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Early report

Micropapillary components in a lung adenocarcinoma predict stump recurrence 8 years after resection: A case report

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ABSTRACT

We report a rare case of lung adenocarcinoma in which micropapillary components were considered to cause stump recurrence. A woman in her fifties was diagnosed with lung cancer in the right middle lobe with invasion to the upper lobe, which was treated by a right middle lobectomy together with upper lobe partial resection. The cancer was pathologically diagnosed as adenocarcinoma and had a free surgical margin. There was no recurrence during the following 5 years and 8 months, and thus periodical surveillance, including computed tomography, was stopped. However, 2 years and 7 months after this, she was discovered to have an abnormal shadow on chest radiography, and a thorough examination revealed a 3-cm-sized tumor involving the previous surgical margin. Therefore, she underwent right upper lobectomy. We pathologically re-evaluated the first tumor and found that it was an adenocarcinoma with a micropapillary component in the periphery, 6 mm away from the surgical margin. In addition, a few tiny clusters of tumor cells were found to be floating within the alveolar spaces near the margin. The first and second tumors showed almost the same histological mixture of components of adenocarcinoma and the same EGFR mutation. From these results, we concluded the second tumor was a stump recurrence originating from the first tumor resection. This case illustrates the importance of careful pathological investigation when an autosuture instrument is used for a partial resection in a case of lung adenocarcinoma with micropapillary components. In such cases, it is particularly important to clarify if micropapillary components are floating near a stump.

1. Introduction

Generally, when a primary lung cancer invades another lobe, a combined lobectomy with partial resection is performed if a tumor is sufficiently distant from the stump of the resection. Despite this precaution, a stump recurrence sometimes occurs due to unknown causes. Primary lung cancers exhibit a variable histology and a high degree of pleomorphism. This complexity has recently been added to because the presence of micropapillary components consisting of floating tumor cells in alveolar spaces has been classified in the International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society International Multidisciplinary Classification of lung adenocarcinoma (New IASLC classification) [1]. This histological finding has been shown to associate with a poor prognosis [2–4]. However, relatively little is known about the relationship between this micropapillary component and stump recurrence. Here we report a case of stump recurrence of a primary nonmucinous adenocarcinoma, which may be closely associated with the micropapillary component identified during pathological and molecular investigation.

2. Case

A woman in her fifties was discovered to have an abnormal chest shadow on radiography, and computed tomography revealed a tumor in the right middle lobe of size 25 mm × 25 mm, with spiculation and invasion of the right upper lobe. Treatment consisted of a right middle lobectomy combined with an upper lobe partial resection. An intraoperative pathological examination for a surgical margin around the partially resected material was

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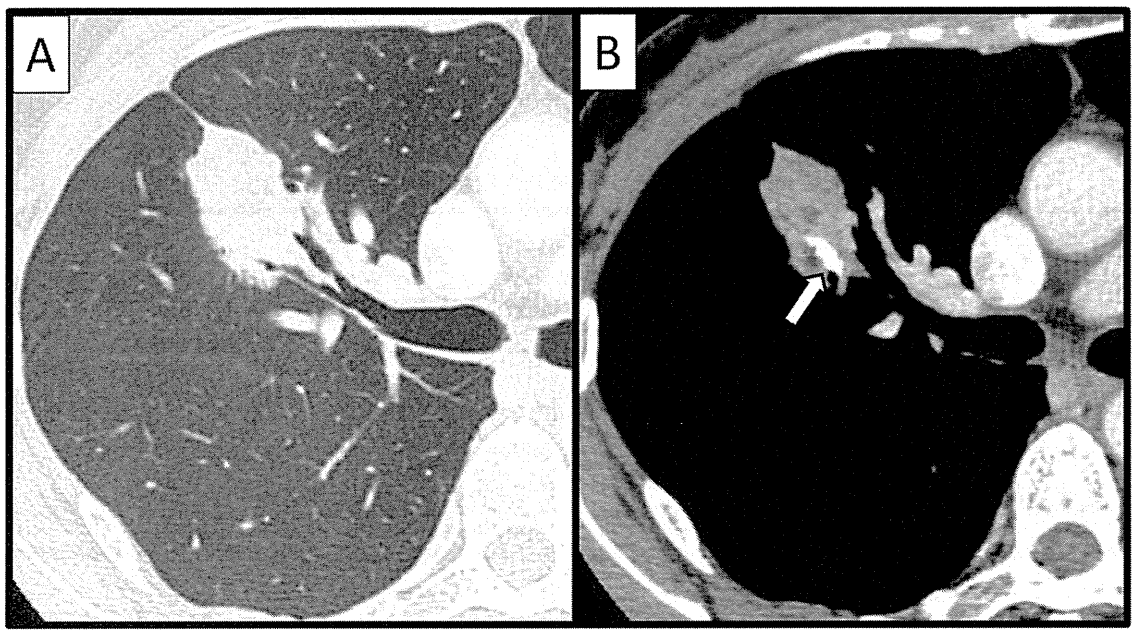

Fig. 1. Chest CT of the second tumor 8 years and 3 months after surgery (A: lung window and B: mediastinal window). The tumor involves the stapler line of the first operation at the right upper lobe (arrow).

not performed. Pathological analysis revealed the tumor to be an invasive adenocarcinoma, 26 mm × 19 mm × 23 mm in size, and predominantly composed of acinar tissue (50%), along with lepidic (15%), papillary (20%), micropapillary (10%), and solid tissues (5%). The tumor showed moderate lymph duct permeation with vascular invasion. We diagnosed patholohical T2aN0M0 lung adenocarcinoma in IB stage. She was followed up for 5 years and 8 months after surgery without adjuvant chemotherapy and showed no findings of recurrence; therefore, her routine follow-up was ended. However, 2 years and 7 months later, she again presented with an abnormal chest shadow on chest radiography, and medical examination and chest CT revealed a tumor in the right upper lobe involving a staple line of the first operation (Fig. 1). She was diagnosed with lung cancer and underwent a right upper lobectomy.

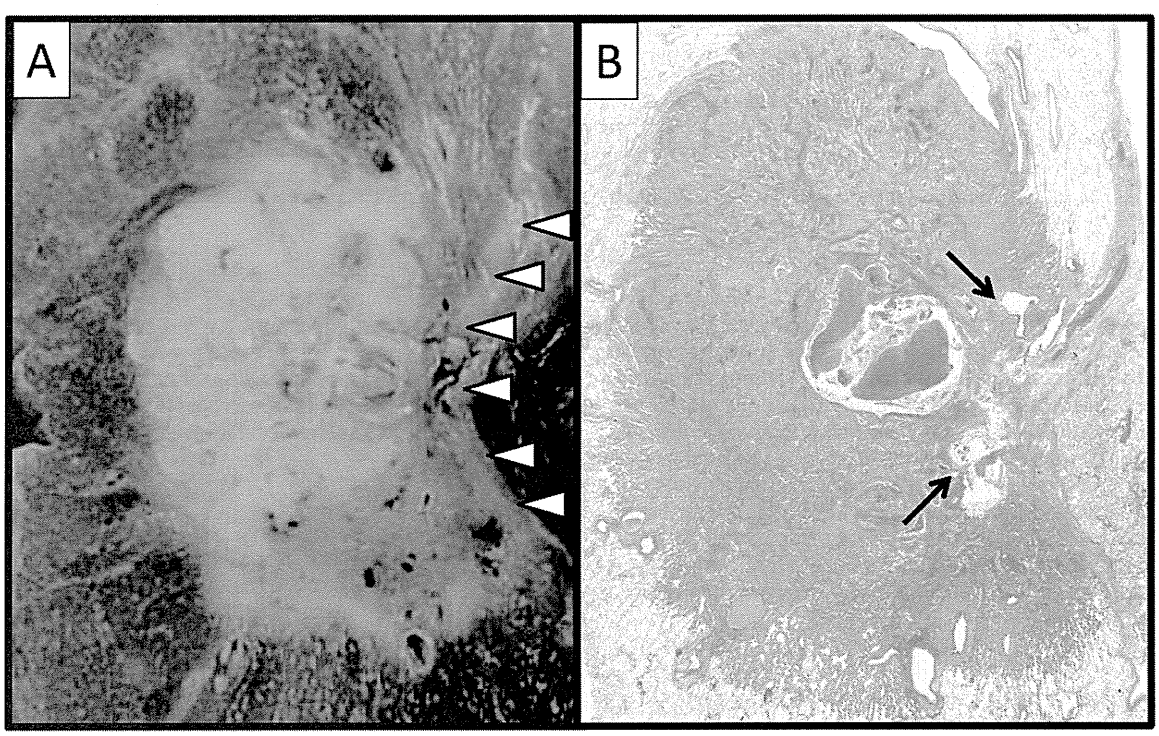

Fig. 2. The second tumor involved the previous surgical margin. Macroscopically, white arrowheads indicate the staple line of the last operation (A). Staples were removed during a specimen processing and the defects were microscopically left in the fibrotic line (B, black arrows).

