Table 1: Patient characteristics and distribution of ITCs in PV blood

	Total	PV cytology			*P*-value
		N	S	C	
Number	130	34	53	43	
Gender					
Male	74	22	26	26	0.8
Female	56	12	27	17	
Age in years (mean ± SD)	68 ± 9.0	67 ± 9.2	67 ± 9.3	69 ± 11.0	0.3
P-stage					
I	98	30	42	26	0.01
II	20	3	5	12	
III or IV	12	1	6	5	
Tumour histology					
Adenocarcinoma	92	21	41	30	0.8
Squamous cell carcinoma	26	8	10	8	
Miscellaneous	12	5	2	5	

PV: pulmonary vein; N: no tumour cells; S: singular tumour cells; C: clustered tumour cells.

MATERIALS AND METHODS

Detection of ITCs

The methods used for the detection of ITCs were reported in detail in our previous study [12]. Briefly, 50 µl/ml of a RosetteSep® Human CD45 Depletion Cocktail (Stemcell Technologies, Inc., Vancouver, Canada) was added to individual whole blood samples and mixed well. After incubation at room temperature, the mixture was diluted with an equal volume of phosphate buffered saline plus 2% fetal bovine serum and mixed gently. The diluted sample was then layered on the top of a Ficoll-Paque™ PLUS and centrifuged, then enriched ITCs were removed from the Ficoll-Paque™ PLUS–plasma interface. The cells were centrifuged down to polylysine-coated glass slides using a cytospin device. Cells on the slides were subjected to Papanicolaou staining.

Patients

One hundred and thirty consecutive patients (56 males, 74 females; range 28–88 years old, median 68.0 years) with primary non-small-cell lung cancer who did not undergo preoperative chemotherapy and/or radiation therapy were evaluated using our method (Table 1). Written informed consent was obtained from all enrolled patients. This study conformed to the ethical guidelines of Osaka University Graduate School of Medicine and was approved by the institutional review board of Osaka University Medical Hospital. All patients underwent a segmentectomy ($n = 11$), lobectomy or bilobectomy ($n = 119$) during systematic mediastinal lymphadenectomy procedures performed from August 2008 to 2010 at Osaka University Medical Hospital.

Postoperative staging of all patients was determined according to the tumour-node-metastasis (TNM) classification of the Union for International Cancer Control, ver. 7, 2009 (Table 1). The median follow-up duration was 19 (6–22 months). In follow-up examinations, all patients were evaluated at 3-month intervals. Each evaluation included a physical examination, chest X-ray and blood tests including tumour markers, while additional thoraco-abdominal CT scans were generally performed at 6-month intervals.

Blood samples, and ITC detection and enrichment

All blood samples were collected immediately after tumour resection by gently aspirating from the tumour-draining PV using an 18-gauge needle and placed in 10-ml ethylenediaminetetraacetic acid tubes. ITCs were isolated using a negative selection method from 1-ml blood samples using the method described in Section 2.1.

Evaluation and classification of clusters

Using all of the samples, one glass slide containing enriched ITCs from each patient was prepared and assessed by Papanicolaou staining. These examinations were performed independently by 2 cytologists (Eiichi Morii and Hideo Yoshimura) who were unaware of the patient's clinical data. For morphological assessment, each cytologist distinguished cancer cells from normal cells by light microscopy based on their morphological appearance, such as cell size and shape, nuclear size and shape and nuclear-cytoplasmic ratio. Furthermore, for cluster formation assessment, patterns of ITCs were classified into the following three types: no tumour cells (N), singular tumour cells (S) and clustered tumour cells (C). The correlations of patient characteristics with the distribution of ITC morphology in pulmonary venous blood are shown in Table 1. There were no significant differences regarding patient characteristics excluding p-staging among the three groups (N, S and C).

Clinical-pathological analyses according to morphological appearance

To reveal the relationships between clinical-pathological findings and ITC morphology, we examined the following parameters: serum CEA, size of solid primary tumour in CT findings, pathological stage and BVI evaluated by haematoxylin and eosin or elastic van Gieson staining. In patients with CEA ≥5 ng/ml, solid lesion size ≥20 mm in diameter and SUV-max ≥2.5 were considered to be elevated values [7, 13]. Statistical analysis was

performed using commercially available statistics software (JMP version 9, SAS Institute). A *P*-value <0.05 was considered to indicate a statistically significant difference. The characteristics of the patients were compared using the *t*-test, chi-square test and Fisher's exact test. Tukey–Kramer was used to compare the mean values of groups. For the analysis of follow-up data, survival curves were calculated using the Kaplan–Meier method, and survival distributions were compared with a log-rank test. In addition to examining the relationships among ITC morphology and clinical-pathological findings, we utilized logistic regression analysis. Furthermore, to examine the risk ratios of recurrence, a multivariate Cox's hazard model was used.

