Histologic Parameters Predicting Survival of Patients with Multiple Non-small Cell Lung Cancers

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Background: In multiple lung cancers (MLCs), distinction between intrapulmonary metastases and multiple primary tumors is important for staging and prognosis. In this study, we have investigated histopathologic prognostic factors of patients with MLCs. **Methods:** Histologic subtype, size differences, lobar location, lymphovascular invasion (LVI), size of the largest tumor, nodal status, number of tumors, morphology of tumor periphery, and immunohistochemical profiles using eight antibodies, were analyzed in 65 patients with MLCs. **Results:** There was no significant difference in the survivals of patients with multiple primary tumors and intrapulmonary metastases, as determined by the Martini-Melamed criteria (p = 0.654). Risk grouping by four histologic parameters, LVI, margin morphology, size differences, and lobar locations of paired tumors were prognostic. The patients with one or two of aforementioned parameters had significantly longer survival than those with three or four parameters (p = 0.017). In patients with largest mass (≤ 5 cm), the risk grouping was found to be an independent prognostic factor (p = 0.022). However, differences in immunohistochemical staining were not related to patients' survival. **Conclusions:** A risk grouping of MLC patients by using combinations of histologic parameters can be a useful tool in evaluating the survival of patients with MLCs, and may indicate clonal relationship between multiple tumors.

Key Words: Multiple lung cancers; Martini-Melamed criteria; Clinicopathologic parameters; Prognostic factors

The incidence of multiple lung cancers (MLCs) in patients with non-small cell lung cancer (NSCLC) has been reported to range from 3.7 to 8.0%,[1-5] and is constantly increasing due to the widespread use of computed tomography (CT) for screening of MLCs in lung cancer patients. In patients with MLC, distinguishing between intrapulmonary metastases and independent primary tumors is of great importance for determining tumor stage, patient prognosis and management, including the type of adjuvant chemotherapy.[6] In the revised 7th tumor, node and metastasis (TNM) staging system for lung cancer, separate tumor nodules in the same lobe were classified as T3 and nodules in different lobes as T4. These changes resulted in restaging of patients with MLCs from stage IIIB to stage IIB or IIIA, and from stage IV to stage IIIA or IIIB. Because patients with stage IIIB or stage IV NSCLCs are regarded as having poor prognoses, they are often treated with chemotherapy or chemoradiotherapy, but do not undergo surgical resection.

Pathologic evaluation has been an important criterion for distinguishing intrapulmonary metastases from independent primary tumors.[6] In 1975, Martini and Melamed[7] developed clinicopathologic guidelines for the diagnosis of MLCs; these guidelines include determinations of tumor location, histologic type, lymphovascular invasion and foci of carcinoma *in situ*. However, these criteria cannot be completely applied to adenocarcinoma. More recently, several molecular methods, including mutational profiling and comparative genomic hybridization, have been used to evaluate the clonal relationships amongst multiple tumors.[8] These molecular methods, however, are neither cost- nor time-effective, and only a limited number of patients can be assessed by molecular tests during routine clinical practice. Diagnosis is therefore primarily based on the histologic characteristics of the tumors, such as histologic pattern, location, presence or absence of carcinoma *in situ*, vascular invasion, and other empirical features. Thus, a practically applicable and clinically relevant histologic approach is needed for appropriate management of patients with MLCs. We therefore analyzed the association between various histologic parameters, both individually and in combination, and clinical outcomes in patients with MLC to determine a practical method of subdividing patients into groups based on good and poor prognosis.

MATERIALS AND METHODS

Patient selection

A review of the pathology files at the Asan Medical Center, Seoul, Korea, identified 98 patients who had been diagnosed with MLCs and underwent surgery from 1990 to 2007. Clinical information and hematoxylin and eosin (H&E)-stained slides of surgically resected lungs were available for 65 of these patients. Their clinicopathologic data were obtained from a retrospective review of medical records. All patients had been staged clinically according to the 6th and 7th editions of the American Joint Committee on Cancer (AJCC) TNM staging system.

Histologic assessment

We retrospectively reviewed H&E-stained slides of the 65 patients with MLCs to evaluate eight histopathologic parameters (H1 to H8). The parameter, H1 indicated the presence (H1-positive) or absence (H1-negative) of lymphovascular inva-

