) The fibrotic focus area consists mainly of fibroblasts arranged in a storiform pattern. (**c**) A fibrotic focus measuring 10.2 × 7.3 mm is visible within the tumor (panoramic view, arrows). The fibrotic focus has a fibrosclerotic core and is surrounded by invasive ductal carcinoma cells. Small residual tumor islands are present within the fibrotic focus. (**d**) The fibrotic focus consists of fibroblasts and hyalinized collagen fibers in a storiform arrangement.

an autoclave, and each specimen was immersed in citrate buffer and incubated at 121°C for 10 min. Immunoperoxidase staining was performed using a labeled streptavidin biotin staining kit (BioGenex) according to the manufacturer's instructions. The antibodies used were the anti-estrogen receptor mouse monoclonal antibody ER88 (BioGenex), the anti-progesterone receptor mouse monoclonal antibody PR88 (BioGenex), the anti-HER2 mouse monoclonal antibody CB11 (BioGenex), and the p53 mouse monoclonal antibody DO7 (Dako, Glostrup, Denmark). ER88, PR88, and CB11 were previously diluted, and DO7 was applied at a dilution of 1:100. After immunostaining, the sections were counterstained with hematoxylin. Sections of the IDCs that were positive for estrogen receptor, progesterone

receptor, HER2, and p53 were used each time as a positive control. As a negative control, the primary antibody was replaced with normal mouse immunoglobulin.

Assessment of ER, PR, p53, and HER2 Expression

Slides of the tumor cells immunostained for estrogen receptor, progesterone receptor, and p53 were scored using the Allred scoring system, as described previously,[26–28] and the Allred scores for estrogen receptor, progesterone receptor, and p53 expression in the tumor cells were classified into the following three categories[19]: (1) Allred score for estrogen receptor in tumor cells (0 or 2, 3–6, and 7 or 8); (2) Allred score for progesterone receptor in tumor cells (0 or 2, 3–6, and 7 or 8); and (3) Allred scores for p53 in tumor cells (0 or 2 or 3, 4–6, and 7 or 8). We modified the Allred scoring system to assess the nuclear expression of p53 in the tumor-stromal fibroblasts forming and not forming fibrotic foci,[19,20] and the Allred scores for p53 expression in tumor-stromal fibroblasts forming and not forming fibrotic foci were classified into the following categories: (1) Allred scores for p53 in tumor-stromal fibroblasts forming fibrotic foci (0, 2, 3, and 4–8); and (2) Allred scores for p53 in tumor-stromal fibroblasts not forming fibrotic foci (0 or 2, 3, and 4–8) (Figures 2 and 3). Of the 1039 IDCs, 373 IDCs had fibrotic foci; we could not assess the Allred scores for p53 in tumor-stromal fibroblasts forming a fibrotic focus in 97 of the 373 IDCs with fibrotic foci because the immunohistochemistry examinations for these specimens were performed using tumor tissue sections that did not contain a fibrotic focus at the time of routine examination. The HER2 status of the tumor cells was semiquantitatively scored on a scale of 0–3 according to the level of HER2 protein expression,[29] and it was classified into three categories: 0 or 1, 2, and 3.

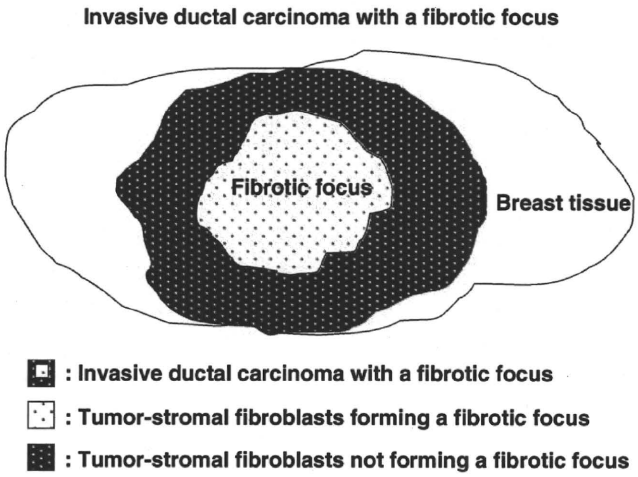


Figure 2 Schematic illustration of an invasive ductal carcinoma with a fibrotic focus.

Patient Outcome and Statistical Analysis

Survival was evaluated using a median follow-up period of 52 months (range: 18–102 months) until February 2009. Of the 1039 IDC patients, 910 patients were alive and well, 129 had developed tumor recurrences, and 58 had died of their disease. The tumor recurrence-free survival and overall survival periods were calculated using the time of surgery as the starting point. Tumor relapse was considered to have occurred whenever evidence of metastasis was found.

The Mann–Whitney test was used to compare the Allred scores for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci, and the correlation analyses were performed using Cochran-Mantel-Haenszel statistics.

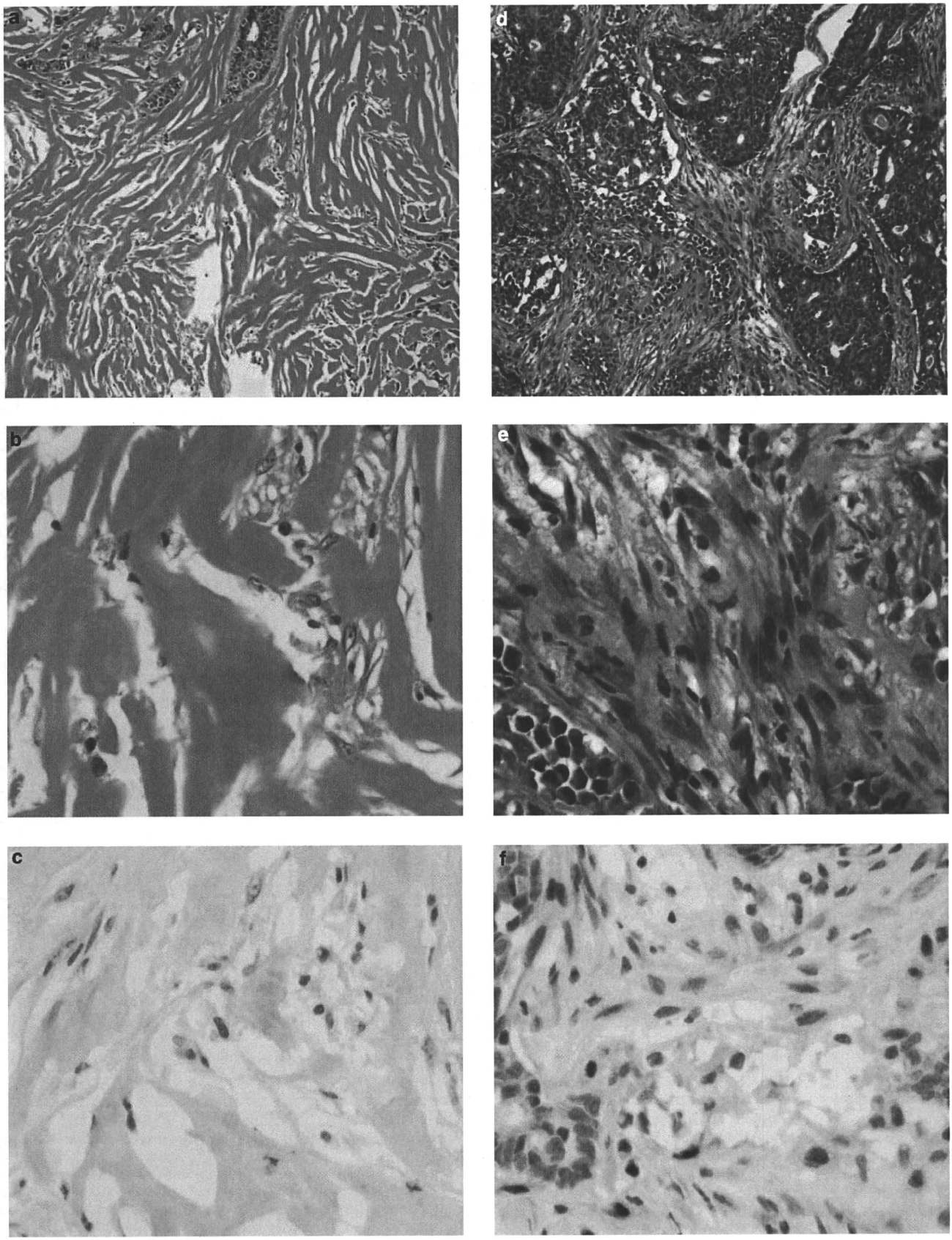
We analyzed the outcome predictive power of the eight histological factors, the grading system for lymph vessel tumor emboli;[21,22] the Allred scores for estrogen receptor; progesterone receptor, and p53 in tumor cells; the category of HER2 expression in tumor cells; the Allred score risk classification for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci, adjuvant therapy (yes or no); age ($\leq$39 years and $>$39 years); and the UICC-pathological nodal status (N factor, ie, no nodal metastasis, N0; 1–3 nodal metastases, N1; 4–9 nodal metastases, N2; and 10 or more nodal metastases, N3)[23] for tumor recurrence, and tumor-related death in univariate analyses using the Cox proportional hazard regression model. The factors significantly associated with outcome in the univariate analyses were then entered together into the multivariate analyses using the Cox proportional hazard regression model according to the UICC pTNM stage. The case-wise and step-down method was applied until all the remaining factors were significant at a *P*-value of below 0.05. Because fewer than 10 tumor-related deaths occurred among the UICC stage I IDC patients (Table 2), it was impossible to perform multivariate analyses for tumor-related death in this group. All the analyses were performed using Statistica for Windows software (StatSoft, Tulsa, OK, USA).