RESULTS

ITC classification and prognosis

ITCs classified as S were detected in 53 of the 130 patients (40%), while those classified as C were found in 43 (33%). During the median follow-up period of 19 months, 33 patients suffered cancer relapse, 21 in the C group, 9 in S and 3 in N. In the relapsed cases, exclusive local recurrence occurred in 11 (pleura in 7, chest wall in 2, hilar lymph node in 2, mediastinal lymph node in 1), local and distant metastases in 8 (bilateral lungs in 3, mediastinal lymph node and neck lymph node in 4, mediastinal lymph node and bone in 1) and exclusive distant metastasis in 13 (contralateral lung in 4, adrenal grand in 3, brain and liver in 2, other organs in 4). Relapse-free survival curves demonstrated that survival in the C group was significantly worse (Fig. 1). Table 2 also shows the 2-year recurrence free rate according to clinical parameters obtained during the follow-up period. From those results, all of the examined parameters showed an adequate predictive value.

Relationships of ITC morphology with clinical and histopathological findings

The relationships among ITC morphology, clinical background and histopathological findings are shown in Fig. 2. There were significant differences regarding ITC morphology for the clinical parameters serum CEA, positive BVI ratio and advanced p-stage. In cases classified as C, the levels of those parameters were elevated when compared with the N and S cases. In contrast, there were no significant differences regarding ITC morphology for elevated SUV-max and solid lesion size (Fig. 2). In addition, multivariate analysis revealed BVI to be one of the most important factors to determine the ITC morphology (Table 3).

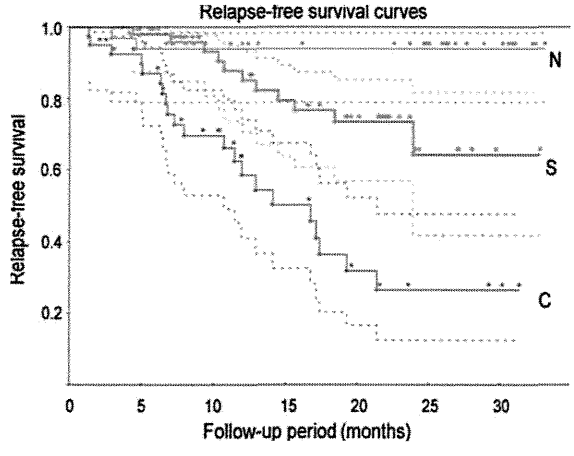

Figure 1: Relapse-free survival curves according to ITC morphology. N: no tumour cells; S: singular tumour cells; C: clustered tumour cells.

DISCUSSION

The present findings of blood samples taken from the PV of resected lungs of patients with lung cancer showed that the morphological pattern of ITCs was correlated with the clinical-pathological parameters for BVI. These results may provide new and important information regarding ITCs in the blood of affected patients.

The presence of ITCs in blood has been reported to be a useful biomarker in lung cancer patients [8–11]. We previously reported that ITCs in the PV were detected using a CD45-negative selection method and found that not only their presence but also cluster formation may be a prognostic indicator for early recurrence of lung cancer [12]. In cases with singular cancer cells, the recurrence rate was low when compared with those with cluster formation. However, it remains unclear whether ITC morphology is related to histopathological findings or clinical background. In the present study, we mainly investigated the relationships between the ITC morphological appearance and clinical pathological findings.

Recent studies have found that ITCs in blood have morphological and biological diversity. Hou *et al.* [14] used a filtration method (isolation by size of epithelial tumor cells [ISET]) to detect and enrich circulating tumour cells (CTCs) according to

Table 2: Relapse-free survival according to clinical-pathological parameters

	Variables	*N*	2Y-RFS (%)	*P*-value
1	ITCs in PV			<0.001
	None (N)	34	93	
	Singular (S)	53	64	
	Clustered (C)	44	26	
2	P-stage			<0.001
	Early (I)	98	71	
	Advanced (II, III, IV)	32	35	
3	Serum CEA			0.03
	Low (<5 ng/ml)	103	66	
	High (≥5 ng/ml)	25	43	
4	SUV-max			<0.001
	Low (<5)	51	77	
	High (≥5)	43	31	
5	BVI			<0.001
	Negative	71	92	
	Positive	59	30	
6	Solid lesion			<0.001
	<20 mm	81	87	
	≥20 mm	49	46	

2Y-RFS: 2-year relapse-free survival; ITCs: isolated tumour cells; PV: pulmonary vein; SUV: standardized uptake value; BVI: blood vessel invasion; Solid lesion: size of solid lesion in CT findings.