sion in any tumor (Fig. 1A), a factor related to poor prognosis.[9] The parameter, H2 indicated the morphology of the tumor-normal interface (Fig. 1B, C); if the interface of a smaller mass possessed pushing feature or desmoplastic reaction with adjacent normal parenchyma (H2-positive), the tumor was more likely to be an intrapulmonary metastasis with poorer patient prognosis than the tumor revealing a lepidic pattern at its margins (H2-negative).[10] The parameter, H3 indicated the difference in size between larger mass and smaller mass(es), because a size difference may reflect their respective growth and may distinguish metastasis from a second primary tumor; we subjectively defined H3-positive as a tumor, 20% smaller than the second tumor. The parameter, H4 specified the lobar locations of tumors, because patients with multiple tumors in one lobe (H4-positive) may have a poorer prognosis than patients with tumors in different lobes (H4-negative).[9] The parameter, H5 specified the size of the largest tumor; tumors with size > 5 cm were defined as H5-positive and ≤ 5 cm as H5-negative. The parameter, H6 indicated histologic subtype, with multiple tumors having the same and different histologic types or subtypes
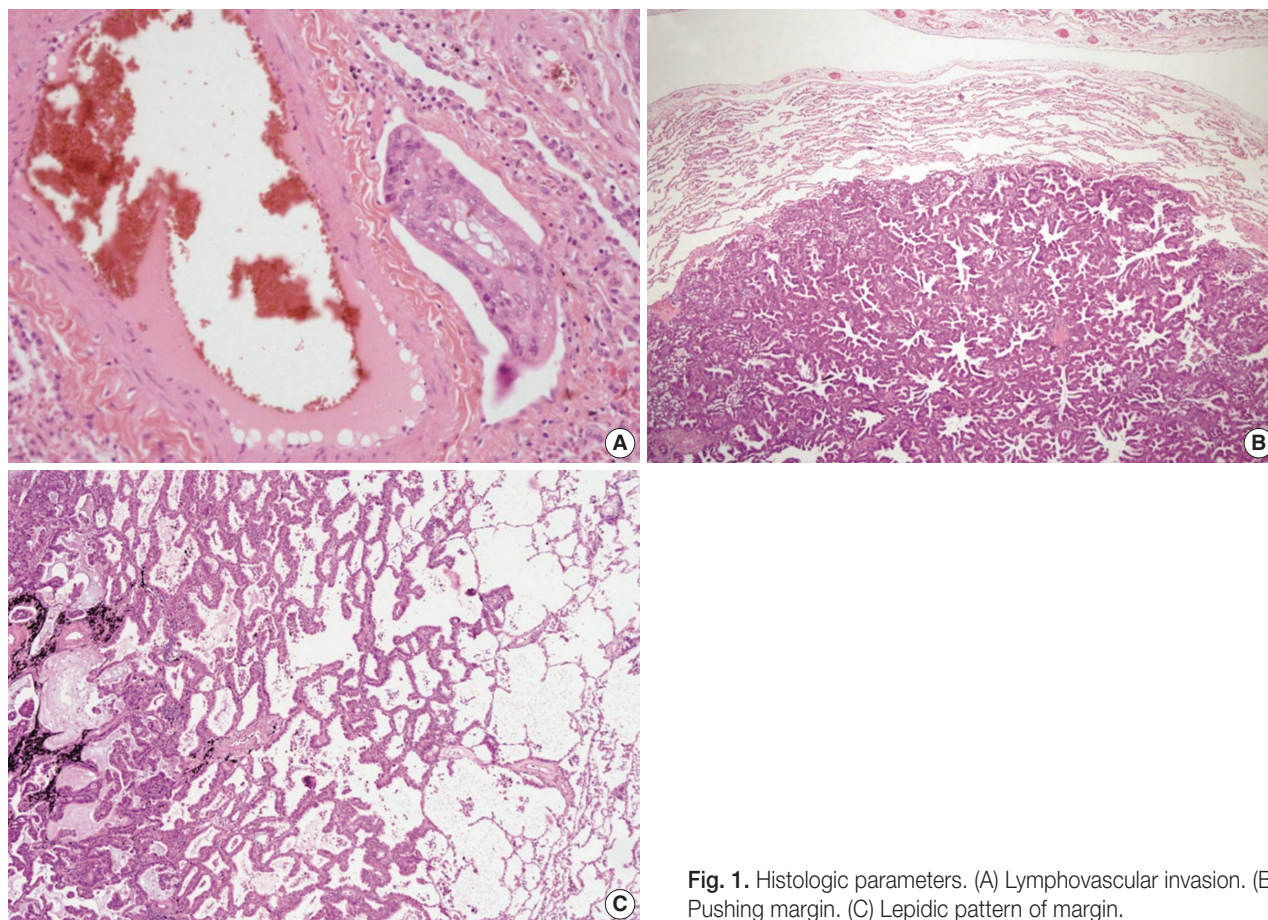
Fig. 1. Histologic parameters. (A) Lymphovascular invasion. (B) Pushing margin. (C) Lepidic pattern of margin.

defined as H6-positive and -negative, respectively; patients with adenocarcinoma were classified according to predominant subtypes, whereas patients with squamous cell carcinomas and other types were evaluated based on cytologic features and stromal characteristics. The parameter, H7 indicated the number of tumors; patients having < 3 and ≥ 3 tumors were classified as H7-negative and -positive, respectively. And, the parameter, H8 specified the presence (H8-positive) or absence (H8-negative) of lymph node metastasis.

