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

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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

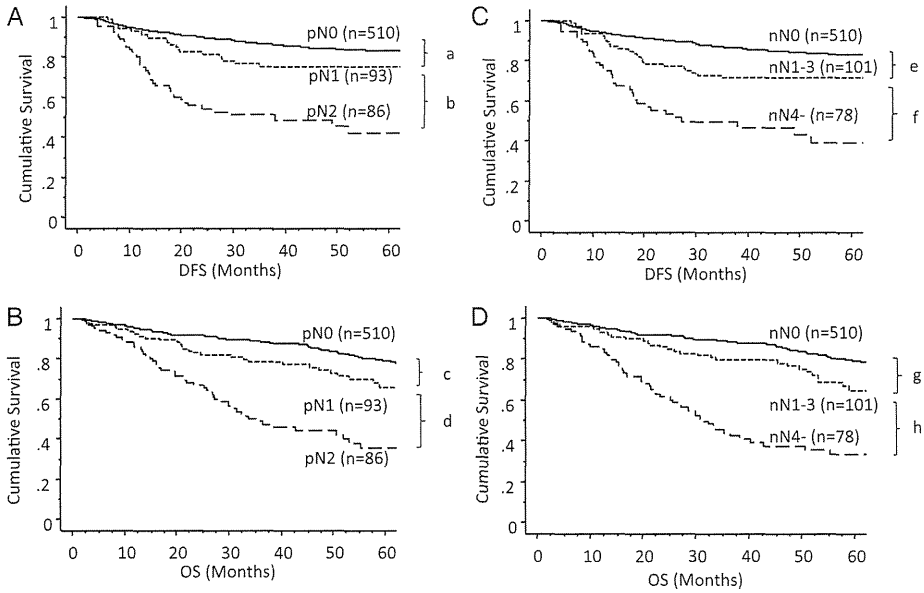

FIGURE 1. DFS and OS according to pN status and nN status. A, DFS curve according to pN status. The 5-year DFS rates for pN0, pN1, and pN2 were 83.0%, 75.3%, and 31.1%, respectively. a, pN0 vs pN1, $P = .0464$; b, pN1 vs pN2, $P < .0001$. B, OS curve according to pN status. The 5-year OS rates for pN0, 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

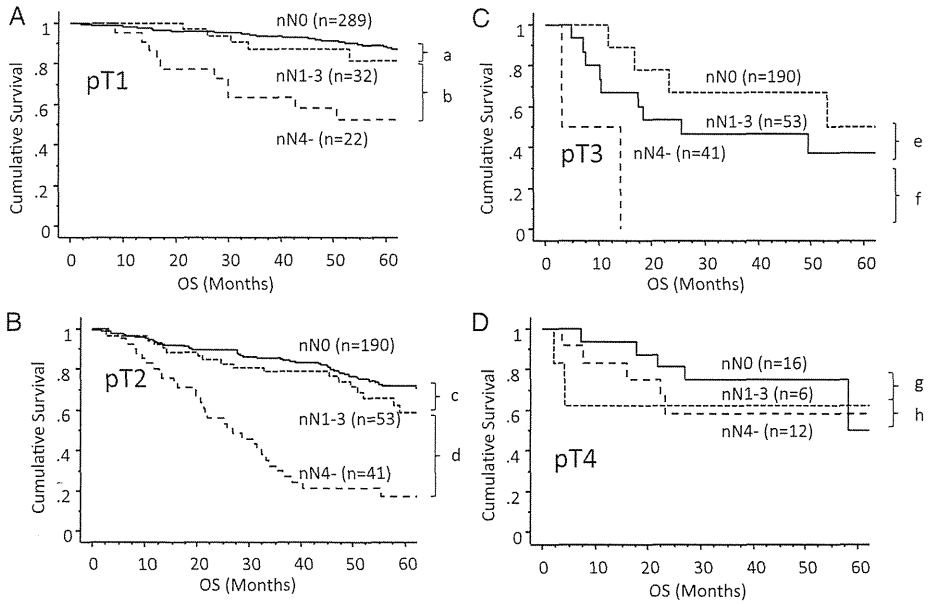

FIGURE 2. OS curves according to nN status across each pT category. A, OS curve according to nN status in pT1 patients. The 5-year OS rates for nN0, nN1, and nN2-3 were 88.5%, 81.4%, and 52.1%, respectively. a, nN0 vs nN1-3, $P = .6757$; b, nN1-3 vs nN4−, $P = .0024$. B, OS curve according to nN status 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