Results

Allred Scores for p53 in Tumor-Stromal Fibroblasts Forming and Not Forming Fibrotic Foci

Although a significant association was observed between the Allred scores for p53 in tumor-stromal fibroblasts forming and those not forming fibrotic foci ($P<0.001$; Figure 4a), the latter value (mean value, 2.2; standard deviation, 2.1) was significantly higher than the former (mean value, 1.6; standard deviation, 2.0; $P=0.001$). The Allred scores for p53 in tumor-stromal fibroblasts forming fibrotic foci were also significantly associated with the fibrotic focus diameter, and in IDCs with a fibrotic focus

diameter >8 mm, the number of IDCs with Allred scores of 4–8 for p53 in tumor-stromal fibroblasts forming fibrotic foci was larger than that of IDCs with Allred scores of 0, 2, or 3 for p53 in tumor-stromal fibroblasts forming fibrotic foci (Figure 4b).



Allred Score Risk Classification for p53 in Tumor-Stromal Fibroblasts Forming and not Forming Fibrotic Foci in Patients with Invasive Ductal Carcinoma with and without Fibrotic Foci

We devised an Allred score risk classification for p53 in tumor-stromal fibroblasts in IDCs based on the combined Allred scores for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci (Table 1). This classification was successfully used to classify IDC patients with or without fibrotic foci into three risk classes (low risk, intermediate risk, and high risk) according to the ratios for tumor recurrence and tumor-related death (Table 2; Figure 5). Among the UICC pTNM stage I IDC patients, the patients in the intermediate- and high-risk classes showed a significantly higher tumor recurrence rate than the patients in the low-risk class (Table 2). Among the UICC pTNM stage II IDC

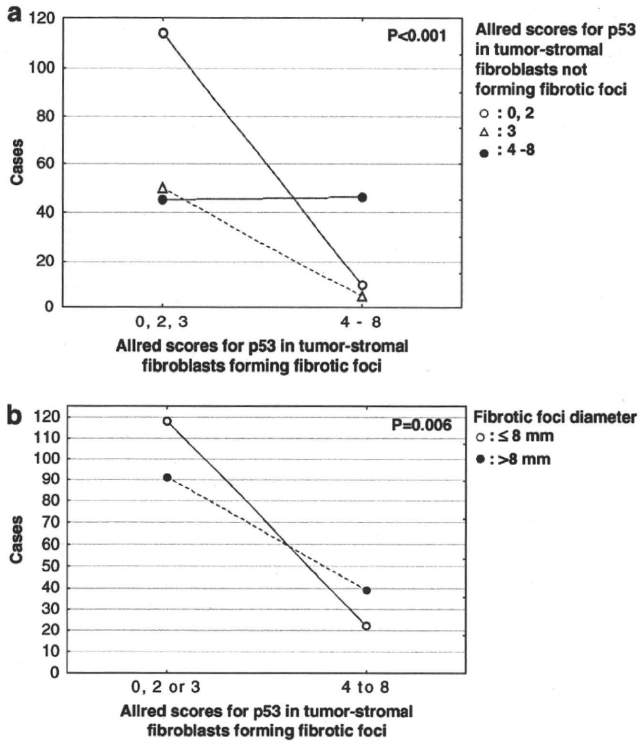


Figure 4 (**a**) Associations between the Allred scores for p53 in tumor -stromal fibroblasts forming and not forming fibrotic foci; the scores were significantly associated with each other ($P<0.001$). (**b**) Associations between the Allred scores for p53 in tumor-stromal fibroblasts forming fibrotic foci and the diameter of the fibrotic foci. Invasive ductal carcinomas with fibrotic foci >8 mm in diameter had a significantly higher Allred score for p53 in tumor-stromal fibroblasts forming fibrotic foci than those with fibrotic foci ≤8 mm in diameter ($P=0.006$).

Table 1 Overall Allred score classification of p53 in tumor-stromal fibroblasts forming and not forming a fibrotic focus

Invasive ductal carcinoma with a fibrotic focus	
A) The Allred scores of p53 in tumor-stromal fibroblasts forming a fibrotic focus	Score class
0, 2, or 3	0
4–8	2
B) The Allred scores of p53 in tumor-stromal fibroblasts not forming a fibrotic focus	Score class
0 or 2	0
3	1
4–8	2
Total (A+B)	0–4
Invasive ductal carcinoma without a fibrotic focus	
The Allred scores of p53 in tumor-stromal fibroblasts not forming a fibrotic focus	Score class
0 or 2	0
3	1
4–8	2
Total	0–2
The Allred score risk classes for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci	Score class
Low-risk class	0 and 1
Intermediate-risk class	2 and 3
High-risk class	4

Table 2 Tumor recurrence and tumor-related death rates according to the Allred score risk classes for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci in patients with invasive ductal carcinoma with or without a fibrotic focus

Risk classes	*Cases*	*TRR (%)*	P-*value*	*MR (%)*	P-*value*
Invasive ductal carcinoma patients as a whole					
Low-risk	648	36 (6)		9 (1)	
Intermediate-risk	232	52 (22)	<0.001	24 (10)	<0.001
High-risk	46	24 (52)	<0.001	15 (33)	0.001
Total	926	112 (12)		48 (5)	
UICC pTNM stage I invasive ductal carcinoma patients					
Low-risk	239	5 (2)		0	
Intermediate-risk	69	10 (15)	<0.001	4 (6)	<0.001
High-risk	6	2 (33)	0.295	0	0.454
Total	314	17 (5)		4 (1)	
UICC pTNM stage II invasive ductal carcinoma patients					
Low-risk	309	18 (6)		5 (2)	
Intermediate-risk	120	23 (19)	<0.001	7 (6)	0.045
High-risk	24	11 (46)	0.041	6 (25)	0.012
Total	453	52 (12)		18 (4)	
UICC pTNM stage III invasive ductal carcinoma patients					
Low-risk	100	13 (13)		4 (4)	
Intermediate-risk	43	19 (44)	<0.001	13 (30)	<0.001
High-risk	16	11 (69)	0.054	9 (56)	0.042
Total	159	43 (27)		26 (16)	

TRR, tumor recurrence rate; MR, mortality rate.