Immunohistochemical staining

Tissue microarray (TMA) slides were prepared from paraffin-embedded tissue blocks of multiple tumors of each patient. Two core tissues (2-mm diameter) were taken from each tumor donor block and arranged in recipient paraffin blocks. Tissue sections of 4-μm thickness were stained with antibodies against cytokeratin 7 (CK7; 1:400, Dako, Glostrup, Denmark), cytokeratin 20 (CK20; 1:200, Dako), thyroid transcription factor-1 (TTF-1; 1:200, Novo, Newcastle, UK), epidermal growth factor receptor (EGFR; 1:100, Zymed, San Francisco, CA, USA), caudal type homeobox transcription factor 2 (CDX-2; 1:100, Cell Marque, Rocklin, CA, USA), p53 (1:3,000, Dako), p16 (1:10, Pharmingen, Franklin Lakes, NJ, USA), and Ki-67 (1:100, Zymed). The expression levels of each protein were scored as zero, in the case of no staining; 1, for 1-20% staining; 2, for 21-40%; 3, for 41-60%; 4, for 61-80%; or 5, for 81-100%. The expression of each protein was compared in paired tumors, by using two cores per tumor, and the staining patterns were recorded. If the staining difference is 2 or more of the 8 proteins, the paired tumors were defined as different staining pattern. The cut off value for both, was based on the maximum area under the receiver operating characteristic curve.

Statistical analysis

Overall survival was determined from the date of surgery to the date of last follow-up or death. The Kaplan-Meier method was used to construct survival curves, which were compared by using the log-rank test. $p < 0.05$ was considered statistically significant.

K-mode clustering, a non-parametric approach for deriving clusters from categorical data of histological parameters and immunohistochemical staining patterns was performed by using klaR, a package of R: A language and environment for statistical computing. The number of clusters and the maximum number of iterations were set at 2 and 10,000, respectively.

Decision tree analysis was performed to display algorithms showing as how the clusters were determined. SPSS ver. 17.0 (SPSS Inc., Chicago, IL, USA) was used for decision tree with Chi-square automatic interaction detector (CHAID) as a growing method. The minimum numbers of parent and child nodes were five and two, respectively. Ten-fold cross-validation was used to estimate the accuracy of the decision tree algorithm.

RESULTS

Clinicopathologic characteristics of patients with MLCs

The clinicopathologic characteristics of the 65 patients with MLC are summarized in Table 1. All the 65 patients had a total of 153 tumors, including 48 patients with 2 tumors each, 12 with 3 tumors, 4 with 4 tumors, and 1 with 5 tumors. The tumors were synchronous in 61 patients and metachronous in 4 patients. Of the 153 tumors, 111 (72.6%) were diagnosed as adenocarcinomas, 31 (20.2%) as squamous cell carcinomas, 5 (3.2%) as large cell carcinomas, 4 (2.6%) as undifferentiated carcinomas, 1 (0.7%) as an adenosquamous carcinoma, and 1 (0.7%) as a pleomorphic carcinoma. Of the 65 patients, 38 were

Table 1. Clinicopathologic features of patients with multiple lung tumors

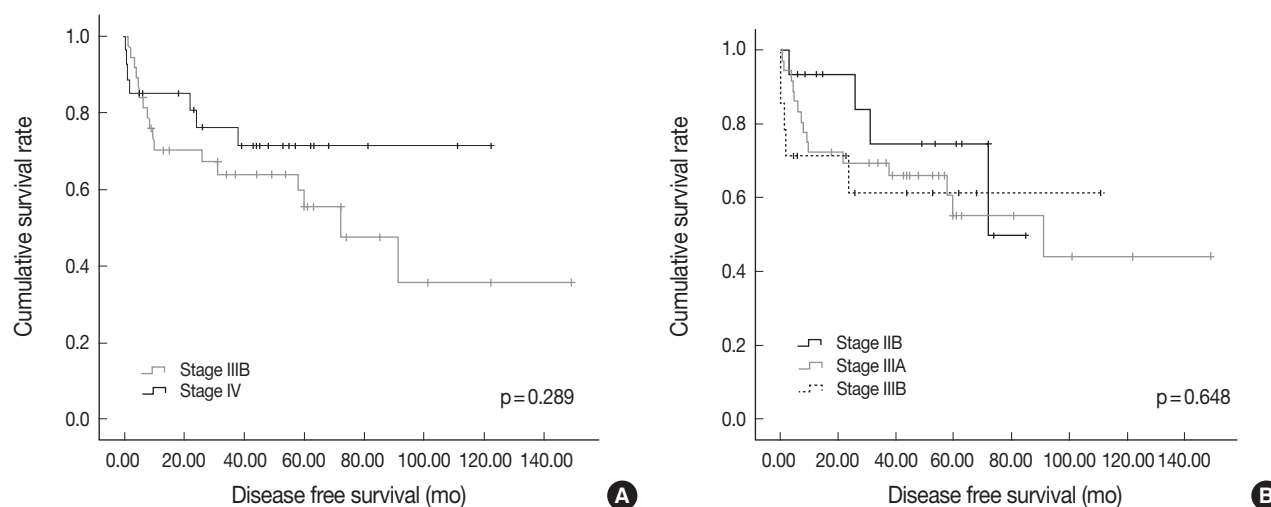| Characteristic	No. of patients (%)
Mean age (range, yr)	58.9 (39-84)
Sex	
Male	40 (61.5)
Female	25 (38.5)
No. of tumors	
2	48 (73.9)
3	12 (18.5)
4	4 (6.1)
5	1 (1.5)
Size of the largest tumor (cm)	
≤ 2	13 (20.0)
>2 and ≤ 3	14 (21.5)
>3 and ≤ 5	25 (38.5)
>5 and ≤ 7	6 (9.2)
>7	7 (10.8)
Synchronous	61 (93.8)
Metachronous	4 (6.2)
Histologic type	
Adenocarcinoma	111 (72.6)
Squamous cell carcinoma	31 (20.2)
Others	11 (7.2)
Pathologic stage	
IIIB	38 (58.4)
IV	27 (41.6)

