Clinicopathological and Survival Analysis of Japanese Patients with Resected Non–Small-Cell Lung Cancer Harboring *NKX2-1*, *SETDB1*, *MET*, *HER2*, *SOX2*, *FGFR1*, or *PIK3CA* Gene Amplification

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Introduction: Gene amplification is an important genetic change in cancer cells. We investigated the prevalence, clinicopathological characteristics, and prognostic value of *NKX2-1* (also known as *TTF-1*), *SETDB1*, *MET*, *HER2*, *SOX2*, *FGFR1*, and *PIK3CA* amplification in Japanese patients with non–small-cell lung cancer (NSCLC).

Methods: The copy numbers of the seven above-mentioned genes were assessed using fluorescence in situ hybridization in a tissue microarray containing 282 surgically resected NSCLC specimens (164 adenocarcinoma [AC], 99 squamous cell carcinoma [SCC], and 19 others). Clinicopathological information were obtained from the medical records.

Results: *NKX2-1*, *SETDB1*, *MET*, *HER2*, *SOX2*, *FGFR1*, and *PIK3CA* gene amplification were observed in 30 of 277 (10.8%), 16 of 280 (5.7%), 38 of 278 (13.7%), 8 of 270 (3.0%), 34 of 278 (12.2%), 18 of 282 (6.4%), and 53 of 278 (19.1%) cases, respectively. Coamplification was detected in 16 of 156 (10.3%) AC patients and 35 of 93 (37.6%) SCC patients ($p < 0.0001$). *NKX2-1* amplification was significantly related to an AC histology ($p = 0.004$), whereas *SOX2*, *FGFR1*, and *PIK3CA* amplifications were related to a SCC histology ($p < 0.0001$). Within the ACs, *NKX2-1* and *SETDB1* amplifications were markers of a shorter survival period. A multivariate Cox proportional hazards model revealed that *NKX2-1* amplification was an independent predictor of poor survival (hazard ratio, 2.938; 95% confidence interval, 1.434–6.022; $p = 0.003$). Coamplification had impact on patient outcome in AC but not in entire NSCLC and SCC.

Conclusions: The amplification status differed among the histological types of NSCLC. *NKX2-1* amplification was an independent and the most practically important predictor of a poor prognosis among Japanese patients with AC.

Key Words: Non–small-cell lung cancer, Gene amplification, Coamplification, *NKX2-1*, *SETDB1*.

(*J Thorac Oncol.* 2015;10: 1590–1600)

Lung cancer is the most frequent cause of cancer-related deaths worldwide. Non–small-cell lung cancer (NSCLC) accounts for nearly 80% of all lung cancer cases. Adenocarcinoma (AC) and squamous cell carcinoma (SCC) are the two major subtypes of NSCLC. Until recently, therapeutic approaches for NSCLC have been largely guided by the tumor stage only, and treatment options have been limited, regardless of whether the patients had AC or SCC. During the past decade, however, chromosomal and genomic changes in NSCLC such as mutations, deletions, translocations, and amplifications have been vigorously explored.

Gene amplification is a copy number gain of a specific locus of a chromosome arm, and various loci of copy number gains, especially on chromosomes 1q, 3q, 5p, 8q, 11q, 16p, and 17q, have been reported for NSCLC.[1] Recently, several groups have conducted fluorescence in situ hybridization (FISH)-based assays for the *NKX2-1* (otherwise known as thyroid transcription factor 1 [*TTF-1*]),[2–5] *MET*,[6–8] *HER2*,[9–11] *SOX2*,[12,13] *FGFR1*,[13–15] and *PIK3CA*[13,16,17] genes to assess their clinical significance. In addition, amplified SET domain, bifurcated 1 (*SETDB1*) has recently been characterized as a key player in human lung tumorigenesis.[18] The profiles of copy number amplification, especially those of the above-mentioned genes (with the exception of *SETDB1*) related to

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Disclosure: The authors have declared no conflicts of interest.

This work was supported by grants from the Ministry of Health, Labour and Welfare (19–19, 10103838), the Japan Society for the Promotion of Science (22590356, 23790396), the Ministry of Education, Culture, Sports, Science and Technology (S-001), the National Cancer Center Research and Development Fund (25-A-1), and Research on global health issues from Ministry of Health, Labor and Welfare.

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DOI: 10.1097/JTO.0000000000000685

ISSN: 1556-0864/15/1011-1590

biologically important signaling pathways in lung carcinogenesis, have been enthusiastically investigated. However, most of the previous studies only examined a limited number of genes, and the interpretations of the results were sometimes inconsistent and remain to be elucidated. From the standpoint of clinical feasibility, the types of probes used for specific purposes should be eventually prioritized in clinical settings, where testing resources are limited. Considering the different clinical profiles of lung cancer among populations, comprehensive studies in Asian populations are necessary.

In this study, we evaluated the copy numbers of the *NKX2-1*, *SETDB1*, *MET*, *HER2, SOX2*, *FGFR1*, and *PIK3CA* genes in resected NSCLCs and investigated their prognostic relevance and their associations with clinicopathological characteristics.

MATERIALS AND METHODS

Tumor Collection and Tissue Microarray Construction

A total of 282 patients with primary NSCLC (AC, n = 164; SCC, n = 99; others, n = 19) who underwent curative surgical resection in the First Department of Surgery at Hamamatsu University Hospital (Japan) between January 1990 and July 2011 were recruited for the study. Clinical and pathological information including age, sex, tumor stage, surgical procedure, smoking history, and outcomes were retrospectively obtained from a review of the patients' medical records. All the subjects provided written informed consent for the use of resected tissues for medical research. The study design was approved by the Institutional Review Board of Hamamatsu University School of Medicine. FISH analyses were performed on tissue microarray (TMA) sections according to a previously reported protocol.[19,20] Briefly, we selected a representative portion of the lung cancer tissue after careful screening for the presence of tumor cells by experienced pathologists. The pathologists marked the location, and we used a cylinder with a diameter of 3 mm to obtain a core from the donor blocks using a standard procedure and instrumentation (Azumaya, Tokyo, Japan). After the TMAs were made, all the TMA cores were again confirmed to contain a sufficient number of tumor cells by reviewing adjacent hematoxylin and eosin–stained sections before the FISH procedures were applied.

Clinical Profiles and Pathological Classification

The clinical profiles of the subjects are summarized in Tables 1 and 2. Two board-certified pathologists (K.S. and H.S.) histologically classified the lung cancers according to the World Health Organization classification (7th edition).

