

Relationship among Genetic Alterations, DNA Content, and Clinicopathological Features in Primary Lung Adenocarcinomas

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Abstract. *Background:* Only fragmentary information is available about the genomic imbalances affecting the malignant potential of lung cancers. *Patients and Methods:* Chromosomal DNA sequence copy number aberrations (DSCNAs) and DNA content (ploidy status) were examined in 34 resected tumor specimens, using comparative genomic hybridization and laser scanning cytometry, respectively. *Results:* Twenty-seven tumors showed DNA aneuploidy. Gains of 9q22-33 and 10q26-qter and loss of 15q14-qter were the significant indicators of lymph node metastasis. These DSCNAs were identified only in aneuploid tumors. Aneuploid tumors with any of these DSCNAs were associated with a worse prognosis than those not associated with any of these DSCNAs. The DNA index was a significant prognosticator in aneuploid tumors, although it was not dependent on the level of genetic alterations. *Conclusion:* Clinically-relevant genetic alterations were identified predominantly in aneuploid tumors. The DNA content also affected the prognosis of patients with aneuploid tumors. Thus, a comprehensive genomic study of aneuploid lung adenocarcinomas should be of great clinical value.

Lung cancer is the leading cause of cancer death in developed countries (1, 2). Adenocarcinoma, the most common pathological subtype of non-small cell lung cancer, shows great variation in biological characteristics, such as cell proliferation, lymph node or distant metastasis, and resistance to chemo-radiotherapy. The biological

behavior of a tumor is affected primarily by genetic abnormalities of the tumor cells. Genetic alterations in overt cancers are often different even among tumors with similar histological features, which makes it difficult to evaluate the biological behavior of each cancer by histopathological examination alone. These differences are partly attributed to genomic instability in each cancer (3-5). However, only fragmentary information is available about the genomic changes underlying the malignant potential of lung cancers.

The present study was conducted to comprehensively examine the genomic changes in lung adenocarcinoma, by comparative genomic hybridization (CGH) and laser scanning cytometry (LSC). The results were correlated with each other and with the clinicopathological features.

Patients and Methods

Tumor specimens. This study protocol was approved by the Ethical Review Committee of Gene Analysis Research at the Yamaguchi University School of Medicine, Japan. Signed informed consent was obtained from all the patients or their families. Thirty-four fresh-frozen specimens of primary lung adenocarcinomas were obtained from patients undergoing surgery at Yamaguchi University Hospital and its affiliated hospitals (Table I). The study population consisted of 22 men and 12 women with an average age of 65 years. According to the World Health Organization classification (2), the carcinomas were histologically classified as acinar adenocarcinoma (n=3) or papillary adenocarcinoma (n=31); and graded as well-differentiated (G1; n=10), moderately-differentiated (G2; n=19), or poorly-differentiated adenocarcinoma (G3; n=5). There were 16 T1, 14 T2, 2 T3 and two T4 tumors, all diagnosed pathologically. The disease stage was diagnosed pathologically as N0 in 23 patients, N1 in six, and N2 in five. None of the patients had received chemotherapy or radiation therapy before surgery. A small tissue fragment was taken from a representative area of each tumor. All tissue specimens were stored at -80°C until examination.

Microdissection and DNA extraction. Lung cancer, like other solid tumors, consists of large amounts of non-carcinoma cells; therefore, it is necessary to extract high-quality carcinoma DNA (6,

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Key Words: Lung adenocarcinoma, comparative genomic hybridization, laser scanning cytometry, DNA ploidy, prognosis, lymph node metastasis.

Table I. Clinicopathological characteristics of the study population (n=34).

| Variables | | Number or Mean±SD |
|----------------------|--------------------|-------------------|
| Age | (Years) | 65±11 |
| Sex | (Male/Female) | 22/12 |
| Histological subtype | (Papillary/Acinar) | 31/3 |
| Differentiation | (G1/G2/G3) | 10/19/5 |
| Tumor size | (mm) | 31±11 |
| p-T status | (T1/T2/T3/T4) | 16/14/2/2 |
| p-N status | (N0/N1/N2) | 23/6/5 |

p-T status=pathological-T status; p-N status=pathological-N status.