Fig. 2. Lack of association of the (A) 6th (p = 0.289) and (B) 7th (p = 0.648) American Joint Committee on Cancer (AJCC) staging systems with survival in patients with multiple lung cancer.
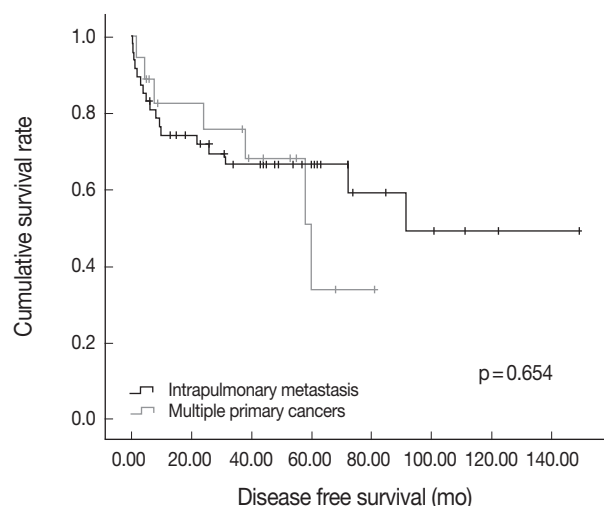

Fig. 3. Lack of association between the Martini-Melamed criteria and survival analysis in patients with multiple lung tumors (p = 0.654).

Table 2. The center of clusters of histologic parameters

Cluster	H2	H3	H4	H6	H7
A	Negative	Negative	Negative	Positive	Negative
B	Positive	Positive	Positive	Positive	Negative

cytologic atypia. Although we expected that the prognosis of the 18 patients with multiple primary tumors would be better than that of the 47 patients with intrapulmonary metastases, we found that the median survival times of these two groups were 60.0 and 91.3 months, respectively, which was statistically not significant (p = 0.654) (Fig. 3).

Pathologic parameters and survival analysis

By employing K-mode clustering, we sorted out patients into two groups according to the eight histopathologic parameters as described in the Materials and Methods section. Since, five parameters (H2, H3, H4, H6, and H7) were sufficient for clustering into two groups; we excluded three parameters (H1, H5, and H8) from this analysis. As shown in Table 2, patients in cluster A (n = 36) were more likely to be negative for the five parameters, whereas patients in cluster B (n = 29) were more likely to be positive for them. Mean survival time tended to be longer in patients in cluster A than in cluster B (111.4 ± 10.8 months vs 57.6 ± 9.6 months) (Fig. 4A), and 5-year survival rates of 30.6% vs 24.1% (p = 0.068), suggested that cluster A represented a lower risk group and cluster B a higher risk group. Construction of a decision tree showed that the overall percentage of correctness was 98.5% with a risk ± standard error of

classified as stage IIIB and 27 as stage IV. After restaging according to the 7th edition of AJCC, 15 patients were classified as stage IIB, 36 as stage IIIA and 14 as stage IIIB. Survival analysis showed that neither of the staging system was relevant to clinical outcome (Fig. 2).