Fluorescence In Situ Hybridization Analysis

FISH analyses were performed using formalin-fixed and paraffin-embedded tumor samples according to the manufacturers' instructions with minor modifications, as described previously.[19,20] Spectrum Orange-labeled bacterial artificial chromosome (BAC) clones, RP11-1083E2 (14q13, *NKX2-1*), RP11-316M1 (1q21, *SETDB1*), RP11-51M22 (7q31, *MET*), RP11-275H4 (3q26, *SOX2*), RP11-106B16 (8p12, *FGFR1*), and RP11-245C23+RP11-355N16 (3q26, *PIK3CA*) (Advanced GenoTechs Co., Tsukuba, Japan), were used as locus-specific FISH probes. Spectrum Green-labeled control probes for the near-centromere locus on chromosome 1 (RP5-832K2), 3 (RP11-91A15), 7 (RP11-90C3), 8 (RP11-12L15), and 14 (RP11-14J7) (Advanced GenoTechs Co.) were also used to enumerate chromosomes 1, 3, 7, 8, and 14 in the FISH experiments. 4′,6-Diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) was used for nuclear staining. *HER2* FISH was performed using Histra (Jokoh Co., Tokyo, Japan), which includes a *HER2*-specific (17q11.2-q12) probe and the chromosome 17 centromeric probe and is frequently used in clinical settings for other cancers. For further validation, we performed a FISH assay on the TMA sections next to the initial ones for each probe panel. This validation was especially useful for obtaining a more confident interpretation in several cases that had insufficiently clear signals during the first hybridization. The FISH slide was interpreted without reference to any information regarding the clinicopathological features and prognosis through the use of anonymously coded specimens. After screening all the sections, the probe signals for at least 50 tumor cell nuclei were randomly counted in at least five representative images per case. The overlapping nuclei were excluded from the analysis. Cores in which the tumor cell signals were too weak were excluded from the interpretation. Copy number amplification was defined based on the criteria that the mean target BAC signal/centromere enumeration probe (CEP) signal ratio was greater than or equal to 2.0. Among the target gene-amplified cases, the median value of the mean target BAC/CEP ratios was calculated for each gene set. Tumors with a mean target BAC/CEP ratio of the median value or higher were defined as "high amplification," whereas tumors with a mean target BAC/CEP ratio greater than or equal to 2.0 and less than the median value were defined as "low amplification." Polysomy or an average target gene copy number/CEP ratio of <2.0 was scored as negative for amplification. "Single amplification" was defined as any gene amplification alone at the seven loci tested, whereas "coamplification" was defined as the simultaneous amplification of a combination of any two or more loci in the same tumor. The FISH slides were examined under a fluorescence microscope (BZ-9000; KEYENCE, Osaka, Japan). The image contrast was adjusted for the entire area. All the probes used in this study had been validated by hybridization to the chromosomal metaphase spread of normal lymphocytes to verify the chromosomal numbers and loci.

Statistical Analysis

Demographic information and associations with clinical characteristics were evaluated using the Fisher exact test (categorical variables) or the Mann–Whitney *U* test (for continuous variables). Overall survival (OS) was calculated as the time from operation to death or last contact. Standard methods for time-to-event data, such as the Kaplan–Meier method and the log-rank test, were used to analyze differences in survival time based on the gene amplification. The method of Holm was used to adjust the *p* values in multiple comparisons.

TABLE 1. Association of Clinicopathological Features and Focal Gene Amplification of *NKX2-1*, *SETDB1*, *MET*, and *HER2*

		NKX2-1 Amplification Status			*SETDB1* Amplification Status			*MET* Amplification Status			*HER2* Amplification Status		
Characteristic	**Total (*N* = 282), N (%)**	**Amp. (*N* = 30), N (%)**	**No-amp. (*N* = 247), N (%)**	***P* Value**	**Amp. (*N* = 16), N (%)**	**No-amp. (*N* = 264), N (%)**	***P* Value**	**Amp. (*N* = 38), N (%)**	**No-amp, (*N* = 240), N (%)**	***P* Value**	**Amp. (*N* = 8), N (%)**	**No-amp. (*N* = 262), N (%)**	***P* Value**
Age, yr													
Median (range)	67 (33–86)	67 (33–83)	67 (33–86)	0.562	66.5 (33–83)	67 (33–86)	0.678	66 (50–79)	67 (33–86)	0.938	70.5 (49–78)	67 (33–86)	0.401
Sex													
Male	202 (71.6)	18 (60.0)	179 (72.5)	0.199	14 (87.5)	186 (70.5)	0.167	34 (89.5)	164 (68.3)	0.007	7 (87.5)	183 (69.8)	0.443
Female	80 (28.4)	12 (40.0)	68 (27.5)		2 (12.5)	78 (29.5)		4 (10.5)	76 (31.7)		1 (12.5)	79 (30.2)	
Smoking status													
Never	79 (28.0)	14 (46.7)	65 (26.3)	0.028	3 (18.8)	76 (28.8)	0.496	7 (25.9)	71 (29.6)	0.274	4 (50.0)	72 (27.5)	0.113
Ever	190 (67.4)	14 (46.7)	172 (69.7)		12 (75.0)	176 (66.7)		30 (70.4)	157 (65.4)		3 (37.5)	178 (67.9)	
Unknown	13 (4.6)	2 (6.6)	10 (4.0)		1 (6.2)	12 (4.5)		1 (3.7)	12 (5.0)		1 (12.5)	12 (4.6)	
Histology													
Adenocarcinoma	164 (58.2)	26 (86.7)	137 (55.4)	0.004	9 (56.3)	154 (58.3)	0.119	27 (71.0)	135 (56.2)	0.260	6 (75.0)	153 (58.4)	0.202
Squamous cell carcinoma	99 (35.1)	4 (13.3)	93 (37.7)		4 (25.0)	95 (36.0)		9 (23.7)	89 (37.1)		1 (12.5)	93 (35.5)	
Others	19 (6.7)	0 (0)	17 (6.9)		3 (18.7)	15 (5.7)		2 (5.3)	16 (6.7)		1 (12.5)	16 (6.1)	
p-T													
1/2/3/4	126/103/30/23	12/9/6/3	114/91/22/20	0.266	8/6/0/2	117/96/30/21	0.493	16/12/6/4	109/89/23/19	0.544	6/2/0/0	117/95/28/22	0.546
p-N													
0/1/2/3	203/27/47/5	20/3/7/0	179/24/39/5	0.700	6/2/8/0	195/25/39/5	0.005	30/4/4/0	170/23/42/5	0.692	8/0/0/0	188/25/44/5	0.461
Pathological stage													
I	179 (63.5)	15 (50.0)	161 (65.2)	0.183	6 (37.5)	171 (64.8)	0.044	23 (60.5)	153 (63.7)	0.812	8 (100)	165 (63.0)	0.112
II	35 (12.4)	6 (20.0)	28 (11.3)		2 (12.5)	33 (12.5)		6 (15.8)	29 (12.1)		0 (0)	32 (12.2)	
III	68 (24.1)	9 (30.0)	58 (23.5)		8 (50.0)	60 (22.7)		9 (23.7)	58 (24.2)		0 (0)	65 (24.8)	
Adjuvant chemotherapy													
Yes	72 (25.5)	7 (23.3)	64 (25.9)	1.000	9 (56.3)	63 (23.9)	0.007	11 (33.3)	60 (25.0)	0.689	0 (0)	65 (24.8)	0.205
No	210 (74.5)	23 (76.7)	183 (74.1)		7 (43.7)	201 (76.1)		27 (66.7)	180 (75.0)		8 (100)	197 (75.2)	