7). We used a microdissection technique was used to prepare 7-μm tissue sections from frozen carcinoma tissue specimens to minimize the contamination by non-carcinoma tissue (8-10). The carcinoma components were carefully microdissected from the stromal tissue and lymphocytes by using a LM200 laser capture microdissector (Arcturus Engineering, Inc., CA, USA). High molecular weight DNA was extracted from approximately 50 to 100 carcinoma cells, using a SepaGene DNA extraction kit (Sankoujunyaku, Tokyo, Japan), according to the manufacturer's instructions. Control DNA was also extracted from peripheral blood lymphocytes of healthy male and female donors.

Degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR). The DNA from carcinoma cells and normal lymphocytes were amplified by degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR) using the universal primer 6-MW(5'-CCGACTCGAGNNNNNNATGTGG-3') on a thermocycler (ASTEC, Fukuoka, Japan), as described previously (10-12). Briefly, a 1-ml aliquot of carcinoma or reference DNA was pretreated with 1 unit of topoisomerase-I (Promega, WI, USA) for 30 min at 37°C. The topoisomerase pretreatment was followed by five treatment cycles (1 min at 94°C, 2 min at 30°C and 2 min at 37°C) using 20 units of thermosequase (Amersham, OH, USA) and one cycle at 95°C for 10 min. Then, 45 μl of 1xPCR buffer containing 2.5 units of TaqDNA polymerase (Takara, Tokyo, Japan) was added, followed by 35 cycles at 94°C for 1 min, 56°C for 1 min and 72°C for 3 min, with a final extension at 72°C for 5 min.

Comparative genomic hybridization (CGH). DOP-PCR-amplified DNA from the tumor tissues and reference normal lymphocytes was labelled with SpectrumGreen-dUTP and SpectrumRed-dUTP (Vysis, IL, USA) by standard nick-translation reaction, respectively. Each DNA sample (200 ng), labelled with 10 mg of Cot-1 DNA (Life Technologies, MD, USA), was dissolved in 10 ml of hybridization buffer and hybridized onto normal denatured metaphase chromosomes for 72 h at 37°C. The hybridized chromosomes were mounted with an antifade solution containing 0.15 mg/ml 4,6-diamino-2-phenylindole. The fluorescence images were captured with an Olympus BX 50 fluorescence microscope equipped with a X100 UplanApo objective and a SenSys 1400 CCD camera (Photometrics, AZ, USA). Gains and losses in the DNA sequence copy number were defined by tumor to reference ratios of >1.2 and <0.8, respectively. Amplifications (high-level gains) were defined by a tumor to reference ratio of >1.4.

Laser scanning cytometry (LSC). LSC was performed as previously described (9, 13, 14). Briefly, touch smears were prepared by touching thawed pulmonary adenocarcinoma tissue specimens on glass slides, which were then fixed in ethanol at room temperature. The cells were stained in propidium iodide (Sigma, MO, USA) solution containing 0.1% RNase (Sigma). The nuclear DNA content was measured by a LSC101 laser scanning cytometer (Olympus, Tokyo, Japan). More than 5,000 cells in each sample were examined and the DNA index (DI) calculated.

Statistical analysis. Fisher's exact test was used to evaluate the differences in the frequencies of genetic alterations and other factors between the two groups. Correlations between the two indices were evaluated by Spearman's rank correlation test. The survival curves were drawn by the Kaplan-Meier method, and the difference between the groups were evaluated by the log rank test. Differences of $p < 0.05$ were considered significant.

Results

Comparative genomic hybridization. DNA sequence copy number gains were frequent at 1q21-23 (20/34, 59%), 5p15 (13/34, 38%), 6p24-21 (12/34, 35%), 7p15-14 (13/38, 38%), 7q11 (12/34, 35%), 8q22 (11/34, 32%), 8q24 (13/34, 38%), 11q13 (13/34, 38%), 16pter-12 (13/34, 38%) and 20q (13/34, 38%) (Figure 1). Amplifications were detected at 5p15 (3/34, 9%), 8q23-qter (3/34, 9%), 16p (5/34, 15%), 20q (3/34, 9%) and 22q (3/34, 9%). Losses were detected at 3p12 (12/34, 35%), 4p15 (12/34, 35%), 4q13-28 (12/34, 35%), 6q12-21 (11/34, 32%), 8p22-11 (12/34, 35%), 9p23-13 (14/34, 41%), 13q21 (18/34, 53%), 17p (13/34, 38%) and 18q12-qter (14/34, 41%).

