

# SNP (–617C>A) in ARE-Like Loci of the NRF2 Gene: A New Biomarker for Prognosis of Lung Adenocarcinoma in Japanese Non-Smoking Women

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## Abstract

**Purpose:** The transcription factor NRF2 plays a pivotal role in protecting normal cells from external toxic challenges and oxidative stress, whereas it can also endow cancer cells resistance to anticancer drugs. At present little information is available about the genetic polymorphisms of the *NRF2* gene and their clinical relevance. We aimed to investigate the single nucleotide polymorphisms in the *NRF2* gene as a prognostic biomarker in lung cancer.

**Experimental Design:** We prepared genomic DNA samples from 387 Japanese patients with primary lung cancer and detected SNP (c.–617C>A; rs6721961) in the ARE-like loci of the human *NRF2* gene by the rapid genetic testing method we developed in this study. We then analyzed the association between the SNP in the *NRF2* gene and patients' overall survival.

**Results:** Patients harboring wild-type (WT) homozygous (c.–617C/C), SNP heterozygous (c.–617C/A), and SNP homozygous (c.–617A/A) alleles numbered 216 (55.8%), 147 (38.0%), and 24 (6.2%), respectively. Multivariate logistic regression models revealed that SNP homozygote (c.–617A/A) was significantly related to gender. Its frequency was four-fold higher in female patients than in males (10.8% female vs 2.7% male) and was associated with female non-smokers with adenocarcinoma. Interestingly, lung cancer patients carrying *NRF2* SNP homozygous alleles (c.–617A/A) and the 309T (WT) allele in the *MDM2* gene exhibited remarkable survival over 1,700 days after surgical operation (log-rank  $p = 0.021$ ).

**Conclusion:** SNP homozygous (c.–617A/A) alleles in the *NRF2* gene are associated with female non-smokers with adenocarcinoma and regarded as a prognostic biomarker for assessing overall survival of patients with lung adenocarcinoma.

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## Introduction

Lung cancer is the leading cause of cancer-related death in many industrial countries. It is classified into two major types, namely, small-cell lung carcinoma (SCLC) and non-small-cell lung carcinoma (NSCLC). While long-term exposure to cigarette smoke is the most common cause of lung cancer (80–90% of lung cancers), non-smokers account for 10–15% of lung cancer cases,

which are often attributed to a combination of genetic and environmental factors [1–3]. The transcription factor NF-E2-related factor 2 (NRF2) is known to control cellular adaptation/protection to reactive oxygen species and electrophiles by inducing antioxidation and detoxification genes [4–6] as well as mediate cancer cell proliferation and drug resistance [7–12]. We have undertaken the present study to examine the clinical impact of the

*NRF2* gene and its genetic polymorphisms on the risk and prognosis of lung cancer.

NRF2 is a “cap'n'collar” basic region-leucine zipper (CNC-bZip) transcription factor and plays a pivotal role in the induction of antioxidant response element (ARE)-regulated genes [4–13]. Under non-stressed conditions, NRF2 protein is associated with Kelch-like ECH associating protein 1 (*KEAP1*) [14]. KEAP1 is known to be a negative regulator of NRF2 by retrieving it in the cytoplasm. Oxidative stress and/or electrophilic attack lead to the dissociation of NRF2 from KEAP1 and thereby the NRF2 protein is translocated into the nucleus. NRF2 together with small multiple alignment format (MAF) sequences binds to ARE sequences [15]. Many genes encoding detoxifying and antioxidant enzymes have been found to be regulated by NRF2 [4–6,15–18]. It has recently been reported that NRF2 contributes to malignant phenotypes of cancer cells *in vitro*, including aggressive cell proliferation, drug resistance, and metabolic re-programming [7–11,19,20]. In this context, the *NRF2* gene is considered to play split roles, for example, in the protection of normal cells and progression of cancer malignancy.

In 2004, Yamamoto and colleagues first reported the structure of the *NRF2* gene and found three SNPs (–653A>G, –651G>A, and –617C>A) and one triplet repeat polymorphism in its regulatory region [21]. Three years later, Marzec *et al.* examined the impact of those SNPs on the regulation of *NRF2* gene expression [22]. In transient transfection assays, they found that the –617C>A SNP significantly affects basal NRF2 protein levels and its function *in vitro* [22]. SNP –617C>A was found to be associated with a higher risk of oxidant-induced acute lung injury in humans [22]. It has been reported that a SNP (c.–617C>A) in the ARE-like loci of the human *NRF2* gene is important for self induction of the *NRF2* gene. *NRF2* regulates the transcription of numerous phase II drug-metabolizing enzymes and phase III drug-transporters (*e.g.*, ABCC1, ABCC2, ABCC4, and ABCG2) in response to oxidative stress *via* direct binding to the ARE sequences in those target genes [23–26]. At present, however, little information is available as to the clinical impact of genetic polymorphisms of the *NRF2* gene and the prognosis of lung cancer.

To gain insight into the genetic polymorphisms of the *NRF2* gene, we have developed rapid genotyping primer sets by utilizing the SmartAmp method, an isothermal DNA amplification process [27,28]. Among a total of 387 lung cancer patients, we found that SNP (c.–617C>A) in the *NRF2* gene is a prognostic biomarker for assessing the gender (female)-related risk of lung adenocarcinomas in the Japanese non-smoking sub-population of lung cancer patients. The epidermal growth factor receptor (*EGFR*) gene was frequently mutated in those female patients harboring the SNP homozygous SNP allele (–617A/A), suggesting a potential link between the SNP homozygote (–617A/A) and *EGFR* gene mutations. Furthermore, NRF2 reportedly regulates expression of the *MDM2* gene that encodes a negative regulator of p53, and this study shows that lung cancer patients with homozygous SNP alleles (–617A/A) in the *NRF2* gene and the 309T (WT) allele in the *MDM2* gene had markedly better overall survival. This is the first report providing clinical evidence that homozygous SNP (–617A/A), as one of the intrinsic genetic polymorphisms in the *NRF2* gene, is associated with the overall survival of lung cancer patients. Our clinical research data strongly suggest that the SNP homozygous allele (–617A/A) is a useful biomarker for clinical diagnosis.

## Results

### Clinicopathological Characterization

The clinicopathological characterization data for the 387 primary lung cancer patients are summarized in Table 1. The patient population comprised 221 men and 166 women, with an overall mean age of 66 years (range 35 to 87 years). The histological type of lung cancer was determined according to the protocol of the third World Health Organization/International Association for the Study of Lung Cancer Classifications [29]. Among the lung cancer patients, 298 were classified as having adenocarcinomas and 89 non-adenocarcinomas. The p-stage was determined by pathological examination of surgical specimens for 376 patients, and their tumours were staged according to the tumor nodes metastasis (TNM) classification of malignant tumours: 292, 46, 35, and 3 patients were respectively classified into stages I, II, III, and IV. For the remaining 11 patients, the p-stage could not be determined. The smoking history was obtained from each lung cancer patient at Kanagawa Cancer Center Research Institute: 154 patients had no smoking history, whereas 233 patients were smokers.

### Preparation of Genotyping Primers for Detection SNP (c.–617 C>A) in *NRF2* Gene

The *NRF2* gene is located on the negative strand of genomic DNA at q31.2 of chromosome 2. To create the template for preparation of genotyping primers, we synthesized double stranded DNA encoding a 424-bp region of nt.178129735 to nt.178130158, including the SNP (c.–617 C>A) in the *NRF2* gene by means of PCR with human genomic DNA. PCR primers used for the DNA synthesis were GACCACTCTCCGACCTAAAGG (forward) and CGAGATAAAGAGTTGTTTGCAGAA (reverse).

**Table 1.** Clinicopathological characterization of primary lung cancer patients.

Variable	No. of patients	(%)
<b>Gender</b>		
Male	221	(57.1)
Female	166	(42.9)
<b>Age (years old)</b>		
≤50	25	(6.4)
>50	362	(93.5)
<b>Histopathology</b>		
Adeno	298	(77.0)
Non-adeno	89	(23.0)
<b>Smoking</b>		
Non-smoker	154	(39.8)
Smoker	233	(60.2)
<b>p-Stage</b>		
I	292	(75.4)
II	46	(11.9)
III	35	(9.0)
IV	3	(0.8)
Undetermined	11	(2.8)

Ages of all patients, 66.4±9.9 (mean±S.D.).

Abbreviation: Adeno, adenocarcinoma.

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The resulting PCR product was then inserted into the TA-cloning site of the pGEM<sup>®</sup>-T Easy Vector (Promega, Madison, WI, USA). The vector was amplified in JM109 High Efficiency Competent Cells (Promega), and vector DNA was purified by using the GeneGET<sup>™</sup> Plasmid Miniprep Kit (Fermentas, Thermo Fisher Scientific Inc., Waltham, MA, USA). The sequence of the inserted DNA was analyzed with a laser-based automated DNA sequencer (ABI PRISM 3100 DNA Analyzer, Applied Biosystems Ltd., Tokyo, Japan). We designed a number of SNP-typing primer candidates and repeatedly tested them by using the vector-inserted DNA as a template until we obtained the best primer set.

The schematic illustration in Figure 1A shows the annealing sites of the best primer set, which comprises four different primers, *i.e.*, TP, OP, FP, and BP. The TP primer was designed such that its turn-back region can discriminate the nucleotide of C or A at c.-617 in the negative DNA strand extended from the 3'-end of the primer (see Figure 1A). Furthermore, two SNPs, c.-651G>A and c.653A>G, were not included in the annealing sites of the four primers. Figure 1B depicts the sequences of primers in the WT (-617C)-typing and SNP (-617)-typing sets. Exciton dye was linked to thymine in the FP primer as symbolized by "Z" in the lower panel of Figure 1B.

Figure 1C shows the time courses of genotyping reactions as functions of fluorescence intensity. By using the genotyping primer sets, we could detect the WT homozygote (-617C/C), WT/SNP heterozygote (-617C/A), and SNP homozygote (-617A/A) in genomic DNA samples. These results were verified by DNA sequence analysis. Namely, we performed sequencing analysis for determination of the WT homozygote (-617C/C), WT/SNP heterozygote (-617C/A), and SNP homozygote (-617A/A) in those genomic DNA samples. We tested 24 samples for each group (*i.e.*, C/C, C/A, and A/A) and confirmed the 100% accordance between the SmartAmp method and the DNA sequencing analysis method.

### Detection of SNP (c.-617 C>A) in the *NRF2* Gene in Lung Cancer Patients

Using the genomic DNA samples prepared from a total of 387 lung cancer patients, we have detected WT and SNP alleles in the *NRF2* gene by the rapid genotyping method described above. Table 2 summarizes these results, showing that 216, 147, and 24 patients could be typed as WT homozygote (-617C/C), WT/SNP heterozygote (-617C/A), and SNP homozygote (-617A/A), respectively. Accordingly, the allele frequency was calculated to be 74.8% and 25.2% for WT (c.-617C) and SNP (c.-617A), respectively, when both male and female patients were grouped together. It is of interest to note, however, that the allele frequency of SNP (c.-617A) was 28.6% for female patients, compared with 22.6% for male patients. Indeed, 18 female patients carried the SNP homozygote (-617A/A), a number three-fold higher than that of male patients. The ratio of homozygous SNP (-617A/A) was 10.8% for female patients, about four-fold higher than the 2.7% found in males (Table 2;  $P=0.004$ ). In contrast, the ratios of WT homozygote (-617C/C) and heterozygote (-617 C/A) were moderately higher in male patients than in female patients (Table 2). On the other hand, with respect to smoking experience, the ratio of homozygous SNP (-617A/A) was 10.4% in the non-smoker sub-population, being about three times ( $P=0.021$ ) higher than the ratio (4.5%) observed in the smokers (Table 2).