P values were obtained using the Mann–Whitney U test and the Fisher exact test.

Five, two, four, and six patients were excluded from the analyses because of weak probe signals during FISH when the *NXK2-1*, *SETDB1*, *MET*, and *HER2* probe sets were used, respectively. Six patients were excluded from analysis using the *HER2* set because of the loss of cores in the tissue microarrays as a result of the entire specimen having been used.

Amp, amplification; FISH, fluorescence in situ hybridization.

TABLE 2. Association of Clinicopathological Features and Focal Gene Amplification of *SOX2*, *FGFR1*, and *PIK3CA*

Characteristic	Total (N = 282), *N* (%)	*SOX2* Amplification Status			*FGFR1* Amplification Status			*PIK3CA* Amplification Status		
		Amp (*N* = 34), N (%)	No-amp (*N* = 244), N (%)	*P* Value	Amp (*N* = 18), N (%)	No-amp (*N* = 264), N (%)	*P* Value	Amp (*N* = 53), N (%)	No-amp. (*N* = 225), N (%)	*P* Value
Age, yr										
Median (range)	67 (33–86)	67.5 (39–84)	67 (33–86)	0.111	65 (43–84)	67 (33–86)	0.695	67 (39–84)	67 (33–86)	0.582
Sex										
Male	202 (71.6)	32 (94.1)	166 (68.0)	0.001	16 (88.9)	186 (70.5)	0.110	49 (92.5)	149 (66.2)	<0.001
Female	80 (28.4)	2 (5.9)	78 (32.0)		2 (11.1)	78 (29.5)		4 (7.5)	76 (33.8)	
Smoking status										
Never	79 (28.0)	1 (2.9)	77 (31.6)	<0.001	1 (5.6)	78 (29.5)	0.028	2 (3.8)	77 (34.2)	<0.001
Ever	190 (67.4)	32 (94.1)	155 (63.5)		15 (83.3)	175 (66.3)		50 (94.3)	136 (60.5)	
Unknown	13 (4.6)	1 (2.9)	12 (4.9)		2 (11.1)	11 (4.2)		1 (1.9)	12 (5.3)	
Histology										
Adenocarcinoma	164 (58.2)	1 (2.9)	161 (66.0)	<0.001	1 (5.6)	163 (61.7)	<0.001	4 (7.5)	158 (70.2)	<0.001
Squamous cell carcinoma	99 (35.1)	31 (91.2)	67 (27.5)		17 (94.4)	82 (31.1)		46 (86.8)	52 (23.1)	
Others	19 (6.7)	2 (5.9)	16 (6.5)		0 (0)	19 (7.2)		3 (5.7)	15 (6.7)	
p-T										
1/2/3/4	126/103/30/23	13/14/5/2	112/86/25/21	0.662	4/8/4/2	122/95/26/21	0.096	12/25/10/6	114/74/20/17	0.001
p-N										
0/1/2/3	203/27/47/5	25/4/5/0	174/23/42/5	0.953	12/3/3/0	191/24/44/5	0.709	31/11/11/0	168/16/36/5	0.015
Pathological stage										
I	179 (63.5)	21 (61.8)	154 (63.1)	0.927	9 (50.6)	170 (64.4)	0.414	26 (49.1)	149 (66.2)	0.057
II	35 (12.4)	5 (14.7)	30 (12.3)		3 (16.7)	32 (12.1)		10 (18.9)	25 (11.1)	
III	68 (24.1)	8 (23.5)	60 (24.6)		6 (33.3)	62 (23.5)		17 (32.1)	51 (22.7)	
Adjuvant chemotherapy										
Yes	72 (25.5)	5 (14.7)	66 (27.0)	0.145	5 (27.8)	67 (25.4)	0.785	8 (15.1)	63 (28.0)	0.056
No	210 (74.5)	29 (85.3)	178 (73.0)		13 (72.2)	197 (74.6)		45 (84.9)	162 (72.0)	

P values were obtained using the Mann–Whitney U test and the Fisher exact test.
Four patients were excluded from the analyses because of weak probe signals during FISH using both the *SOX2* and *PIK3CA* probe sets.
Amp, amplification; FISH, fluorescence in situ hybridization.

Univariate and multivariate Cox proportional hazards models were fitted to calculate the hazard ratios (HRs) of death with adjustments for other potential confounding factors. The *p* values less than 0.05 were considered to be statistically significant. The statistical analyses were performed using the software EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan),[21] which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria, version 3.0.2).