The average number of DNA sequence copy number aberrations (DSCNAs) was 15.0 per tumor. The average number of gains was 8.2 per tumor, including 0.9 amplifications per tumor, while the average number of losses was 6.8 per tumor. There was no significant difference in the average number of DSCNAs between large tumors (with a maximum diameter greater than 30 mm; n=14) and small tumors (with a maximum diameter of 30 mm or less; n=20). Although the number of DSCNAs did not affect the nodal status, gains of 9q22-33 and 10q26-qter and losses of 15q14-qter were significant indicators of lymph node metastasis ($p=0.0087$, $p=0.0005$, $p=0.0477$, respectively, Table II). Of the eleven (82%) patients with lymph node metastasis, nine had at least one of these indicators, and of the 23 (91%) patients without lymph node metastasis, 21 had none of these indicators (Table II). Patients with any of these indicators (n=9) had significantly shorter disease-free survival than patients with none of these indicators (n=25, $p < 0.001$, Table II, Figure 1). The total number of DSCNAs for patients with any of these indicators was greater than that for patients with none of these indicators ($p < 0.05$, Figure 1).

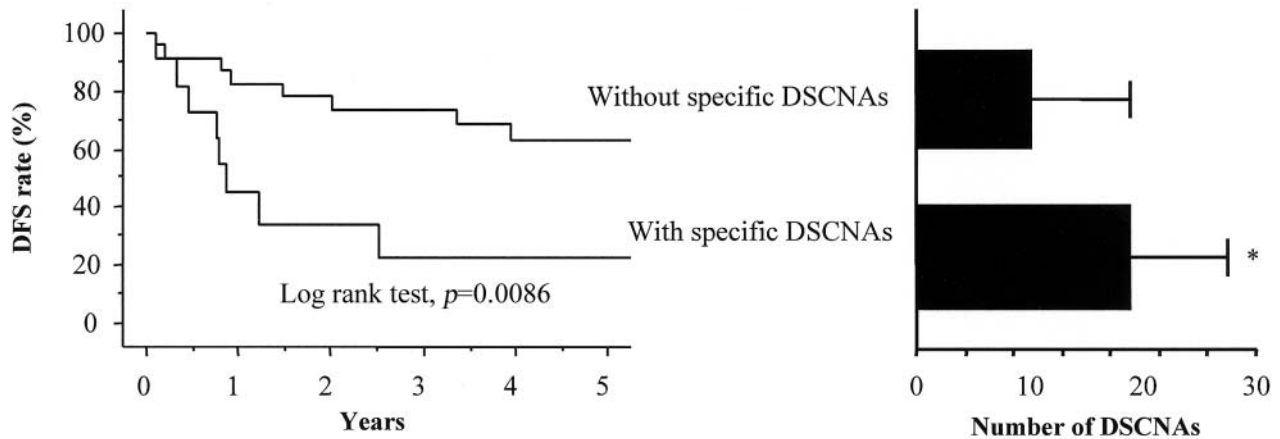


Figure 1. Patients with any of the specific DSCNAs ($n=9$) had significantly worse disease-free survival than patients without any of the specific DSCNAs ($n=25$, $p<0.001$). The total number of DSCNAs in patients with any of the specific DSCNAs was greater than that in patients without any of the specific DSCNAs ($p<0.05$). (DSCNAs, DNA sequence copy number aberrations, specific DSCNAs, gains of 9q22-33 and 10q26-qter and losses of 15q14-qter, $^*p<0.05$)

Table II. Specific DNA sequence copy number aberrations in relation to lymph node metastasis and recurrence.