By multivariate analysis, the SNP homozygote (-617A/A) in the *NRF2* gene was found to be independently associated with gender (Table 3). Among a total of 20 adenocarcinoma patients (females+males) carrying the SNP homozygote (-617A/A), 16 patients were females who had no cigarette-smoking experience

(Table 4). In contrast, there were no male adenocarcinoma patients in the sub-group of non-smokers carrying the SNP homozygote (-617A/A) (Table 4). These results demonstrate a marked gender difference in terms of non-smoking patients carrying the SNP homozygote (-617A/A).

To gain more insight into the gender difference among 24 patients homozygous for the SNP (c.-617A/A), we have analyzed the genetic polymorphisms of human *CYP2A6\*4* (whole gene deletion) and the numbers of (GT)<sub>n</sub> repeats in the *HO-1* gene 5'-flanking region. These data are summarized in Table 5. *CYP2A6\*4/\*4* (whole gene deletion) was found in only one female patient in this subgroup (Table 5), whereas among 387 lung cancer patients, *CYP2A6\*4/\*4* was detected in seven patients (1.8%) with lung adenocarcinoma. The number of (GT)<sub>n</sub> repeats in the *HO-1* gene 5'-flanking region varied greatly (14 to 34 repeats) among these 24 patients.

Interestingly, as shown in Table 5, either exon 19 or exon 21 of the *EGFR* gene was frequently mutated in female patients who were non-smokers and had homozygous SNP alleles (-617A/A). Two of those patients were diagnosed as p-stage IIA (cases 1 and 16). They were treated first surgically and then with gefitinib. These patients had no relapse over 1879 days (case 1) and 939 days (case 16).

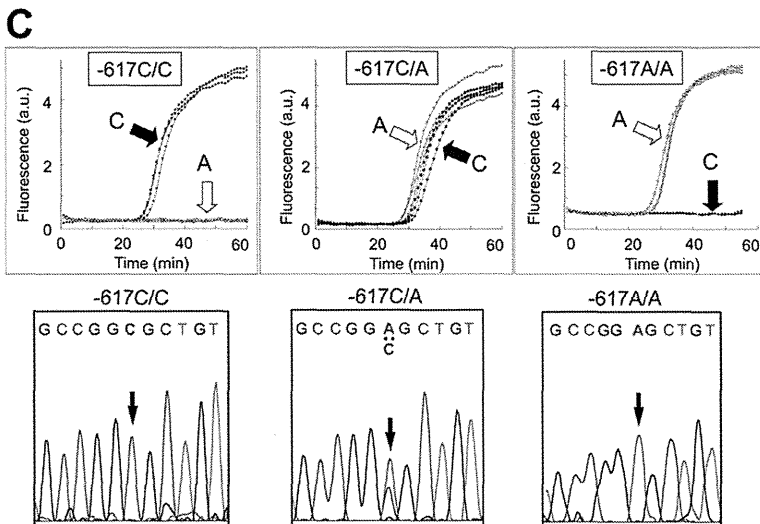
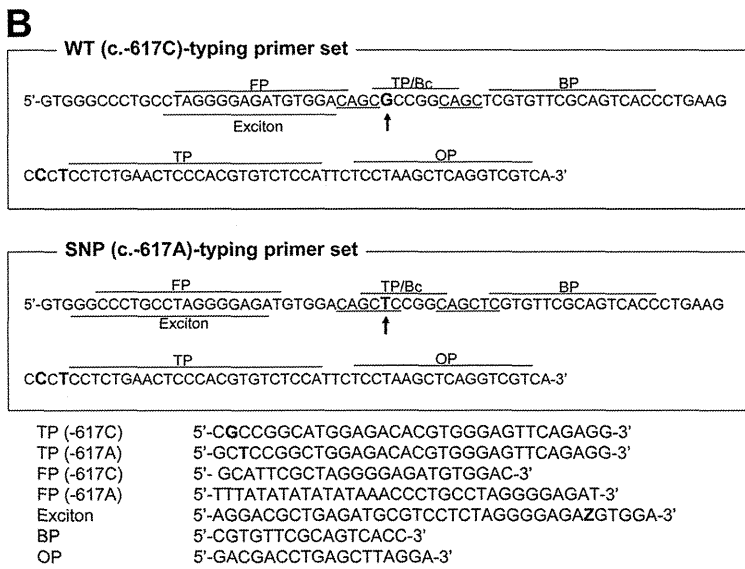
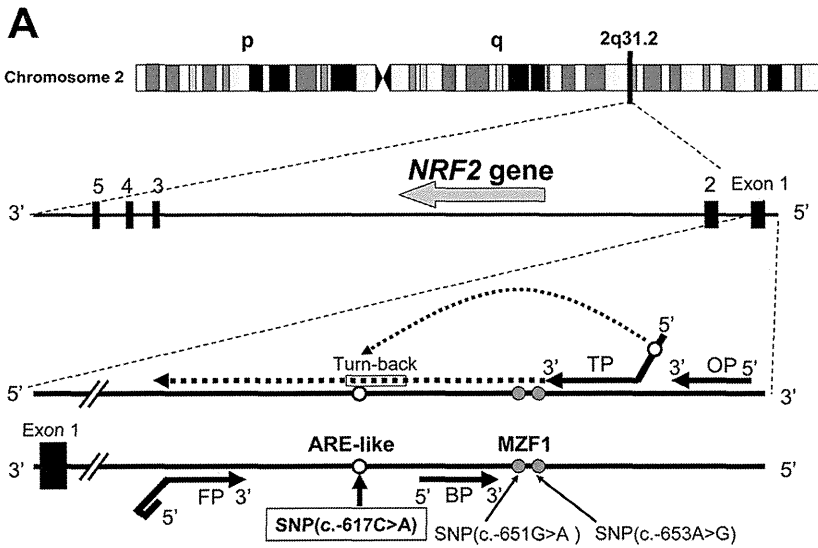
### Association of SNP (c.-617 C>A) in the *NRF2* Gene with Overall Survival of Lung Cancer Patients

We investigated a potential association between SNP (c.-617 C>A) in the *NRF2* gene and the overall survival of lung cancer patients, since we could obtain follow-up information on 369 patients among the total of 387 lung cancer patients over 1,700 days after surgical operation at Kanagawa Cancer Center. A survival Kaplan-Mayer plot is shown in Figure 2A. Our univariate analysis has revealed that lung cancer patients (p-stages I to IV) carrying homozygous SNP alleles (-617A/A) in the *NRF2* gene experienced significantly better overall survival, as compared with patients with heterozygous alleles (c.-617C/A) (log-rank  $P=0.021$ ). In contrast, no association was found between patients with homozygous WT alleles (-617C/C) and those with heterozygous alleles (c.-617C/A) (Figure 2A). It is important to mention that one female patient with the homozygous SNP (-617A/A) (case 5 in Table 5) died due to primary pancreatic cancer during the follow-up study.

We further analyzed the associations between the *NRF2* genotypes and patients' overall survival in the p-stage I of non-small cell lung cancer (NSCLC) including adenocarcinoma, squamous cell cancer, and large cell cancer. In the case of stage I, patients ( $n=285$ ) were treated by surgical excision of the tumor, with no follow-up treatment by adjuvant therapy or chemotherapy. Nonetheless, patients harboring homozygous alleles (-617A/A) in the *NRF2* gene exhibited the best record of overall survival among the members of these three different allele groups (Figure 2B).

### Potential Link between *NRF2* and *MDM2* Genotypes

*NRF2* reportedly regulates expression of the *MDM2* gene that encodes a crucial negative regulator of p53. To gain insight into a potential link between *MDM2* and *NRF2* genotypes, we have analyzed the SNP (c.309T>G) in the *MDM2* gene as well as the SNP (-617C>A) in the *NRF2* gene using the genomic DNA samples from lung cancer patients. Table 6 summarizes the corresponding results, where the number of patients harboring T/T, T/G, or G/G genotype in the *MDM2* gene has been given for each genotype of the *NRF2* gene (*i.e.*, -617C/C, C/A, or A/A). In the genotype groups of -617C/C and -617C/A, the 309G (SNP



**Figure 1. SmartAmp-based detection of SNP (c.-617C>A) in the NRF2 gene.** SNP (c.-617C>A) resides in the promoter region of the NRF2 gene on chromosome 2q31.2. Panel A presents a schematic illustration of annealing sites of the TP, FP, and OP primers. Panel B shows cDNA encoding a partial sequence of the NRF2 gene and primer annealing sites. Panel C depicts the results of SNP detection. a.u. = arbitrary unit. doi:10.1371/journal.pone.0073794.g001

allele frequency was 0.606 and 0.541, respectively. In contrast, the SNP allele frequency was found to be markedly lower (0.333) in the genotype group of NRF2 -617A/A. In the case of adenocarcinoma, female patients harboring 309T/T, T/G, and G/G genotype in the MDM2 gene were 7, 8, and 1, respectively (Table S1); most patients were harboring the 309T (WT) allele in the MDM2 gene.

## Discussion

### SNP (c.-617C>A) in the NRF2 Gene and Female Non-smokers with Adenocarcinoma

Recent genome-wide association studies (GWAS) have identified several loci associated with lung cancer susceptibility in never-smoking women in Asia; they were, 5p15.33 (rs2736100) [30], 6p21.3 (rs3817963) [30], 3q28 (rs10937405 and rs4488809) [31], 10q25.2 (rs7086803) [32], 6q22.2 (rs9387478) [32], and 6p21.32 (rs2395185) [32].

In the present study, differing from those reports, we found that SNP (c.-617C>A; rs6721961) in the NRF2 gene located on chromosome 2q31.2 is associated with Japanese non-smoking female patients with adenocarcinoma and their overall survival. While the allele frequency of SNP c.-617C>A in the NRF2 gene was estimated to be 25.2%, non-smoking females harboring homozygous alleles (-617A/A) had a markedly higher incidence of adenocarcinoma (Table 4, Table 5), as compared with non-smoking males harboring the same genotype. In other words, the -617 C>A SNP in the ARE-like loci of the human NRF2 gene seems to be associated with female non-smokers with adenocar-

cinoma. Furthermore, it is noteworthy that the EGFR gene was frequently mutated in female patients who were non-smokers and had homozygous SNP alleles (-617A/A) in the NRF2 gene (Table 5), suggesting a potential link between the SNP homozygote (-617A/A) and EGFR gene mutations.