RESULTS

Correlations of *NKX2-1*, *SETDB1*, *MET*, *HER2*, *SOX2*, *FGFR1*, and *PIK3CA* Amplification Statuses with Clinicopathological Data

Out of 282 NSCLC specimens that were examined, FISH was successful using the *NKX2-1*, *SETDB1*, *MET*, *HER2*, *SOX2*, *FGFR1*, and *PIK3CA* probe sets in 277, 280, 278, 270, 278, 282, and 278 cases, respectively (the reasons for failure are noted at the bottoms of Tables 1 and 2). Figure 1 shows representative images of tumor cells with gene amplification. The prevalences of the individual amplification of the *NKX2-1*, *SETDB1*, *MET*, *HER2*, *SOX2*, *FGFR1*, and *PIK3CA* genes were 10.8% (n = 30), 5.7% (n = 16), 13.7% (n = 38), 3.0% (n = 8), 12.2% (n = 34), 6.4% (n = 18), and 19.1% (n = 53) among the patients with NSCLC, respectively (Tables 1 and 2). The median values for the average ratio gene/CEP signals among the amplified tumors were 3.3 (range, 2.1–21.3), 2.5 (range, 2.0–6.3), 2.5 (range, 2.1–11.2), 2.2 (range, 2.1–16.3), 3.4 (range, 2.0–16.7), 3.3 (range, 2.0–5.6), and 2.9 (range, 2.0–10.5), for *NKX2-1*, *SETDB1*, *MET*, *HER2*, *SOX2*, *FGFR1*, and *PIK3CA*, respectively. Tumors from 266 of the patients were evaluable using FISH analyses for all seven gene sets. Of these patients, a total of 126 patients (47.4%) had tumors harboring at least one gene amplification among the seven genes (54 of the 156 patients [34.6%] with AC versus 64 of the 93 patients [68.8%] with SCC; $p < 0.0001$). Coamplification was found in 54 (20.3%) NSCLC patients (Supplementary Table 1, Supplementary Digital Content 1, http://links.lww.com/JTO/A896), among whom nine had tumors harboring the coamplification of greater than or equal to three gene loci. Coamplification was detected in

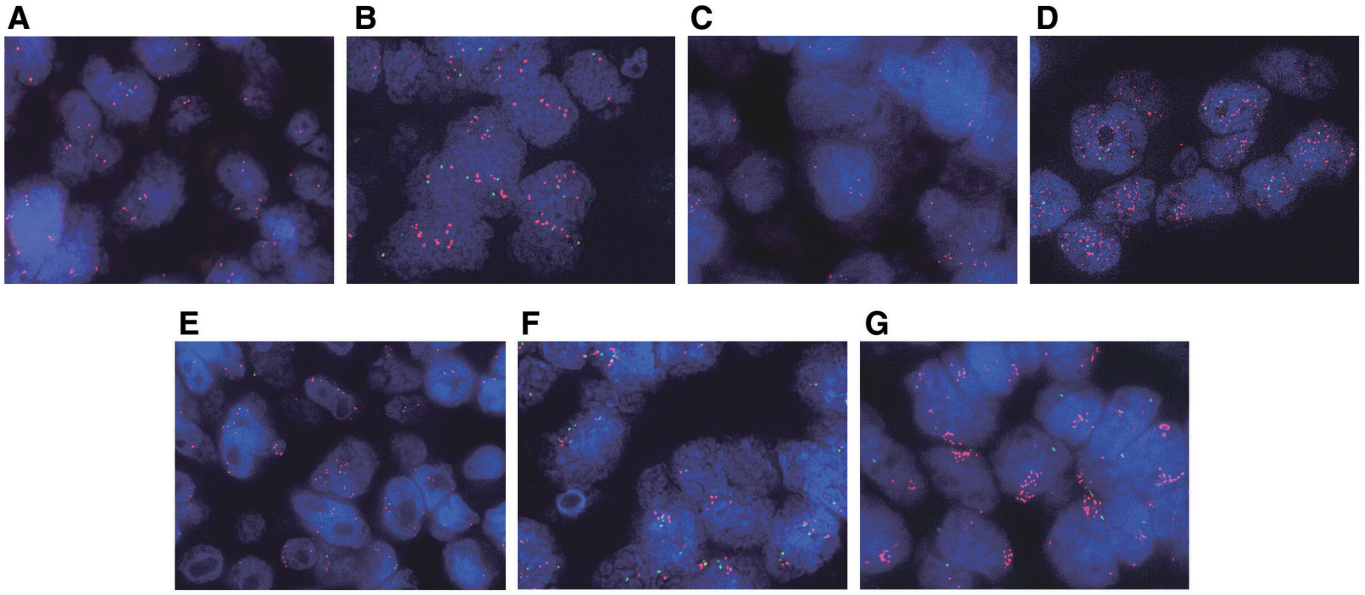

FIGURE 1. Representative fluorescence in situ hybridization (FISH) images showing the amplifications of the *NKX2-1* (*A*), *SETDB1* (*B*), *MET* (*C*), *HER2* (*D*), *SOX2* (*E*), *FGFR1* (*F*), and *PIK3CA* (*G*) genes (in orange). The corresponding centromere enumeration probes (CEP) are shown in green.

35 (37.6%) patients with SCC, but only 16 (10.3%) patients with AC exhibited coamplification ($p < 0.0001$). Among the patients with amplified *NKX2-1*, *SETDB1*, *MET*, *HER2*, *SOX2*, *FGFR1*, or *PIK3CA* genes, coamplification with another gene locus was detected in 41.4% (12 of 29), 71.4% (10 of 14), 39.5% (15 of 38), 62.5% (5 of 8), 90.9% (30 of 33), 55.6% (10 of 18), and 72.5% (37 of 51), respectively (Supplementary Fig. 1, Supplementary Digital Content 2, http://links.lww.com/JTO/A897). The most frequent coamplification combinations were *NKX2-1* and *MET* in AC, and *SOX2* and *PIK3CA* in SCC (Supplementary Table 1, Supplementary Digital Content 1, http://links.lww.com/JTO/A896).

The *MET*, *SOX2*, and *PIK3CA* amplification statuses in NSCLC were significantly associated with sex, as shown in Tables 1 and 2. *NKX2-1* amplification was negatively correlated with the smoking history (Table 1). In contrast, *SOX2*, *FGFR1*, and *PIK3CA* amplification were positively correlated with the smoking history (Table 2). No correlation was found between *SETDB1* gene amplification and patient sex or smoking history (Table 1). *NKX2-1* gene amplification was more frequent in AC than in SCC (Table 2). *SOX2*, *FGFR1*, and *PIK3CA* gene amplification were more frequent in SCC than in AC (Table 2). *SETDB1* amplification was associated with an advanced pathological stage (Table 1). Intriguingly, the proportion of patients who received adjuvant chemotherapy was significantly higher for the *SETDB1*-amplified patients (Table 1), compared with the other patients. *HER2* amplification was not significantly associated with any clinicopathological characteristics (Table 1). We assessed the differences in the clinicopathological features between the high amplification cases and the low amplification cases for each gene (Supplementary Tables 2 and 3, Supplementary Digital Content 3, http://links.lww.com/JTO/A898 and Supplementary Digital Content 4, http://links.lww.com/JTO/A899). High-level amplification of the *SETDB1* gene was observed almost exclusively in AC. In contrast, low-level *SETDB1* amplification was more commonly observed in SCC. No significant differences were observed in the other six gene profiles.