Figure 3 Tumor-stromal fibroblasts forming (**a**, **c**, **e**) and not forming a fibrotic focus (**b**, **d**, **f**). A fibrotic focus consists of tumor-stromal fibroblasts and hyalinized collagen fibers (**a** and **c**) and many tumor-stromal fibroblasts show a moderately intense nuclear staining pattern for p53. The Allred score for p53 in these tumor-stromal fibroblasts forming a fibrotic focus is 7 (intensity score, 2; proportion score, 5) (**e**). Carcinoma cells invade in irregular-shaped nests with a tubular structure (**b**) and tumor-stromal fibroblasts with oval nuclei not forming a fibrotic focus are seen (**d**). Many tumor-stromal fibroblasts not forming a fibrotic focus show a faint, moderate or strong intense nuclear staining pattern for p53, whereas tumor cells showing a faint intense nuclear staining pattern for p53 are visible (**f**). The Allred score for p53 in these tumor-stromal fibroblasts not forming a fibrotic focus is 8 (intensity score, 3; proportion score, 5).

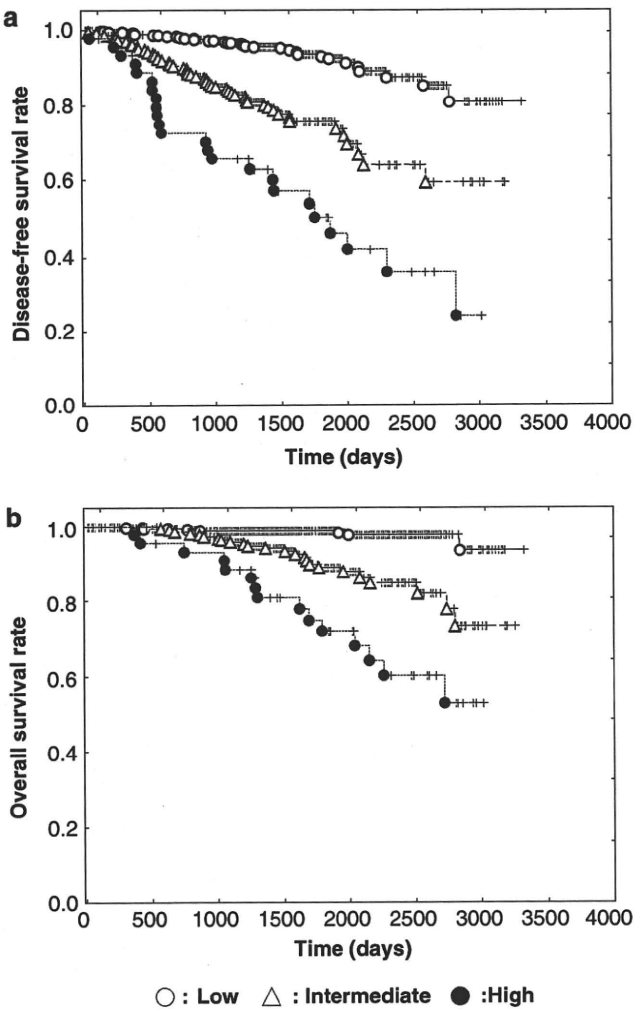


Figure 5 Disease-free survival curves and overall survival curves of invasive ductal carcinoma (IDC) patients overall (**a** and **b**) according to the Allred score risk classification for p53 in tumor-stromal fibroblasts forming and not forming a fibrotic focus (FF). The disease-free survival time (**a**) and the overall survival time (**b**) of the IDC patients significantly decrease with the risk class of the Allred score risk classification for p53 in tumor-stromal fibroblasts forming and not forming FF.

patients, the tumor recurrence rate and the mortality rate for each risk class were significantly increased according to the risk classes of the classification (Table 2). Among the UICC pTNM stage III IDC patients, the patients in the intermediate-risk class showed a significantly higher tumor recurrence rate and mortality rate than the patients in the low-risk class, and the patients in the high-risk class showed a marginally significantly higher tumor recurrence rate and a significantly higher mortality rate than the patients in the intermediate-risk class (Table 2).

Overall, the Allred score risk classification for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci (trend hazard rate, 2.9; trend 95% confidence interval, 1.6–5.2; *P*-value, <0.001) was superior to the Allred scores for p53 in tumor-stromal fibroblasts not forming fibrotic foci alone (trend hazard rate, 1.5; trend 95% confidence interval, 0.8–2.6; *P*-value, 0.172) for accurately predicting tumor-related death among patients with IDC, as shown in a multivariate analysis.

Factors Significantly Associated with Tumor Recurrence and Tumor-Related Death

Among the patients with UICC pTNM stage I IDC, an intermediate-risk class (hazard rate, 6.2; 95% confidence interval, 2.1–18.5; *P*-value, 0.001) and a high-risk class (hazard rate, 11.6; 95% confidence interval, 2.1–63.8; *P*-value, 0.005) for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci and a histological grade of 3 (hazard rate, 2.9; 95% confidence interval, 1.1–7.6; *P*-value, 0.034) significantly increased the hazard rates for tumor recurrence in a multivariate analysis.

Among the patients with UICC pTNM stage II IDC, an intermediate-risk class and a high-risk class for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci significantly increased the hazard rates for tumor recurrence and tumor-related death in the multivariate analyses (Table 3). Grades 2 and 3 lymph vessel tumor emboli and the presence of blood vessel invasion significantly increased the hazard rates for tumor recurrence in the multivariate analysis (Table 3). A UICC pN1 category and a fibrotic focus diameter >8 mm significantly increased the hazard rates for tumor-related death and an Allred score of 7 or 8 for the progesterone receptors in the tumor cells significantly decreased the hazard rate for tumor-related death in the multivariate analyses (Table 3).

Among the patients with a UICC pTNM stage III IDC, an intermediate-risk class and a high-risk class for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci, grade 3 lymph vessel tumor emboli and a UICC pN3 category significantly increased the hazard rates for tumor recurrence and tumor-related death in the multivariate analysis (Table 4). A fibrotic focus diameter >8 mm significantly increased the hazard rate for tumor recurrence and an Allred score of 7 or 8 for estrogen receptor in the tumor cells significantly decreased the hazard rate for tumor-related death in the multivariate analysis (Table 4).