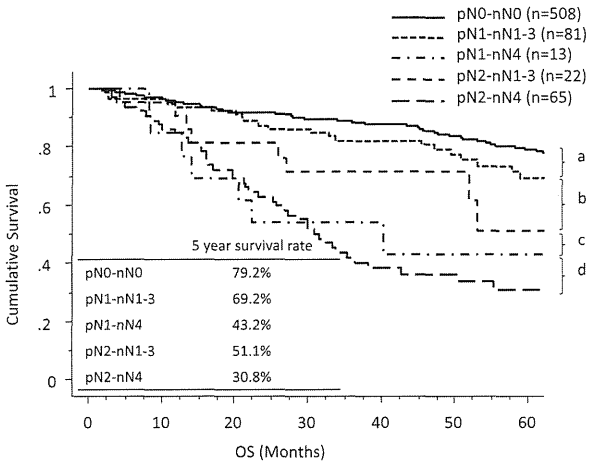

FIGURE 3. OS curves according to combinations between nN status and pN status. Patients with pN2-nN1-3 (n = 22) had better prognoses than patients with pN1-nN4− (n = 13). However, there was no statistically significant difference between the two groups due to the small populations. The survival curve 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.

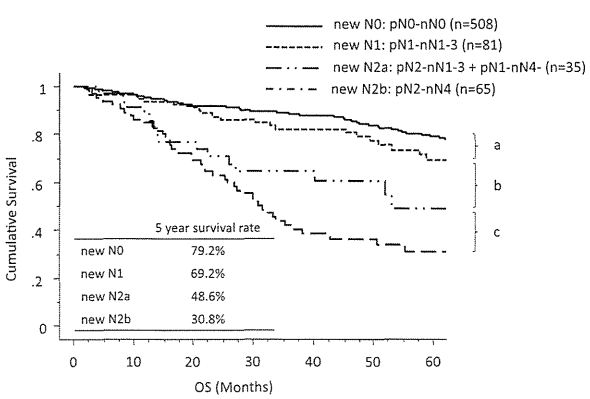

FIGURE 4. OS curves according to combinations of nN status and pN status. We propose a new classification based on combined pN and nN categories: namely, N0 becomes pN0-nN0, N1 becomes pN1-nN1-3, N2a becomes pN2-nN1-3 + pN1-nN4− and N2b becomes pN2-nN4. Each survival curve was proportional and well distributed. 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.
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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