Survival Analysis of Gene Amplification in NSCLC

We evaluated the prognostic roles of *NKX2-1, SETDB1, MET, HER2, SOX2, FGFR1*, and *PIK3CA* gene amplification. With a median follow-up of 3.5 years, the median survival time (MST) in the whole population was 11.5 years (95% confidence interval [CI], 9.1 to not reached [NR]). Concerning OS, a significantly poor prognosis was observed for NSCLC patients with *NKX2-1* amplification, compared with those lacking a *NKX2-1* copy number gain (log-rank, $p = 0.045$, Fig. 2*A*). No significant differences were observed when comparing patients with and those without a copy number gain for the *SETDB1*, *MET*, *HER2*, *SOX2*, *FGFR1*, or *PIK3CA* gene (log-rank, $p = 0.12$, 0.32, 0.74, 0.63, 0.23, and 0.14, respectively), Figure 2*B–G*. Coamplification also did not influence the OS when compared with patients harboring no-amplification and single amplification (log-rank, $p = 0.078$, Supplementary Fig. 2A, Supplementary Digital Content 5, http://links.lww.com/JTO/A900).

Survival Analysis of *NKX2-1*, *SETDB1*, *MET*, and *HER2* Amplification in Adenocarcinoma

We performed subset survival analyses for gene amplification according to the different subtypes of NSCLC.

In AC, statistically significant differences in OS were observed between patients with or without *NKX2-1* amplification and those with or without *SETDB1* amplification (log-rank, $p = 0.0003$ and 0.011, respectively, Fig. 3*A* and *B*). The

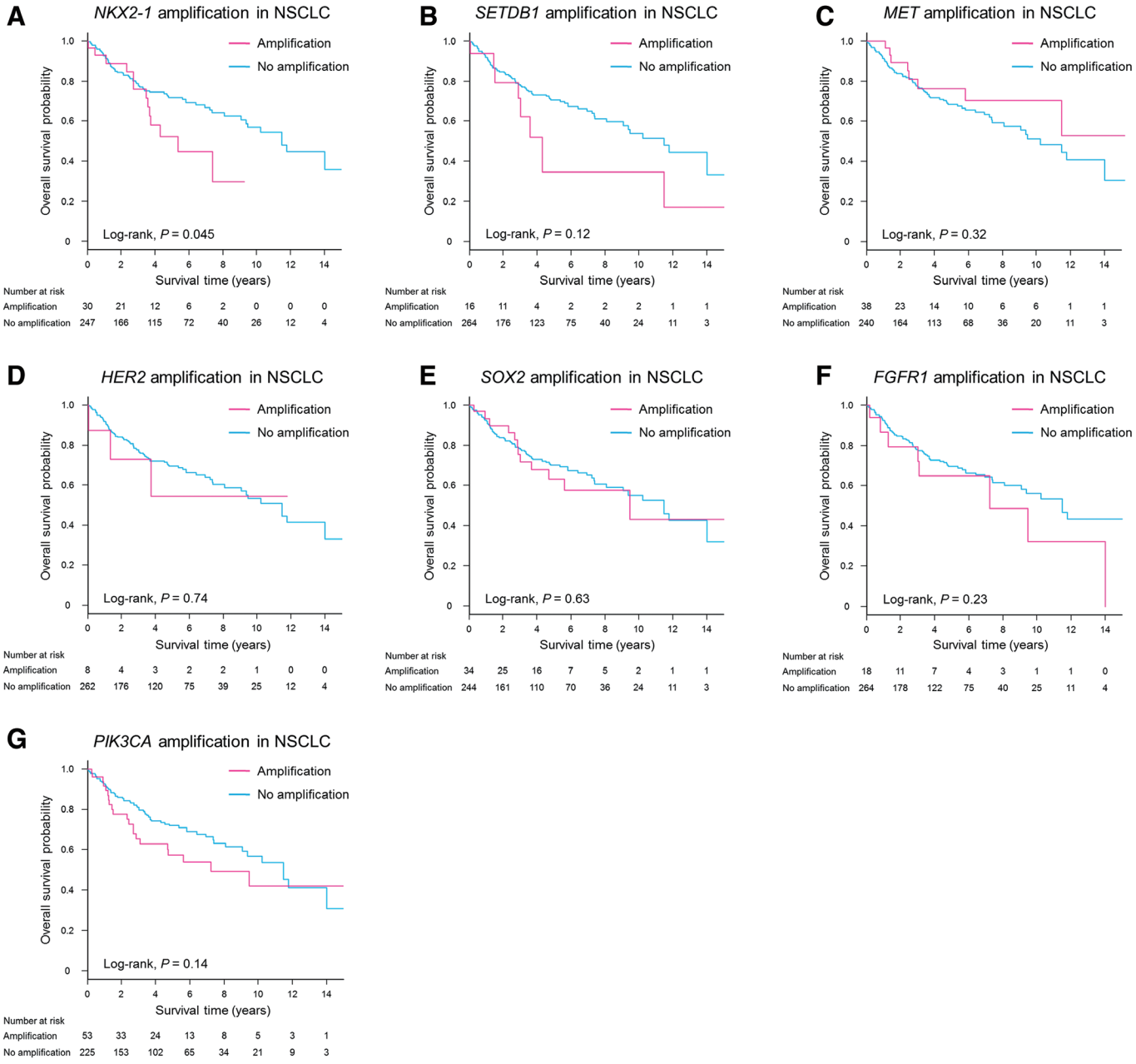

FIGURE 2. Kaplan–Meier curves for overall survival (years) stratified according to the gene amplification status of *NKX2-1*, *SETDB1*, *MET*, *HER2*, *SOX2*, *FGFR1*, and *PIK3CA* in non–small-cell lung cancer (NSCLC) patients. *A*, A significantly poorer outcome was observed for patients with *NKX2-1* amplification, compared with those without amplification (log-rank, $p = 0.045$). *B–G*, No significant differences in overall survival were observed between patients with and those without *SETDB1*, *MET*, *HER2*, *SOX2*, *FGFR1*, and *PIK3CA* gene amplifications, respectively.