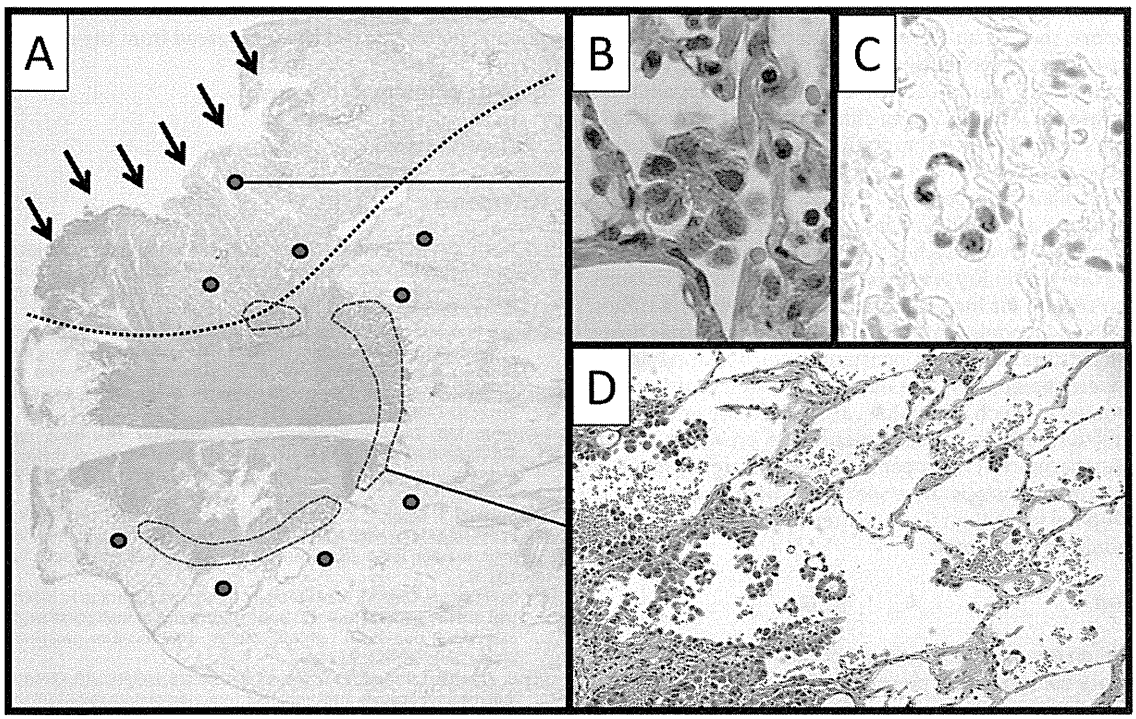

Fig. 3. Histlogical findings of first tumor. A: Photomicrograph of the first tumor. A dotted line locates in interlobar pleura, black arrows indicate the surgical margin of the upper lobe, the areas surrounded by a red dotted line consist of micropapillary component and the red circles mark the location of floating tumor cells (FTCs) (B). FTCs are positive for p53 in immunohistochemical staining (C). FTCs disperse from the tumor into surrounding alveolar apaces (D).

The tumor was found to be 30 mm × 20 mm × 30 mm in size, encompassing the staples, and had a necrotic area. Pathologically, the tumor was an invasive adenocarcinoma and the predominant component was acinar (60%), along with lepidic (10%), papillary (20%), and micropapillary tissues (10%). This composition was similar to that of the previous tumor (Fig. 2). The tumor showed mild lymph duct permeation and vascular invasion, and the surgical margins were negative. Immunohistochemically, both the original and subsequent tumor were positive for thyroid transcription factor-1, surfactant apoprotein-A, p53, p63, cyclinD1, epidermal growth factor receptor (EGFR), and carcinoembryonic antigen, and negative for urine protein-1, human mucin antigen (HIK1083), and anaplastic lymphoma kinase. The Ki67 (MIB-1) index was about 20% in both tumors. Written informed consent for genetic analysis of the tumor cells was obtained from the patient. Genetically, both tumors showed the same 15-base deletion (p.E749-A750del) in the *EGFR* gene at exon 19. We re-analyzed the first operative specimen and found that the surgical margin was 6 mm from the main tumor but a few tumor cell clusters of a micropapillary component were floating within alveolar spaces only 2.5 mm away from the margin (Fig. 3). Finally, we diagnosed the second tumor as a stump recurrence. The patient was discharged having had no significant complications; however, she presented with multiple pulmonary and third lumber metastasis a year and 6 months after the second surgery and is still being treated with chemoradiotherapy 2 years and 10 months after the surgery.

3. Discussion

Here we have presented a case of lung cancer recurrence at the stump, which appears to be associated with the presence of a micropapillary component near the margin. As described above, the pathological re-analysis of tissue removed during the first operation revealed that a few tumor cell clusters of the micropapillary component were floating within the alveolar spaces adjacent to the surgical margin, despite the main tumor being 6 mm away from the margin. As the first and second surgery were performed 8 years apart, and the tumor discovered in the second operation had a lepidic pattern, which suggests an in situ lesion, and could be metachronous to the primary tumor. However, it seems likely that the second tumor was not a metachronous primary carcinoma but a recurrent one, because the second tumor occurred from an excision stump of the first tumor and both tumors had similar component patterns of adenocarcinoma subtypes and immunohistochemical staining and the same EGFR mutation.

Lung adenocarcinoma has wide histological polymorphism and commonly consists of various histological components within the same tumor. Therefore, Travis et al. has proposed in the New IASLC classification that not only the most predominant component of a carcinoma be reported, but also the proportion of the other histologic components [1]. Girard N et al. reported that comprehensive assessment of histologic components is a powerful diagnostic tool that seems to be a promising method for determining whether multiple lung adenocarcinomas are metastatic or multiple primaries. Recently a micropapillary component was added as a new subtype in the classification as it associated with a poor prognosis [2–4]. An adenocarcinoma with a micropapillary component can spread further from the main tumor than would otherwise be expected from macroscopic evaluation by aerogenous dissemination. However, few studies have considered the relationship between the micropapillary component and stump recurrence. To date, the stump distance has only been examined in surgery for lung cancer [5–7]; however, there has been no detailed report about the stump distance from the primary tumor in combined lobar and partial resections, such as that reported here. In the present case, although the stump was located 6 mm from the main tumor in the first operation, the tumor had a micropapillary component and minute

tumor cell clusters were floating within alveolar spaces close to the stump. Therefore, there is an extremely high possibility that scattered micropapillary components were also present in the residual side of the pulmonary stump because aerogenous dissemination forms discontinuous lesions. Strong similarities in the histological components and an identical mutation in the two tumors also suggest that the recurrence at the surgical margin originated from floating micropapillary components.

How should we surgically manage an adenocarcinoma that has invaded one lobe from another and which has micropapillary components? One possibility would be to perform a rapid intraoperative frozen section for lung tumors to define whether a tumor has micropapillary components and, if it does, whether it has floating tumor cell clusters close to an excision stump. If floating tumor cell clusters were remote from the main tumor, stump processing should be avoided as much as possible, and lobectomy or anatomical segmentectomy might be recommended to prevent a stump recurrence. Furthermore, if micropapillary clusters are present at a surgical margin in the postoperative specimen, there should be very close follow-up for more than 5 years after surgery or the second operation should be considered.

4. Conclusion

To perform an operation for lung adenocarcinoma safely when a micropapillary component is present, it is necessary to exercise more care than in cases of adenocarcinomas without a micropapillary component. In addition, further examination is needed to clarify the safety margin in the case of pulmonary adenocarcinoma with micropapillary components as it is not known how far the floating neoplastic cell clusters spread from the main tumor.

Conflict of interest statement

None declared.

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CHEST Original Research
LUNG CANCER

A Proposal for Combination of Total Number and Anatomical Location of Involved Lymph Nodes for Nodal Classification in Non-small Cell Lung Cancer

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Background: **We previously reported the prognostic impact of the number of involved lymph nodes (LNs) on survival in non-small cell lung cancer (NSCLC). However, it remains unknown whether the total number or anatomic location of involved LNs is a superior prognostic factor.**
Methods: **A total of 689 patients with NSCLC who underwent complete resection involving dissection of the hilar and mediastinal LNs with curative intent of ≥ 10 LNs were enrolled. The association between the total number of LNs (nN) involved and survival was assessed by comparison with the anatomic location of LN involvement (pathologic lymph node [pN]), the present nodal category.**
Results: **We classified the patients into five categories according to the combined pN and nN status as follows: pN0-nN0, pN1-nN1-3, pN1-nN4−, pN2-nN1-3, and pN2-nN4. Although there was no statistically significant difference between the pN1-nN4− and pN2-nN1-3 categories, pN2-nN1-3 had better prognoses than pN1-nN4−. On multivariate analysis, the nN category was an independent prognostic factor for overall survival and disease-free survival (vs nN4−; the hazard ratios of nN0 and nN1-3 for overall survival were 0.223 and 0.369, respectively, $P < .0001$ for all), similar to the pN category. We propose a new classification based on a combination of the pN and nN categories: namely, N0 becomes pN0-nN0, the N1 category becomes pN1-nN1-3, the N2a category becomes pN2-nN1-3 + pN1-nN4−, and the N2b category becomes pN2-nN4. Each survival curve was proportional and was well distributed among the curves.**
Conclusions: **A combined anatomically based pN stage classification and numerically based nN stage classification is a more accurate prognostic determinant in patients with NSCLC, especially in the prognostically heterogeneous pN1 and pN2 cases. Further large-scale international cohort validation analyses are warranted.** ***CHEST 2013; 143(6):1618–1625***

Abbreviations: DFS = disease-free survival; LN = lymph node; nN = number of lymph nodes; NSCLC = non-small cell lung cancer; OS = overall survival; pN = pathologic lymph node; pT = pathologic tumor

Various pathologic and molecular markers have been assessed regarding their usefulness in identifying patients at high risk for recurrence. However, the TNM system remains the most important determinant of staging. Because the prognosis of lung cancer is directly proportional to the presence of lymph node (LN) metastasis, accurate LN assessment is crucial in determining treatment. Accurate staging of non-small cell lung cancer (NSCLC) requires assessment of the hilar and mediastinal LNs with pathologic evaluation. However, the present nodal classification still contains some limitations particularly concerning

For editorial comment see page 1527

heterogeneous pN1 and pN2 disease and the lack of a clear biologic definition of the distinguishing of N1 and N2.[1-4]

The seventh edition of the TNM classification for NSCLC[5] has been updated, with some modifications from the sixth edition.[6] However, the LN descriptor in the new classification remains the same as in the previous edition, and depends solely on the anatomic extent of LN involvement, despite the changes in the nodal map. In some other solid tumors, such as breast, gastric, and colorectal tumors, the number of metastatic lymph nodes has been included in the TNM staging system.

In our previous report,[7] we demonstrated that resection of ≥ 10 LNs influenced survival and that the number of involved LNs (four and more) is a strong independent prognostic factor in NSCLC. This may provide new information for determining the N category in the next TNM classification. However, it remains unknown whether the total number or anatomic location of involved LNs is a superior prognostic factor in NSCLC. Therefore, we retrospectively compared or combined the number of metastatic LNs (nN) category and the classic pathologic LNs (pN) category on survival in patients with completely resected NSCLC in whom ≥ 10 LNs had been harvested.