Recent studies have demonstrated that mutations in the tyrosine kinase domain of the EGFR are frequently found among non-smoker patients with NSCLC [33]. Approximately 90% of these mutations are exon 19 deletions or exon 21 L858R point mutations in the tyrosine kinase domain [34]. In the vast majority of cases, EGFR mutations are non-overlapping with other oncogenic mutations (e.g., KRAS mutations, ALK rearrangements) found in NSCLC [34]. A large randomized clinical study named the "IRESSA Pan-Asian Study (IPASS)" has reported that high rates of mutations in the EGFR gene were observed in female NSCLC patients without smoking experience [35]. A high incidence of EGFR gene mutations was reported in female non-smokers with adenocarcinoma of lung: 30–40% in East Asians, as compared with 15% in Caucasians [36–38]. Both EGFR gene mutations and homozygous SNP alleles (-617A/A) in the NRF2 gene were frequently observed in Japanese female adenocarcinoma patients without smoking experience (Table 4). As shown in Table 7, ethnic group-dependent difference was observed in the NRF2 genotype, where the frequency of the -617A allele is high in Japanese, Taiwanese, and Chinese populations. Thus, it is of great interest to investigate the link between the SNP homozygote (-617A/A) and EGFR gene mutations and to gain insight into the underlying molecular mechanism.

### SNP (c.-617C>A) in the NRF2 Gene as a Biomarker for Prognosis of Lung Cancer

The NRF2 gene is regarded as a double-edged sword. It plays an important role in protecting normal cells from external toxic challenges and oxidative stress, whereas it can also endow cancer cells resistance to anticancer drugs. Recently it has been reported that NRF2 contributes to the malignant phenotypes of cancer cells *in vitro*, including aggressive cell proliferation, drug resistance, and metabolic re-programming [8,20]. Indeed, NRF2 activation is involved in the emergence of cancer resistance to various anticancer drugs by transcriptionally activating a battery of self-defense genes, such as those encoding antioxidant enzymes, phase

**Table 2. Classification of primary lung cancer patients with respect to NRF2 genotypes, gender, and histopathology.**

	NRF2 gene SNP (-617)			P-value*
	C/C	C/A	A/A	
Patients	216 (55.8)	147 (38.0)	24 (6.2)	
Gender*				
Male	127 (57.5)	88 (39.8)	6 (2.7)	
Female	89 (53.6)	59 (35.5)	18 (10.8)	0.004
Histopathology				
Adeno	164 (55.0)	114 (38.3)	20 (6.7)	
Non-adeno	52 (58.4)	33 (37.0)	4 (4.5)	0.687
Smoking behavior*				
Smoker	133 (58.4)	92 (37.0)	8 (4.5)	
Non-smoker	83 (53.9)	55 (35.7)	16 (10.4)	0.021
p-Stage				
I	156 (53.4)	114 (39.0)	22 (7.5)	
II	28 (60.9)	16 (34.8)	2 (4.3)	
III	23 (65.7)	12 (34.3)	0 (0)	
IV	1 (33.3)	2 (66.7)	0 (0)	0.459

The number of patients (%).

\*P- values were calculated by Fisher's exact test.

Abbreviation: Adeno, adenocarcinoma.

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**Table 3. Logistic regression analysis for evaluation of the association among homozygous SNP alleles (-617A/A) in the NRF2 gene and gender/smoking experience of lung cancer patients.**

Variable	P	Odds Ratio	95% CI
Gender	0.041	3.48	1.05 to 11.51
Smoking	0.463	0.66	0.22 to 2.00

Abbreviation: CI, confidence interval.

Gender code: 1 = female; 0 = male.

Smoking experience code: 1 = smoker; 0 = non-smoker.

The multivariate logistic regression analysis was performed under two categories, i.e., the gender (female and male) and the smoking experience (smoker and non-smoker).

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**Table 4.** Classification of primary lung cancer patients with respect to *NRF2* genotypes, smoking behavior, adenocarcinoma, and gender.

	C/C	NRF2 gene SNP (-617)		P-value*
		C/A	A/A	
Patients (M+F)	216	147	24	
Smoking behavior				
Smoker (M)	114	75	6	
Smoker (F)	19	17	2	
Non-smoker (M)	13	13	0	
Non-smoker (F)	70	42	16	0.014
Adenocarcinoma				
Smoker (M)	75	48	4	
Smoker (F)	15	16	0	
Non-smoker (M)	11	13	0	
Non-smoker (F)	63	37	16	0.003

\*P-values were calculated by Fisher's exact test.

Abbreviation: M, male; F, female.

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II detoxifying enzymes, and ABC transporters [23–25]. ABCG2 is known to mediate the efflux of gefitinib (Iressa®) from cancer cells [39], and its expression is regulated by NRF2 [26] and the EGFR-tyrosine kinase cascade [40,41].

As revealed in the Kaplan-Meier plot (Figure 2), lung cancer patients (both females and males) with homozygous SNP alleles (-617A/A) in the *NRF2* gene had markedly high overall survival. Univariate analysis showed a significant difference between the -617 A/A and -617 C/A groups in terms of overall survival (log-rank  $P=0.021$ ). It is important to note that, except for one patient (case 5 in Table 5) who died because of primary pancreatic cancer, all of the adenocarcinoma patients with homozygous SNP alleles (-617A/A) in the *NRF2* gene survived over 1,000 days after surgical excision of the tumor that was followed up with neither adjuvant therapy nor chemotherapy, even when p-stage I patients were considered alone (Figure 2B). To our knowledge, this is the first report providing clinical evidence that homozygous SNP (-617A/A), as one of the intrinsic genetic polymorphisms in the *NRF2* gene, is associated with overall survival of lung cancer patients.

SNP -617C>A is considered to play a pivotal role in the positive feedback loop of transcriptional activation of the *NRF2* gene to regulate the NRF2 protein level (Figure 3). Since the SNP (c.-617A) in the ARE-like loci of the human *NRF2* gene decreases the binding affinity to the transcription factors of NRF2/small

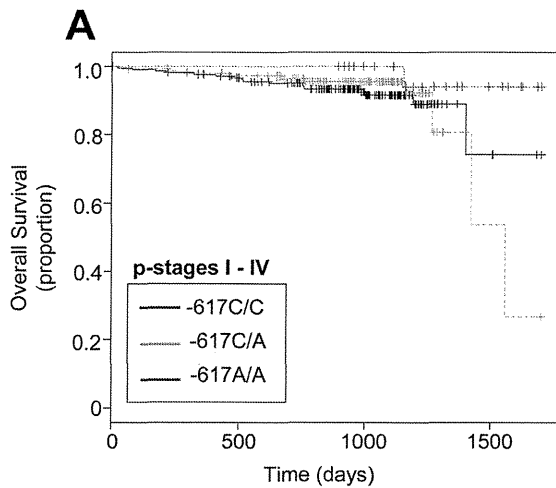
**Table 5.** Clinicopathological profiling of 24 patients harboring homozygous SNP alleles (-617A/A) in the *NRF2* gene.

Case	Histology	p stage	Age	Gender	smoker	(GT)n repeats	CYP2A6	EGFR mutation	Gefitinib therapy
1	Ad	IIA	74	F	non-smoker	19,30	Wt	Exon 21	Yes
2	Ad	IA	53	F	non-smoker	23	*4/*4	Exon 19	-
3	Ad	IB	70	F	non-smoker	24	Wt	Exon 21	-
4	Mix	IB	63	F	non-smoker	34	Wt	Exon 21	-
†5	Ad	IB	61	F	non-smoker	23	Wt	Exon 19	-
6	Ad	IB	72	F	non-smoker	22	Wt	Exon 21	-
7	Ad	IA	40	F	non-smoker	19	Wt	Exon 19	-
8	Ad	IA	71	F	non-smoker	17	Wt	Exon 21	-
9	Ad	IA	45	F	non-smoker	30	Wt	Exon 21	-
10	Ad	IA	74	F	non-smoker	30	Wt	Exon 21	-
11	Ad	IA	73	F	non-smoker	22	Wt	Exon 19	-
12	Ad	IA	69	F	non-smoker	22	Wt	Exon 21	-
13	Ad	IA	62	F	non-smoker	28	Wt	None	-
14	Ad	IA	73	F	non-smoker	14	Wt	Exon 19	-
15	Ad	IA	63	F	non-smoker	15	Wt	Exon 19	-
16	Ad	IIA	73	F	non-smoker	30	Wt	None	Yes
17	Sq	IA	72	F	smoker	20	Wt	None	-
18	Ple	IA	75	F	smoker	29	Wt	None	-
19	Ad	IA	74	M	smoker	23,27	Wt	None	-
20	Sq	IA	78	M	smoker	23	Wt	None	-
21	Ad	IA	65	M	smoker	30	Wt	Exon 21	-
22	Sq	IB	75	M	smoker	20	Wt	None	-
23	Ad	IA	77	M	smoker	28,31	Wt	Exon 21	-
24	Ad	IA	80	M	smoker	29,30	Wt	Exon 19	-

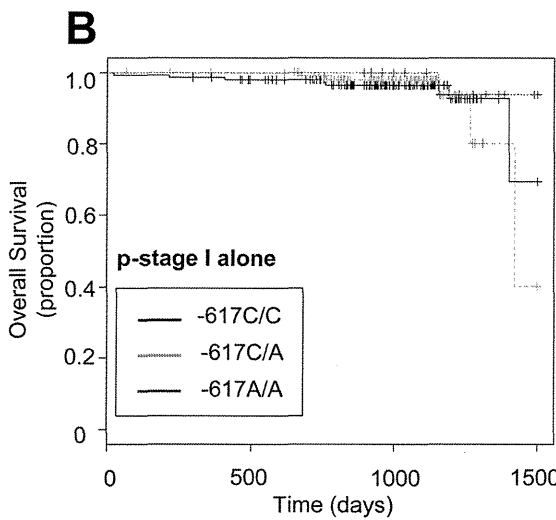
Abbreviation: Ad, adenocarcinoma; Mix, adenocarcinoma and squamous cell carcinoma; Ple, pleomorphic carcinoma; Sq, squamous cell carcinoma; F, female; M, male; Wt, wild type.

†Patient (case 5) died because of primary pancreatic cancer.

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Number at risk				
	0	500	1000	1500
A/A	24	24	24	8
C/A	141	132	68	1
C/C	204	187	92	4
Time (days)	0	500	1000	1500



Number at risk				
	0	500	1000	1500
A/A	22	22	22	7
C/A	111	111	56	1
C/C	152	143	70	3
Time (days)	0	500	1000	1500

**Figure 2. Kaplan-Meier plots showing the overall survival of patients harboring the WT homozygote (-617C/C), WT/SNP heterozygote (-617C/A), or SNP homozygote (-617A/A) in the NRF2 gene.** Patients with p-stages I to IV (A) and p-stage I only NSCLC (B). The number of patients at times 0, 500, 1000, or 1500 days after surgical operation is described along with genotypes of the NRF2 gene. doi:10.1371/journal.pone.0073794.g002

MAF [22], it is anticipated that the homozygote -617A/A significantly attenuates the positive feedback loop of transcriptional activation of the NRF2 gene.