MST was 5.3 years (95% CI, 3.5 to NR) for the patients with *NKX2-1*-amplified AC and was NR for those with *NXK2-1* normal copy number AC. Multivariate Cox regression analysis revealed that *NKX2-1* gene amplification was an independent prognostic factor (HR, 2.938; 95% CI, 1.434–6.022), with smoking status (HR, 2.020; 95% CI, 1.024–3.987) and pathological disease stage (HR, 2.156; 95% CI, 1.478–3.145), in AC (Table 3). *SETDB1* amplification was not an independent predictor of poor survival (HR, 1.037; 95% CI, 0.372–2.892, Table 3). *MET* amplification had neither prognostic impact (p log-rank = 0.53, univariate analysis HR, 1.320; 95% CI, 0.550–3.171) in AC (Fig. 3*C*; Table 3), nor *HER2* amplification (p log-rank = 0.67, univariate analysis HR, 1.369; 95% CI, 0.327–5.740) (Fig. 3*D*; Table 3). A Kaplan–Meier curve showed a significant difference in OS between patients with coamplification and those with no amplification ($p = 0.040$, Supplementary Fig. 2*B*, Supplementary Digital Content 5, http://links.lww.com/JTO/A900). We next evaluated whether

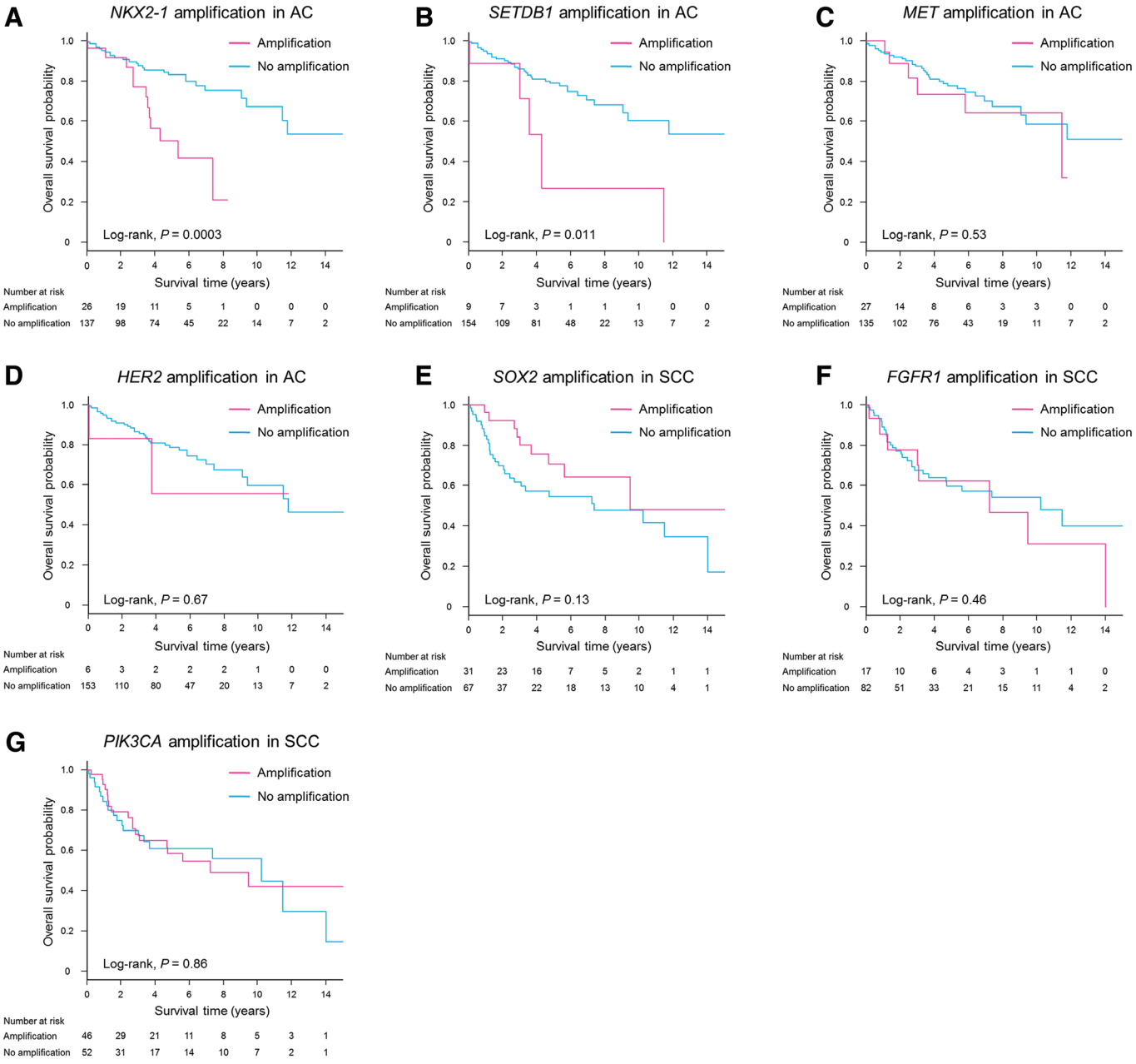

FIGURE 3. Kaplan–Meier estimates for overall survival (years) stratified according to *NKX2-1*, *SETDB1*, *MET*, and *HER2* gene amplification among patients with adenocarcinoma (AC) (*A–D*) and stratified according to *SOX2*, *FGFR1*, and *PIK3CA* gene amplification among patients with squamous cell carcinoma (SCC) (*E–G*). *A* and *B*, Significant survival differences were observed for patients with *NKX2-1* and *SETDB1* amplification, compared with those without each of the gene amplifications (log-rank, $p = 0.0003$ and 0.011, respectively). *C* and *D*, *MET* amplification and *HER2* amplification had no survival impact on patients with adenocarcinoma (log-rank, $p = 0.53$ and 0.67, respectively). *E*, Patients with *SOX2* amplification tended to have a better survival outcome than the patients without a *SOX2* copy number gain (log-rank, $p = 0.13$). *F* and *G*, No differences in survival were observed between SCC patients with or without *FGFR1* or *PIK3CA* gene amplification (log-rank, $p = 0.46$ and 0.86, respectively).