Materials and Methods

Patient Eligibility

A total of 1,311 consecutive patients who underwent surgical resection for primary lung cancer at our institution from 2000 to 2007 were examined retrospectively. The patients with clinical stages IA to IIIA, including patients with stage cN2 with single-station nodal metastasis, underwent surgery. Cases of induction therapy, incomplete resection, and limited resection were excluded from this study. Patients with tumors classified histologically as small-cell lung cancer or low-grade malignancies were also excluded. In addition, those in whom nine or fewer LNs were harvested were also excluded in the present analysis because our previous study suggested that resection of at least 10 LNs was necessary to maintain the optimal quality of surgery and accurate staging.[7] Finally, a total of 689 patients with NSCLC who underwent complete resection involving dissection of ≥ 10 hilar and mediastinal lymph nodes with curative intent, consisting of lobectomy or more extensive resection, were eligible.

Data Collection

The patient charts, including the pathologic diagnosis and operative reports, were reviewed. Staging was determined according to the sixth edition of the TNM staging system.[8] The histologic tumor type was determined according to the World Health Organization classification (third edition).[9] LNs were dissected with the adipose connective tissue of the corresponding anatomic regions, as designated by the surgeon intraoperatively. All dissected LNs were examined pathologically and classified on the basis of anatomic location by the numbering system described in the Naruke map.[10] The number of resected and involved LNs from each defined anatomic location was confirmed on the basis of the pathologic report provided by Drs Nomura, Matsubayashi, and Nagao. We performed two different stratifications of LN status assessment: the absence or presence and anatomic extent of nodal metastases (pN categories), and the number of regional LNs with metastases (nN categories). Based on our previous results, four or more involved LNs is the best benchmark of prognostic variables.[7] Therefore, we classified involved LNs into the three nN categories as follows: nN0, no LN metastasis; nN1-3, metastasis in one to three nodes; and nN4−, metastasis in four or more LNs. The pathologists were blinded to the clinical outcome.

We chose overall survival (OS) and disease-free survival (DFS) as end points and investigated the associations between the nN categories and these endpoints compared with standard pN categories. OS was calculated from the date of surgery to the time of death. Observations were censored at final follow-up if the patient was alive. DFS was defined as the time from surgery to locoregional relapse or distant metastasis of lung cancer, and in cases without relapse, any deaths due to causes other than lung cancer were censored. Patients were examined at intervals of 3 months for the first 2 years and at intervals of 6 months for the next 3 years or thereafter on an outpatient basis. The follow-up evaluation involved the following procedures: physical examination, chest radiography, CT scan of the chest and abdomen, and blood examination, including that of pertinent tumor markers. Further evaluations, including brain MRI or CT scan, bone scintigraphy, and integrated PET scan, were performed on the first appearance of any symptoms or signs of recurrence. The median follow-up time was 3.5 years.

Statistical Analysis

Survival curves were plotted using the Kaplan-Meier method. Differences in survival among the groups were examined using the log-rank test. A two-category comparison was performed using the Student t test for quantitative data. Multivariate analysis was performed using the Cox proportional hazards model to examine any possible association between the total number of involved LNs and survival, with adjustment for the effects of other potential prognostic factors, including age, sex, histology, tumor factor, and type of surgery performed. All tests were two-sided, and P values of $< .05$ were considered to indicate statistically significant differences. StatView 5.0 software (SAS Institute Inc) was used for statistical analysis.

Ethical Considerations

The approval of the institutional review board of Tokyo Medical University was obtained (project approval no. 965). But, as this was a retrospective study, the need to obtain written informed consent from either the patients or their representatives was waived, in accordance with the American Medical Association.

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Results

Patient Characteristics

The characteristics of patients were as follows: median age: 64.5 years; sex: 417 men (60.5%) and 272 women (39.5%); histopathologic diagnosis: 497 adenocarcinomas (72.1%), 140 squamous cell carcinomas (20.3%), 42 large cell carcinomas (6.1%), and 10 others (1.5%); pathologic stages: 480 stage I (69.7%), 94 stage II (13.6%), and 115 stage III (14.1%); pN factors: 510 pN0 (74.0%), 93 pN1 (13.5%), and 86 pN2 (12.5%); nN factors: 510 nN0 (74.0%), 101 nN1-3 (14.5%), and 78 nN4− (11.4%). The mean number of resected LNs was 18.1 (right side, 18.5; left side, 17.6). The mean number of involved LNs was 4.5 (range, 1-22) in LN-positive cases (Table 1).

Table 1—*Patient Characteristics (N = 689)*

Variable/Category	No. (%)
Age, y	
Mean	64.5
Range	26-87
Sex	
Male	417 (60.5)
Female	272 (39.5)
Histology	
Adenocarcinoma	497 (72.1)
Squamous cell	140 (20.3)
Large cell	42 (6.1)
Other	10 (1.5)
Tumor location	
Right	452 (65.6)
Upper/middle/lower	274/31/147
Left	237 (34.4)
Upper/lower	134/103
Surgical procedure	
Lobectomy	637 (92.4)
Bilobectomy	37 (5.4)
Pneumonectomy	15 (2.2)
p Stage	
I	480 (69.7)
II	94 (13.6)
III	115 (14.1)
pT factor	
pT1	344 (50.0)
pT2	283 (41.1)
pT3	27 (3.9)
pT4	34 (5.0)
pN factor	
pN0	510 (74.0)
pN1	93 (13.5)
pN2	86 (12.5)
nN factor	
nN0	510 (74.0)
nN1-3	101 (14.6)
nN4−	78 (11.4)
Total No. of resected LNs	
Mean (range)	18.1 (10-49)
10-19	450 (65.3)
20-29	192 (37.9)
≥30	47(6.8)
No. involved LNs in positive cases	
Mean (range)	4.5 (1-22)

LN = lymph node, nN = number of lymph nodes; pN = pathologic lymph node; pT = pathologic tumor.

Survival Analysis

First, we classified the patients into three nN categories: nN0, no LN metastasis; nN1-3, metastasis in one to three nodes; and nN4−, metastasis in four or more LNs. We then assessed the OS and DFS in each pN stage classification and nN category (Fig 1). The survival curves showed clear differences in the OS and DFS of each subgroup of both the pN and nN classifications. There was also a significant difference in OS and DFS for each of the nN categories (the 5-year OS rates for nN0, nN1-3, and nN4− were 79.2%, 64.8%, and 39.2%, respectively, $P = .0426$ and $P < .0001$ for nN0 vs nN1-3 and nN1-3 vs nN4−, respectively; the 5-year DFS rates were 83.0%, 71.6%, and 32.9%, respectively, $P = .0024$ and $P = .0002$ for nN0 vs nN1-3 and nN1-3 vs nN4−, respectively).

Second, we performed validation of the nN category in terms of OS for each pathologic tumor (pT) category (Fig 2). Although the differences between each pair of nN categories were not always significant, there was a tendency toward the deterioration of OS from the nN0 to the nN4− subgroup. Similar results were found in terms of DFS (data not shown).

Third, we classified the patients into five categories of combinations of the pN and nN status to compare the prognostic significance of the pN and nN status. The five N categories were as follows: pN0-nN0, pN1-nN1-3, pN1-nN4−, pN2-nN1-3, and pN2-nN4. As shown in Figure 3, patients with pN2-nN1-3 (n = 22) had better prognoses than patients with pN1-nN4− (n = 13). However, there was no statistically significant difference between these two groups due to the small populations. The survival curve of pN2-nN1-3 patients was similar to that of pN1-nN1-3 patients, which is an operable population, while the survival curves of pN1-nN4− patients were similar, but still superior to that of pN2-nN4− patients.

Because of the strong correlation between the pN and nN categories, we performed multivariate analysis for each category to confirm each prognostic impact for OS and DFS.[11] On multivariate analysis, the nN category was an independent prognostic factor for OS and DFS (vs nN4−; the hazard ratios of nN0 and nN1-3 for OS were 0.223 and 0.369, respectively, $P < .0001$ for all categories) as was the case for the pN category (Tables 2, 3). Therefore, both the pN and nN categories were identified as strong prognostic factors for OS and DFS in NSCLC. Moreover, the populations of the pN1-nN1-3 and pN2-nN1-3 categories were small, and the OS of patients within these two groups did not statistically differ. And, there were significant differences between pN1 and pN2

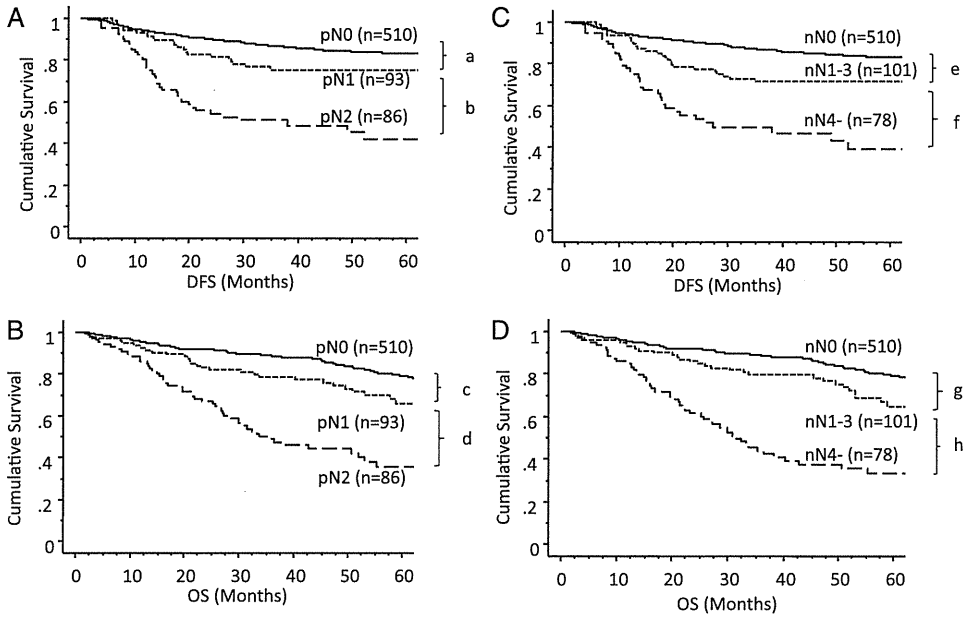

FIGURE 1. DFS and OS according to pN status and nN status. A, DFS curve according to pN status. The 5-year DFS rates for pN0, pN1, and pN2 were 83.0%, 75.3%, and 31.1%, respectively. a, pN0 vs pN1, $P = .0464$; b, pN1 vs pN2, $P < .0001$. B, OS curve according to pN status. The 5-year OS rates for pN0, pN1, and pN2 were 79.2%, 65.9%, and 35.4%, respectively. c, pN0 vs pN1, $P = .0181$; d, pN1 vs pN2, $P < .0001$. C, DFS curve according to nN status. The 5-year DFS rates for nN0, nN1, and nN2-3 were 83.0%, 71.6%, and 39.2%, respectively. e, nN0 vs nN1, $P = .0024$; f, nN1 vs nN2-3, $P = .0002$. D, OS curve according to nN status. The 5-year DFS rates for nN0, nN1, and nN2-3 were 79.2%, 64.8%, and 32.9%, respectively. g, nN0 vs nN1, $P = .0426$; h, nN1 vs nN2-3, $P < .0001$. DFS = disease-free survival; nN = number of lymph nodes; OS = overall survival; pN = pathologic lymph node.