Discussion

This study clearly showed that the values of the Allred scores for p53 in tumor-stromal fibroblasts not forming fibrotic foci were significantly higher than those in tumor-stromal fibroblasts forming fibrotic foci. Fibrotic foci are fibrotic scar-like lesions that mainly consist of tumor-stromal fibroblasts admixed with various numbers of tumor cells; some fibrotic foci do not contain any tumor cells.[1,2] In contrast, tumor-stromal fibroblasts not forming fibrotic foci commonly admix with many tumor cells that show stromal invasion. This difference

Table 3 Multivariate analyses for tumor recurrence and tumor-related death in UICC pTNM stage II invasive ductal carcinoma patients ($n=453$)

Factors	*Tumor recurrence*		*Tumor-related death*	
	HR (95% CI)	*P-value*	*HR (95% CI)*	*P-value*
p53 Allred score risk classes of tumor-stromal fibroblasts forming and not forming a fibrotic focus				
Low-risk	Referent		Referent	
Intermediate-risk	3.5 (1.4–4.4)	0.003	3.3 (1.0–10.5)	0.043
High-risk	5.2 (1.8–6.5)	<0.001	4.7 (1.3–17.3)	0.021
Grading system for lymph vessel tumor emboli				
Grade 0	Referent		Referent	
Grade 1	1.5 (0.8–3.0)	0.226	0.5 (0.1–2.5)	0.421
Grades 2 and 3	2.5 (1.4–4.4)	0.003	2.0 (0.6–6.3)	0.275
Blood vessel invasion				
Absent	Referent		Referent	
Present	2.1 (1.1–3.8)	0.017	1.1 (0.3–3.8)	0.914
The Allred scores for progesterone receptors in tumor cells				
0 or 2	Referent		Referent	
3–6	—		0.8 (0.2–3.0)	0.729
7 or 8	—		0.2 (0.07–0.7)	0.009
UICC pN category				
pN0	Referent		Referent	
pN1	—		14.7 (1.9–113.1)	0.010
Fibrotic focus, diameter				
Absent	Referent		Referent	
≤8 mm	—		1.3 (0.2–8.5)	0.763
>8 mm	—		3.4 (1.2–9.8)	0.025

HR, hazard rate; CI, confidence interval; —, not significance in univariate analysis.
The multivariate analysis for tumor recurrence was performed using the p53 Allred score risk classes in tumor-stromal fibroblasts forming and not forming a fibrotic focus, grading system for lymph vessel tumor emboli, blood vessel invasion, histological grade, and age.
The multivariate analysis for tumor-related death was performed using the p53 Allred score risk classes in tumor-stromal fibroblasts forming and not forming a fibrotic focus, grading system for lymph vessel tumor emboli, blood vessel invasion, the Allred scores for progesterone receptors in tumor cells, UICC pN category, fibrotic focus diameter, and age.

strongly suggests that the tumor cell–stromal cell interaction occurs more frequently in the outer area of a fibrotic focus than in the inner area of a fibrotic focus within IDCs,[10,11] probably resulting in the higher Allred scores for p53 in tumor-stromal fibroblasts not forming fibrotic foci. However, the Allred scores for p53 in tumor-stromal fibroblasts forming fibrotic foci were significantly associated with those for p53 in tumor-stromal fibroblasts not forming fibrotic foci. Thus, the tumor cell–stromal cell interaction probably occurs more frequently in IDCs with fibrotic foci than in IDCs without fibrotic foci.

We and others have already reported that the fibrotic focus diameter is a significant outcome predictor among patients with IDC who have fibrotic foci,[1–5] and our previous study showed that a fibrotic focus diameter of greater than 8 mm, similar to the Allred score for p53 in tumor-stromal fibroblasts not forming a fibrotic focus, was a significant outcome predictor for patients with IDC independent of the UICC pTNM stage.[19] In this study, a fibrotic focus diameter was also a significant outcome predictor for IDC patients of UICC pTNM stage II and IDC patients of UICC pTNM stage III, and IDCs with fibrotic foci greater than 8 mm in diameter showed a significantly higher Allred score for p53 in tumor-stromal fibroblasts forming fibrotic foci than IDCs with fibrotic foci of 8 mm or less in diameter. Thus, one can conclude that p53-expressing tumor-stromal fibroblasts located in both the inner and outer regions of fibrotic foci heighten the malignant potential of IDCs, probably accounting for the prognostic value of the fibrotic focus diameter. In addition, the grading system for lymph vessel tumor emboli significantly increased the hazard rates for tumor recurrence or tumor-related death in multivariate analyses performed for IDC patients with UICC pTNM stage II and UICC stage III. Therefore, the fibrotic focus diameter and the grading system for lymph vessel tumor emboli are likely to be very important histological outcome predictors for patients with IDC.

The results of this study clearly show that the Allred score risk classification for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci had a greater outcome predictive power than the Allred scores for p53 in tumor-stromal fibroblasts not forming fibrotic foci alone. Furthermore, the Allred score risk classification for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci is a very important outcome predictor for patients with IDC

Table 4 Multivariate analyses for tumor recurrence and tumor-related death in UICC pTNM stage III invasive ductal carcinoma patients

Factors	*Tumor recurrence*		*Tumor-related death*	
	HR (95% CI)	P-*value*	*HR (95% CI)*	P-*value*
p53 Allred score risk classes of tumor-stromal fibroblasts forming and not forming a fibrotic focus				
Low-risk	Referent		Referent	
Intermediate-risk	2.9 (1.3–6.3)	0.009	5.2 (1.6–17.2)	0.007
High-risk	6.0 (2.6–13.9)	<0.001	20.1 (5.8–69.0)	<0.001
Grading system for lymph vessel tumor emboli				
Grade 0	Referent		Referent	
Grade 1	0.6 (0.2–1.8)	0.340	0.5 (0.1–3.1)	0.480
Grade 2	0.6 (0.2–1.6)	0.281	1.7 (0.5–5.8)	0.426
Grade 3	6.5 (2.9–14.4)	<0.001	2.6 (1.0–6.7)	0.045
UICC pN category				
pN0	Referent		Referent	
pN1	6.3 (0.5–81.3)	0.166	8.8 (0.4–203.7)	0.171
pN2	6.9 (0.6–70.2)	0.108	5.0 (0.3–80.1)	0.256
pN3	2.8 (1.5–5.3)	0.001	3.3 (1.4–7.8)	0.005
Fibrotic focus, diameter				
Absent	Referent		Referent	
≤8 mm	1.6 (0.6–4.3)	0.383	1.3 (0.2–8.6)	0.777
>8 mm	2.8 (1.3–6.2)	0.009	2.1 (0.5–9.5)	0.337
The Allred scores for estrogen receptor in tumor cells				
0 or 2	Referent		Referent	
3–6	0.7 (0.3–1.9)	0.488	1.2 (0.3–5.0)	0.836
7 or 8	0.6 (0.2–1.5)	0.257	0.4 (0.2–0.9)	0.033

HR, hazard rate; CI, confidence interval; pN, pathological regional lymph node; N0, no nodal metastasis; N1, 1–3 nodal metastases; N2, 4–9 nodal metastases; N3, 10 or more nodal metastases.
The multivariate analysis for tumor recurrence was performed using the p53 Allred score risk classes in tumor-stromal fibroblasts forming and not forming a fibrotic focus, grading system for lymph vessel tumor emboli, UICC pN category, fibrotic focus diameter, the Allred scores for estrogen receptors in tumor cells, the Allred scores for progesterone receptors in tumor cells, the Allred scores for p53 in tumor cells, invasive tumor size, tumor necrosis, and histological grade.
The multivariate analysis for tumor death was performed using the p53 Allred score risk classes in tumor-stromal fibroblasts forming and not forming a fibrotic focus, grading system for lymph vessel tumor emboli, UICC pN category, fibrotic focus diameter, the Allred scores for estrogen receptors in tumor cells, the Allred scores for p53 in tumor cells, HER2 category in tumor cells, age, invasive tumor size, and histological grade.

and an intermediate-risk or high-risk classification significantly increased the hazard rates for tumor recurrence and tumor-related death independent of the UICC pTNM stage in multivariate analyses that included well-known prognostic factors. Thus, we can conclude that the Allred score risk classification based on the Allred score for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci appears to be an excellent histological predictor of outcome among patients with IDC with or without fibrotic foci. However, as we could not analyze the outcome predictive power of the Allred score risk classification for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci among patients with IDC according to the types of adjuvant therapy (chemotherapy, endocrine therapy, and chemoendocrine therapy) in detail, the predictive power of the Allred score risk classification for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci should be analyzed separately among IDC patients treated with chemotherapy, endocrine therapy, and chemoendocrine therapy in the future.