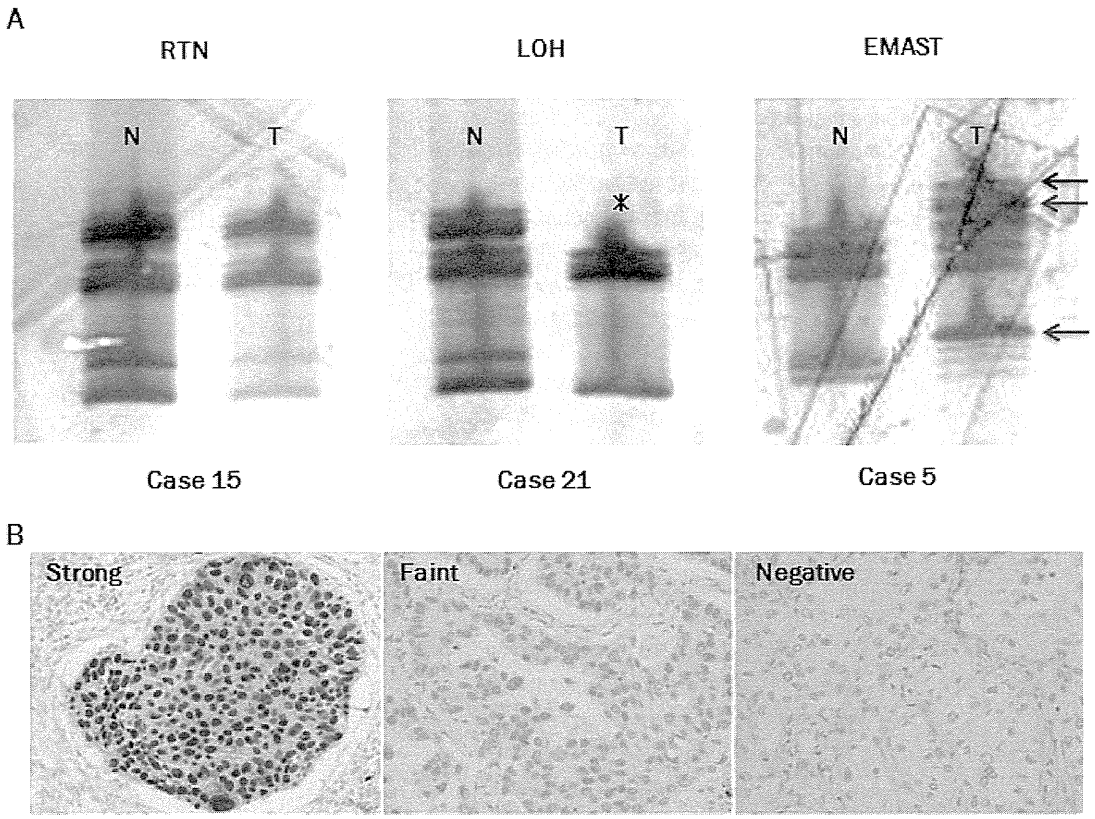

Figure 1. A. Representative results of alterations at a selected tetra-nucleotide repeating region (D9S303). PCR products were separated by polyacrylamide gel electrophoresis and visualized by silver stain. Case 5 (right panel) exhibits elevated microsatellite alternations at selected tetra-nucleotide (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.

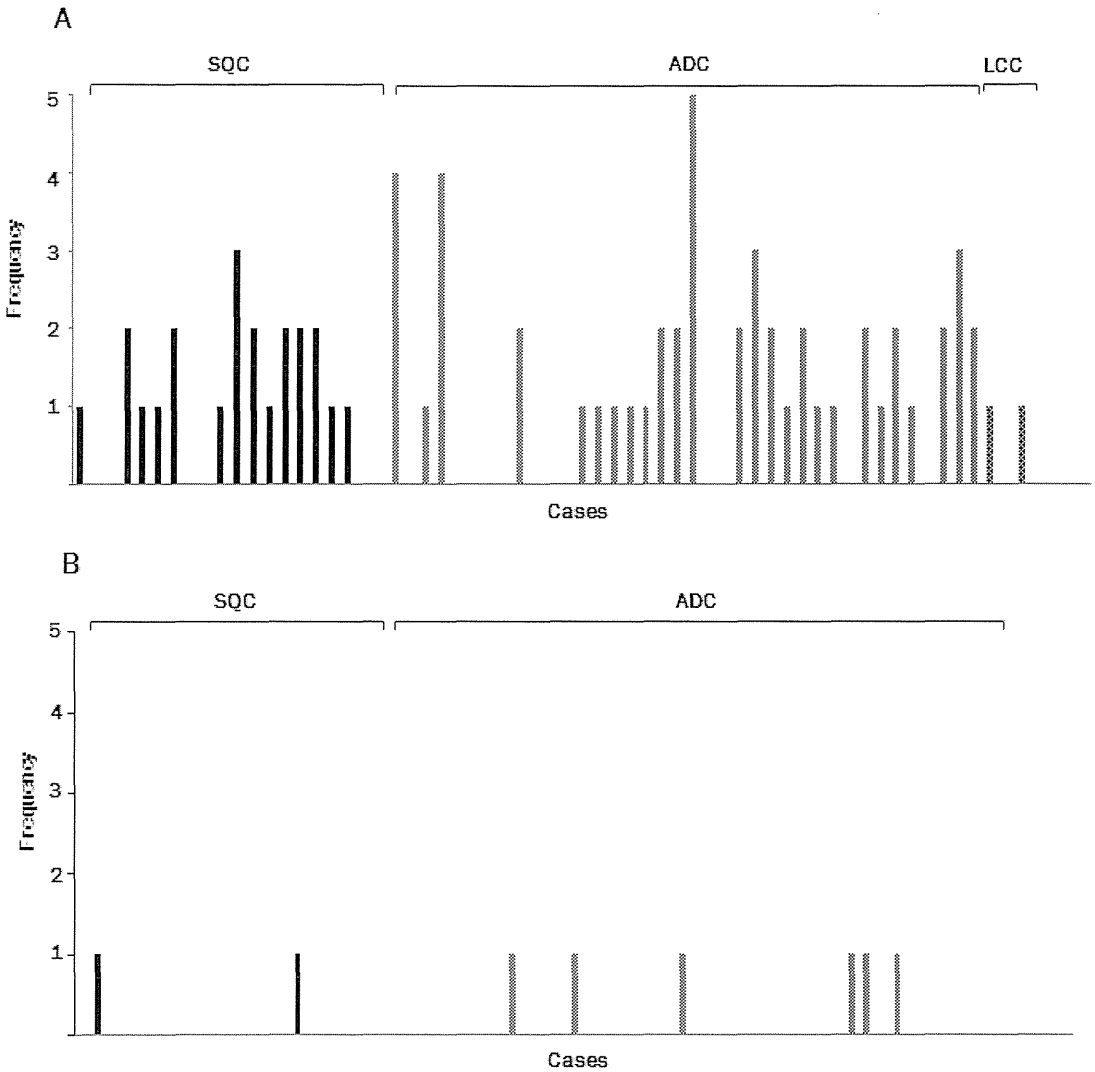

Figure 2. A. Frequency of EMAST (the number of regions where instability occurred among the ten selected tetranucleotide-repeating regions) in each case. B. Frequency of traditional microsatellite instability (MSI) (number of regions where instability occurred among the five regions of the Bethesda panel) 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