(Figs 1A, 1B) and between nN1-3 and nN4− (Figs 2A, 2B), which mean by a still subcategory exist. We propose a new classification for testing, based on combined pN and nN categories: namely, the new N0 category becomes pN0-nN0, the new N1 category becomes pN1-nN1-3, the new N2a category becomes pN2-nN1-3 + pN1-nN4−, and the new N2b category becomes pN2-nN4. Figure 4 shows the survival curves of the new classifications, which were proportional and well distributed among the curves.

Discussion

The TNM stage classification was developed to provide high specificity for patients with similar prognoses and treatment options. Nodal status is a major determinant of stage and survival of patients with NCSLC after surgery. The seventh TNM staging system included notable changes in the T and M descriptors and in the nodal map, while the N descriptor remained the same as in the previous version and depended solely on the anatomic extent of involved LNs. The anatomically based pN classification has some unsatisfactory aspects. Of these, the heterogeneity of pN1 and pN2 with regard to prognosis is the most notable. Therefore, some subclassifications have been proposed.[1-4,12-17] In addition, differences among surgeons in the labeling of LN stations intraoperatively will occur regardless of the use of a new nodal map. This indicates that it is necessary to refine the currently used pN stage classification and has justified attempts to identify alternative nodal classification methods. In some other solid tumors, such as breast, gastric, and colorectal tumors, the number of metastatic lymph nodes has been included in the TNM staging system. The number of metastatic LNs, when classified into several categories, has been shown to be a prognostic factor for resected NSCLC.[11,15,18] Wei and colleagues[11] evaluated this issue and suggested that the nN category is a better prognostic determinant than the location-based pN stage classification. However, to date, it has remained unknown whether the nN category or the pN stage classification is a better prognostic factor in lung cancer.

It is important to consider how many or to what extent LNs should be harvested for the accurate assessment of nodal status and to maintain the optimal quality of surgery in NSCLC before evaluating the effectiveness of prognostic determinants among the pN and nN categories. The number of resected LNs in early NSCLC has been proven to be a prognostic factor which has influenced survival, similar to that in colorectal, breast, and bladder cancer.[19-24] Some reports have suggested that the optimal number of removed LNs is 11 to 16 in order to accurately assess stage I lung cancer.[24,25] In another study, the removal

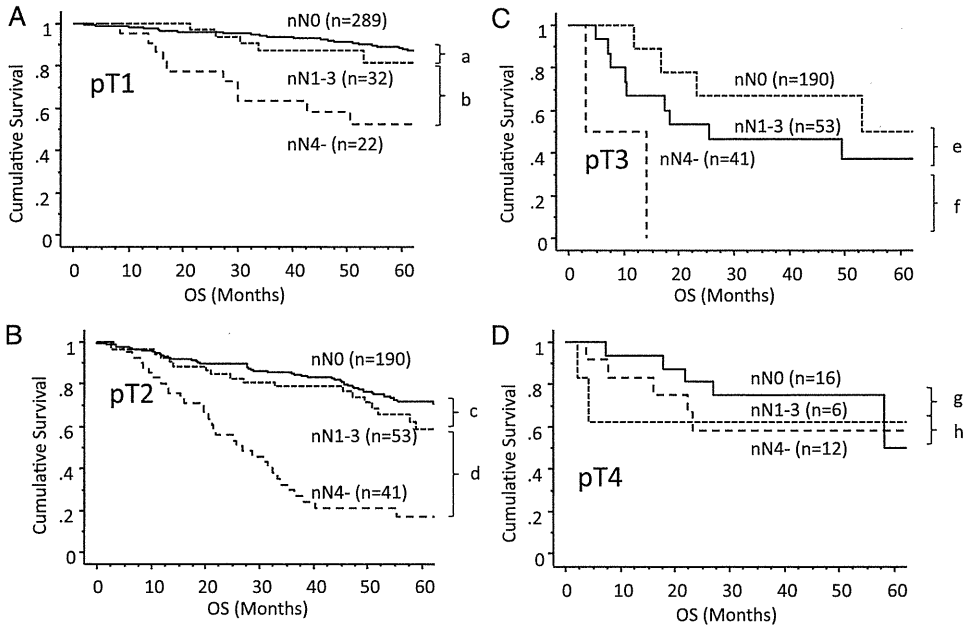

FIGURE 2. OS curves according to nN status across each pT category. A, OS curve according to nN status in pT1 patients. The 5-year OS rates for nN0, nN1, and nN2-3 were 88.5%, 81.4%, and 52.1%, respectively. a, nN0 vs nN1-3, $P = .6757$; b, nN1-3 vs nN4−, $P = .0024$. B, OS curve according to nN status in pT2 patients. The 5-year OS rates for nN0, nN1-3, and nN4− were 71.2%, 58.7%, and 16.7%, respectively. c, nN0 vs nN1-3 $P = .6083$; d, nN1-3 vs nN4− $P < .0001$. C, OS curve according to nN status in pT3 patients. The 5-year OS rates for nN0, nN1-3, and nN4− were 37.3%, 50.0%, and 0%, respectively. e, nN0 vs nN1-3, $P = .2537$; f, nN1-3 vs nN4−, $P = .0046$. D, OS curve according to nN status in pT4 patients. The 5-year OS rates for nN0, nN1-3, and nN4− were 50.0%, 62.5%, and 58.3%, respectively. g, nN0 vs nN1-3 $P = .4305$; h, nN1-3 vs nN4−, $P = .8623$. pT = pathologic tumor. See Figure 1 legend for expansion of other abbreviations.

of 11 LNs was set as a threshold for inclusion.[18] The Staging Manual in Thoracic Oncology of the International Association for the Study of Lung Cancer (IASLC) recommends that at least six LNs/stations be removed or sampled and histologically confirmed to be free of disease in order to define pN0 status.[5] We previously demonstrated that the resection of 10 or more LNs influenced survival while maintaining the quality of surgery.[7] Therefore, in the present analysis, we excluded those for whom < 10 LNs were harvested. In the present series, 617 of 689 cases (89.6%) met this criterion. In the TNM classification for some other tumors, the number of positive LNs has been included in the definition of pN categories.[26] The number of metastatic LNs, when classified into several categories, has been shown to be a prognostic factor for resected NSCLC.[11,15,18] There was a significant difference in OS and DFS among each nN category as well as the pN categories. The OS and DFS survival curves of each nN category are well distributed and proportional (Fig 1). Moreover, as Figure 2 shows, a clear tendency toward the deterioration of OS from nN0 to nN4− in the same pT category was observed when we attempted to validate the results for each pT category. The curves were evenly distributed over pT1, pT2, and pT3. However, the curves were closer in the higher pT stage of pT4, perhaps due to the small population size. Another reason may be that the prognosis of the higher pT category was already poor, regardless of the presence of metastatic LNs. On multivariate analysis, not only the pN status, but also the nN status, was demonstrated to be a major independent prognostic factor for both OS and DFS in the current series, which is consistent with a previous report.[11] These results showed that both pN and nN categories have a powerful discriminative ability concerning the prognosis of NSCLC.

In general, patients with NSCLC with pN1 or pN2 disease are known to exhibit prognostic heterogeneity.[1-4,12-17] The OS and DFS curves of pN1 and pN2 were widely distributed in the current series, indicating that there are some subclassifications required to distinguish the two curves. To evaluate these subgroups and demonstrate which is the most accurate prognostic factor, the anatomic location of involved LNs, or the total number of involved LNs, we classified the patients into five categories combining the pN and nN status as follows: pN0-nN0, pN1-nN1-3, pN1-nN4−, pN2-nN1-3, and pN2-nN4−. Patients with pN2-nN1-3 (n = 22) had better prognoses than patients with pN1-nN4− (n = 13). However, there was no statistically significant difference between the

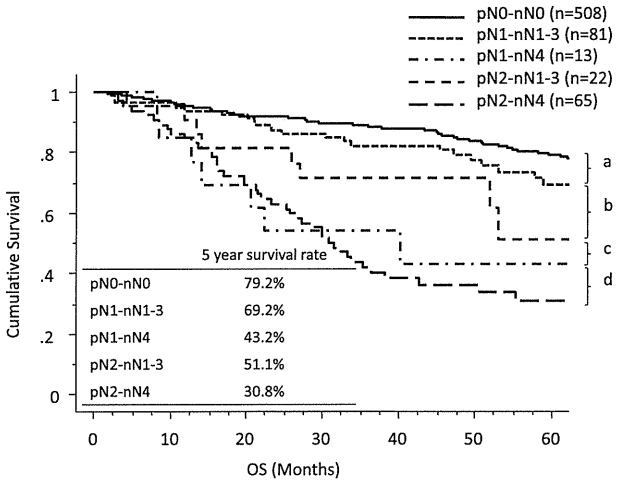

FIGURE 3. OS curves according to combinations between nN status and pN status. Patients with pN2-nN1-3 (n = 22) had better prognoses than patients with pN1-nN4− (n = 13). However, there was no statistically significant difference between the two groups due to the small populations. The survival curve of pN2-nN1-3 was similar to that of pN1-nN1-3, while the survival curves of pN1-nN4− were similar to that of pN2-nN4, a population with worse prognoses. a, pN0-nN0 vs pN1-nN1-3, $P = .2908$; b, pN1-nN1-3 vs pN2-nN1-3, $P = .1102$; c, pN2-nN1-3 vs pN1-nN4, $P = .1292$; d, pN1-nN4− vs pN2-nN4, $P = .7810$. See Figure 1 legend for expansion of abbreviations.

two groups due to the small numbers of patients. This result indicates that the nN category might be used to further subdivide the pN category into two prognostically distinct subgroups. Finally, we propose combining patients with pN2-nN1-3 with those who have a better prognosis into the pN2 category and patients in the pN1-nN4− category with those who have a worse prognosis into a single pN category. Therefore, we reclassified the patients in the current series into four categories as shown in Figure 4. Each OS survival curve of the new classification appears to be proportional with a significant tendency to differ between the new N1 and new N2a and between the new N2a and new N2b categories ($P = .0028$ and $P = .0726$, respectively).