It has recently been reported that NRF2 regulates the basal expression of the murine double minute-2 (*Mdm2*) gene [42]. Since human MDM2 is an oncoprotein that binds to p53 protein and inactivates the tumor suppressor activity of p53 [43], NRF2 can indirectly contribute to p53-mediated cell cycle control and/or

**Table 6. Classification of primary lung cancer patients with respect to genotypes of NRF2 and MDM2 genes.**

	NRF2 (-617)		
	C/C	C/A	A/A
Patients (N)	216	147	24
MDM2 (c.309)	N (%)	N (%)	N (%)
T/T	35 (16.2)	36 (24.5)	11 (45.8)
T/G	100 (46.3)	63 (42.9)	10 (41.7)
G/G	81 (37.5)	48 (32.6)	3 (12.5)

N, the number of patients; % in parentheses. doi:10.1371/journal.pone.0073794.t006

apoptosis [44]. One SNP in the MDM2 promoter region, a T-to-G change at nucleotide c.309 (rs2279744) in the first intron, increases the binding affinity toward stimulatory protein 1 (Sp1) and results in higher expression levels of MDM2 protein [45]. This, in turn, attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans [45]. Asians, including Japanese, have higher frequencies of the 309G allele as compared with African-Americans and Caucasians [46]. It has been reported that this polymorphism in the MDM2 gene is associated with the prognosis for several types of tumors, including lung cancer [47].

As demonstrated in Table 6, the 309G (SNP) allele frequency of the MDM2 gene was markedly lower (0.333) in the genotype group of NRF2 -617A/A, as compared with those observed in the genotype groups of NRF2 -617C/C and -617C/A. It is suggested that lung cancer patients harboring both the 309T (WT) allele in the MDM2 gene and the -617A allele in the NRF2 gene have better prognosis owing to well-controlled tumor suppression via

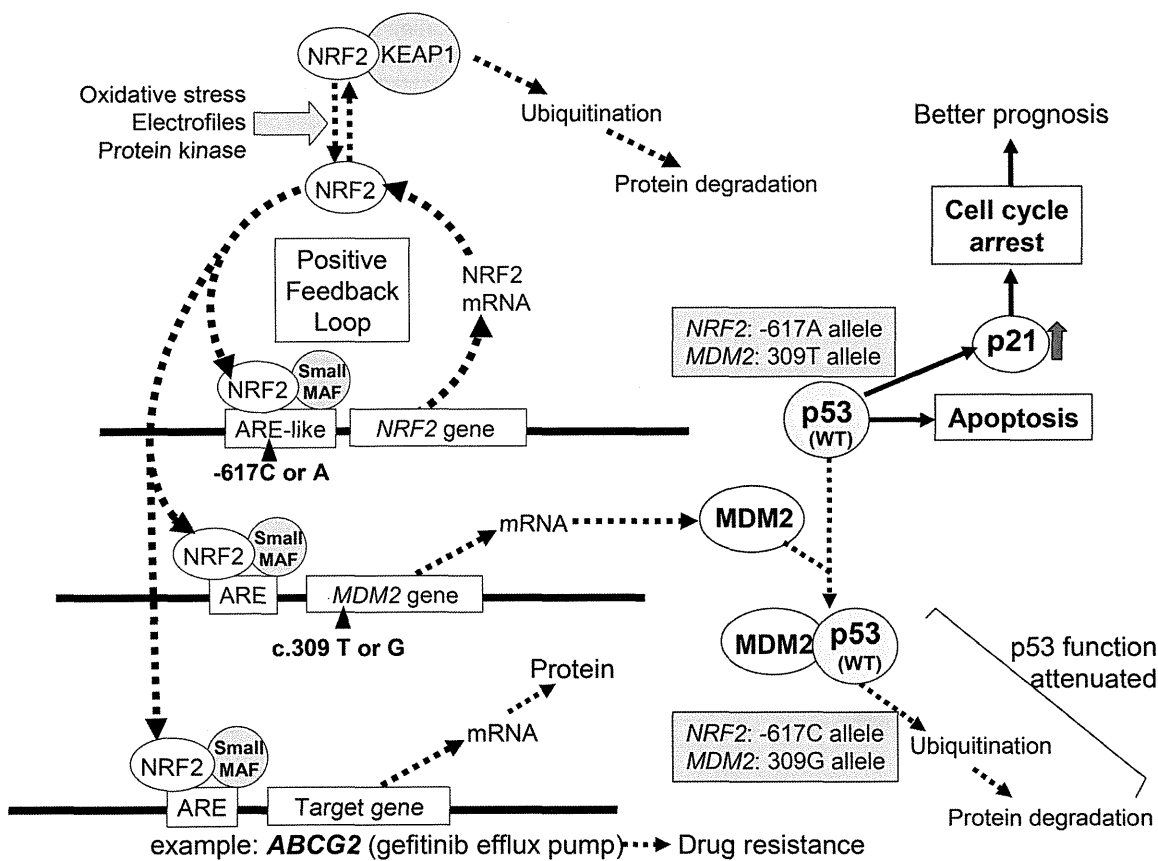
**Table 7. Frequencies of wild type (-617C) and SNP (-617A) alleles in the NRF2 gene among different ethnic groups.**

Ethnic group	Allele frequency NRF2 (-617)					N	Data source
	C	A	C/C	C/A	A/A		
African	0.925	0.075	0.850	0.150	0.000	246	*
African-American	0.893	0.107	0.787	0.213	0.000	61	*
European	0.883	0.117	0.778	0.208	0.013	379	*
American in Utah	0.888	0.112	0.788	0.200	0.012	85	*
American mixed	0.862	0.138	0.757	0.210	0.033	181	*
Mexican in Los Angeles	0.803	0.197	0.667	0.273	0.061	66	*
Japanese	0.775	0.225	0.618	0.315	0.067	89	*
Japanese (lung cancer)	0.748	0.252	0.558	0.380	0.062	387	This study
Taiwanese	0.726	0.274	0.524	0.405	0.071	168	This study
Chinese in Beijing	0.722	0.278	0.515	0.412	0.072	97	*
Southern Han Chinese	0.710	0.290	0.500	0.420	0.080	100	*

N, the number of subjects.

\*1000 Genomes. [http://browser.1000genomes.org/Homo\\_sapiens/Variation/Population?db=core;r=2:178129537-178130537;v=rs6721961;vdb=variation;vf=4574214](http://browser.1000genomes.org/Homo_sapiens/Variation/Population?db=core;r=2:178129537-178130537;v=rs6721961;vdb=variation;vf=4574214).

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**Figure 3. Schematic illustration showing the effect of *NRF2* SNP-617C>A and *MDM2* SNP c.309 T>G on the p53-mediated suppression of cancer cell proliferation and drug resistance.** In response to oxidative stress, electrophiles challenge, or protein kinase-mediated phosphorylation (e.g., via the PI3K-Akt pathway), the NRF2 protein is released from KEAP1 and then translocated into the nuclei. The SNP-617C>A in the ARE-like motif is considered to play a role in the positive feedback loop of transcriptional activation of the *NRF2* gene. The SNP homozygote (-617 A/A) significantly attenuates the positive feedback loop and also expression of *NRF2*-target genes, such as *MDM2* and *ABCG2*. In the case of *MDM2* gene expression, the SNP (c.309 T>G) in the first intron of the *MDM2* gene increases the binding affinity toward Sp1 and results in higher expression levels of MDM2 protein. MDM2 protein, thus highly expressed, binds to p53 (wild type; Wt) protein and leads to ubiquitination and proteasomal degradation of p53 (Wt) protein. Combination of the 309G (SNP) allele of the *MDM2* gene and the -617C (Wt) allele of the *NRF2* gene may have negative impacts on p53 (Wt)-mediated tumor suppression. On the other hand, lung cancer patients harboring both the 309T (Wt) allele of the *MDM2* gene and the -617A (SNP) allele of the *NRF2* gene may have better prognosis due to the tumor suppressor function of p53 (Wt), such as apoptosis and p21<sup>WAF1/cip1</sup>-mediated cell cycle arrest. Expression of the *ABCG2* gene is known to be up-regulated by NRF2. Gefitinib, an inhibitor of EGFR tyrosine kinase, is extruded by ABCG2 out of cancer cells. Thus, NRF2-mediated induction of ABCG2 expression can confer cancer cells with acquired resistance to gefitinib and other anticancer drugs. doi:10.1371/journal.pone.0073794.g003

cell cycle arrest and/or apoptosis mediated by p53 (WT); refer to Figure 3. This may explain, in part, our finding that the lung cancer patients with homozygous SNP alleles (-617A/A) in the *NRF2* gene had markedly high overall survival rates (Figure 2).

In cancer tissues, somatic mutations take place frequently. In addition to the above-mentioned genetic polymorphisms as the “intrinsic” mechanism, mutations in the *KEAP1* and/or *NRF2* genes are the “acquired” mechanisms that lead to constitutive activation of NRF2. In fact, mutations in the *NRF2* and *KEAP1* genes have been found in carcinomas of the lung [12], breast [48], liver [49], and stomach [49]. Abnormalities in NRF2 activity were correlated with poor prognosis, when measured either as recurrence-free or overall 5-year survival. A recent immunohistochemical study has revealed that increased expression of NRF2 protein and decreased expression of KEAP1 protein are common abnormalities in NSCLC and are associated with poor prognosis [50]. Importantly, abnormal expression of NRF2 and KEAP1 proteins was more common than that of the corresponding gene

mutations [50], suggesting the involvement of other mechanisms such as intrinsic genetic polymorphisms of those genes. To bridge the gap between the homozygous SNP alleles (-617A/A) in the *NRF2* gene and the high overall survival of lung cancer patients shown in this study, we need to carry out further clinical follow-up studies with lung cancer patients (p-stages III and IV) who have been subjected to chemotherapeutic treatments. As exemplified in the present study, genetic polymorphisms/mutations and fine balances among NRF2, KEAP1, MDM2, p53, p21<sup>WAF1/cip1</sup> and other genes are likely to contribute to the progression of cancer and, consequently, the prognosis of cancer patients.

#### Development of a Rapid Genotyping Method for Personalized Cancer Therapy

One of the challenges in lung cancer management is to identify biomarkers for personalized cancer therapy. To effectively advance personalized medicine, cost-effective methods should be developed for genotyping. It would be desirable to include such



information in each patient's record as guidance for medial doctors to provide individualized treatment. In the present study, we have developed a rapid genetic testing method to elucidate the impact of genetic polymorphisms in the *NRF2* gene on the risk and survival of patients with primary lung cancer. The method enables the detection of genetic polymorphisms in target genes within 30 to 45 minutes under isothermal conditions that do not require DNA isolation and PCR amplification. Thus, this genotyping method would provide a simple and practical tool for personalized cancer therapy and assessment of prognosis.

## Materials and Methods

### Patients and Sample Collection

This clinical research was conducted according to the Declaration of Helsinki Principles. Under written informed consent, we collected blood samples from patients with primary lung cancer who received surgical operation at the Kanagawa Cancer Center. Protocols for sample collection, anonymity, storage, and transportation to RIKEN Yokohama Institute required for the present study were approved by both the Institutional Review Board of the Kanagawa Cancer Center Research Institute and the Research Ethics Committee at RIKEN Yokohama Institute. Procedures for analyzing the *HO-1* gene 5'-flanking region sequence as well as for genotyping the *NRF2*, *CYP2A6*, and *MDM2* genes in the genomic DNA samples were approved by the Research Ethics Committee at RIKEN Yokohama Institute.