In this study, we did not investigate the associations of the Allred scores for p53 with the presence of *p53* gene abnormalities in tumor-stromal fibroblasts. Although *p53* mutations in tumor-stromal fibroblasts are relatively common among primary breast cancers and other cancers and have been reported to exert a positive effect on cancer growth,[12–15] some studies have not shown any p53 mutations in the tumor-stroma of breast cancer.[16–18] We have already reported that fibroblasts forming fibrotic foci show significantly higher proliferative activities than those not forming fibrotic foci and found that no significant association exists between the proliferative activity of fibroblasts forming fibrotic foci and the fibrotic foci diameter.[7] In contrast, the Allred scores for p53 in tumor-stromal fibroblasts forming fibrotic foci were significantly lower than the Allred scores for p53 in tumor-stromal fibroblasts not forming fibrotic foci, and a significant association between the increase in the Allred scores for p53 in tumor-stromal fibroblasts forming fibrotic foci and the fibrotic foci diameter was observed in this study. Thus, although the mechanism that increases the malignant potential of IDCs through the expression of p53 in tumor-stromal fibroblasts should be investigated from the viewpoint of

p53 gene abnormalities, p53 immunoreactivity in tumor-stromal fibroblasts produced by tumor cell–stromal cell interactions inside and outside fibrotic foci might in fact reflect specific reactive changes other than the proliferative activity of fibroblasts forming fibrotic foci within the stroma that might be correlated with the prognosis.

In conclusion, this is the first study to show clearly that p53 expression in tumor-stromal fibroblasts forming and not forming fibrotic foci is strongly associated with the outcome of IDC patients. Because p53 expression in tumor-stromal fibroblasts forming and not forming fibrotic foci might be important in tumor progression in IDCs, p53 expression could be a very important target for tumor gene therapy for IDCs, suppressing tumor cell–stromal cell interactions arising from *p53* gene abnormalities or p53-related tumor microenvironment reactions.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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Isoflavone intake and risk of lung cancer: a prospective cohort study in Japan[1–3]

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ABSTRACT

Background: Although case-control studies support the idea that soy foods or isoflavone intake is associated with a decreased risk of lung cancer, little evidence is available from prospective cohort studies. Moreover, no prospective study has addressed this association in men.

Objective: We investigated the association between isoflavone intake and lung cancer incidence.

Design: We conducted a population-based prospective cohort study in 36,177 men and 40,484 women aged 45–74 y with no history of cancer at baseline in 1995–1999. Participants responded to a validated questionnaire, which included 138 food items. We used Cox proportional hazards regression analysis to estimate the hazard ratios (HRs) and 95% CIs of lung cancer incidence according to isoflavone intake, which was estimated by genistein content from soy foods.

Results: During 11 y (671,864 person-years) of follow-up, we documented 481 male and 178 female lung cancer cases. In men we found an inverse association between isoflavone intake and risk of lung cancer in never smokers (n = 13,051; multivariate HR in the highest compared with the lowest quartile of isoflavone intake: 0.43; 95% CI: 0.21, 0.90; P for trend = 0.024) but not in current or past smokers. A similar, nonsignificant inverse association was seen in never-smoking women (n = 38,211; HR: 0.67; 95% CI: 0.41, 1.10; P for trend = 0.135). We also tested effect modification by smoking status (P for interaction = 0.085 in men and 0.055 in men and women combined).

Conclusion: In a large-scale, population-based, prospective study in Japan, isoflavone intake was associated with a decreased risk of lung cancer in never smokers. *Am J Clin Nutr* 2010;91:722–8.

INTRODUCTION

Isoflavones, of which genistein and daidzein are the 2 major forms, are obtained primarily from soy or soy products in Asian diets. Because their structure is similar to that of the human female hormone 17-β-estradiol, isoflavones have particular affinity for the β-estrogen receptor (1) and can therefore act as estrogen agonists and antagonists that compete for estradiol at the receptor complex (2). In fact, isoflavones have been proposed to lower the risk of several sites of cancer, such as breast (3, 4) and prostate (5, 6), that are considered hormone related.

On the basis of findings that estrogen receptors are expressed in healthy lung tissue and lung tumors (7) and that estrogen induces cell proliferation in vivo and in vitro (8), a possible role for sex hormones in lung carcinogenesis has been proposed. Furthermore, several epidemiologic studies reported that hormone replacement therapy is associated with lung cancer risk, albeit that the direction of the association was not consistent (9–12). Thus, isoflavone intake may be related to the risk of lung as well as other hormone-related cancers.

Although 8 case-control studies in Asian populations on the association between soy foods or isoflavone intake and lung cancer risk (13–19) have been reported, the association remains controversial, particularly with regard to subgroup analyses by sex, smoking status, or histologic type. Moreover, little evidence is available from prospective cohort studies in Asia (20), and no prospective study has addressed this association in men. Recent observations suggest that lung cancer in never smokers is a distinct entity (21), and sex differences in lung cancer susceptibility have been debated (22). Given this background, prospective investigation of the association of isoflavone intake with lung cancer risk by smoking status and sex would be informative.

We investigated the association between isoflavone intake and lung cancer risk among Japanese men and women on the basis of a large-scale, population-based, prospective study with an 11-y follow-up period.

SUBJECTS AND METHODS

Study population

The Japan Public Health Center–based Prospective Study was launched during 1990–1994. We defined the study population as all registered Japanese inhabitants in 11 public health

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center (PHC) areas aged 40–69 y at the start of the baseline survey (23) (n = 140,420). The study protocol was approved by the Institutional Review Board of the National Cancer Center, Japan. For the present analysis, we excluded 2 PHC areas (Tokyo and Osaka) because data on cancer incidence were not available and a different definition of study population was used in Tokyo and Osaka, respectively (n = 23,524).

Participants in the present study were those aged 45–74 y who responded to a self-administered 5-y follow-up questionnaire, which included demographic data, personal medical history, smoking and alcohol drinking, diet (via a food-frequency questionnaire [FFQ]), and other lifestyle factors in 1995–1999. We initially identified 116,672 participants as the study population at the baseline survey after exclusion of 224 participants who were found to be ineligible due to non-Japanese nationality (n = 51), late report of emigration that occurred before the start of the follow-up period (n = 166), incorrect birth date (n = 3), and duplicate registration (n = 4). Furthermore, we excluded the 1631 participants who had died, moved out of the study area, or were lost to follow-up before the 5-y follow-up questionnaire survey. The remaining 115,041 participants were considered eligible for the present study. A total of 91,239 responded to the questionnaire, which gave a response rate of 79%.

We then excluded participants with incomplete information on soy food intake (n = 1115), those who reported daily energy intakes at the upper or lower 2.5% ends of the range (980 and 4222 kcal for men and 826 and 3693 kcal for women, respectively) (n = 4504), and those with incomplete information on smoking status (n = 5807). We also excluded 3152 participants who reported or were diagnosed with cancer before the 5-y follow-up questionnaire survey. Finally, a total of 76,661 participants (36,177 men and 40,484 women) were included in the analysis.