Martini-Melamed criteria and survival analysis

We used the Martini and Melamed criteria, with minor modifications, to distinguish multiple primary lung cancers from intrapulmonary metastases. The presence of carcinoma *in situ* in patients with adenocarcinomas can be determined by the occurrence of a lepidic pattern around the tumor periphery with mild
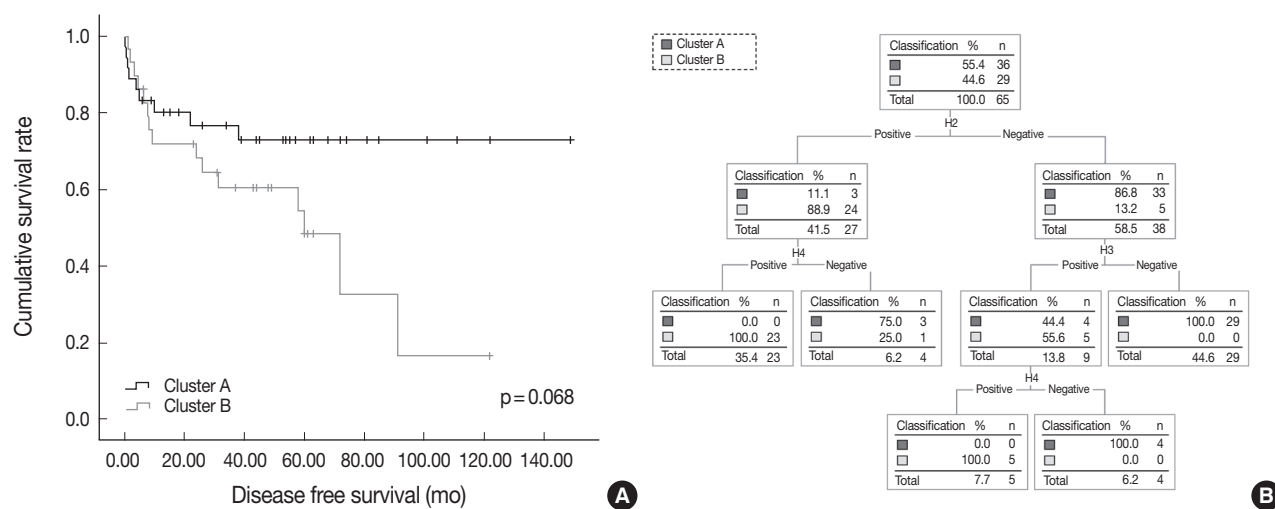
Fig. 4. K-mode clustering and decision tree analysis of five histopathologic parameters. (A) The 5-year survival rates of clusters A and B are 30.6% vs 24.1%, respectively (p=0.068). (B) A decision tree, showing an overall percentage of correctness of 98.5% and a risk±standard error of 1.5±1.5%
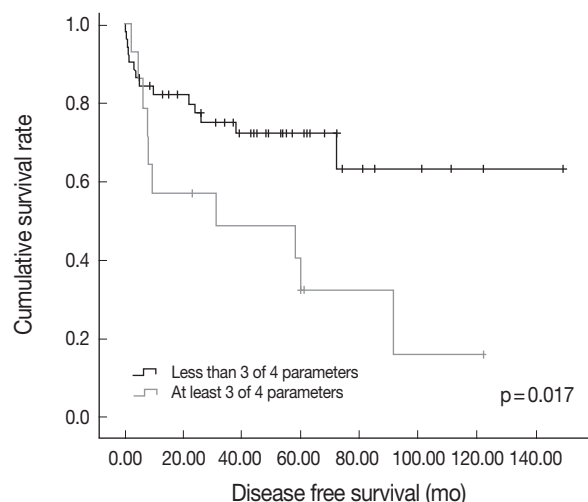

Fig. 5. Survival analysis of patients relative to grouped parameters. The survival times of patients with ≥3 and <3 clinicopathologic parameters differ significantly (p=0.017).

1.5±1.5% (Fig. 4B). The risk±standard error of 10-fold cross-validation was found to be 4.6±2.6%.

When we analyzed the prognostic significance of each individual histologic parameter, we found that none of them was prognostic for patient survival except for size of the largest tumor (H5). Because clustering showed prognostic power, we grouped the patients according to the number of positive parameters, by using various combinations. We found that the combination of parameters H1-4 was statistically significant. Based on these four parameters, we divided patients into two groups, those positive for one or two parameters (low-risk group)

Table 3. Patient survival relative to clinicopathologic parameters

Clinicopathologic parameter		No. of cases	p-value
Lymphovascular invasion (H1)	Absent	50	0.691
	Present	15	
Pushing margin (H2)	Absent	38	0.056
	Present	27	
Size differences (H3)	≥20%	44	0.196
	<20%	21	
Tumor locations (H4)	Different lobe	22	0.807
	Same lobe	43	
Size of the largest tumor (H5) (cm)	≤5	52	0.001
	>5	13	
Histologic features (H6)	Different	17	0.903
	Same	48	
No. of separate tumors (H7)	2	48	0.855
	>2	17	
Lymph node metastasis (H8)	Absent	31	0.235
	Present	34	
Age (yr)	≤55	23	0.052
	>55	42	
Sex	Female	25	0.022
	Male	40	
Histologic parameters (among H1, H2, H3, and H4)	Less than 3 of 4	51	0.017
	At least 3 of 4	14	

and those positive for three or four parameters (high-risk group). There was a significant difference in the survival times of these two groups (p=0.017) (Fig. 5). Survival data are shown in Table 3.