The Taiwanese samples (N=168) used in this study were randomly selected from the Han-Chinese Cell and Genome Bank in Taiwan described previously [51], in which more than 3,300 healthy controls were collected and randomly selected through registry.

### Preparation of Genomic DNA

Peripheral venous blood samples from lung cancer patients were collected into tubes containing Na<sub>2</sub>EDTA. Genomic DNA was extracted by the use of the QIAamp blood kit (QIAGEN K.K., Tokyo, Japan) according to the manufacturer's instructions.

### Genotyping

Based on the SmartAmp method, rapid genotyping primers were developed for detecting both the SNP (c.-617C/A) in the ARE-like loci of the human *NRF2* gene (Figure 1) and the *CYP2A6\*4* genotype (whole gene deletion) [51]. We used exciton-controlled hybridization-sensitive fluorescent primers for optical detection of genotyping reactions (Figure 1). After genomic DNA was denatured at 98°C for 3 min, the genotyping reactions were allowed to proceed isothermally at 60°C for 60 min in a Mx3000P PCR system (Agilent Technologies, Santa Clara, CA, USA). The SNP c.309T>G in of the *MDM2* gene were detected by the Duplex SmartAmp method, as described previously [46].

### Analysis of Length Variability of (GT)<sub>n</sub> Repeats in the *HO-1* Gene Promoter

The partial genomic DNA including (GT)<sub>n</sub> repeats located in the 5'-flanking region of the *HO-1* gene was amplified by the polymerase chain reaction (PCR) [53,54] with a fluorescence probe-labeled sense primer p1-s (5'-AGAGCCTGCAGCTTCT-CAGA-3') and an unlabeled anti-sense primer p1-as (5'-

ACAAAGTCTGGCCATAGGAC-3'). These primers were designed according to the previously reported sequences [55]. The PCR cycle program of 95°C for 30 seconds, 63°C for 30 seconds, and 60°C for 30 seconds was carried out for a total of 30 cycles. The resulting PCR products were visualized by electrophoresis in 3% agarose gels containing ethidium bromide. The electrophoresis revealed two differently-sized PCR products attributable to two alleles with different (GT)<sub>n</sub> repeat sequences in the *HO-1* gene. The (GT)<sub>n</sub> repeats in the PCR products were analyzed with a laser-based automated DNA sequencer (ABI PRISM 3100 DNA Analyzer, Applied Biosystems Ltd., Tokyo, Japan).

### Analysis of Mutation Status in the *EGFR* Gene

DNA samples were isolated from frozen tissues or formalin-fixed and paraffin-embedded tissue sections that had been surgically excised from lung cancer loci. Epidermal growth factor receptor (*EGFR*) gene exons 19 and 21 were analyzed for their mutational status by the loop-hybrid mobility shift assay, a PCR-based heteroduplex analysis method, as described previously [56,57].

### Statistical Analysis

The association of lung cancer with the allele frequency of the gene of interest was assessed by considering the confounding effects derived from known risk factors, such as age, gender, smoking history, and histopathology. After preliminary bivariate analysis by using Fisher's exact test or  $\chi^2$  test, the multivariate logistic regression method was performed to estimate independent variables associated with the SNP homozygote (-617A/A) in the *NRF2* gene. Furthermore, the Kaplan-Meier method was used to estimate survival curves for overall survival. Log-rank tests were used to compare the survival curves of patients in different *NRF2* subgroups. The statistical significance of all the data was tested by the analysis of variance. We performed statistical analysis with the SPSS statistics program (v.19.0; SPSS Inc., Chicago, USA). Values of  $P < 0.05$  were considered to indicate statistical significance.

## Supporting Information

**Table S1 Classification of female adenocarcinoma patients with respect to genotypes of *NRF2* and *MDM2* genes.**  
(DOC)

## Acknowledgments

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## Author Contributions

Conceived and designed the experiments: YO UN AL YI YN TK YH. Performed the experiments: YO UN YE AL HK. Analyzed the data: YO TI SM MT. Contributed reagents/materials/analysis tools: YO UN YE AL HK. Wrote the paper: YO TI. Wrote the research plan: TI. Contributed to the sample collection: YM HN MTML TY. Contributed to the clinical study: YM HN MTML TY.

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# Combination Chemotherapy with Irinotecan and Cisplatin for Large-Cell Neuroendocrine Carcinoma of the Lung

## A Multicenter Phase II Study

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**Introduction:** We conducted a phase II study of combination chemotherapy with irinotecan (CPT) and cisplatin (CDDP) in patients with advanced large-cell neuroendocrine carcinoma (LCNEC) of the lung.

**Methods:** Patients received irinotecan (60 mg/m<sup>2</sup>, days 1, 8, and 15) and cisplatin (60 mg/m<sup>2</sup>, day 1) every 4 weeks for up to four cycles. The primary endpoint was the response rate. Expected and threshold values for the primary endpoint were 50% and 30%.

**Results:** Forty-four patients were enrolled between January 2005 and November 2011. The response rate (RR) was 54.5% (95% confidence interval [CI], 38.8–69.6%). The median progression-free survival time was 5.9 months (95% CI, 5.5–6.3), and the median survival time was 15.1 months (95% CI, 11.2–19.0). A central pathological review of specimens from 41 patients demonstrated that 30 patients had LCNEC but that 10 patients had small-cell lung cancer (SCLC) and one had non-small-cell lung cancer with a neuroendocrine structure. The RR was 46.7% (95% CI, 28.3–65.7%) in the LCNEC group and 80% (95% CI, 44.4–97.5%) in the SCLC group ( $p = 0.0823$ ). The median survival time was 12.6 months (95% CI, 9.3–16.0) in the

LCNEC group and 17.3 months (95% CI, 11.2–23.3) in the SCLC group ( $p = 0.047$ ).

**Conclusions:** Combination chemotherapy with irinotecan and cisplatin was active in patients with LCNEC, but the RR and the overall survival period among the patients with LCNEC seemed to be inferior to those among the patients with SCLC. Small numbers of patients were a major limitation in this study.

**Key Words:** Cisplatin, Irinotecan, Large-cell neuroendocrine carcinoma, Lung.

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Large-cell neuroendocrine carcinoma (LCNEC) and small-cell lung cancer (SCLC) are recognized as high-grade neuroendocrine carcinoma of the lung, and account for approximately 15% of all forms of lung cancer. A considerable overlap in nuclear size was observed between LCNEC and SCLC in a morphometric analysis.<sup>1</sup> Thus, a differential diagnosis between LCNEC and SCLC using tiny biopsy specimens is often difficult. Consequently, prospective clinical trials on chemotherapy in advanced LCNEC have not been reported. LCNEC shares many similarities with SCLC in terms of not only structure, immunohistochemistry, and molecular biology, but also treatment;<sup>2</sup> several studies have shown that LCNEC responds to cisplatin-based chemotherapeutic regimens similar to those used for SCLC.<sup>3,4</sup> However, as LCNEC is a poorly recognized and underdiagnosed entity, it is frequently mistaken for poorly differentiated non-small-cell lung cancer (NSCLC), atypical carcinoid, or intermediate cell-type SCLC.<sup>5,6</sup> In one previous study that included 75 patients, only 44 (53%) were correctly diagnosed as having LCNEC at the outset, whereas 31 (47%) were misdiagnosed as having other NSCLCs.<sup>7</sup> Such difficulty is attributable to the obscure structure of neuroendocrine tumors at the light microscopy level, especially when cytology or small biopsy samples are being examined.<sup>5</sup> As a result, no optimal treatment for patients with LCNEC has been indicated as yet, and no evidence exists as to whether affected patients might benefit from chemotherapeutic protocols designed for NSCLC or SCLC.

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**TABLE 1.** Patient Characteristics

Characteristic	No. of Patients
Sex	
Men	35
Women	9
Age (yr)	
Median	62.5
Range	28–74
PS	
0	21
1	23
Clinical stage <sup>a</sup>	
IIIB	5
IV	28
Recurrence after surgery	11

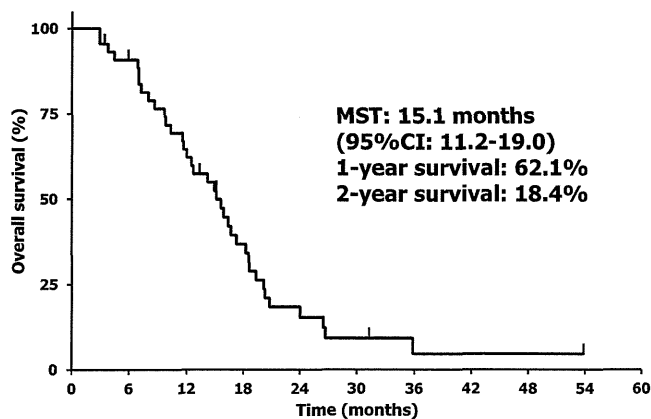
<sup>a</sup>Clinical stage was based on the 5th edition of the tumor, node, metastasis staging system of the Union Internationale Centre le Cancer.  
PS, performance status.

Combination chemotherapy consisting of irinotecan (CPT) and cisplatin (CDDP) is active against both NSCLC and SCLC.<sup>8–12</sup> Taking these rationales into consideration, we conducted a multicenter phase II study of CPT and CDDP in patients with advanced LCNEC.

## PATIENTS AND METHODS

### Patient Population and Treatment Plan

Patients were required to have histologically confirmed advanced-stage LCNEC. Recurrences after surgical resection were permitted. Other criteria included an age of 20 to 75 years, an Eastern Cooperative Oncology Group performance status of 0 or 1, measurable disease, a PaO<sub>2</sub> at room air 65 Torr or more, and adequate organ function. Key exclusion criteria included prior chemotherapy, interstitial pneumonia as determined by chest radiograph, and symptomatic brain metastases. All the patients were required to provide written informed consent, and the Institutional Review Board approved the protocol.



**FIGURE 1.** Overall survival curve including all 44 eligible patients. MST, median survival time; CI, confidence interval.

Patients received 60mg/m<sup>2</sup> of CDDP on day 1 and 60mg/m<sup>2</sup> of CPT on days 1, 8, and 15 every 4 weeks for up to four cycles if neither unacceptable toxicity nor tumor progression were observed.

### Study Evaluation

Imaging studies were scheduled to assess the objective response every 2 months. Diagnostic specimens including hematoxylin-eosin staining and immunohistochemistry for neuroendocrine markers were centrally reviewed by six expert pathologists (TK, MN, YI, KI, GI, and KT) who were blinded to the patients' clinical information. The pathology panel members performed the pathology review independently, and a final diagnosis was established by mutual consent. Histologic diagnostic criteria for LCNEC included organoid nesting, trabecular growth, and rosette-like and palisading patterns, which suggest neuroendocrine differentiation. The neuroendocrine features need to be confirmed by immunohistochemical markers, such as chromogranin, synaptophysin, and CD56.

The Response Evaluation Criteria in Solid Tumors guidelines, version 1.0, was used to evaluate antitumor activity.<sup>13</sup> Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria, version 2.0.