Exposure data

The FFQ used in the 5-y follow-up questionnaire survey was designed to estimate dietary intake from 138 food items and was validated for the estimation of various nutrients and food groups. The participants were asked about how often they consumed the individual food items (frequency of intake) and representative relative sizes compared with standard portions during the previous year (24). Of the 138 food items, 8 items (standard portion size) dealt specifically with consumption of soy and isoflavones: miso soup (150 g), soymilk (200 g), tofu for miso soup (20 g), tofu for other dishes (75 g), *yushidofu* (predrained tofu; 150 g), *koyadofu* (freeze-dried tofu; 60 g), *aburaage* (deep-fried tofu; 2 g), and *natto* (fermented soybeans; 50 g). These 8 items contributed 95.9% of total genistein intake in the estimates from dietary records in our validation study (25).

For miso soup, the FFQ included questions on the frequency of consumption (almost never, 1–3 d/mo, 1–2 d/wk, 3–4 d/wk, 5–6 d/wk, or daily) and on the daily amount consumed (number of bowls: <1, 1, 2, 3, 4, 5, 6, 7–9, or10). For soymilk, the FFQ included questions on 10 frequency categories only: almost never, 1–3 times/mo, 1–2 times/wk, 3–4 times/wk, 5–6 times/wk, 1 glass/d, 2–3 glasses/d, 4–6 glasses/d, 7–9 glasses/d, or >9 glasses/d. For other soy foods, the FFQ contained questions on frequency (almost never, 1–3 times/mo, 1–2 times/wk, 3–4 times/wk, 5–6 times/wk, 1 time/d, 2–3 times/d, 4–6 times/d, or ≥7 times/d) and sizes relative to a standard portion (small [50% smaller than standard], medium [same as standard], or large [50% larger than standard]).

Daily intake of each food item was calculated by multiplying frequency by standard portion and, if available, the relative portion size for each item in the FFQ. Intake of genistein and daidzein was calculated by using values in a specially developed food composition table of Japanese foods (26), which contained measured values of soy foods (27, 28). This allowed for the effect of food processing on genistein content, including fermentation, to be taken into consideration in estimating intake. We did not collect information on the use of isoflavone supplements. Intake of food and nutrients was log-transformed and adjusted for total energy intake by the residual model (29). Because the estimates of genistein and daidzein intake were highly correlated (Spearman's rank correlation coefficient = 0.996), the results for genistein are provided as representative for isoflavones.

To evaluate the validity and reproducibility of energy-adjusted genistein intake, we compared the estimates from the FFQ with 28-d (or 14-d for the Ishikawa PHC area) dietary records and with the FFQ (1-y interval), respectively, obtained from a subsample of the cohort (6, 30–32). Spearman's rank correlation coefficients were 0.65 (cohort I) and 0.48 (cohort II) in men and 0.55 (cohort I) and 0.45 (cohort II) in women for validity and 0.75 (cohort I) and 0.51 (cohort II) in men and 0.69 (cohort I) and 0.41 (cohort II) in women for reproducibility. Moreover, Spearman's rank correlation coefficient for genistein between energy-adjusted intake from the FFQ, from serum concentration was 0.22, and from creatinine-adjusted urinary excretion was 0.30 (25).

Follow-up

We followed study participants until 31 December 2005. Participants who died or moved to other municipalities were identified annually through residential registers in the respective PHC areas. Cause of death was confirmed by using mortality data from the Ministry of Health, Labor, and Welfare. Among study participants, 6027 (7.9%) died, 2781 (3.6%) moved away, and 248 (0.3%) were lost to follow-up during the study period.

We identified lung cancer incidence by voluntary reports from local major hospitals in the study areas, and data linkage with population-based cancer registries, with permission. We used death certificate information as a supplementary information source. In our cancer registry system, the proportion of cases for which information was obtained from death certificates only was 4.7% during the study period. During 671,864 person-years of follow-up (median follow-up period: 8.0 y), a total of 659 (481 in men and 178 in women) newly diagnosed lung cancer cases were identified.

The site of origin and histologic type were coded by using the *International Classification of Diseases for Oncology, Third Edition* (C34.0–C34.9) (33). Diagnosis of lung cancer was confirmed by histologic or cytologic examination in 84% of cases (n = 556) and was based on clinical findings or unspecified evidence in the remaining 16% of cases. Histologic type was classified into adenocarcinoma (n = 289; 44%), squamous cell carcinoma (n = 144; 22%), small cell carcinoma (n = 71; 11%), and other histologic types according to the World Health Organization's histologic classification of lung tumors (34).

Statistical analysis

We prospectively counted the number of person-years of follow-up for each participant from the date of completion of the 5-y follow-up questionnaire until the date of diagnosis of lung cancer, date of death, movement out of the study area, or 31 December 2005, whichever occurred first.

Cox proportional hazards regression analysis was used to calculate the hazard ratios (HR) and 95% CIs of lung cancer incidence according to quartile of isoflavone intake and to adjust for potentially confounding variables by using SAS statistical software, version 9.1 (SAS Institute Inc, Cary, NC) (35). Dummy variables were created for quartiles of isoflavone intake, and the lowest quartile used as the reference category. We calculated *P* values for the analysis of linear trends by assigning ordinal values for categories of isoflavone intake and entering the number as a continuous term in the regression model. All reported *P* values are 2-tailed.

Multivariate-adjusted HRs were adjusted for age (in y), study area (9 PHC areas), smoking status (never; past; current: <10, 10–19, 20–29, 30–39, or ≥40 cigarettes/d in men and <20 or ≥20 cigarettes/d in women), and alcohol consumption (nondrinker; current drinker: 1–150, 151–300, 301–450, or ≥451 g ethanol/wk in men and nondrinker or current drinker in women), menopausal status in women (premenopausal, natural, or induced postmenopausal), and total intake of vegetables, fruit, and fish (quartiles). We did not include use of exogenous female hormones as a covariate because we identified only 2 lung cancer cases in current users of exogenous female hormones.

We conducted stratified analysis by smoking status on the association between isoflavone intake and lung cancer risk. We then tested effect modification by smoking status through the addition of cross-product terms to the multivariate model. Among never smokers, we performed additional analyses by using information on passive smoking or age at menarche in the baseline survey because the 5-y follow-up questionnaire did not include these questions. For age at menarche, we included combined categories of age at menarche (<16 or ≥16 y old) and menopause (≤50 or >50 y old) in the multivariate model among never-smoking postmenopausal women on the basis of our previous finding (10). All analyses were repeated after the exclusion of participants diagnosed in the first 3 y of follow-up.

RESULTS

The characteristics of participants according to isoflavone intake are shown in **Table 1**. Those with higher intakes were less likely to be current smokers and more likely to be postmenopausal and to consume more vegetables, fruit, and fish.

The association between isoflavone intake and risk of lung cancer is shown in **Table 2**. We found no significant association in men or women, although the point estimates of multivariate-adjusted HRs in the highest quintile of isoflavone intake were below unity. After adjustment for age, study area, smoking status, alcohol consumption, menopausal status in women, and total vegetable, fruit, and fish intake, the multivariate HRs of lung cancer for the highest compared with the lowest quartile of isoflavone intake were 0.89 (95% CI: 0.67, 1.19; *P* for trend = 0.451) in men and 0.83 (95% CI: 0.54, 1.29; *P* for trend = 0.409) in women.