When we subdivided the pN1 and pN2 categories into two subgroups according to the nN category, there was no statistically significant difference between the two groups, but patients with pN2-nN1-3 had better prognoses than patients with pN1-nN4−. This result indicates a possible limitation of the present pN classification for nodal status. The overall disease burden, rather than the anatomic location of LN involvement, has the most relevance in prognosis.[11,27] However, the present pN classification is a major independent prognostic factor in operative NSCLC, as was the nN classification on multivariate analysis in the present series. Therefore, we propose a new nodal classification combination of the pN (anatomic location) and nN (total number) status of LN involvement, which may reflect the survival of operable NSCLC cases more accurately than any single category.

There are some limitations in this study, despite the benefits of the addition of the nN category for predicting survival. First, this was a retrospective and single-institution analysis. Second, it is difficult to accurately estimate the number of LN sites involved both preoperatively and in inoperable patients by CT scan or any other diagnostic imaging methods. The scope of this study involved only the definition of prognosis based on the p stage and not on the c stage, which

Table 2—*Multivariate Analysis of OS and DFS Including pN Classification*

	OS			DFS		
Variable/Category	HR	95% CI	*P* Value	HR	95% CI	*P* Value
Age, y						
<70	...	...	...	...	...	...
≥70	1.018	0.759-1.366	.9053	0.928	0.650-1.325	.6806
Sex						
Men	...	...	...	...	...	...
Women	0.768	0.548-1.076	.1245	1.025	0.709-1.480	.8965
Histopathology						
Nonadenocarcinoma	...	...	...	...	...	...
Adenocarcinoma	0.560	0.409-0.768	.0003[a]	1.063	0.712-1.588	.7653
pT factor						
T4	...	...	<.0001[a]	...	...	.0002[a]
T1	0.378	0.205-0.696	.0018[a]	0.460	0.227-0.935	.0319[a]
T2	0.863	0.482-1.545	.619	1.074	0.547-2.103	.8366
T3	1.192	0.564-2.522	.212	1.180	0.450-3.093	.7362
pN factor						
pN2	...	...	<.0001[a]	...	...	<.0001[a]
pN0	0.274	0.194-0.386	<.0001[a]	0.257	0.172-0.33	<.0001[a]
pN1	0.297	0.188-0.468	<.0001[a]	0.351	0.206-0.599	.0001[a]

DFS = disease-free survival; HR = hazard ratio; OS = overall survival. See Table 1 for expansion of other abbreviations.
[a]Statistically significant.

Table 3—*Multivariate Analysis of OS and DFS Including nN Classification*

Variable/Category	OS			DFS		
	HR	95% CI	*P* Value	HR	95% CI	*P* Value
Age, y						
<70	...	...	...	...	...	...
≥70	1.023	0.764-1.372	1.023	0.919	0.644-1.311	0.6404
Sex						
Men	...	...	...	...	...	...
Women	0.776	0.556-1.082	0.1344	1.016	0.705-1.463	0.9332
Histopathology						
Nonadenocarcinoma	...	...	...	...	...	...
Adenocarcinoma	0.583	0.425-0.799	0.0008[a]	1.157	0.775-1.728	0.4760
pT factor						
T4	...	...	<.0001[a]	...	...	<.0001[a]
T1	0.473	0.256-0.873	0.0167[a]	0.551	0.268-1.131	0.1040
T2	1.120	0.624-2.010	0.7036	1.319	0.665-2.618	0.4284
T3	2.114	0.977-4.573	0.5730	1.654	0.616-4.447	0.3182
nN factor						
nN4−	...	...	<.0001[a]	...	...	<.0001[a]
nN0	0.200	0.141-0.284	<.0001[a]	0.223	0.146-0.339	<.0001[a]
nN1-3	0.197	0.123-0.315	<.0001[a]	0.369	0.219-0.623	0.0002[a]

See Table 1 and 2 legends for expansion of abbreviations.
[a]Statistically significant.

is a limitation of this investigation. Technical improvements in preoperative evaluation to accurately identify all metastatic LN sites are necessary. Although there are various clinical markers to evaluate potential malignant lesions, there is as yet no reliable method or evidence suggesting that PET scans or tumor markers can definitively indicate malignancy. Therefore, this is the reason why we decided to concentrate on the p stage as a step toward establishing preoperative clinical evaluation. Third, the definition of the optimal category in terms of the number of metastatic lymph nodes needs to be further explored; because the definitions, and, therefore, the data, differ according to the institution, it is difficult to determine the optimal category definition. Further multiinstitution studies using identical protocols are needed.

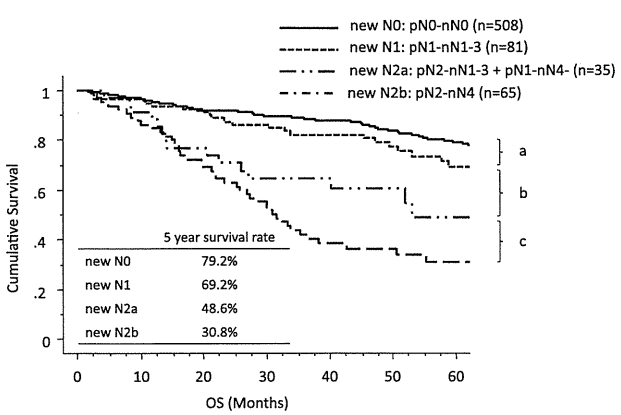

FIGURE 4. OS curves according to combinations of nN status and pN status. We propose a new classification based on combined pN and nN categories: namely, N0 becomes pN0-nN0, N1 becomes pN1-nN1-3, N2a becomes pN2-nN1-3 + pN1-nN4− and N2b becomes pN2-nN4. Each survival curve was proportional and well distributed. a, New N0 vs new N1a, $P = .2908$; b, new N1a vs new N2a, $P = .0028$; c, new N2a vs new N2b, $P = .0726$. See Figure 1 legend for expansion of abbreviations.

CONCLUSION

The current results demonstrate that combined anatomically based pN and numerically based nN stage classification as proposed in this study is a better prognostic determinant in pN1 and pN2 prognostically heterogeneous patients with NSCLC. Further large-scale cohort studies, including global prospective validation analyses and multiinstitution studies, are warranted to demonstrate the validity of this proposal for the next TNM classification.

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Dr Saji: contributed to the design and coordination of the study, statistical analysis, preparing the manuscript, and revising the article for important intellectual content and read and approved the final manuscript.
Dr Tsuboi: contributed to preparing the manuscript and read and approved the final manuscript.
Dr Shimada: contributed to data collection and analysis and read and approved the final manuscript.
Dr Kato: contributed to data collection and analysis and read and approved the final manuscript.
Dr Yoshida: contributed to data collection and analysis and read and approved the final manuscript.
Dr Nomura: contributed to pathologic analysis and read and approved the final manuscript.
Dr Matsubayashi: contributed to pathologic analysis and read and approved the final manuscript.
Dr Nagao: contributed to pathologic analysis and read and approved the final manuscript.

Dr Kakihana: contributed to data collection and analysis and read and approved the final manuscript.
Dr Usuda: contributed to preparing the manuscript and read and approved the final manuscript.
Dr Kajiwara: contributed to preparing the manuscript and read and approved the final manuscript.
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Original Article

Elevated microsatellite alterations at selected tetra-nucleotide (EMAST) in non-small cell lung cancers—a potential determinant of susceptibility to multiple malignancies

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Abstract: The present study evaluated the potential clinicopathologic significance of elevated microsatellite alteration at selected tetra-nucleotide (EMAST) in non-small cell lung cancer (NSCLC). Sixty-five NSCLCs (19 squamous cell carcinomas, 39 adenocarcinomas, one adenosquamous cell carcinoma, and 6 large cell carcinomas) were examined for EMAST in the ten selected tetra-nucleotide markers. Traditional microsatellite instability (MSI) in the five mono- or di-nucleotide markers of the Bethesda panel was also examined, and compared with EMAST. The incidence of EMAST was higher than that of traditional MSI, as 64.6% (42/65) and 12.3% (8/65) tumors respectively exhibited EMAST and traditional MSI in at least one marker. EMAST and traditional MSI appear to occur independently, as no significant association in their incidence was found (Fisher's exact test, $P = 0.146$). Subjects who exhibited EMAST in two or more markers had a significantly higher incidence of history of other malignant neoplasms (42.9% [9/21]), compared to those with less than two markers (16.3% [7/43] (Chi-square test, $P = 0.021$)). Taken together, impairment of molecular machinery for maintaining stable replication of the tetra-nucleotide-repeating regions, which would differ from machinery for mono- or di-nucleotide-repeating regions, may elevate susceptibility to NSCLCs and certain neoplastic diseases. Elucidation of the potential molecular mechanism of EMAST is expected to lead to a discovery of a novel genetic background determining susceptibility to NSCLC and other multiple neoplasms. This is the first report describing a clinicopathologic significance of EMAST in NSCLC.

Keywords: Non-small cell lung cancer, elevated microsatellite alteration at selected tetra-nucleotide, microsatellite instability, chromosomal instability, loss of heterozygosity, multiple malignant neoplasms

Introduction

Lung cancer is one of the most common causes of cancer-related death in the developed world [1, 2]. Even among patients with early stage diseases, a substantial proportion die due to recurrent disease (the 5-year survival rate is 66.0–83.9% in stage IA and 53.0–66.3% in stage IB for non-small cell lung cancer [NSCLC]) [3-5]. Understanding the biological properties and molecular mechanism of NSCLCs is important for the development of a novel therapeutic strategy.