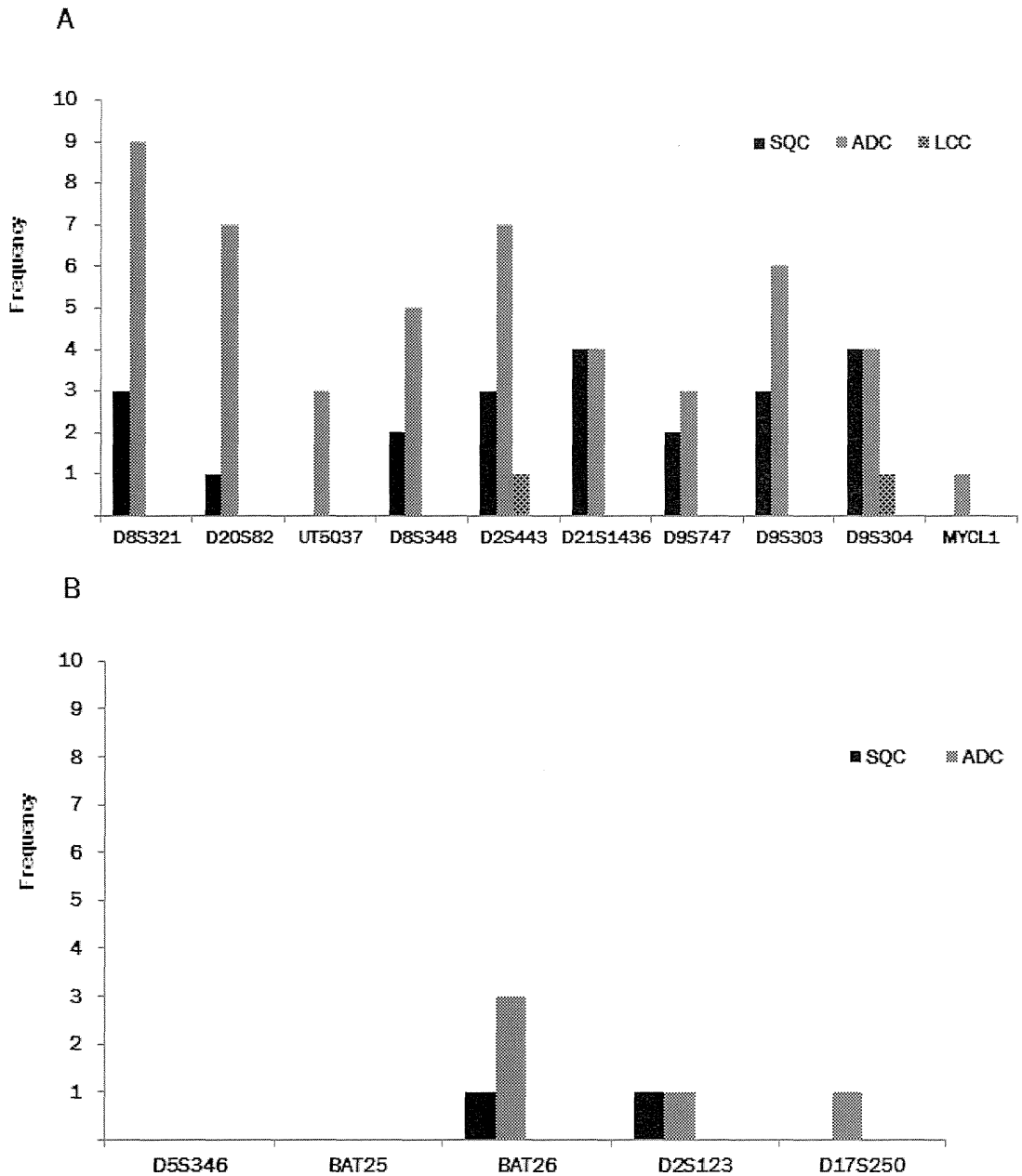

Figure 3. A. Frequency of EMAST (number of tumors exhibiting instability in tumors that could be examined) in each region. B. Frequency of MSI (number of tumors exhibiting instability in tumors that could be examined) in each region SQC, squamous cell carcinoma; ADC, adenocarcinoma; LCC, large cell carcinoma.