The results of stratified analysis by smoking status on the association of isoflavone intake with lung cancer risk are shown in **Table 3**. In men, an inverse association was found among

TABLE 1
Baseline characteristics of participants according to quartile of isoflavone intake (n = 76,661)[1]

	Men (n = 36,177)					Women (n = 40,484)				
	Quartile of isoflavone intake					Quartile of isoflavone intake				
	Lowest	Second	Third	Highest	*P* for trend[2]	Lowest	Second	Third	Highest	*P* for trend[2]
No. of participants	9044	9044	9045	9044		10,121	10,121	10,121	10,121	
Age (y)	55.8 ± 7.9[3]	56.0 ± 7.6	56.1 ± 7.5	57.1 ± 7.4	<0.001	56.2 ± 8.2	56.2 ± 7.7	56.7 ± 7.4	57.3 ± 7.3	<0.001
Current smokers (%)	50.0	48.1	46.1	41.4	<0.001	6.2	4.7	4.1	3.9	<0.001
No. cigarettes/d[4]	24 ± 16	22 ± 12	22 ± 11	21 ± 11	<0.001	15 ± 9	15 ± 8	15 ± 10	14 ± 10	0.078
Current drinkers (%)	74.3	75.3	75.5	71.8	<0.001	17.8	17.8	18.1	15.5	<0.001
Postmenopausal status (%)	—	—	—	—	—	71.3	74.4	77.7	81.2	<0.001
Current use of exogenous female hormones (%)	—	—	—	—	—	3.0	2.6	2.7	2.8	0.432
Dietary intake[5]										
Energy (kcal/d)	2180 ± 664	2169 ± 629	2219 ± 659	2160 ± 631	0.644	1865 ± 589	1872 ± 568	1903 ± 568	1839 ± 538	0.941
Vegetables (g/d)	167 ± 125	188 ± 117	199 ± 118	221 ± 135	<0.001	202 ± 129	223 ± 126	233 ± 122	245 ± 133	<0.001
Fruit (g/d)	149 ± 149	168 ± 144	179 ± 141	191 ± 145	<0.001	220 ± 181	233 ± 163	238 ± 156	243 ± 160	<0.001
Fish (g/d)	81 ± 57	86 ± 50	92 ± 52	93 ± 53	<0.001	79 ± 51	84 ± 46	86 ± 45	86 ± 47	<0.001
Miso soup (mL/d)	145 ± 112	259 ± 148	298 ± 171	318 ± 187	<0.001	125 ± 100	214 ±134	246 ± 149	267 ± 158	<0.001
Soy food (g/d)	34 ± 15	63 ± 18	90 ± 28	162 ± 113	<0.001	35 ± 15	63 ± 19	89 ± 28	162 ± 110	<0.001
Genistein (mg/d)	9 ± 3	17 ± 2	26 ± 3	48 ± 22	<0.001	9 ± 3	17 ± 2	26 ± 3	48 ± 21	<0.001
Daizein (mg/d)	6 ± 2	11 ± 1	16 ± 2	30 ± 13	<0.001	6 ± 2	11 ± 1	16 ± 2	29 ± 12	<0.001

[1] Results for isoflavone are reported in terms of those for genistein because of the high correlation between intake estimates for genistein and daidzein.
[2] Determined by using Jonckheere-Terpstra or Cochran-Armitage test.
[3] Mean ± SD (all such values).
[4] Among current smokers.
[5] Energy-adjusted by using the residual method (29).

TABLE 2
Age- and study area– and multivariate-adjusted hazard ratios (HRs) for lung cancer incidence according to intake of isoflavones (n = 76,661)[1]

	Quartile of isoflavone intake				
	Lowest	Second	Third	Highest	P for trend
Men (n = 36,177)					
No. of cases	117	129	128	107	—
Person-years	76,442	78,112	79,298	78,351	—
Age- and area-adjusted HR (95% CI)	1.00 (reference)	1.04 (0.81, 1.34)	1.02 (0.78, 1.32)	0.78 (0.59, 1.04)	0.090
Multivariate-adjusted HR[2] (95% CI)	1.00 (reference)	1.09 (0.84, 1.41)	1.10 (0.84, 1.44)	0.89 (0.67, 1.19)	0.451
Women (n = 40,484)					
No. of cases	49	44	42	43	—
Person-years	87,644	90,034	90,817	91,165	—
Age- and area-adjusted HR (95% CI)	1.00 (reference)	0.89 (0.59, 1.34)	0.86 (0.56, 1.31)	0.87 (0.56, 1.35)	0.527
Multivariate-adjusted HR[2] (95% CI)	1.00 (reference)	0.88 (0.58, 1.33)	0.85 (0.55, 1.30)	0.83 (0.54, 1.29)	0.409

[1] A Cox proportional hazards model was used to estimate HRs and 95% CIs. Results for isoflavone are reported in terms of those for genistein because of the high correlation between intake estimates for genistein and daidzein.

[2] Adjusted for age (in y), study area (9 public health center areas), smoking status (never; past; current: <10, 10–19, 20–29, 30–39, or ≥40 cigarettes/d in men and <20 or ≥20 cigarettes/d in women), alcohol consumption (nondrinker; current drinker: 1–150, 151–300, 301–450, or ≥451 g ethanol/wk in men and nondrinker or current drinker in women), menopausal status in women (premenopausal, natural, or induced postmenopausal), and total intake of vegetables, fruit, and fish (quartiles).

never smokers (multivariate HR for the highest compared with lowest quartile: 0.43; 95% CI: 0.21, 0.90; P for trend = 0.024), whereas no association was found among current or past smokers. In women, the corresponding HR among never smokers was 0.67 (95% CI: 0.41, 1.10; P for trend = 0.135). We were unable to show an association in current and past smoking women because of the small number of lung cancer cases (n = 17 and 4, respectively). We also tested effect modification by smoking status on the association between isoflavone intake and lung cancer risk among men (P for interaction = 0.085) and men and women combined (P for interaction = 0.055).

Our analyses on the association of individual soy foods with lung cancer risk in never smokers showed similar a direction to that of the association with total isoflavone intake: the

TABLE 3
Age- and study area– and multivariate-adjusted hazard ratios (HRs) for lung cancer incidence according to intake of isoflavones by smoking status[1]

	Quartile of isoflavone intake				
	Lowest	Second	Third	Highest	P for trend
Men					
Never smokers (n = 13,051)					
No. of cases	22	20	19	13	—
Person-years	26,234	27,405	29,196	30,796	—
Age- and area-adjusted HR (95% CI)	1.00 (reference)	0.78 (0.42, 1.45)	0.68 (0.36, 1.29)	0.42 (0.20, 0.87)	0.019
Multivariate-adjusted HR[2] (95% CI)	1.00 (reference)	0.84 (0.45, 1.56)	0.72 (0.38, 1.38)	0.43 (0.21, 0.90)	0.024
Current smokers (n = 16,792)					
No. of cases	74	90	85	69	—
Person-years	38,012	37,581	36,546	32,536	—
Age- and area-adjusted HR (95% CI)	1.00 (reference)	1.22 (0.89, 1.66)	1.16 (0.84, 1.61)	0.99 (0.69, 1.40)	0.864
Multivariate-adjusted HR[2] (95% CI)	1.00 (reference)	1.25 (0.91, 1.72)	1.21 (0.86, 1.69)	1.03 (0.72, 1.48)	0.955
Past smokers (n = 6334)					
No. of cases	21	19	24	25	—
Person-years	12,195	13,126	13,555	15,019	—
Age- and area-adjusted HR (95% CI)	1.00 (reference)	0.87 (0.46, 1.63)	1.20 (0.65, 2.22)	1.01 (0.54, 1.89)	0.768
Multivariate-adjusted HR[2] (95% CI)	1.00 (reference)	0.82 (0.44, 1.54)	1.18 (0.63, 2.21)	0.96 (0.50, 1.82)	0.858
Women					
Never smokers (n = 38,211)					
No. of cases	43	40	41	33	—
Person-years	81,395	85,302	86,617	87,044	—
Age- and area-adjusted HR (95% CI)	1.00 (reference)	0.89 (0.57, 1.38)	0.90 (0.58, 1.40)	0.71 (0.44, 1.15)	0.197
Multivariate-adjusted HR[2] (95% CI)	1.00 (reference)	0.88 (0.57, 1.36)	0.88 (0.56, 1.38)	0.67 (0.41, 1.10)	0.135

[1] A Cox proportional hazards model was used to estimate HRs and 95% CIs. Results for isoflavone are reported in terms of those for genistein because of the high correlation between intake estimates for genistein and daidzein.