| Specific DSCNAs | | Lymph node metastasis | | | Prognosis | |
|-----------------|----------|------------------------|------------------------|-----------|-----------------|--------|
| | | Positive ($n=11$) | Negative ($n=23$) | P | 5-year DFS rate | P |
| 9q22-33 (+) | (yes/no) | 3/8 | 0/23 | 0.0087 | 0%/53% | 0.0732 |
| 10q26-qter (+) | (yes/no) | 5/6 | 0/23 | 0.0005 | 0%/57% | 0.0006 |
| 15q14-qter (-) | (yes/no) | 4/7 | 2/21 | 0.0477 | 33%/55% | 0.4055 |
| Any | (yes/no) | 9/2 | 2/21 | <0.0001 | 23%/63% | 0.0086 |

DSCNAs=DNA sequence copy number aberrations; DFS=disease-free survival;

(+)=over-representation; (-)=loss; Any=any of 9q22-33 (+) , 10q26-qter (+) , or 15q14-qter (-).

Laser scanning cytometry. Tumors with a DNA index of 1.0 were defined as diploid and all others as aneuploid. Among the 34 tumors, seven (21%) were diploid and 27 (79%) were aneuploid. DNA ploidy did not correlate with the tumor size, nodal status, or disease-free survival. The number of DSCNAs was greater in aneuploid tumors than in diploid tumors ($p=0.0032$, Table III). With respect to the specific DSCNAs associated with nodal status (gains of 9q22-33 and 10q26-qter and losses of 15q14-qter), all these alterations were identified in aneuploid tumors, but not in diploid tumors (Table III). Aneuploid tumors could be further divided into two subgroups according to the DNA index (<2 or ≥ 2) on the basis of prognostic outcome, although the number of DSCNAs was not significantly different between the groups (Table IV). The disease-free survival rate of patients with aneuploid tumors with a DNA index ≥ 2 was significantly shorter than that of patients with aneuploid tumors with a DNA index <2 , and that of

patients with diploid tumors (Table IV, Figure 2). The total number of DSCNAs for aneuploid tumors with a DNA index ≥ 2 was similar to that of aneuploid tumors with a DNA index <2 , while the total number of DSCNAs for both aneuploid tumor groups was greater than that for diploid tumors (both $p<0.05$, Figure 2).

Discussion

An abnormal DNA index (aneuploidy) was found to be associated with a high level of genetic alteration detected by CGH. Furthermore, in this series, clinically-relevant genetic alterations were detected only in aneuploid tumors. These findings suggest that aneuploid tumors are cytogenetically different from diploid tumors and that aneuploid tumors are more unstable and aggressive than diploid tumors. In addition to the ploidy pattern, aneuploid tumors with a high DNA index were associated with a worse surgical outcome

Table III. Relationship between DNA ploidy status and DNA sequence copy number aberrations.

| | DNA ploidy | | P |
|------------------------|----------------------|-------------------------|---------|
| | Diploid tumors (n=7) | Aneuploid tumors (n=27) | |
| Type of DSCNA (number) | Mean±SD | Mean±SD | |
| (+) | 3.1±3.6 | 9.5±7.7 | 0.0418a |
| Amplifications | 0.3±0.5 | 1.0±1.7 | 0.2721a |
| Gains | 2.9±3.7 | 8.5±6.9 | 0.0459a |
| (-) | 2.4±1.7 | 7.9±4.6 | 0.0042a |
| Total | 5.6±4.2 | 17.4±11 | 0.0106a |
| Specific DSCNAs | yes/no | yes/no | |
| 9q22-33 (+) | 0/7 | 3/24 | 0.3557b |
| 10q26-qter (+) | 0/7 | 5/22 | 0.2177b |
| 15q14-qter (-) | 0/7 | 6/21 | 0.1693b |
| Any | 0/7 | 11/16 | 0.0400b |