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

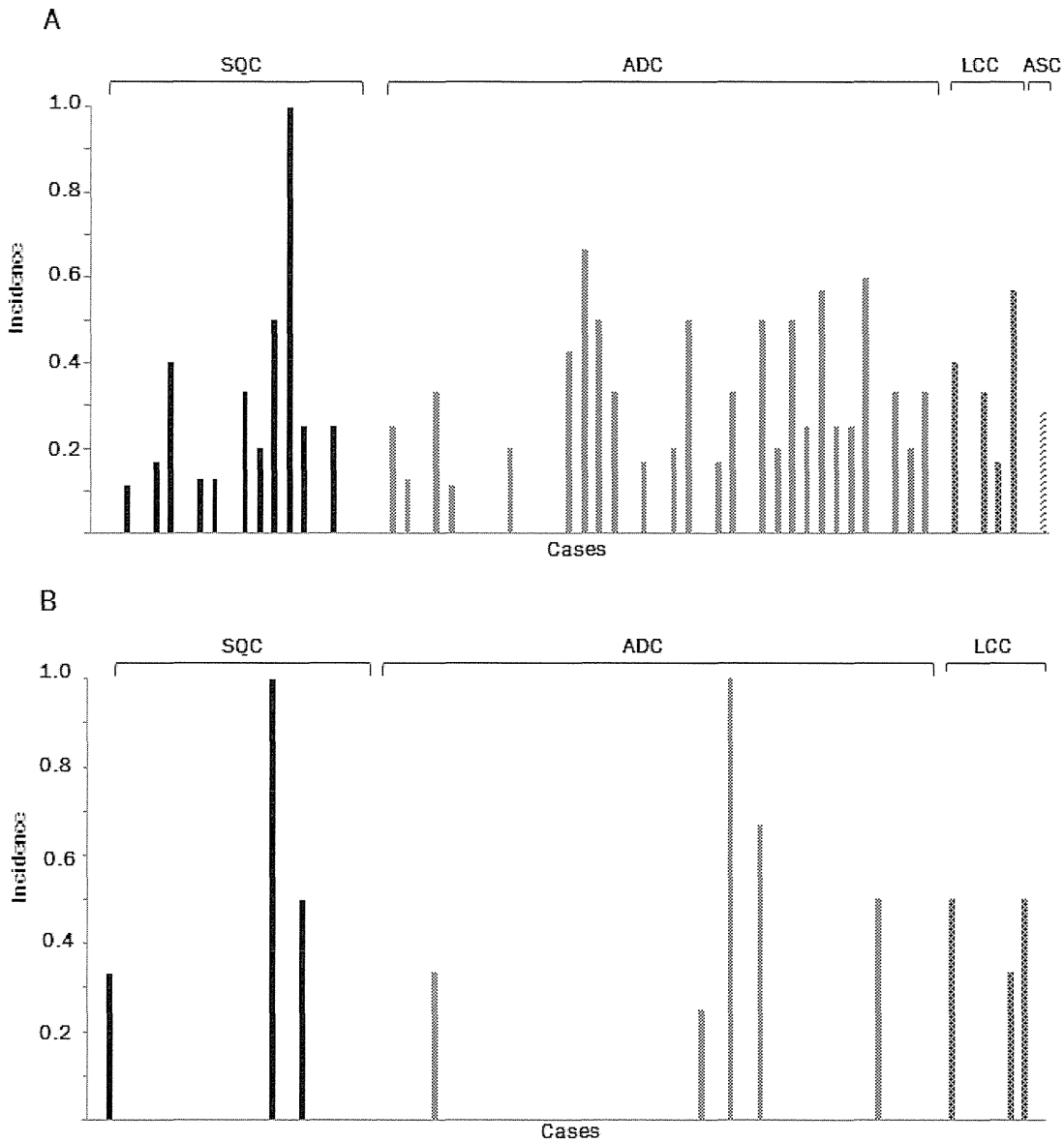

Figure 4. A. Incidence of loss of heterozygosity (LOH) in the selected tetra-nucleotide-repeating regions informative in each case. B. Incidence of LOH in the regions informative of the Bethesda panel in each case. SQC, squamous cell carcinoma; ADC, adenocarcinoma; LCC, large cell carcinoma.