### Study Design and Statistical Analysis

This trial was designed as a multicenter, prospective, single-arm phase II study. The primary endpoint of this study was the response rate (RR) in the eligible patients with advanced LCNEC diagnosed by institutional pathologists. The secondary endpoints included overall survival (OS), progression-free survival (PFS), and toxicity. In accordance with the minimax two-stage phase II study design reported by Simon,<sup>14</sup> the treatment program was designed to refuse RRs of 30% (P0) and to provide a significance level of 0.05 with a statistical power of 80% for assessing the activity of the regimen as a 50% RR (P1). The upper limit for first-stage drug rejection was six responses in the 19 assessable patients; the upper limit for second-stage rejection was 16 responses in a cohort of 39 assessable patients.

The OS was defined as the interval between enrollment in this study and death or the final follow-up visit. The OS and PFS were estimated using the Kaplan–Meier analysis method. Exploratory subgroup analyses for RR, OS, and PFS were planned according to the results of the central pathological review. Survival data were compared among groups using a log-rank test. All the reported *p* values were two-sided. This study was registered with the UMIN Clinical Trials Registry (number UMIN000004796).

## RESULTS

A total of 44 patients from 11 Japanese institutes were enrolled between January 2005 and November 2011. All 44 patients were eligible for inclusion. The patient characteristics are listed in Table 1. Thirty patients (68%) completed four cycles of chemotherapy, whereas three patients received three cycles, seven patients received two cycles, and four patients

**TABLE 2.** Efficacy of Combination Chemotherapy with Irinotecan and Cisplatin

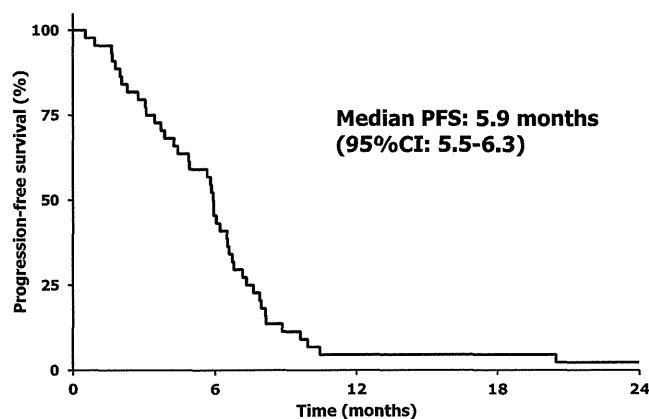
Type of Response	Central Pathological Review (n = 40)					
	All Patients (N = 44)		Large-Cell Neuroendocrine Carcinoma (n = 30)		SCLC (n = 10)	
	No. of Patients	%	No. of Patients	%	No. of Patients	%
CR	0	0	0	0	0	0
PR	24	54.5	14	46.7	8	80
CR + PR	24	54.5	14	46.7	8	80
95%CI	38.8–69.6		28.3–65.7		44.4–97.5	
Stable disease	12	27.3	10	33.3	1	10
Progression	7	15.9	6	20	1	10
Not evaluable	1	2.3	0	0	0	0
Median progression-free survival (mo) (95%CI)	5.9 (5.5–6.3)		5.8 (3.8–7.8)		6.2 (5.2–7.2)	
Median survival time (mo) (95%CI)	15.1 (11.2–19.0)		12.6 (9.3–16.0)		17.3 (11.2–23.3)	

Four patients of 14 responders in the large-cell neuroendocrine carcinoma group and four patients of eight responders in the SCLC group received full planned dose of chemotherapy. CR, complete response; PR, partial response; CI, confidence interval; SCLC, small-cell lung cancer.

received one cycle. Reasons for the discontinuation of the protocol treatment for the four patients who received only one cycle included progressive disease (n = 2), deterioration of PS (n = 1), and liver dysfunction prolonged for more than 2 weeks (n = 1). The median of relative dose intensity for CDDP or CPT was 94.3% (range, 66.2%–102.1%) and 82.4% (range, 33.3%–100%), respectively.

Twenty-four partial responses were seen among the 44 eligible patients, yielding an objective RR of 54.5% (95% confidence interval [CI], 38.8%–69.6%; Table 2). All 24 responders received four cycles of chemotherapy. Of them, eight patients received full planned dose of chemotherapy. The dose intensity of CDDP or CPT was not associated with RR (data not shown).

Figure 1 shows the OS curve for all 44 eligible patients. At 8 months after the last enrollment, the median survival time (MST) was 15.1 months (95% CI, 11.2–19.0 months). The 1-year and 2-year survival rates for all 44 eligible patients were 62.1% and 18.4%, respectively. The median PFS was 5.9 months (95% CI, 5.5–6.3 months; Fig. 2).



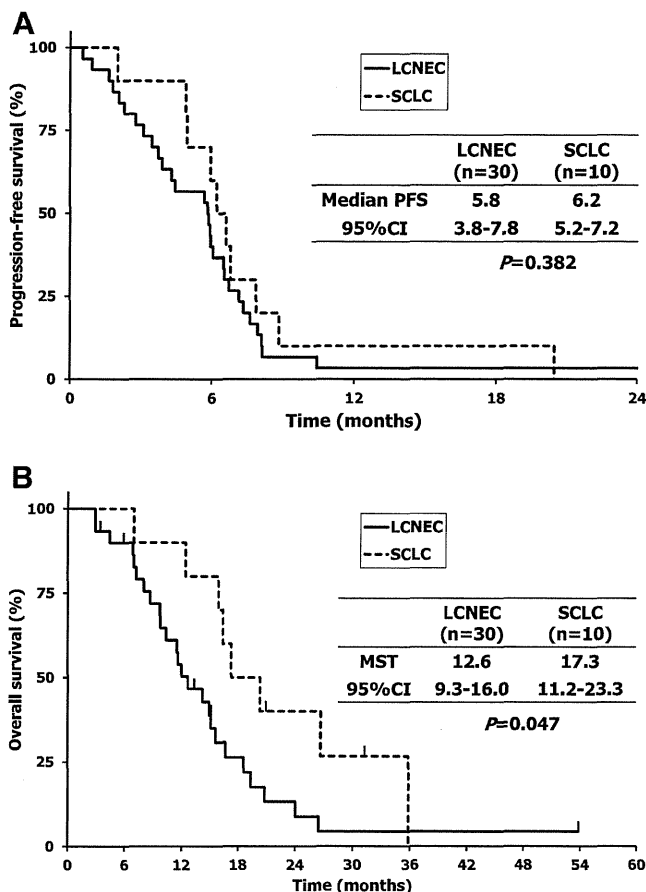
**FIGURE 2.** PFS curve including all 44 eligible patients. CI, confidence interval; PFS, progression-free survival.

Toxicity was evaluated in all eligible patients. The most common toxicity was neutropenia (Table 3). Twenty-four patients (55%) experienced grade 3 or 4 neutropenia, but only three patients (7%) developed neutropenic fever. Three patients (7%) developed grade 3 infection with neutropenia, and another three patients (7%) developed grade 3 infection without neutropenia. Two patients (5%) developed grade 3 diarrhea. No treatment-related deaths occurred in this series.

**TABLE 3.** Maximum Toxicity Grades

Toxicity	Toxicity Grade				% 3-4
	1	2	3	4	
Leukopenia	8	16	6	4	23
Neutropenia	3	9	11	13	55
Anemia	18	18	6	1	16
Thrombocytopenia	13	2	0	0	0
Febrile neutropenia	—	—	3	0	7
Bilirubin	6	2	0	1	2
AST	13	2	2	0	5
ALT	14	1	2	0	5
Creatinine	10	2	1	0	2
Hyponatremia	27	0	4	1	11
Hypokalemia	9	0	1	0	2
Hyperkalemia	10	1	1	0	2
Nausea	17	9	3	—	7
Vomiting	8	4	1	0	2
Appetite loss	21	12	3	0	7
Diarrhea	17	12	2	0	5
General fatigue	17	7	2	0	5
Constipation	3	2	0	0	0
Alopecia	17	9	0	0	0
Infection with neutropenia	1	0	3	0	7
Infection without neutropenia	1	1	3	0	7
Fever (noninfectious)	5	1	0	0	0

AST, aspartate transaminase; ALT, alanine transferase.



**FIGURE 3.** A, PFS and (B) overall survival curves for 30 patients diagnosed as having LCNEC and 10 patients diagnosed as having SCLC based on a central review. MST, median survival time; CI, confidence interval; PFS, progression-free survival; LCNEC, large-cell neuroendocrine carcinoma; SCLC, small-cell lung cancer; MST, median survival time.

A total of 36 patients (82%) received second-line chemotherapy. Twenty-five patients received amrubicin, four patients received platinum-based chemotherapy (CDDP + CPT [ $n = 2$ ], CDDP + etoposide [ $n = 1$ ], and carboplatin + paclitaxel [ $n = 1$ ]), and six patients received docetaxel.

Pathological specimens for central review were available in 41 patients. Pathological specimens in three patients were not available because these specimens were returned to other institutions, where biopsy was conducted. Thirty patients were diagnosed as having LCNEC, whereas 10 patients were diagnosed as having SCLC, and one patient was diagnosed as having NSCLC with a neuroendocrine structure. The RR was 47% (95% CI, 28.3%–65.7%) for patients diagnosed as having LCNEC and 80% (95% CI, 44.4%–97.5%) for patients diagnosed as having SCLC ( $p = 0.082$ ). The median PFS was 5.8 months (95% CI, 3.8–7.8 months) in the LCNEC group and 6.2 months (95% CI, 5.2–7.2 months) in the SCLC group ( $p = 0.382$ ) (Fig. 3A). The MST was 12.6 months (95% CI, 9.3–16.0 months) in the LCNEC group and 17.3 months (95% CI, 11.2–23.3 months) in the SCLC group ( $p = 0.047$ ) (Fig. 3B).

## DISCUSSION

This trial is the first to evaluate the use of combination chemotherapy consisting of CPT and CDDP prospectively in patients with advanced LCNEC. The lower limit of the CI for the RR exceeded 30%, which was the predefined threshold. The MST was 15.1 months (95% CI, 11.2–19.0 months), and the median PFS was 5.9 months (95% CI, 5.5–6.3 months). LCNEC is currently categorized as a variant form of large-cell carcinoma, that is NSCLC, but LCNEC has neuroendocrine features, similar to SCLC. Combination chemotherapy consisting of CPT and CDDP seems to be promising as a first-line chemotherapy for patients with advanced LCNEC.

According to the central pathological review, which was blinded to all clinical information, 30 patients (73%) were diagnosed as having LCNEC, and 10 patients (24%) were diagnosed as having SCLC. The PFS was similar between the LCNEC and SCLC patients; however, the OS of the SCLC patients was significantly longer than that of the LCNEC patients. The RR was also superior in the SCLC patients. A previous retrospective study examined the clinical outcome of the patients with high-grade neuroendocrine carcinoma, probable LCNEC, which was diagnosed on the basis of biopsy results. The RR for second-line chemotherapy was 17% among the patients with HGNEC-probable LCNEC (2/12), and 43% among the patients with SCLC (45 of 102) ( $p = 0.12$ ).<sup>15</sup> The chemosensitivity of LCNEC is considered to be lower than that of SCLC, especially in response to second-line chemotherapy. Consequently, the OS of the LCNEC patients was inferior to that of the SCLC patients.