the magnitude of gene amplification had an impact on the postoperative survival outcomes. Patients with AC exhibiting a relatively high amplification of the *NKX2-1* gene tended to have a poorer prognosis than those who had tumors with a low amplification of the *NKX2-1* gene (MST, 7.4 versus 3.7 years), but the difference was not significant ($p = 0.26$, Supplementary Fig. 3*A*, Supplementary Digital Content 6, http://links.lww.com/JTO/A901). No survival difference according to the magnitude of *MET* amplification was observed (Supplementary Fig. 3*B*, Supplementary Digital Content 6, http://links.lww.com/JTO/A901). Because most cases with *SETDB1*-amplified AC had a high amplification

TABLE 3. Results of Univariate and Multivariate Cox Proportional Hazards Model Analyses of Overall Survival in Patients with Adenocarcinoma

Characteristic	Per Unit for HR	Univariate HR	95% CI	*P* Value	Multivariate HR	95% CI	*P* value
Age	1 year	1.020	0.989–1.051	0.205			
Sex	Male/female	1.749	0.885–3.457	0.108			
Smoking status	Ever/never or unknown	2.149	1.100–4.198	0.025	2.020	1.024–3.987	0.043
Pathological stage	1-stage	2.228	1.584–3.134	<0.0001	2.156	1.478–3.145	<0.0001
Adjuvant chemotherapy	Yes/no	2.095	1.113–3.944	0.022			
NKX2-1 amplification	Yes/no	3.388	1.667–6.884	<0.001	2.938	1.434–6.022	0.003
SETDB1 amplification	Yes/no	3.198	1.238–8.263	0.016	1.037	0.372–2.892	0.944
MET amplification	Yes/no	1.320	0.550–3.171	0.534			
HER2 amplification	Yes/no	1.369	0.327–5.740	0.667			

HR, hazard ratio; CI, confidence interval.

status (7 of 9, Supplementary Table 2, Supplementary Digital Content 3, http://links.lww.com/JTO/A898), we could not assess the influence of the dosage level of *SETDB1* amplification. We also could not determine the prognostic role of the magnitude of *HER2* amplification because of the very small number of patients with *HER2*-amplified tumors.

Survival Analysis of *SOX2*, *FGFR1*, and *PIK3CA* Amplification in Squamous Cell Lung Cancer

In SCC, we evaluated the prognostic impact of the amplification of three genes (*SOX2*, *FGFR1*, and *PIK3CA*) because the amplification of these genes was observed in SCC almost exclusively (Table 2). Patients with *SOX2*-amplified tumors showed a trend toward better survival than the negative group (log-rank, $p = 0.13$, Fig. 3*E*). The MST was 9.5 years (95% CI, 4.7 to NR) for the patients with *SOX2*-amplified SCC and 7.4 years (95% CI, 2.4–14.0) for the SCC patients without *SOX2* amplification. Neither the *FGFR1* nor the *PIK3CA* gene amplification status affected OS among the SCC cases (log-rank, $p = 0.46$ and 0.86, respectively), Figure 3*F* and *G*. Apart from AC, coamplification had no prognostic impact among the patients with SCC (log-rank, $p = 0.82$, Supplementary Fig. 2C, Supplementary Digital Content 5, http://links.lww.com/JTO/A900). The degrees of gene amplification of the *SOX2*, *FGFR1*, and *PIK3CA* genes did not affect the survival outcomes of patients with SCC (Supplementary Fig. 3*C–E*, respectively; Supplementary Digital Content 6, http://links.lww.com/JTO/A901).

DISCUSSION

In this study, we comprehensively explored whether the amplification of seven genes, *NKX2-1*, *SETDB1*, *MET*, *HER2*, *SOX2*, *FGFR1*, and *PIK3CA*, was related to the clinical factors and outcomes of Japanese patients with resected NSCLC. This study is noteworthy in that it evaluated the amplification of multiple genes within the same cohort; furthermore, to the best of our knowledge, this is the first report to investigate the prognostic significance of *SETDB1* amplification in lung cancer. We showed that AC was more common among patients with *NKX2-1* amplification than among those without. *SOX2*, *FGFR1*, and *PIK3CA* gene amplification were significantly associated with a squamous histology, consistent with the findings of previous studies.[1,13,15–17,22–24] Our study showed that *NKX2-1* amplification in AC was an independent predictor of a poor prognosis. Furthermore, a correlation between the postoperative survival period of AC patients and the magnitude of *NKX2-1* amplification was suggested. Our data also indicated that postoperative OS was shorter among cases with AC harboring *SETDB1* amplification. In contrast, patients with *SOX2*-amplified tumors in SCC tended to have a better survival outcome than those without, as described in previous reports.[12,13] Survival analyses performed according to copy number variations in the *MET*, *HER2*, *FGFR1*, and *PIK3CA* genes showed no significant differences in our cohort. We also demonstrated that coamplification was more frequent in SCC (37.6%) than in AC (10.3%), and the combination of *SOX2* and *PIK3CA* was the most prevalent among the seven genes that were examined; this result is not unexpected, based on their positional proximity at chromosome 3q26. Unlike the mutual exclusive status among gene alterations such as *epidermal growth factor receptor* (*EGFR*) mutations, v-Ki-*ras*2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutations, and anaplastic lymphoma kinase (*ALK*) rearrangements in NSCLC,[25] the phenomena of gene amplification were less mutually exclusive, although a part of them were also recognized as a cause of oncogenic addiction. In AC, we also showed that coamplification might be a stronger prognostic indicator than single gene amplification.

Genomic amplification is a hallmark of carcinogenesis and progression.[26] Some amplified genes have been considered to be targets for molecular specific therapies,[18,24,27] such as the use of trastuzumab against *HER2* amplification in breast[28] and gastric[29] cancer. The exact mechanistic explanation of gene amplification remains debatable, although some researchers[30] have claimed that amplicons are generated by replication timing switch. Amplification is more prominent in cases with greater environmental burdens (smoking, male sex, and others).[31] The disruption of the mitosis maintenance system also seems to be related to gene amplification in human lung cancer.[32]