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-

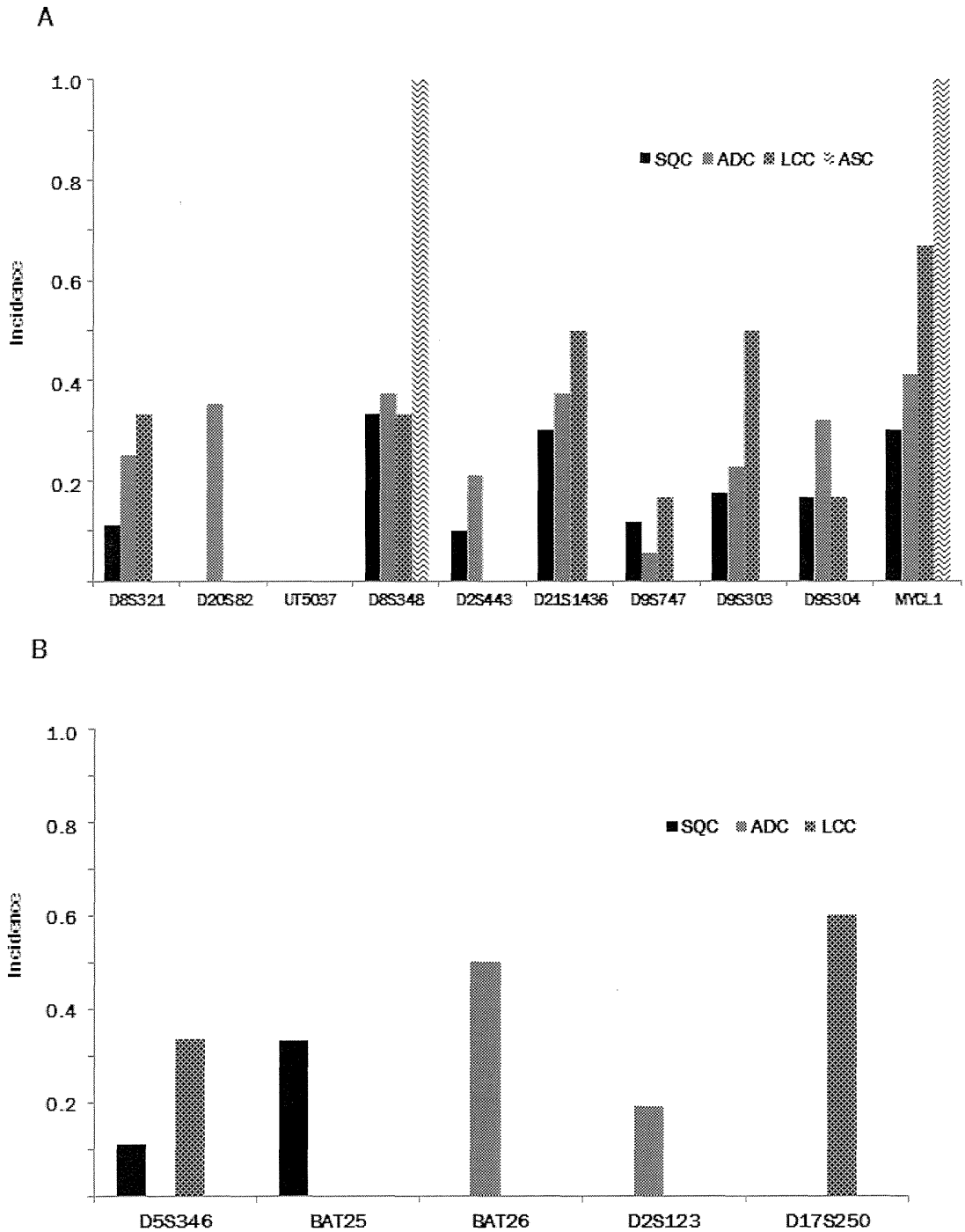

Figure 5. A. Incidence of tumors exhibiting LOH in the selected tetra-nucleotide-repeating regions informative in each region. B. Incidence of tumors exhibiting LOH in the regions informative of the Bethesda panel in each region. SQC, squamous cell carcinoma; ADC, adenocarcinoma; LCC, large cell carcinoma.

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,

Table 3. Associaton between EMAST and clicopathologic subjects

			EMAST-low	EMAST-high	*P* value
Sex					0.743
Male		(46)	31	15	
Female		(19)	12	7	
Age (year)	mean±SD		67.4 ± 9.6	66.4 ± 11.1	0.713
Smoking history					0.233
Smoker		(52)	36	16	
Non-smoker		(13)	7	6	
Brinkmann index	mean±SD		928.7 ± 891.3	994.5 ± 1057.8	0.793*
Medical history of malignant neoplasm		(64)			0.021
Present		(16)	7	9	
Absent		(48)	36	12	
Family history of malignant neoplasm		(59)			0.926
Present		(30)	20	10	
Absent		(29)	19	10	
Histological subtype					0.905
SQC		(19)	12	7	
ADC		(39)	24	15	
ASC		(1)	1	0	
LCC		(6)	6	0	
pT factor					0.621
pT1		(37)	25	12	
pT2		(26)	16	10	
pT3		(2)	2	0	
Vascular invasion		(65)			0.161
Present		(19)	15	4	
Absent		(46)	28	18	
Lymphatic invasion		(65)			0.349
Present		(14)	11	3	
Absent		(51)	32	19	
Proliferaitve activity#		(61)			0.747
Low (Ki67 index ≤ 10%)		(21)	14	7	
High (Ki67 index > 10%)		(40)	25	15	
Ki-67 index	mean±SD		23.9% ± 19.8	27.2% ± 22.8	0.557*
p53 immunohistochemical expression		(65)			0.663
Low (p53 socre ≤ 1.13)		(33)	21	12	
High (p53 score > 1.13)		(32)	22	10	
p53 score	mean±SD		20.5 ± 31.4	22.7 ± 28.9	0.784*
p53 LOH		(35)			0.213
Present		(6)	3	3	
Absent		(29)	22	7	