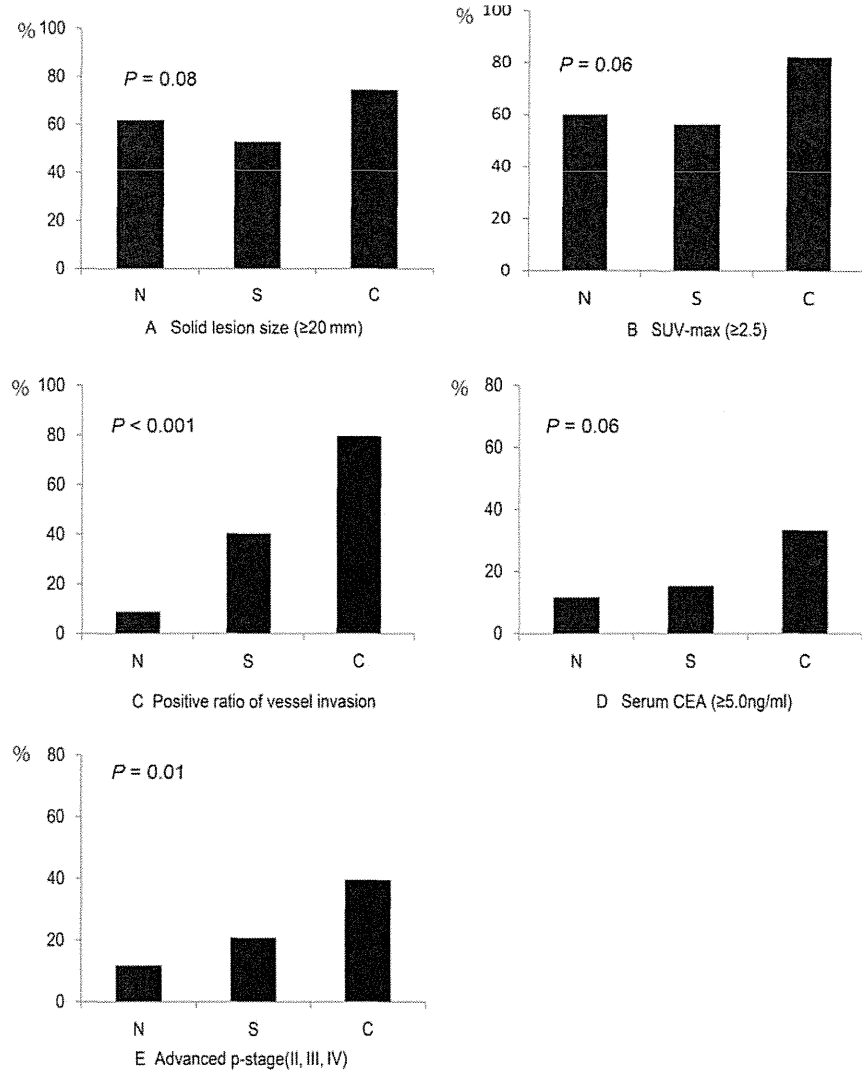

Figure 2: Relationships among ITC morphology and clinical parameters. The vertical axis indicates a high or positive ratio of each individual parameter. (**A**) Solid lesion size in CT findings (≥20 mm). (**B**). SUV-max in PET findings (≥5). (**C**) Blood vessel invasion. (**D**) Serum CEA (≥5 ng/ml). (**E**) Advanced pathological stage (II/III/IV).

the cell size obtained from the blood of lung cancer patients and then analysed their biological characteristics. Based on immunochemical staining results, they showed that clustered CTCs survive longer and have greater anti-apoptosis potential when compared with singular cells. Their findings correspond to our previous results.

As for other predictive factors, histopathological analyses have also provided important information about the biological characteristics of primary cancer. Table 2 shows the factors indicated to have a significant predictive value in the present study, with BVI found to be one of the most revealing prognostic biomarkers. Several groups have reported that BVI was a useful predictive factor for recurrence and metastasis [4, 5]. However, the related mechanisms have not been fully elucidated. In the present study, we found that the presence of clustered cancer cells was strongly related to BVI by primary tumours, as the positive ratio of BVI was significantly higher in cases with clustered ITCs when compared with the N and S groups. Our results showed that clustered ITCs in blood were seen in most cases with BVI, whereas singular cancer cells were also found in cases without BVI. This discrepancy may be because singular ITCs are more easily shed from microvascular vessels around the primary tumour that are too small to be diagnosed as positive BVI, while clustered ITCs may enter the bloodstream mainly as a result of large vessel invasion in an amount adequate for a BVI positive diagnosis. Our findings suggest that BVI indicates not only the presence of singular cancer cells in the bloodstream, but also clustered cancer cells with metastasis potential. In addition, clustered cancer cells may be easily shed from the invaded vein into the bloodstream in cases with vessel invasion, resulting in metastasis. For further analysis of the prognostic recurrent value of these clinical parameters, risk ratios after excluding apparent local recurrence cases were calculated using the Cox's proportional hazard model (Table 4). Those results also revealed BVI as