NKX2-1 (*TTF-1*) is a homeodomain-containing transcription factor located at chromosome 14q13.3. This gene is known to be amplified specifically in a considerable proportion of lung tumors[33,34] and has been shown to be a lineage-specific oncogene in lung cancer.[34,35] *NKX2-1* is also known

to have a tumor suppressive function.[36,37] The depletion of *NKX2-1* in lung AC enhanced the transforming growth factor- β–mediated epithelial-to-mesenchymal transition,[36] the tumor seeding ability, and the metastatic proclivity.[37] *NKX2-1* gene amplification is related to NKX2-1 protein expression,[2,4] which is an indicator of a favorable prognosis in lung AC.[2–4,38] The prognostic impact of *NKX2-1* gene amplification, however, remains controversial. Interestingly, Tang et al.[2] reported that *NKX2-1* amplification displayed a significant positive correlation with the presence of *KRAS* mutations, and NSCLC patients with *NKX2-1* amplification tended to have a poorer survival outcome than those lacking amplification. Barletta et al.[3] reported that there was no difference in survival between *NKX2-1*-amplified ACs and not-amplified ACs, and they also showed that patients with no NKX2-1 expression or NKX2-1 expression and *NKX2-1* amplification had a significantly higher risk of death than patients with ACs with NKX2-1 expression and without *NKX2-1* amplification. Lee et al.[5] reported that *NKX2-1* amplification was a poor prognostic factor for OS in patients with resected ACs with NKX2-1 protein expression and *EGFR* mutations. Conversely, Perner et al.[4] demonstrated that ACs with a high level of *NKX2-1* amplification tended to have an increased OS, compared with other AC patients. In our study, *NKX2-1* amplification was associated with a significantly increased risk of death among patients with AC, regardless of the NKX2-1 expression status or the *EGFR* mutation status. The reason for this discrepancy is unknown, and the double-edged characteristics of *NKX2-1* as both an oncogene and a tumor suppressor gene should be noted.

This study demonstrated, for the first time, that *SETDB1* amplification was a marker of poor survival outcome, although we failed to identify *SETDB1* amplification as an independent prognostic factor in multivariate analysis. The amplification of *SETDB1* at 1q21.3[39] has been detected in various cancers including lung cancer,[18,40] and *SETDB1* has been recognized as an oncogene. Reports on the prevalence of *SETDB1* amplification in NSCLC are few, and the total number of known cases of amplification is only 24 (8 out of 40 reported by Rodriguez-Paredes et al.[18] and 16 in the present cohort). Further confirmation in a larger patient sample is warranted to determine the clinical significance of *SETDB1* amplification on patient outcome.

MET amplification is known to be one of the mechanisms by which *EGFR* mutated lung tumors become resistant to EGFR tyrosine kinase inhibitors (TKIs).[41] Previous studies have shown that *MET* gene amplification was observed in 2.1% to 21% of NSCLC patients.[6–8,42] The variability of these studies may depend on differences in the criteria used to define amplification, the methods used to determine amplification, disease stage, or race. *MET* amplification was reported as being an independent negative prognostic factor among patients with resected NSCLC in a study from Italy,[6] but the OS was not affected in the present study, in agreement with the findings of another previous study.[42]

In addition to *MET* amplification, *HER2* amplification is also known to be involved in acquired resistance to EGFR TKI therapy.[41] In this study, *HER2* amplification was seen in only 3% of NSCLCs, and no correlation was observed between the *HER2* amplification status and the survival outcome. In NSCLC, the prevalence of *HER2* amplification has been reported to range from 2% to 9.9%[9,10,43] by FISH and 19% by chromogenic in situ hybridization,[44] using almost the same definition of gene amplification as that used in this study. The prognostic significance of *HER2* amplification in NSCLC remains uncertain. A recent study showed that *HER2* amplification was associated with a poor postoperative OS outcome when analyzed only among patients with *HER2*-mutated NSCLC.[44] Because the efficacy of HER2-targeted therapy, such as trastuzumab and irreversible TKIs targeting HER2 and EGFR, in patients with *HER2*-amplified or *HER2*-mutated NSCLC has not been elucidated, more comprehensive studies evaluating the HER2-targeted therapeutic implications tailored to the *HER2* status are necessary.

The amplification of *FGFR1* at the chromosome 8p11.23 locus has drawn the attention of many lung cancer investigators because *FGFR1* amplification appeared to be a promising target for anti-FGFR treatment,[24,27] although conflicting data have been reported in terms of the prognostic impact.[14,15,45,46] In our study, the relatively small number of *FGFR1* amplified cases (n = 18) make it difficult to conclude that an association between *FGFR1* amplification and patient survival exists.

The 3q26 locus contains the *SOX2* and *PIK3CA* genes, which have been functionally validated as prognosis modifiers for cancer cells.[22,47,48] In addition, SOX2 overexpression is likely to be a triggering event in the lung SCC carcinogenesis sequence.[49,50] Against expectations of an oncogenic effect, the amplification of *SOX2* has been shown to be associated with a better survival outcome in SCC[12] and NSCLC.[13] Similar to these studies, *SOX2* amplification tended to be associated with a better survival outcome in our cohort, although the observation did not reach statistical significance. The reason why *SOX2* amplification can act as a favorable survival indicator remains unclear, but it may be related to the fact that *SOX2* is a differentiation marker.[22,49] *PIK3CA* amplification has been suggested to be a major cause of the overactivation of the PI3K/Akt pathway that promotes lung squamous cell carcinogenesis,[23] but *PIK3CA* amplification was not associated with clinical outcome in patients with either NSCLC or SCC in our cohort. One previous study of Japanese patients with lung cancer showed a shorter OS in *PIK3CA*-amplified cases,[17] whereas another showed no difference in prognosis.[13] One of the reasons confounding the effect of *PIK3CA* amplification might be the high frequency of coamplification with the *SOX2* gene in *PIK3CA*-amplified cases (28 of 53 [52.8%] in NSCLC and 26 of 46 [56.5%] in SCC).

In conclusion, the copy number amplification of seven loci (*NKX2-1*, *SETDB1*, *MET*, *HER2*, *SOX2*, *FGFR1*, and *PIK3CA*), previously reported for a few of these genes in lung cancer, was comprehensively investigated in Japanese subjects. The amplification of these genes was not significantly related to the overall outcomes of the NSCLC patients, with the exception of *NKX2-1* amplification, providing a rationale for prioritizing this test for clinical predictability. The amplification profiles, however, differed according to the histological types. Actually, the correlation between *NKX2-1* amplification

and a poor outcome was prominent among patients with AC. Further replication of these studies may lead to the practical use of these markers in specific settings as outcome predictors in patients with lung cancer.

ACKNOWLEDGMENTS

We thank Mrs. Kiyoko Nagura, Mr. Hisaki Igarashi, and Mr. Shinichiro Kiyose (Hamamatsu University School of Medicine) for their technical assistance and for providing the probes.

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