*Statistical association was analyzed by Fisher's exact test or chi-square test, and difference was analyzed by Student's t test. EMAST, elevated microsatellite alteration at selected tetra-nucleotide; SQC, squamous cell carcinoma; ADC, adenocarcinoma; ASC, Adenosquamous carcinoma; LCC, large cell carcinoma. #Four cases were not available for immonohistochemical examination due to too small tumors.

14 of 19 (73.7%) SQCs, and 2 of 6 (33.3%) LCCs. Among the ten regions, EMAST tended to preferentially occur at D8S321 (12/65, 18.5%), D2S443 (11/65, 16.9%), D9S303 (9/65, 13.8%), D9S304 (9/65, 13.8%), D20S82 (8/65, 12.3%), D21S1436 (8/65, 12.3%), and

Table 4. Associaton between LOH at tetra-nucleotide markers and clicopathologic subjects

			LOH-low	LOH-high	*P* value
Sex					0.928
Male		(46)	32	14	
Female		(19)	13	6	
Age (year)	mean±SD		67.9 ± 10.5	65.0 ± 8.8	0.276*
Smoking history					0.622
Smoker		(52)	36	16	
Non-smoker		(13)	9	4	
Brinkmann index	mean±SD		1000.1 ± 931.6	848.2 ± 981.8	0.548*
Medical history of malignant neoplasm		(64)			0.533
Present		(16)	12	4	
Absent		(48)	32	16	
Family history of malignant neoplasm		(59)			0.275
Present		(30)	20	10	
Absent		(29)	23	6	
Histological subtype					0.880
SQC		(19)	14	5	
ADC		(39)	28	11	
ASC		(1)	0	1	
LCC		(6)	3	3	
pT factor					0.808
pT1		(37)	26	11	
pT2		(26)	19	7	
pT3		(2)	0	2	
Vascular invasion		(65)			0.936
Present		(19)	13	6	
Absent		(46)	31	15	
Lymphatic invasion		(65)			0.260
Present		(14)	8	6	
Absent		(51)	36	15	
Proliferaitve activity#		(61)			0.907
Low (Ki67 index ≤ 10%)		(21)	15	6	
High (Ki67 index > 10%)		(40)	28	12	
Ki-67 index	mean±SD		26.0% ± 20.8	23.2% ± 21.2	0.627*
p53 immunohistochemical expression		(65)			0.857
Low (p53 socre ≤ 1.13)		(33)	22	11	
High (p53 score > 1.13)		(32)	22	10	
p53 score	mean±SD		23.9 ± 31.2	15.3 ± 28.1	0.295*
p53 LOH		(35)			0.329
Present		(6)	3	3	
Absent		(29)	20	9	

*Statistical association was analyzed by Fisher's exact test or chi-square test, and difference was analyzed by Student's t test. LOH, loss of heterozygosity; SQC, squamous cell carcinoma; ADC, adenocarcinoma; ASC, Adenosquamous carcinoma; LCC, large cell carcinoma; #Four cases were not available for immonohistochemical examination due to too small tumors.

D8S348 (7/65, 10.8%) than at UT5037 (3/65, 4.6%), D9S747 (5/65, 7.7%), and MYCL1 (1/65, 1.5%) (**Figure 3A**).