Table 3: Multivariate analysis of clinical-pathological parameters related to morphology of ITCs

	P-value	Odds ratio	95% CI
S/N			
BVI (+)	<0.01	5.85	1.54–30.04
CT (solid >2 cm)	0.25	0.49	0.13–1.65
CEA (>5 ng/ml)	0.31	1.56	0.33–8.68
P-stage (II, III, IV)	0.8	1.19	0.24–6.07
SUV-max (>2.5)	0.8	1.19	0.31–4.75
C/N			
BVI(+)	<0.01	103.38	15.3–2215
CT (solid >2 cm)	0.77	0.73	0.07–5.21
CEA (5 ng/ml)	0.39	2.33	0.34–19.75
P-stage (II, III, IV)	0.56	1.91	0.18–20.3
SUV-max (>2.5)	0.21	0.21	0.008–2.23

CI: confidence interval; N: no tumour cells; S: singular tumour cells; C: clustered tumour cells; BVI: blood vessel invasion; SUV: standardized uptake value.

Table 4: Multivariate analysis of the prognostic value in recurrent cases excluding local recurrence

	P-value	Risk ratio	95% CI
CEA (>5 ng/ml)	0.79	1.16	0.35–3.29
CT (solid >2 cm)	0.31	2.8	0.42–55.54
BVI	0.02	4.27	1.14–20.38
P-stage (II, III, IV)	0.04	2.98	1.01–8.93
SUV-max (>2.5)	0.8	3.54	0.88–24.71

CI: confidence interval; BVI: blood vessel invasion; SUV: standardized uptake value.

an independent predictive factor, while they suggest that the presence of BVI is an important factor to determine the morphology of ITCs in blood and the relationship to distant metastasis.

In conclusion, the presence of ITCs in the PV of resected lungs of lung cancer patients was shown to be related to varied morphologies, while significant relationships were found among clinical factors and histopathological findings. Importantly, BVI may indicate the presence of clustered ITCs. Our results support the notion that BVI is a predictive factor for early recurrence and distant metastasis, as a large number of clustered ITCs with metastatic potential may be disseminated from invaded blood vessels. These findings should be helpful to further elucidate the mechanism of metastasis in lung cancer.

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Conflict of interest: none declared.

REFERENCES

[1] Alberg AJ, Ford JG, Samet JM, American College of Chest Physicians. Epidemiology of lung cancer: ACCP evidence-based clinical practice guidelines (2nd edition). Chest 2007;132:29S–55S

[2] Sawabata N, Miyaoka E, Asamura H, Nakanishi Y, Eguchi K, Mori M *et al.* Japanese lung cancer registry study of 11,663 surgical cases in 2004: demographic and prognosis changes over decade. J Thorac Oncol 2011; 6:1229–35.

[3] Sawabata N, Ohta M, Takeda S, Hirano H, Okumura Y, Asada H *et al.* Serum carcinoembryonic antigen level in surgically resected clinical stage I patients with non-small cell lung cancer. Ann Thorac Surg 2002; 74:174–9.

[4] Gabor S, Renner H, Popper H, Anegg U, Sankin O, Matzi V *et al.* Invasion of blood vessel as significant prognostic factor in radically resected T1–3N0M0 non-small lung cancer. Eur J Cardiothorac Surg 2004;25:439–42.

[5] Kessler R, Gasser B, Massard G, Roeslin N, Meyer P, Wihlm JM *et al.* Blood vessel invasion is a major prognostic factor in resected non-small cell lung cancer. Ann Thorac Surg 1996;62:1489–93.

[6] Hanin F, Lonneux M, Cornet J, Noirhomme P, Coulon C, Distexhe J *et al.* Prognostic value of FDG uptake in early stage non-small cell lung cancer. Eur J Cardiothorac Surg 2008;33:819–23.

[7] Okereke I, Gangadharan S, Kent MS, Nicotera SP, Shen C, DeCamp MM. Standard uptake value predicts survival in non-small cell lung cancer. Ann Thorac Surg 2009;88:911–6.