However, multivariate Cox regression analysis showed that the groupings were not independently prognostic of survival of the patient (p=0.265). However, the size of the largest mass was found to be independently prognostic (p=0.040) (Table 4).

Table 4. Multivariate analysis of factors prognostic of survival among the 4 grouped parameters

Clinicopathologic parameters	No. of cases	HR	95% CI	p-value
Grouped parameters (among H1, H2, H3, and H4)		1.673	0.676-4.137	0.265
Less than 3 of 4	51			
At least 3 of 4	14			
Size of the largest mass (cm)		2.699	1.046-6.965	**0.040**
≤5	52			
>5	13			
Sex		2.335	0.844-6.463	0.103
Female	25			
Male	40			

HR, hazard ratio; CI, confidence interval.

Table 5. Survival analysis of 4 grouped parameters according to tumor sizes

Size of the largest mass (cm)	Grouped 4 parameters (H1, 2, 3, and 4)	No. of cases	p-value
≤5 (n=52)	>3	44	**0.020**
	≤3	8	
>5 (n=13)	>3	7	0.749
	≤3	6	

To exclude the contribution of tumor size, we performed subgroup analysis of the 52 H5-negative patients, which were classified as T1 or T2a stage. The low-risk group showed significantly better prognosis than the high-risk group (p=0.020) (Table 5). Moreover, multivariate analysis revealed that in patients with T1 and T2a stage tumors and regardless of the size of the largest mass, the grouping was an independent prognostic factor (Table 6).

According to the 7th edition of AJCC, 15 patients were classified as stage IIB, 36 as stage IIIA and 14 as stage IIIB. Based on our method, we restaged 65 patients after determining the nature of MLC. In low risk group, which favored multiple primary tumors, the largest tumor was restaged. Thirteen patients were classified as stage IA, 7 as stage IB, 11 as stage IIA, 4 as stage IIB, 28 as stage IIIA and 2 as stage IIIB. This restaging pattern significantly predicted survival of the patients (p=0.050) (Fig. 6).

Immunohistochemical (IHC) staining results

We compared the expression of CK7, CK20, TTF-1, EGFR, CDX-2, p53, and p16 and the Ki-67 labeling index in paired tumors, by using two cores per tumor. When staining pattern is different in at least two of the eight proteins, we defined as

Table 6. Multivariate analysis of prognostic factors in patients with T2a stage tumors

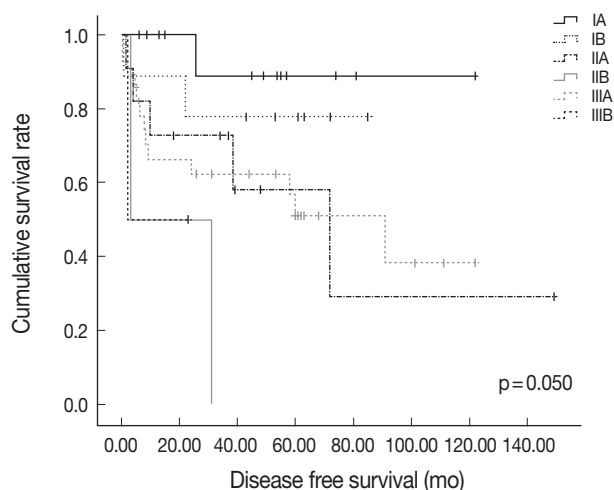| Clinicopathologic parameters	HR	95% CI	p-value
Grouped parameters (among H1, H2, H3, and H4)			
Less than 3 of 4	4.297	1.231-14.993	**0.022**
At least 3 of 4			
Size of the largest mass (cm)			
≤3	0.708	0.191-2.621	0.605
>3 and ≤5			
Sex			
Female	3.757	1.033-13.669	0.045
Male			
Age (yr)			
≤55	1.519	0.487-4.738	0.471
>55			

HR, hazard ratio; CI, confidence interval.

Fig. 6. Restaging of 65 multiple lung cancers (MLC) patients according to the 7th American Joint Committee on Cancer (AJCC) criteria after determination of the nature of each MLC by grouping four parameters. Grouping significantly predicted patient survival (p=0.050).

different clonal origin. However, there was no significant difference in the survival times of patients with and without the aforementioned differences (p=0.174).