^aP-values obtained by Fisher's exact test, ^bP-values obtained by Spearman's rank correlation test, DSCNA=DNA sequence copy number aberration, (+)=over-representation, (-)=loss, Any=any of 9q22-33 (+), 10q26-qter (+), or 15q14-qter (-)

than aneuploid tumors with a low DNA index. Although this result correlates with a previous report on DNA index in hepatocellular carcinoma (15), we consider it to be still possible that a diploid tumor could be falsely defined as an aneuploid tumor in some cases. We believe that the ploidy status was accurately determined in this study because the number of DSCNAs for aneuploidy tumors with a high DNA index was similar to that for those with a low DNA index, and that for diploid tumors was significantly lower than that for both aneuploid tumor groups. Similar results were obtained even when the DNA index was used as a numerical variable: the DNA index for aneuploid tumors was not dependent on the number of DSCNAs (correlation coefficient of 0.119, $p=0.556$), but it impacted on the prognostic outcome (Cox hazard ratio=2.83, 95% CI=1.06-7.53, $p=0.0371$), using linear regression analysis and Cox regression analysis, respectively.

The number of DSCNAs was not associated with the tumor size, pathological nodal status, or prognostic outcome in our series. This finding differs from previous reports, which we attribute to the fact that the number of genetic aberrations usually increases with tumor progression since malignant tumors are genetically unstable, irrespective of the type of genetic instability (3-5). However, our observations do not contradict this notion because lung adenocarcinomas, irrespective of their size, often have mature malignant biological potential and exhibit various genetic alterations at the time of clinical diagnosis. Because

Table IV. Influence of DNA index on DNA sequence copy number aberration, lymph node metastasis, and prognosis in patients with aneuploid tumors (n=27).

| Variables | (Mean±SD) | DNA index | | P |
|------------------------|-----------|-----------|-----------|---------------------|
| | | <2 (n=19) | ≥2 (n=8) | |
| Total number of DSCNAs | | 17.7±11.2 | 16.9±12.3 | 0.7710 ^a |
| Specific DSCNAs | | | | |
| 9q22-33 (+) | (yes/no) | 2/17 | 1/7 | 0.8815 ^b |
| 10q26-qter (+) | (yes/no) | 3/16 | 2/6 | 0.5737 ^b |
| 15q14-qter (-) | (yes/no) | 3/16 | 3/5 | 0.2153 ^b |
| Any | (yes/no) | 6/13 | 5/3 | 0.1354 ^b |
| Lymph node metastasis | (yes/no) | 5/14 | 5/3 | 0.0754 ^b |
| 5-year DFS rate | (%) | 25% | 49% | 0.0161 ^c |

DSCNAs=DNA sequence copy number aberrations; DFS=disease-free survival; (+)=over-representation; (-)=loss; DFS=disease-free survival; ^aP-value obtained by Fisher's exact test; ^bP-values obtained by Spearman's rank correlation test; ^cP-value obtained by log rank test.

the biological characteristics of lung adenocarcinoma differ greatly in individual tumors with similar macroscopic or microscopic appearance, tumor size or nodal status does not always reflect the accumulation of multiple genetic alterations.

In our series, the gains of 9q22-33 and 10q26-qter and the loss of 15q14-qter were associated with lymph node metastasis and early recurrence, even though cancer-related genes have not yet been identified at the loci of 9q22-33, 10q26-qter, and 15q14-qter in lung adenocarcinoma. It is likely that these chromosomal loci harbor genes that contribute to the nodal or distant metastasis of lung adenocarcinoma. According to various studies, an allelic loss of 15q is a marker of metastasis and recurrence in breast cancer (16), whereas the FGFR2 gene on the human chromosome 10q26 is amplified in diffuse-type gastric cancer, and the WDR11 gene on human chromosome 10q26 is disrupted in glial tumors (17).

With respect to the predictive accuracy of lymph node metastasis, nine of the eleven patients with nodal metastasis had at least one of the relevant genetic alterations; namely, gains of 9q22-33 and 10q26-qter and loss of 15q14-qter, showing a sensitivity rate of 82%, whereas 21 of the 23 patients without nodal metastasis had none of these alterations, showing a specificity rate of 91%. According to another study, gains of 8q24 and 20q12-qter were associated with lymph node metastasis of esophageal squamous cell carcinoma, based on the examination of preoperative