We also found that 12.3% (8/65) of tumors (6 of 39 [15.4%] ADCs, 2 of 19 [10.5%] SQCs) exhibited traditional MSI in either of the mono-

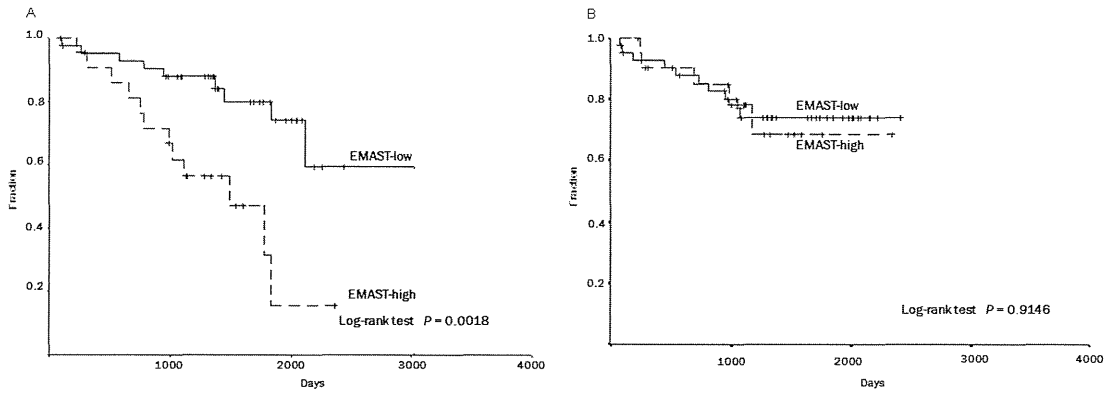

Figure 6. Association between EMAST and 5-year overall survival (A) and 5-year disease-free survival (B). Kaplan-Meier survival curves are shown. EMAST-high: tumors exhibiting EMAST in two or more of the tetra-nucleotide-repeating regions. EMAST-low: tumors exhibiting the EMAST in one or none of the regions. Five-year overall survival rates were 79.9% and 31.4% in EMAST-low and –high groups, respectively (Log-rank test, P = 0.0018) (A). Five-year disease-free survival rates were 73.9% and 68.5% in EMAST-low and –high groups, respectively (Log-rank test, P = 0.9146) (B).

or di-nucleotide-repeating regions of the Bethesda panel (**Figure 2B**). Traditional MSI was found at three markers, BAT 26 (4/65, 6.2%), D2S8123 (2/65, 3.1%) and D17S250 (1/65, 1.5%) (**Figure 3B**).

EMAST and traditional MSI appear to occur independently, as no significant association in their incidence was found (Fisher's exact test, *P* = 0.146).

LOH in selected tetra-nucleotide-repeats and Bethesda panel

All the tumors examined were heterozygous in at least two markers among the ten tetra-nucleotide-repeating regions, and 58 (89.2%) were heterozygous in at least one marker of the Bethesda panel. LOH at the tetra-nucleotide-repeated regions was found in 41 of 65 (63.1%) tumors (25/39 [64.1%] ADCs, 11 of 19 [57.9%] SQCs, 4 of 6 [66.7%] LCCs and one adenosquamous cell carcinoma) (**Figure 4A**). LOH tended to preferentially occur at D8S348 (7/18, 38.9%), D21S1436 (10/28, 35.7%), MYCL1 (11/31, 35.5%), D9S304 (11/44, 25.0%), D9S303 (11/45, 24.4%), D20S82 (6/27, 22.2%), and D8S321 (7/32, 21.9%), than at D2S443 (5/11, 15.2%), D9S747 (5/59, 8.5%), and UT5037 (0/41, 0.0%) (**Figure 5A**).

LOH at the mono- or di-nucleotide regions of the Bethesda panel was found in 11 of 58 (19.0%) tumors (3/19 [15.8%] squamous cell carcinomas, 5 of 39 [12.8%] adenocarcinomas and 3 of 6 [50%] large cell carcinomas) (**Figure 4B**). LOH tended to preferentially occur at BAT25 (2/17, 11.8%), BAT 26 (2/4, 50.0%), and D2S123 (4/38, 10.5%), than at D5S346 (2/27, 7.4%), and D17S250 (3/43, 7.0%) (**Figure 5B**).

Association between EMAST/MSI and clinicopathologic parameters

Tumors exhibiting EMAST in two or more of the tetra-nucleotide-repeating regions were defined as EMAST-high (22/65, 33.8%), and all other tumors were defined as EMAST-low (43/65, 66.2%), according to the previous studies [15, 28]. The level of EMAST showed significant association with medical history of an overlap with other malignant neoplasms (**Table 3**). There were no significant correlations between the level of EMAST and other clinicopathologic parameters (i.e., sex, age, smoking history, family history of malignancies, histological subtype, pathological T factor (pT), vascular and lymphatic invasion, proliferative activity [Ki-67 index], LOH of p53 locus, and immunohistochemical expression of p53 protein) (**Table 3**).

Tumors exhibiting traditional MSI in two or more regions of the Bethesda panel were defined as MSI-high (0/8, 0%), and all other tumors were defined as MSI-low (8/8, 100%). There were no significant correlations between the level of