[2] Adjusted for age (in y), study area (9 public health center areas), smoking status (never; past; current: <10, 10–19, 20–29, 30–39, or ≥40 cigarettes/d in men and <20 or ≥20 cigarettes/d in women), alcohol consumption (nondrinker; current drinker: 1–150, 151–300, 301–450, or ≥451 g ethanol/wk in men and nondrinker or current drinker in women), menopausal status in women (premenopausal, natural, or induced postmenopausal), and total intake of vegetables, fruit, and fish (quartiles).

multivariate HRs of the highest compared with lowest quartile of intake were 0.89 (95% CI: 0.45, 1.75; *P* for trend = 0.860) in men and 0.85 (95% CI: 0.54, 1.36; *P* for trend = 0.575) in women for miso soup, 0.66 (95% CI: 0.33, 1.31; *P* for trend = 0.197) in men and 0.95 (95% CI: 0.59, 1.53; *P* for trend = 0.831) in women for tofu, and 0.95 (95% CI: 0.45, 2.02; *P* for trend = 0.562) in men and 0.81 (95% CI: 0.48, 1.37; *P* for trend = 0.450) in women for natto.

We also investigated the association between isoflavone intake and risk of lung cancer by histologic type among never smokers. Because we did not have a sufficient number of lung cancer cases to analyze individual histologic types [adenocarcinoma (n = 33; 45%), squamous cell carcinoma (n = 16; 22%), and small cell carcinoma (n = 5; 7%) in men; adenocarcinoma (n = 115; 73%) and squamous cell carcinoma (n = 10; 6%) in women], we show the results of adenocarcinoma in women only: the multivariate HR for the highest compared with the lowest quartile among never-smoking women was 0.76 (95% CI: 0.42, 1.37; *P* for trend = 0.386).

We conducted additional analyses among never smokers. When analysis was restricted to participants who were not exposed to passive smoking at the workplace (4493 participants with 25 lung cancer cases in men; 22,499 participants with 96 lung cancer cases in women), similar results were obtained (multivariate HR for the highest compared with the lowest quartile: 0.68; 95% CI: 0.21, 2.20; *P* for trend = 0.257 in men; multivariate HR: 0.56; 95% CI: 0.30, 1.05; *P* for trend = 0.087 in women). In postmenopausal women, further adjustment with the use of combined categories of age at menarche and menopause did not alter the results substantially (data not shown). Results after the exclusion of cases diagnosed in the first 3 y of follow-up were essentially unchanged (data not shown).

DISCUSSION

In this population-based prospective cohort study, we found an inverse association between isoflavone intake and risk of lung cancer in never-smoking men but not in current- or past-smoking men. We saw a similar decrease in risk in never-smoking women. Our findings support the possibility of effect modification by smoking status on the association between isoflavone intake and lung cancer risk (*P* for interaction = 0.085 in men and 0.055 in men and women combined). To our knowledge, this is the first prospective cohort study to report the association between isoflavone intake and lung cancer in men.

Several in vitro and in vivo studies have supported a protective effect of isoflavones on lung carcinogenesis. Lian et al (36) reported that genistein inhibited cell growth and induced apoptosis in non–small cell lung cancer cells. In female athymic mice, soy phytochemicals slowed the in vivo growth of non–small cell xenografts (37).

In previous studies in Asian countries, case-control studies have also suggested a protective effect of soy foods or isoflavone intake against lung cancer incidence (13, 15–19). With regard to prospective studies, however, only one study has been reported, and it was limited to women (20). In the Singapore Chinese Health Study, Seow et al (20) reported an inverse association between isoflavone intake and total lung cancer among nonsmoking women, with a multivariate-adjusted HR for lung cancer incidence in the highest compared with lowest quartile of isoflavone intake of 0.59 (95% CI: 0.38, 0.91), whereas no association was found in ever smokers.

Here, we observed a nonsignificant decrease in the risk of adenocarcinoma and total lung cancer in never-smoking women. It is possible that our data might not have sufficient statistical power to detect an association between isoflavone intake and lung cancer risk among never-smoking women. In addition, lack of information on passive smoking in never-smoking women at the time of exposure assessment may have masked a significant association, albeit that our additional analysis using information from the baseline survey on passive smoking did not materially change the results. Similar to our study, Seow et al's (20) prospective study in nonsmoking women in Singapore reported a nonsignificant inverse association of isoflavone intake with risk of lung adenocarcinoma but a significant inverse association with other histologic types. In contrast, the only case-control study that showed results by histologic type, which was conducted among never-smoking women in Hong Kong, found an inverse association between tofu or soy and lung adenocarcinoma and large cell carcinoma (13). Thus, the results of these studies of risk in never smokers by histologic type were inconsistent.

In addition to its prospective study design, high response rate, and relatively low proportion of loss to follow-up, our study has several other strengths. Participants were recruited from the Japanese general population, which has relatively higher isoflavone intake than Western populations. Isoflavone intake was measured by a questionnaire with a reasonably high level of validity and reproducibility.

Several limitations of the study also warrant mention. First, behavior in adhering to isoflavone intake may have confounded the association between isoflavone intake and lung cancer risk. Previous studies in Japan reported that soy food intake was associated with other traditional or healthy food intakes (38–41). We therefore included intake of vegetables, fruit, and fish (18, 42), which may relate to lung cancer risk, into the multivariate model to investigate an independent association between isoflavone intake and lung cancer risk. Nevertheless, we cannot completely exclude the possibility that isoflavone intake is a marker of unmeasured factors. Second, we did not collect information on isoflavone supplement use. However, a relatively recent 2006 survey on supplement use in Japan showed a low prevalence of isoflavone supplementation (<1.6%) (43), and thus intake from supplements is considered to be negligible. Third, because we assessed isoflavone intake by using an FFQ, some misclassification of isoflavone intake may have arisen in estimating the effect on lung cancer risk. However, such misclassification was likely nondifferential and would tend to result in underestimation of the effect of isoflavone intake.

Although the mechanisms of this putative protective effect of isoflavones on lung carcinogenesis are not fully understood, not only estrogen-dependent (via the mediation of estrogen receptors) but also estrogen-independent mechanisms are possible. Genistein is a potent inhibitor of epidermal growth factor receptor (EGFR) kinase activity (44). In clinical findings, *EGFR* gene mutation in non–small cell lung cancer was a strong predictor of benefit from EGFR tyrosine kinase inhibitors (45). Taken together, these findings suggest that isoflavones may inhibit lung carcinogenesis through EGFR-mediated mechanisms. Because never-smoking status, East Asian ethnicity, and female sex are associated with *EGFR* gene mutation in lung cancers (45), we speculate that the EGFR-mediated mechanisms may be dominant and explain the present difference between smokers and

never smokers in the association of isoflavone with lung cancer. In fact, a case-control study in Japan investigating the association between soy foods and non–small cell lung cancer by *EGFR* mutation status found an inverse association with *EGFR*-mutated lung cancer only (46). Further epidemiologic studies on the association of isoflavone intake with lung cancer risk by using information on *EGFR* mutation status would likely provide a better understanding of the mechanisms.

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