Genetic instability is one of the most essential properties of malignant neoplasm [7-12]. Two different types of genetic instability, microsatellite instability (MSI) and chromosomal instability (CSI), have been well investigated in a variety of malignant neoplasms [8-10, 12-14]. While some types of malignancies preferentially exhibit the MSI phenotype, others preferentially exhibit the CSI phenotype [9-11]. For hereditary non-polyposis colorectal cancer (HNPCC), MSI due to germ line alterations of mismatch repair genes (i.e., *hMLH1*, *hMSH2* and *hMSH6*) is an essential molecular basis of its development

Table 1. Essential information for cases of NSCLCs examined

Sex		
Male		46
Female		19
Age (year)	mean±SD (range)	67±10 (40-82)
Smoking history		
Smoker		52
Non-smoker		13
Brinkmann index	mean±SD (range)	810 ± 943.2 (0-5000)
Medical history of malignant neoplasm		
Present		16
Absent		48
Unknown		1
Family history of malignant neoplasm		
Present		30
Absent		29
Unknown		6
Histological subtype		
SQC		19
ADC		39
ASC		1
LCC		6
pT factor		
pT1		37
pT2		26
pT3		2
Extent of operation		
Lobectomy		43
Segmentectomy		12
Partial resection		10

NSCLC, non-small cell lung cancer; SQC, Squamons cell lung carcinoma; ADC, Adenocarcinoma; ASC, Adenosquamous carcinoma; LCC, Large cell carcinoma.

[10, 11, 14, 15, 32]. On the other hand, for NSCLC, CSI plays an important role in carcinogenesis, as homozygous/heterozygous deletions in certain chromosomal loci frequently occur [9, 10, 13, 17-19]. Participation of MSI in carcinogenesis of the lung has been negatively interpreted based on the results from studies analyzing conventional mono- or di-nucleotide-repeating microsatellite regions (Bethesda panel) [20]. Interestingly, several studies of tetra-nucleotide-repeating microsatellite regions have demonstrated frequent MSI in NSCLC, and have proposed the term, "elevated microsatellite alteration at selected tetra-nucleotide (EMAST)" [21-23]. However, the participation of MSI in NSCLC remains controversial [13, 16-26]. Moreover, these findings imply that the underlying mechanism maintaining replication stability of mono- or di-nucleotide-repeating regions is different from that of tetra-nucleotide-repeating regions [6, 23]. The potential alterations in novel molecules other than known mismatch repair factors (i.e., *hMLH1*, *hMSH2* and *hMSH6*) could be involved in the occurrence of EMAST and promote carcinogenesis of NSCLC. Similar to traditional MSI in mono- or di-nucleotide-repeating regions, EMAST in the unsettled tetra-nucleotide-repeating regions has been evaluated among individual studies [16, 21-23, 26-29]. A recent study proposed ten candidate regions as universal markers for assessment of EMAST [6]. To our knowledge, EMAST in NSCLC in these ten markers has yet to be investigated.

The present study examined 65 NSCLCs for EMAST in the ten markers and analyzed the potential associations between EMAST and a series of clinicopathologic parameters.

Materials and methods

Tumor samples

Sixty-five NSCLCs (19 squamous cell carcinomas [SQCs], 39 adenocarcinomas [ADCs], one adenosquamous cell carcinomas, 6 large cell carcinomas [LCCs]) without lymph node metastasis and preoperative chemotherapy or radiation therapy were investigated. Characteristics of patients are summarized in **Table 1**. All tumors were re-evaluated and diagnosed by

Table 2. Information for microsatellite markers and PCR-primers used

Marker	Chromosomal location	Primer sequence (5'-3')	Annealing temperature (°C)	Size of PCR-product (bp)
Selected tetra-nucleotide makers				
D8S321	Chromosome 8	S:GATGAAAGAATGATAGATTACAG	58	Approx. 245
		A:ATCTTCTCATGCCATATCTGC		
D20S82	20p12.3	S:GCCTTGATCACACCACTACA	61	246-270
		A:GTGGTCACTAAAGTTTCTGCT		
UT5037	Chromosome 8	S:TTCCTGTGAACCATTAGGTCA	60	Approx. 145
		A:GGGAGACAGAGCAAGACTC		
D8S348	8q24.13-8q24.3	S:ACCGACAGACTCTTGCCTCCAAA	58	Approx. 408
		A:TCACTCAGCTCCCATAACTTGGCAT		
D2S443	2p13.2-2p13.1	S:GAGAGGGCAAGACTTGGAAG	58	Approx. 251
		A:ATGGAAGAGCGTTCTAAAACA		
D21S1436	21q21.1	S:AGGAAAGAGAAAGAAAGGAAGG	58	Approx. 178
		A:TATATGATGAAAGTATATTGGGGG		
D9S747	9q32	S:GCCATTATTGACTCTGGAAAAGAC	56	182-202
		A:CAGGCTCTCAAAATATGAACAAAAT		
D9S303	9q21.32	S:CAACAAAGCAAGATCCCTTC	55	Approx. 163
		A:TAGGTACTTGGAAACTCTTGGC		
D9S304	9q21	S:GTGCACCTCTACACCCAGAC	60	Approx. 165
		A:TGTGCCCACACACATCTATC		
MYCL1	1p34.1	S:TGGCGAGACTCCATCAAAG	53	140-209
		A:CTTTTTAAGCTGCAACAATTTC		
Bethesda Panel markers				
D5S346	5q21-22	S:ACTCACTCTAGTGATAAATCGGG	55	96-122
		A:AGCAGATAAGACAGTATTACTAGTT		
BAT25	4q12	S:TCGCCTCCAAGAATGTAAGT	58	Approx. 125
		A:TCTGCATTTTAACTATGGCTC		
BAT26	2p16	S:TGACTACTTTTGACTTCAGCC	58	Approx. 125
		A:AACCATTCAACATTTTTAACCC		
D2S123	2p16	S:AAACAGGATGCCTGCCTTTA	60	197-227
		A:GGACTTTCCACCTATGGGAC		
D17S250	17q11.2-17q12	S:GGAAGAATCAAATAGACAAT	52	151-169
		A:GCTGGCCATATATATATTTAAACC		
p53 loss of heterozygosity marker				
TP53alu	17p.13.1	S:TCGAGGAGGTTGCAGTAAGCGGA	55	Approx. 150
		A:AACAGCTCCTTTAATGGCAG		

Approx., approxymetly.

board-certified pathologists according to UICC classification (7th edition) of tumors [30] and World Health Organization Classification of Tumours of the lung. The study plan was approved by the ethics committee of Yokohama City University Graduate School of Medicine. Inclusive informed consent for research use was obtained from all patients providing materials.

Laser-capture micro-dissection of neoplastic cells and DNA extraction

Neoplastic cells were isolated from paraffin-embedded tissue sections using a laser capture micro-dissection system (PALM MCB, Bernried, Germany). Paired reference DNA was extracted from non-tumoral lung tissue or the regional lymph nodes using the High Pure PCR

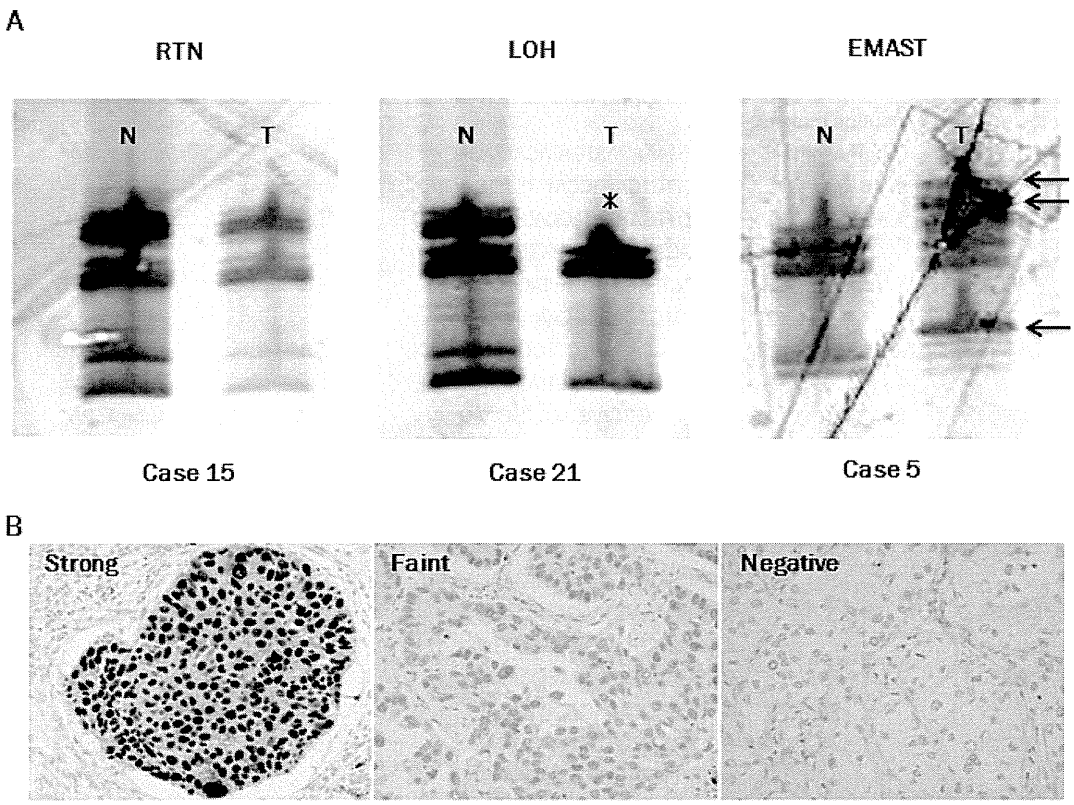

Figure 1. A. Representative results of alterations at a selected tetra-nucleotide repeating region (D9S303). PCR products were separated by polyacrylamide gel electrophoresis and visualized by silver stain. Case 5 (right panel) exhibits elevated microsatellite alternations at selected tetra-nucleotide (EMAST), as shifted bands (arrows) appear in the tumor sample (T) compared to non-tumor sample (N). Case 21 (center panel) exhibits loss of heterozygosity (LOH), as the slower migrating band (asterisk) of the two bands in the non-tumor sample (N) disappeared in the tumor sample (T). Case 15 (left panel) exhibits no alteration (RTN: retain) and serves as a reference. B. Representative results of immunohistochemistry for p53. Examples of strong (left panel), faint (center panel), and negative (right panel) expression are shown. Magnification: x400.