Based on K-mode clustering, we sorted out patients into two groups in terms of their IHC staining patterns. We found that five markers (CK7, TTF-1, EGFR, p53, and the Ki-67 labeling index) were sufficient for clustering. The center of the clusters is shown in Table 7. Cluster A, observed in 20 patients, tended to show different expression patterns in paired tumors, whereas cluster B, observed in 39 patients, tended to show similar expression patterns. The mean survival times in these two groups were 95.3±11.7 and 77.6±12.6 months, respectively (p=0.099) (Fig. 7A), suggesting that cluster A as a low-risk group and
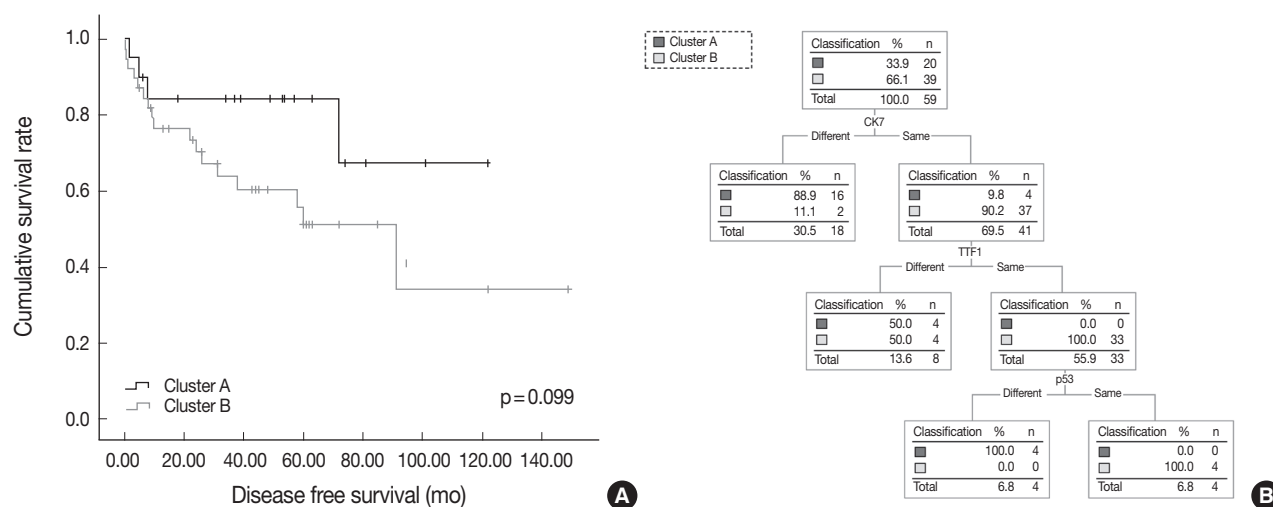
Fig. 7. K-mode clustering and decision tree analysis of five immunohistochemical staining results. (A) The mean survival times of patients in clusters A and B were 95.3 ± 11.7 and 77.6 ± 12.6 mo, respectively (p = 0.099). (B) A decision tree, showing an overall percentage of correctness of 96.6% and a risk ± standard error of 3.4 ± 2.4%.

Table 7. The center of clusters of immunohistochemical staining results

Cluster	CK7	TTF-1	EGFR	p53	Ki-67
A	Different	Different	Same	Different	Same
B	Same	Same	Same	Same	Same

CK7, cytokeratin 7; TTF-1, thyroid transcription factor-1; EGFR, epidermal growth factor receptor.

DISCUSSION

The main purpose of this study was to identify readily available histopathologic prognostic factors in routine pathologic practice, in patients with MLCs, regardless of the clonal origin of the tumors. Grouping of these patients based on 6th and 7th AJCC staging showed that clinical staging alone was not prognostic of patient survival. Therefore, before pathologic staging of MLC, determining whether the tumors are multiple primary cancers or intrapulmonary metastases is critical for predicting patient prognosis. Although molecular methods have been used to determine the clonality of multiple tumors,[11] the reported assays are not feasible in routine pathologic practice, because of their high cost and the time required for the execution.

cluster B as a high-risk group. A decision tree showed that the overall percentage of correctness was 96.6% with a risk ± standard error of 3.4 ± 2.4% (Fig. 7B). The risk ± standard error of 10-fold cross-validation was 6.8 ± 3.3%.

The Martini and Melamed criteria have been used to histologically distinguish the MLCs, but these criteria are associated with discrepancies with respect to molecular results and other clinicopathologic criteria.[6,12] When our 65 patients with MLCs were classified according to the Martini and Melamed criteria as having intrapulmonary metastases or multiple primary cancers, there was no significant difference in the survival between the two groups (p = 0.654). This observation suggests that the criteria alone were limited in predicting the prognosis of patients with MLC. One reason for the discrepancy may be that during the 1970s, when the Martini and Melamed criteria were developed, the most common histologic subtype was squamous cell carcinoma, whereas now it has been transformed into adenocarinoma.[2,13-15]

We therefore evaluated eight histopathologic parameters in our 65 patients with MLCs, both individually and in combination. The evaluation included the assessment of presence of lymphovascular invasion in any tumor (H1), the tumor-normal interface of the smaller mass (H2), size difference > 80% between paired tumors (H3), lobar locations (H4), size of the largest tumor (H5), differences in predominant histologic subtypes (H6), number of separate tumors (H7), and lymph node status (H8).