The present study had several limitations. First, only 30 patients (73%) were diagnosed as having LCNEC, based on a central review. The RR in these 30 patients was 47% (95% CI, 28.3%–65.7%). Thus, the lower limit of the 95%CI for the RR did not exceed the predefined threshold of 30%. Second, data regarding the efficacy of second-line chemotherapy were not available. We could not confirm the chemoresistance of LCNEC in second-line chemotherapy, compared with that of SCLC. Third, the number of patients included in the study was small. The enrollment period exceeded 6 years despite the multicenter design. The relatively low incidence of LCNEC and the difficulty of diagnosing LCNEC pathologically using small biopsy specimens was responsible for the slow accrual.

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## Radiographically determined noninvasive adenocarcinoma of the lung: Survival outcomes of Japan Clinical Oncology Group 0201

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**Objective:** The study objective was to evaluate the long-term survival of patients with radiographically determined noninvasive lung adenocarcinomas.

**Methods:** A prospective, multi-institutional study on image diagnosis to define early (noninvasive) adenocarcinomas of the lung (Japan Clinical Oncology Group 0201) has shown that a consolidation/tumor ratio on thin-section computed tomography 0.25 or less in cT1a ( $\leq 2.0$  cm) could be used as a better radiologic criterion for a noninvasive pathology than a consolidation/tumor ratio 0.50 or less in cT1a-b ( $\leq 3.0$  cm). From the prognostic viewpoints, these criteria were evaluated for 545 patients with adenocarcinoma who underwent lobectomy and lymph node dissection.

**Results:** The subjects consisted of 233 men and 312 women with a median age of 62 years. The median follow-up period among overall patients was 7.1 years (range, 0-8.5 years). The overall and relapse-free 5-year survivals of the overall patients were 90.6% and 84.7%, respectively. When a consolidation/tumor ratio 0.5 or less in cT1a-b was used as a cutoff, the 5-year overall survivals of radiologic noninvasive (121 patients, 22.2%) and invasive (424 patients, 77.8%) adenocarcinomas were 96.7% and 88.9%, respectively, and the difference was statistically significant ( $P < .001$ , log-rank test). With the use of a consolidation/tumor ratio 0.25 or less in cT1a, the 5-year overall survivals of radiologic noninvasive (35 patients, 12.1%) and invasive (254 patients, 87.9%) adenocarcinomas were 97.1% and 92.4%, respectively, and the difference was not statistically significant ( $P = .259$ ).

**Conclusions:** The radiologic criteria of a consolidation/tumor ratio 0.25 or less in cT1a ( $\leq 2.0$  cm) and 0.50 in cT1a-b ( $\leq 3.0$  cm) were both able to define a homogeneous group of patients with an excellent prognosis before surgery. (*J Thorac Cardiovasc Surg* 2013;146:24-30)

Our understanding of the natural history of small, peripheral adenocarcinomas has greatly progressed.<sup>1-5</sup> This understanding is reflected in the recently revised international multidisciplinary classification of lung adenocarcinoma sponsored by the International Association for the Study of Lung Cancer, American Thoracic Society, and European Respiratory Society.<sup>6</sup>

This new classification is characterized by the creation/abandonment of some terminology for early and advanced adenocarcinomas and by a multidisciplinary approach for the application of the new classification in a clinical setting. In particular, the term “bronchioloalveolar carcinoma” is no longer used, and new concepts have been introduced, such as adenocarcinoma in situ and minimally invasive adenocarcinoma. Invasive adenocarcinomas are classified according to the predominant pattern after comprehensive histologic subtyping with lepidic, acinar, papillary, micropapillary, and solid patterns. The term “mixed subtype adenocarcinoma” is no longer used.

In this new proposal, the surgical features have been described as part of a multidisciplinary approach to the comprehensive classification of adenocarcinomas. Especially for early adenocarcinomas, crucial questions have been raised, such as “Is sublobar (limited) resection adequate oncological treatment for some early adenocarcinomas?” and “Can computed tomography (CT) be used to select patients for sublobar resection?”<sup>6,7</sup> The Japan Clinical Oncology Group (JCOG) conducted a prospective, radiologic study of thin-section computed tomography (TSCT) to identify radiologic criteria that predict pathologic noninvasiveness

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**Abbreviations and Acronyms**

CI	= confidence interval
CT	= computed tomography
C/T	= consolidation/tumor
GGO	= ground-glass opacity
JCOG	= Japan Clinical Oncology Group
TSCT	= thin-section computed tomography

in clinical IA lung cancer arising in the periphery of the lung (JCOG 0201).<sup>8</sup> The current study demonstrates the prognosis of the patients who are selected for limited sublobar resection according to the radiologic criteria for noninvasive adenocarcinomas. If we believe that patients with noninvasive adenocarcinomas may be appropriately treated with limited sublobar surgical resection instead of lobectomy, it is crucially important to answer the question, "Can CT be used to select candidates for sublobar resection or those who can benefit from limited sublobar resection?"

In selecting candidates for sublobar resection for early lung adenocarcinomas, however, the preoperative, radiologic prediction of the degree of invasive growth of the tumor has been realized to be a crucial point. Because of the advent of high-resolution CT or TSCT, the radiologic appearance of adenocarcinomas at various stages has been described in detail, and the radiology-pathology correlation has been studied. A localized, nodular lesion characterized by a low-to-moderate increase in CT density that does not obscure lung structures, such as the pulmonary artery/vein and bronchus, is referred to as "ground-glass opacity" (GGO).<sup>3</sup> It has been shown that GGOs are more likely to be an early form of adenocarcinomas, such as bronchioloalveolar carcinoma, adenocarcinoma in situ, or minimally invasive adenocarcinoma. In particular, some GGOs are accompanied by a solid part, and it has been demonstrated that a solid component represents the portion of invasive growth. These observations suggested that the degree of pathologic invasive growth in adenocarcinoma could be quantified according to the proportion of increased solid density in the CT appearance of the lesion.<sup>9</sup>

On the basis of these observations, the JCOG 0201 study was planned to establish radiologic criteria that could be used to identify pathologic early (noninvasive) adenocarcinomas according to the quantification of the solid component in TSCT. For this purpose, a consolidation/tumor (C/T) ratio on TSCT was used, and the hypothesis that a C/T ratio 0.50 or less in cT1a-b ( $\leq 3.0$  cm) in the TNM staging system (7th) indicates a noninvasive pathology was tested with specificity as the primary end point. The pathology-radiology correlation has been described.<sup>8</sup> The present study addresses the evaluation of a radiologic criterion in terms of the prognosis after surgery. If we can combine prognostic and pathologic

features, we may be able to improve the radiologic criteria for defining noninvasive adenocarcinoma.

**PATIENTS AND METHODS****The Japan Clinical Oncology Group 0201 Study**

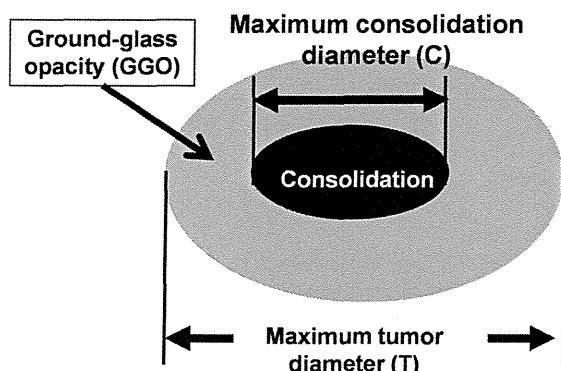
The study design and primary results of the prospective, multi-institutional study entitled JCOG 0201 have been published.<sup>8</sup> The study protocol was approved by the JCOG Clinical Trial Review Committee and the institutional review board of each participating center. The study was originally intended to define the radiologic criteria that indicate the pathologic noninvasiveness of adenocarcinomas arising in the periphery of the lung. If the radiologic selection of early adenocarcinomas with an excellent prognosis could be achieved, candidates for limited resection, not for lobectomy, could be precisely identified. Therefore, the radiologic definition of noninvasive adenocarcinoma needs to be assessed not only according to the radiology-pathology correlation but also according to the prognosis. The current study describes the prognosis of patients with resected adenocarcinoma according to different radiologic criteria. In brief, patients who met the following criteria were enrolled in the JCOG 0201 study: (1) a suspected or diagnosed lung cancer based on the findings from a plain x-ray or CT scan; (2) clinical stage IA (ie, T1N0M0) by thoracic enhanced CT; (3) the center of the tumor located peripherally (ie, in the outer half of the lung field) on CT; (4) measurable in at least 1 dimension in TSCT; (5) age 20 to 75 years; (6) no prior thoracotomy; (7) feasible for pulmonary lobectomy; and (8) obtained written informed consent. Before surgery, all patients underwent a contrast-enhanced, thin-section helical CT scan with 1- to 3-mm collimation, with particular focus on the primary tumor to estimate the size of the entire tumor, including GGO and the solid part (consolidation), where the C/T ratio was defined as a predictor of pathologic early adenocarcinoma (Figure 1). The patients then underwent surgical resection with at least lobectomy and hilar/mediastinal lymph node dissection. In the JCOG 0201 study, the appropriateness of C/T ratios of 0.50 for cT1a-b ( $\leq 3.0$  cm) and 0.25 for cT1a ( $\leq 2.0$  cm) was studied by comparing the C/T ratio and pathology of the resected specimen. The primary end point of the study was the specificity, which was defined as the proportion of patients with radiologically diagnosed invasive lung cancer determined by the central radiologic review among patients with pathologically diagnosed invasive lung cancer. Pathologic noninvasiveness was defined as pN0 disease with neither vascular invasion nor lymphatic permeation on resected specimen.

**Patients**

Between December 2002 and May 2004, we enrolled 811 patients from 31 institutions. There were 357 men and 454 women (age range, 27-75 years; median, 61 years). Of these, 562 patients (69.3%) underwent major lung resection. After 17 patients who were ineligible on the basis of postoperative pathologic findings were excluded, the prognosis of 545 patients (67.2%) was finally studied according to radiologic criteria for noninvasive adenocarcinoma mainly represented by the C/T ratio. The subjects of this study are the same as those in the analysis of pathology-radiology correlation of JCOG0201.

**Statistical Analysis**

The method of sample size calculation has been described.<sup>8</sup> Overall survival was defined as the duration from enrollment to death from any cause. For the patients alive, overall survival was censored at the last visit. Relapse-free survival was defined as the duration from enrollment to first recurrence or death from any cause. For the patients alive and recurrence-free, relapse-free survival was censored at the last visit. The probability of survival was estimated by the Kaplan-Meier method, and survival curves were drawn. The difference in survival between the groups was tested by the log-rank test. All *P* values were 2-sided. All statistical



**FIGURE 1.** Calculation of the C/T ratio to define radiologic noninvasive lung cancer on TSCT. The maximum diameter of consolidation (C) is divided by the maximum tumor diameter (T) to give the C/T ratio. GGO, Ground-glass opacity; C/T, consolidation/tumor; TSCT, thin-section computed tomography.

analyses were performed with SAS software release 9.2 (SAS Institute, Inc, Cary, NC) by the JCOG Data Center.