Template Preparation Kit (Roche GmbH, Mannheim, Germany) according to the manufacturer's instructions.

Analysis of alteration in selected microsatellite markers

The ten selected tetra-nucleotide-repeating markers proposed by the previous study (D8S321, D20S82, UT5037, D8S348, D2S443, D21S1436, D9S747, D9S303, D9S304, and MYCL1) [6] and the Bethesda panel (D5S346, BAT25, BAT26, D2S123, and D17S250) [15] were examined. Primers used and appropriate annealing temperatures are listed in **Table 2**. PCR products were separated by polyacrylamide gel electrophoresis and visualized by silver stain [8]. MSI was judged based on a shift in extra bands in the tumor sample, which was not found in non-tumoral samples (**Figure 1A**). Among the cases with different repeat lengths in the microsatellite regions (informative cases), loss or unequivocally lower signal in either of the two bands in the tumor sample was judged as loss of heterozygosity (LOH) (**Figure 1A**).

Analysis of LOH in p53 gene locus

The deletion of the p53 gene locus on chromosome 17p13.1 was also examined using a microsatellite marker (TP53alu) [33]. Primers used and appropriate annealing temperatures are listed in **Table 2**. LOH was judged in the same manner as described above.

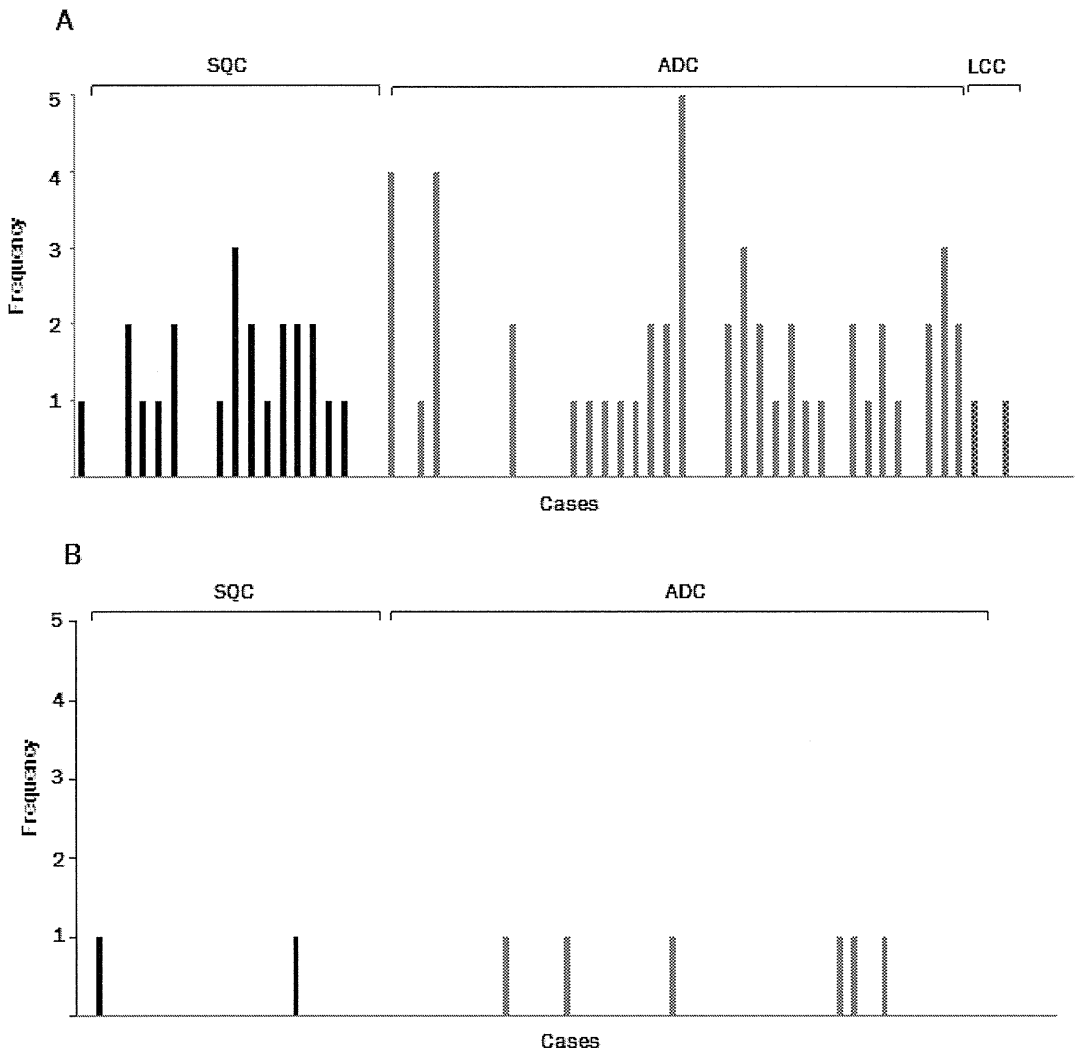

Figure 2. A. Frequency of EMAST (the number of regions where instability occurred among the ten selected tetranucleotide-repeating regions) in each case. B. Frequency of traditional microsatellite instability (MSI) (number of regions where instability occurred among the five regions of the Bethesda panel) in each case. SQC, squamous cell carcinoma; ADC, adenocarcinoma; LCC, large cell carcinoma.

Histopathology

The largest tumor sections were cut from formalin-fixed, paraffin-embedded tissue blocks. The sections were deparaffinized, rehydrated, and incubated with 3% hydrogen peroxide, followed by blocking of endogenous peroxidase activity and non-immunospecific protein binding with 5% goat serum. The sections were boiled in citrate buffer (0.01 M, pH 6.0) for 15 min to retrieve masked epitopes and then incubated with a primary antibody against p53 (DO7, Dako, Ely, UK), Ki-67 (MIB1, Dako), factor VIII-related antigen (F8/86, Dako), and D2-40 (D2-40, Becton Dickinson, San Joes, CA). Immunoreactivity was visualized using an Envision detection system (Dako), and the nuclei were counterstained with hematoxylin. Intensity of immunohistochemical signals of p53 protein was classified into negative (score 0), faint (score 1), and strong (score 2). Strong intensity was defined as an obviously intense signal in the nuclei (**Figure 1B**). Faint intensity was defined as unequivocally less signal, but not negative, in comparison to strong intensity (**Figure 1B**). The p53 expression level was calculated as a percentile of the averaged intensity level, as described elsewhere [34]. Values

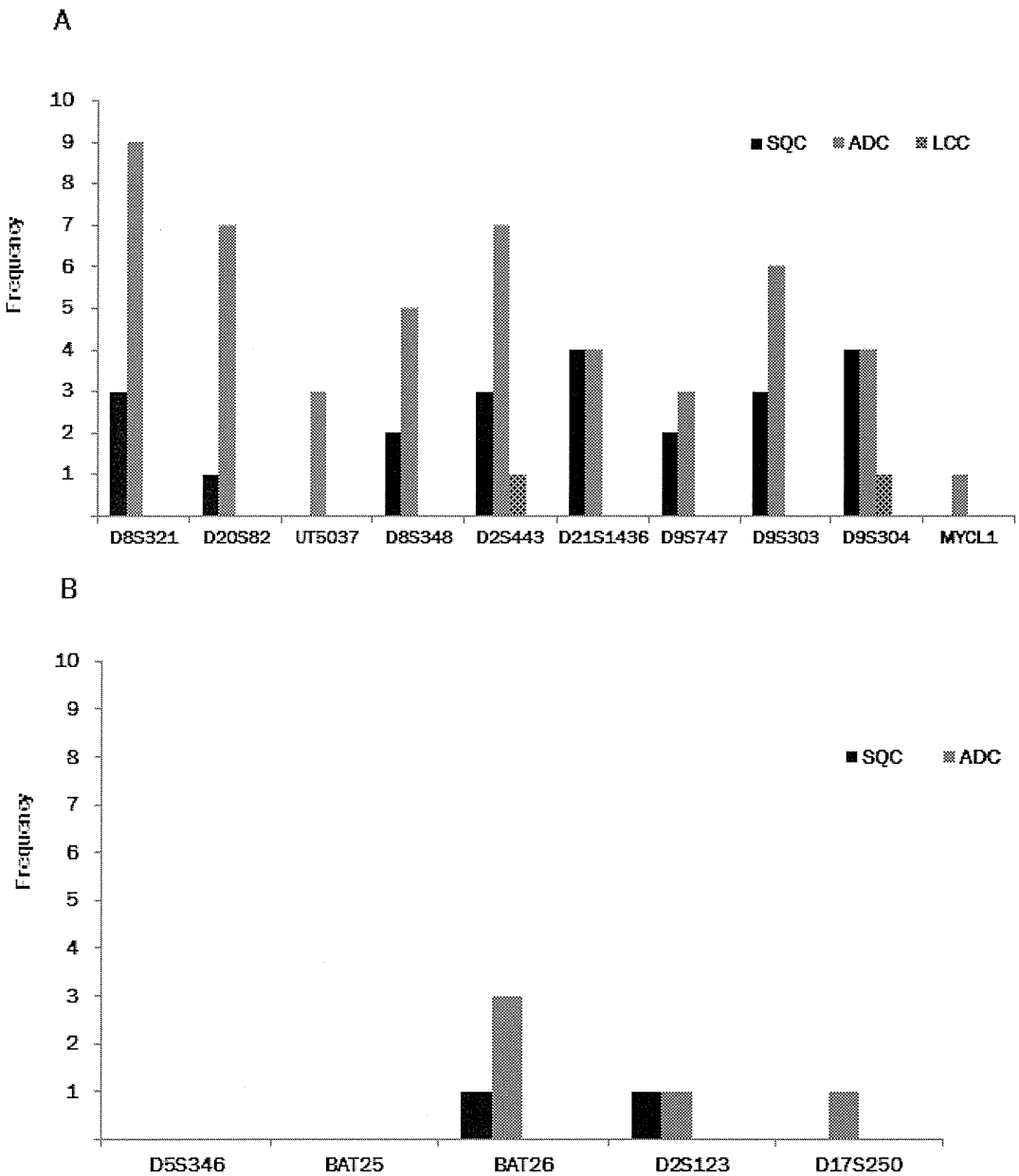

Figure 3. A. Frequency of EMAST (number of tumors exhibiting instability in tumors that could be examined) in each region. B. Frequency of MSI (number of tumors exhibiting instability in tumors that could be examined) in each region SQC, squamous cell carcinoma; ADC, adenocarcinoma; LCC, large cell carcinoma.

of less than or equal to the median value (1.13%) were classified as low expressers and values of more than 1.13% were classified as high expressers. Labeling index of MIB1 was calculated as the proportion of cells with positive nuclei by counting 500–1000 cancer cells. The Ki-67 labeling indices of ≤10% and >10% were classified as low and high levels, according to the results of our previous study [35]. Vascular and lymphatic invasion was evaluated by elastica van Gieson stain, D2-40 Stain and factor VIII-related antigen stain.