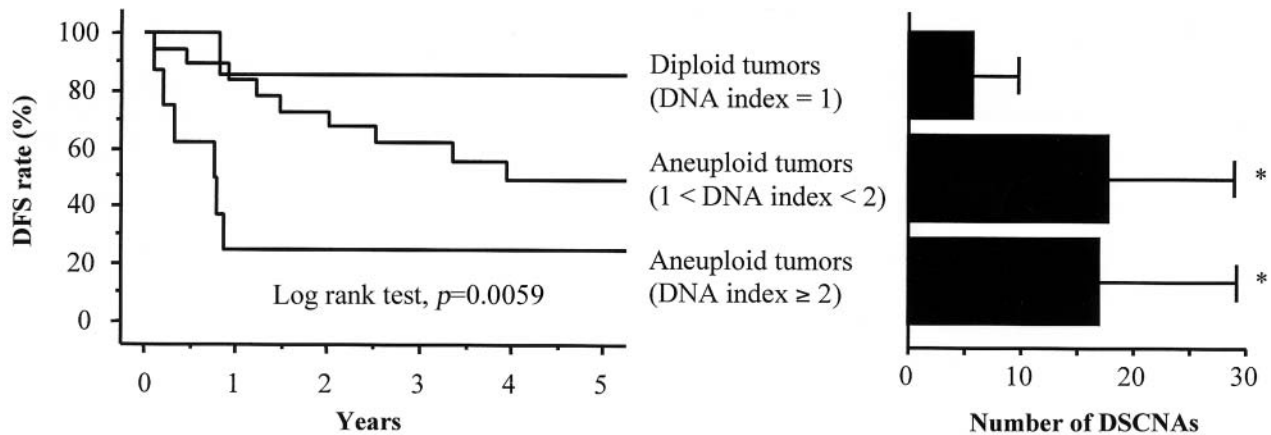


Figure 2. The disease-free survival rate of patients with aneuploid tumors with a DNA index ≥ 2 was significantly worse than that of patients with aneuploid tumors with a DNA index < 2 , and that of patients with diploid tumors. The total number of DSCNAs for aneuploid tumors with a DNA index ≥ 2 was similar to that for aneuploid tumors with a DNA index < 2 . The total number of DSCNAs for both aneuploid tumor groups was greater than that for diploid tumors ($p < 0.05$). (DSCNAs, DNA sequence copy number aberrations; *, $p < 0.05$ versus diploid tumors)

endoscopic biopsy specimens (18). This identification was also found to be useful in additional blinded cases.

The preoperative identification of lymph node-negative disease in patients undergoing lung cancer surgery may allow the surgeon to perform limited surgery. However, a large-scale study is needed to establish a reliable prediction of nodal status *via* genetic assessment because the reproducibility of CGH and intra-tumoral genetic heterogeneity, as described in pancreatic cancers (19), is still uncertain in lung adenocarcinoma.

DNA ploidy status is frequently measured to estimate the biological malignant potential of various cancers. However, in lung cancer, the significance of the DNA ploidy status as a reliable prognostic marker remains controversial (20-22). In the present series, aneuploid tumors were found to be associated with a significantly higher DSCNA number than diploid tumors. Moreover, specific chromosomal regions were involved more frequently in aneuploid tumors than in diploid tumors, with losses of 3q12-13, 6q12-21 9p23-13, 12q21 and 13q21, and gains of 7q11 and 11q13 ($p < 0.05$), which suggests that these specific chromosomal loci harbor genes that check DNA replication and chromosomal separation. This supports the finding that the aneuploidy number is frequently associated with chromosomal instability (3-5).

Apart from the tumor size and histological grade, lymph node metastasis (Cox hazard ratio=13.7, 95% CI=3.72-50.8, $p < 0.0001$) and DNA index (Cox hazard ratio=2.33, 95% CI=1.02-5.36, $p = 0.0459$) were identified as the best independent prognosticators for disease-free survival in our series, according to Cox regression analysis with stepwise variable selection. Thus, routine measurement of the DNA index could improve the prediction of prognosis after lung cancer surgery.

Conclusion

Clinically-relevant genetic alterations are predominantly identified in aneuploid tumors, the associated prognosis of which is affected by the DNA index. Because multiple molecular pathologies are associated with carcinogenesis and the progression of lung adenocarcinoma, composite genomic evaluation will become invaluable.

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