Only one of these parameters was found to be in significant association with survival and that was the size of the largest mass (H5), suggesting that application of the T3 or T4 descriptor of 6th and 7th AJCC staging systems is inferior to this parameter in predicting patient's outcome when the clonal status of multiple tumors is not considered.

As we observed that no single histologic parameter could discriminate multiple primary tumors from intrapulmonary metastasis, we grouped patients based on histologic parameters by using two methods. In K-mode clustering, five parameters (H2, H3, H4, H6, and H7) could distinguish between low- and high-risk groups (p = 0.068). Although the difference was not statistically significant, the criteria were practically useful when applied to a decision tree. Larger studies will enable the histologic risk grouping of patients with MLC.

We also observed that simple grouping of patients according to the number of positive parameters, H1, H2, H3, and H4, was related to survival of the patients. We also observed significant differences in survival between the high-risk group with three or four positive parameters, and the low-risk group with one or two positive parameters (p = 0.017). These results indicate that risk grouping by histologic parameters reflects the nature of MLCs; i.e., whether they are multiple primary tumors or intrapulmonary metastases.

When we included H6 in grouping our patients, we found that risk grouping was not in significant correlation with survival, suggesting that the similarity of predominant histologic subtype may not represent clonal association. These results may reflect problems in sampling histologically heterogeneous tumors and/or low inter-observer reproducibility.[6] Moreover, metastases may derive from a minor subtype of the primary tumor resulting in a large difference in major subtypes between the primary and metastatic lesions.

In multivariate Cox regression analysis, the simple grouping by using the four parameters (H1, H2, H3, and H4) was not independently prognostic of survival (p = 0.265), although the size of the largest tumor remained prognostic (p = 0.040). To eliminate interference of the size factor, we analyzed patients with largest mass ≤ 5 cm (n = 52) and > 5 cm (n = 13) in diameter. We found that the four grouped parameters were significantly associated with survival in patients with largest tumor ≤ 5 cm (p = 0.020), but not in those with largest tumor > 5 cm (p = 0.749). Moreover, multivariate Cox regression analysis of patients with largest tumor ≤ 5 cm showed that the simple grouping was significantly associated with patient survival (p = 0.022). These findings indicate that risk grouping by histologic parameters may be a useful prognostic indicator for MLC patients with largest tumor ≤ 5 cm. CT screening of lung cancer patients may result in greater detection of small MLCs. By employing histologic parameters, more accurate assessment of MLCs can be expected after surgery. Development of preoperative or intraoperative histologic assessments, in correlation with radiologic findings, may refine the treatment of patients with MLC.

Since recent studies have shown that some cancers develop through multistage accumulation of genetic mutations,[16,17] we expected that comparisons of protein expression by paired tumors could differentiate intrapulmonary metastases from multiple primary cancers. Overexpression of p53 and reduced expression levels of p16 have been described as unfavorable prognostic factors in patients with lung cancer, with differences in p53 and p16 expression by paired tumors supplying information on clonality or prognosis.[18-22] In addition, mutations in *EGFR* gene have been associated with predominantly papillary and lepidic pattern adenocarcinomas.[6,23,24] Nonmucinous-type adenocarcinomas are positive for CK7 and negative for CK20.[25,26] TTF-1 is a nuclear transcription factor, which is expressed in thyroid epithelial cells and alveolar pneumocytes, as well as in pulmonary adenocarcinomas differentiating towards terminal respiratory units.[27,28] CDX-2 is expressed by some pulmonary colloid carcinomas and may be associated with MUC-2 expression.[29,30] The Ki-67 labeling index has been widely used as a marker of cell proliferation. We immunohistochemically assayed expression of these eight proteins, p53, p16, EGFR, CK7, CK20, TTF-1, and CDX-2 and Ki-67 labeling index in 59 sets of paired tumors. Although we expected that different expression patterns by paired tumors would be indicative of multiple primary cancers and a better prognosis than intrapulmonary metastases, we found that differences in their expression levels were not significantly associated with survival (p = 0.174). In K-mode clustering, the patients with MLCs could be divided into two groups based on their IHC staining results, with one group showing a similar pattern and the other group showing a different pattern, but there was no difference in survival between the groups. These results indicate that immunostaining pattern may not be useful in distinguishing amongst MLCs, although the TMA cores may have been too small to represent entire tumors, which often show heterogeneity.

In conclusion, we found that no single histologic or immunohistochemical parameter could be used to differentiate between multiple primary tumors and intrapulmonary metastases. Our results indicate that a systematic assessment of MLCs by using combinations of histologic parameters may result in an easy, cost-effective, accurate and practically useful tool in evaluating the survival of patients with MLC, and may indicate the nature of MLCs as multiple primary tumors or intrapulmonary metastases.

ACKNOWLEDGEMENTS

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