Statistical analysis

The possible associations between EMAST/LOH status and various clinicopathologic

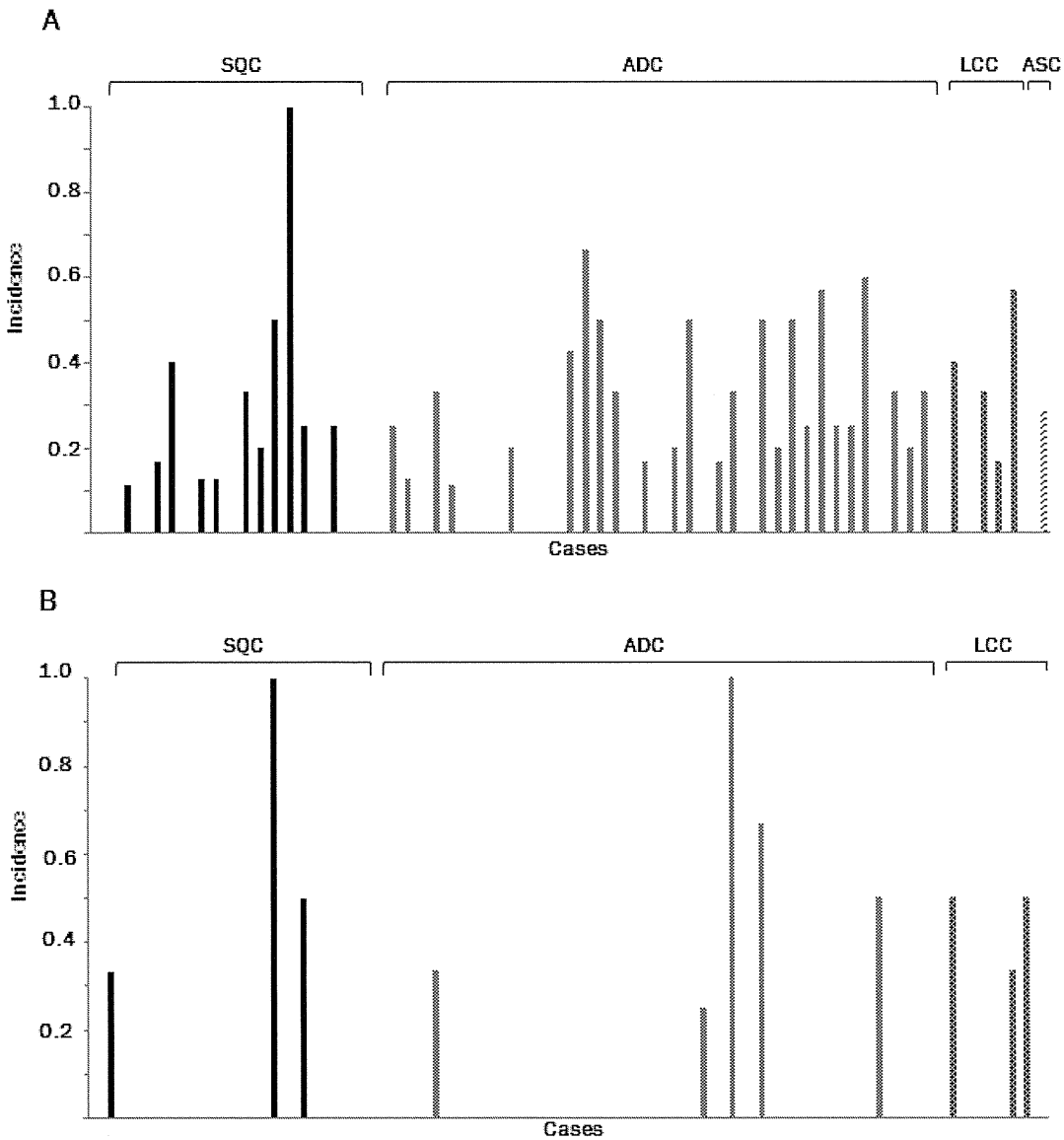

Figure 4. A. Incidence of loss of heterozygosity (LOH) in the selected tetra-nucleotide-repeating regions informative in each case. B. Incidence of LOH in the regions informative of the Bethesda panel in each case. SQC, squamous cell carcinoma; ADC, adenocarcinoma; LCC, large cell carcinoma.

parameters were analyzed with Fisher's exact test or Chi-square test. The post-operative disease-free span was defined as the period from the date of surgery to the date when the recurrence of disease was diagnosed. An observation was censored at the last follow-up if the patient was alive or had died of a cause other than lung cancer. The differences in overall survival rate and in disease-free survival rate were analyzed using log-rank test. *P* values less than 0.05 were considered significant. All statistical analyses were performed using SPSS software (SPSS for Windows Version 11.0 J; SPSS; Chicago, IL).

Results

Instability in selected tetra-nucleotide-repeats and Bethesda panel

Among the 65 tumors examined, 56 could be examined for alteration in all the microsatellite regions. The remaining 9 could not be exam-

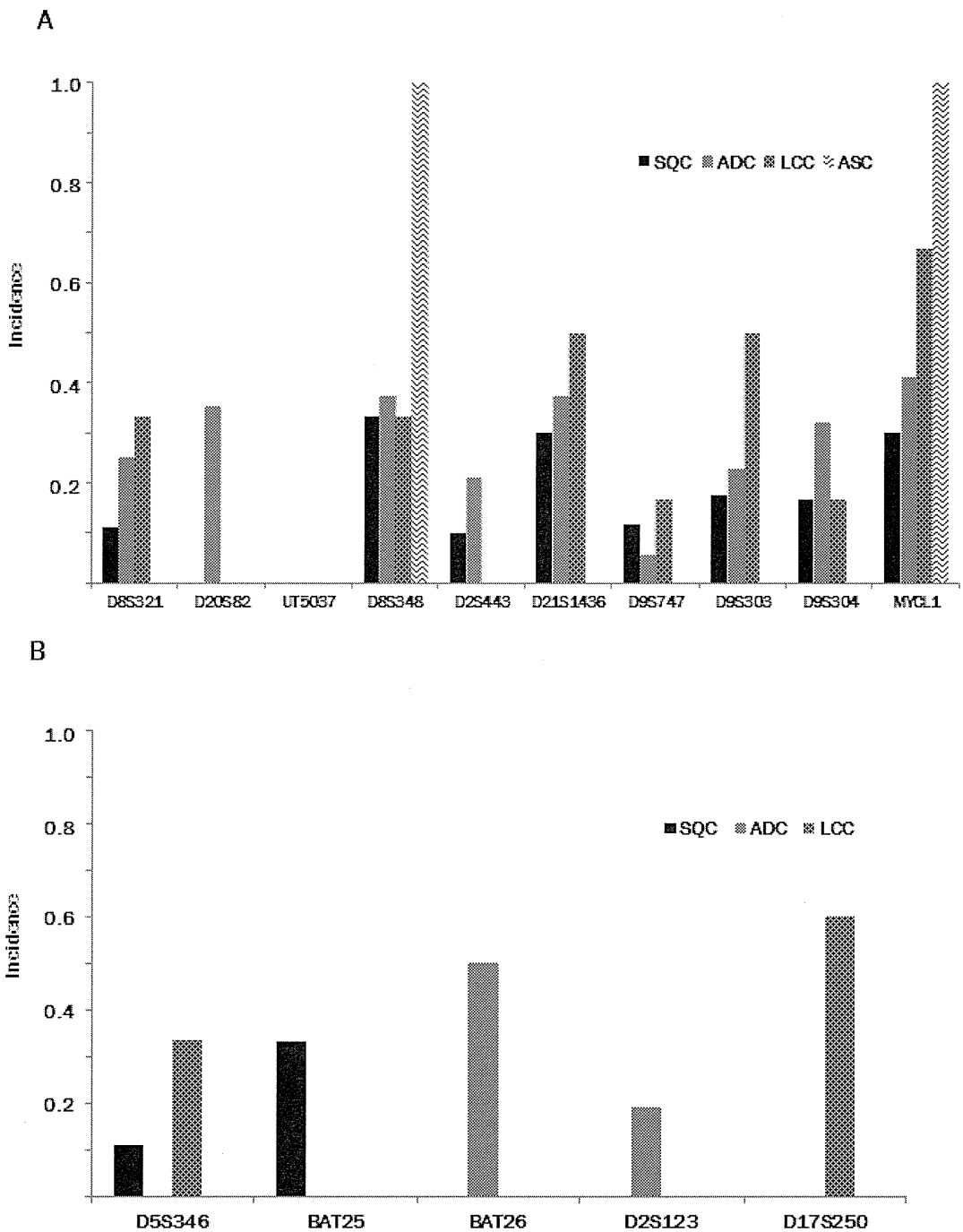

Figure 5. A. Incidence of tumors exhibiting LOH in the selected tetra-nucleotide-repeating regions informative in each region. B. Incidence of tumors exhibiting LOH in the regions informative of the Bethesda panel in each region. SQC, squamous cell carcinoma; ADC, adenocarcinoma; LCC, large cell carcinoma.

ined, 7 in one marker (D2S443, D8S348, D9S303, or D21S1436) and 2 in two markers (D2S443 and D9S304, D8S348 and D9S304).

EMAST in either of the ten tetra-nucleotide-repeating regions was found in 64.6% (42/65) of tumors (**Figure 2A**): 26 of 39 (66.7%) ADCs,