**RESULTS**

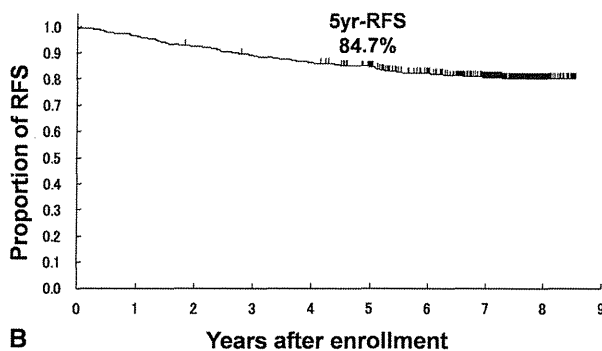
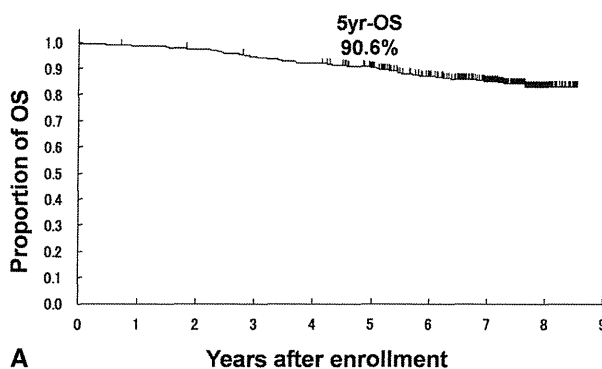
**Prognosis of Patients According to a Consolidation/Tumor Ratio 0.5 or Less (n = 545)**

The patient characteristics of the entire cT1a-b ( $\leq 3.0$  cm) population are shown in Table 1. Among the 545 patients, 233 (42.8%) were men and 312 (57.2%) were women. The median age was 62 years (range, 35-75 years). The median follow-up period among overall patients was 7.1 years after surgery (range, 0-8.6 years). The overall and relapse-free

**TABLE 1. Patient characteristics of the entire cT1a-b ( $\leq 3.0$  cm) population (n = 545)**

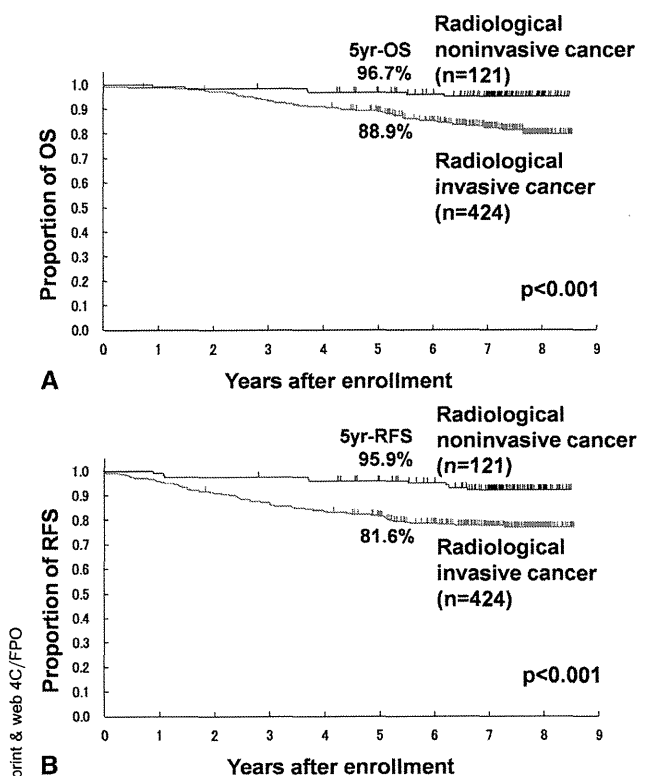
Characteristics	No. of patients (%)
Gender	
Men	233 (42.8)
Women	312 (57.2)
Age (y)	
Median (range)	62 (35-75)
Maximum tumor diameter on TSCT*	
$\leq 2.0$ cm	289 (53.0)
$>2.0$ -3.0	254 (46.6)
Unknown	2 (0.4)
C/T ratio on TSCT* and final pathology†	
$\leq 0.5$ (radiologically noninvasive)	121 (22.2)
Pathologically noninvasive	115 (21.1)
Pathologically invasive	6 (1.1)
$>0.5$ (radiologically invasive)	424 (77.8)
Pathologically noninvasive	263 (48.3)
Pathologically invasive	161 (29.5)

TSCT, Thin-section computed tomography; C/T, consolidation/tumor. \*Maximum tumor diameter and a C/T ratio on TSCT were both evaluated by a central radiologic review board. †Patients with adenocarcinoma that was diagnosed at the time of surgery were eligible, and 16 patients with a different final pathologic diagnosis were included in the pathologically invasive group.



**FIGURE 2.** Overall (A) and relapse-free (B) survival curves for the entire group (cT1a-b,  $\leq 3.0$  cm, n = 545). OS, Overall survival; RFS, relapse-free survival.

survival curves of the cT1a-b ( $\leq 3.0$  cm) population are presented in Figure 2, and the 5-year overall and relapse-free survivals were 90.6% and 84.7%, respectively. Among the 545 adenocarcinomas, when a C/T ratio 0.5 or less in cT1a-b ( $\leq 3.0$  cm) was used as a radiologic criterion for noninvasive cancer, 121 adenocarcinomas (22.2%) were diagnosed as noninvasive and 424 adenocarcinomas (77.8%) were diagnosed as invasive. Among the 121 radiologic noninvasive adenocarcinomas, 115 (95.0%) were precisely determined to be pathologic noninvasive cancer. Among the 424 radiologic invasive adenocarcinomas, 161 (38.0%) were precisely determined to be pathologic invasive cancer. Therefore, the specificity and sensitivity were 96.4% (95% confidence interval [CI], 92.3-98.7) and 30.4% (95% CI, 25.8-35.3), respectively. The overall survival curves for radiologic noninvasive cancer (n = 121) and invasive cancer (n = 424) are presented in Figure 3, A. The 5-year overall survivals for noninvasive cancer and invasive cancer were 96.7% and 88.9%, respectively, and the difference in overall survival was statistically significant ( $P < .001$ ). The relapse-free survival curves for radiologic noninvasive cancer (n = 121) and invasive cancer (n = 424) are presented in Figure 3, B. The 5-year relapse-free survivals for noninvasive and invasive cancer were 95.9% and 81.6%, respectively, and the difference in relapse-free survival was statistically significant ( $P < .001$ ).



**FIGURE 3.** Overall (A) and relapse-free (B) survival curves for radiologically noninvasive (n = 121) and invasive (n = 424) adenocarcinomas based on a C/T ratio of 0.50 or less in cT1a-b ( $\leq 3.0$  cm) for noninvasiveness on TSCT. The differences in overall and relapse-free survival are statistically significant ( $P < .001$  and  $< .001$ , respectively). OS, Overall survival; RFS, relapse-free survival; C/T, consolidation/tumor; TSCT, thin-section computed tomography.

**Prognosis of Patients According to a Consolidation/Tumor Ratio 0.25 or Less for cT1a Adenocarcinomas ( $\leq 2.0$  cm in Size) (n = 289)**

The patient characteristics of this population are shown in Table 2. Among the 289 patients, 129 (44.6%) were men and 160 (55.4%) were women. The median age was 61 years (range, 35-75 years). The median follow-up period among overall patients was 7.1 years after surgery (range, 0-8.5 years). The overall and relapse-free survival curves for the cT1a ( $\leq 2.0$  cm) population (n = 289) are presented in Figure 4, and the 5-year overall and relapse-free survivals were 93.0% and 88.9%, respectively. Among the 289 cT1a ( $\leq 2.0$  cm) adenocarcinomas, when a C/T ratio 0.25 or less was used as a radiologic criterion for noninvasive cancer, 35 adenocarcinomas (12.1%) were diagnosed as noninvasive and 254 adenocarcinomas (87.9%) were diagnosed as invasive. Among the 35 radiologic noninvasive adenocarcinomas, 34 (97.1%) were precisely determined to be pathologic noninvasive cancer. Among the 254 radiologic invasive adenocarcinomas, 78 (30.7%) were precisely determined to be pathologic invasive cancer. Therefore, the

**TABLE 2.** Patient characteristics of cT1a ( $\leq 2.0$  cm) population (n = 289)

Characteristics	No. of patients (%)
Gender	
Men	129 (44.6)
Women	160 (55.4)
Age (y)	
Median (range)	61 (35-75)
C/T ratio on TSCT* and final pathology†	
$\leq 0.25$ (radiologically noninvasive)	35 (12.1)
Pathologically noninvasive	34 (11.8)
Pathologically invasive	1 (0.3)
$> 0.25$ (radiologically invasive)	254 (87.9)
Pathologically noninvasive	176 (60.9)
Pathologically invasive	78 (27.0)

C/T, Consolidation/tumor; TSCT, thin-section computed tomography. \*Maximum tumor diameter and a C/T ratio on TSCT were both evaluated by a central radiologic review board. †Patients with adenocarcinoma that was diagnosed at the time of surgery were eligible, and the patients with a different final pathologic diagnosis were included in the pathologically invasive group.

specificity and sensitivity were 98.7% (95% CI, 93.2-100.0) and 16.2% (95% CI, 11.5-21.9), respectively. The overall survival curves for radiologic noninvasive cancer (n = 35) and invasive cancer (n = 254) are presented in Figure 5, A. The 5-year overall survivals for noninvasive cancer and invasive cancer were 97.1% and 92.4%, respectively, and the difference in overall survival was not statistically significant ( $P = .259$ ). The relapse-free survival curves for radiologic noninvasive cancer (n = 35) and invasive cancer (n = 254) are presented in Figure 5, B. The 5-year relapse-free survivals for noninvasive and invasive cancer were 97.1% and 87.7%, respectively, and the difference in relapse-free survival was not statistically significant ( $P = .106$ ).

**Prognosis of Patients With cT1b Adenocarcinomas ( $> 2.0$ - $3.0$  cm in Size) (n = 254) According to a Consolidation/Tumor Ratio 0.5 or Less**

Two patients with unknown preoperative tumor size were excluded. Among the 254 patients, 104 (40.9%) were men and 150 (59.1%) were women. The median age was 62 years (range, 37-75 years). The median follow-up period among overall patients was 7.0 years after surgery (range, 0-8.5 years). The overall and relapse-free survivals for the cT1b ( $> 2.0$ - $3.0$  cm) population were 87.8% and 79.9%, respectively. When a C/T ratio 0.5 or less was used as a radiologic criterion for noninvasive cancer, 54 adenocarcinomas (21.3%) were diagnosed as noninvasive and 200 adenocarcinomas (78.7%) were diagnosed as invasive. The overall survival curves for radiologic noninvasive cancer (n = 54) and invasive cancer (n = 200) are presented in Figure 6, A. The 5-year overall survivals for noninvasive cancer and invasive cancer were 96.3% and 85.5%, respectively, and the difference in overall survival was statistically significant

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