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*.

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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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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.