[8] Pantel K, Brakenhoff RH, Brandt B. Detection, clinical relevance and specific biological properties of disseminating tumour cells. Nat Rev Cancer 2008;8:329–40.

[9] Sawabata N, Okumura M, Utsumi T, Inoue M, Shiono H, Minami M *et al.* Circulating tumor cells in peripheral blood caused by surgical manipulation of non-small-cell lung cancer: pilot study using an immunocytology method. Gen Thorac Cardiovasc Surg 2007;55:189–92.

[10] Sienel W, Seen-Hibler R, Mutschler W, Pantel K, Passlick B. Tumour cells in the tumour draining vein of patients with non-small cell lung cancer: detection rate and clinical significance. Eur J Cardiothorac Surg 2003;23: 451–6.

[11] Tanaka F, Yoneda K, Kondo N, Hashimoto M, Takuwa T, Matsumoto S *et al.* Circulating tumor cell as a diagnostic marker in primary lung cancer. Clin Cancer Res 2009;15:6980–6.

[12] Funaki S, Sawabata N, Nakagiri T, Shintani Y, Inoue M, Kadota Y *et al.* Novel approach for detection of isolated tumor cells in pulmonary vein using negative selection method: morphological classification and clinical implications. Eur J Cardiothorac Surg 2011;40:322–7.

[13] Okada M, Nishio W, Sakamoto T, Uchino K, Yuki T, Nakagawa A *et al.* Effect of histologic type and smoking status on interpretation of serum carcinoembryonic antigen value in non-small cell lung carcinoma. Ann Thorac Surg 2004;78:1004–9.

[14] Hou J, Krebs M, Ward T, Sloane R, Priest L, Hughes A *et al.* Circulating tumor cells as a window on metastasis biology in lung cancer. Am J Pathol 2011;178:989–96.

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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

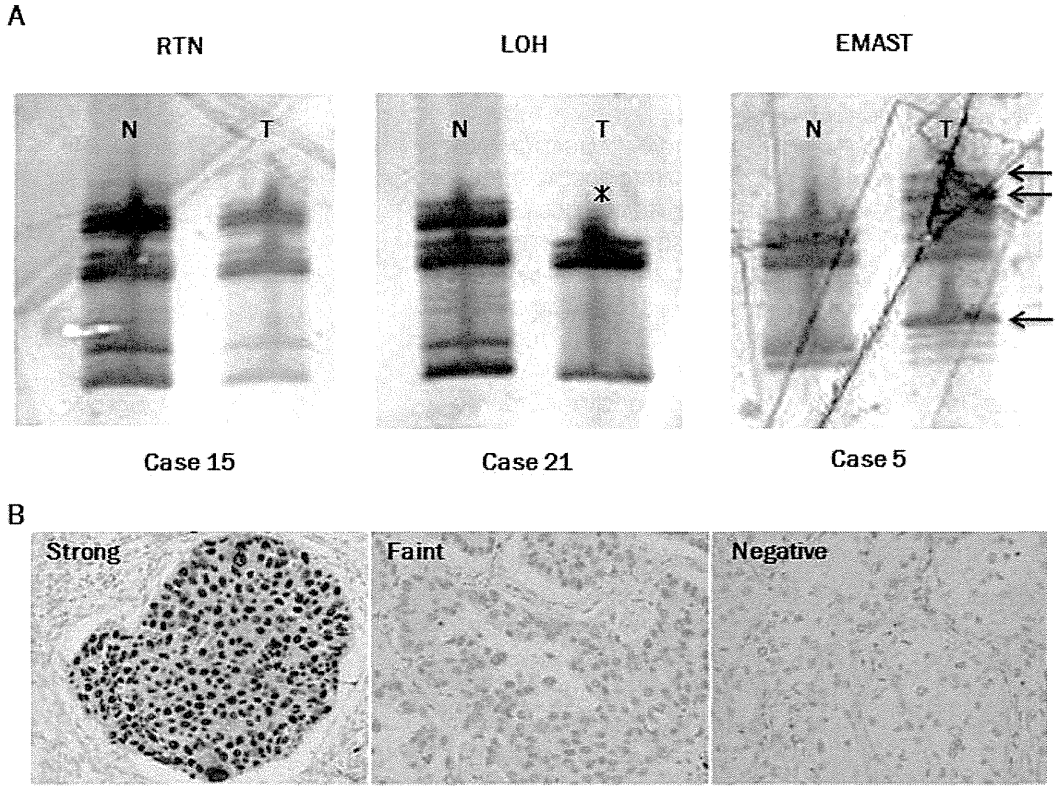

Figure 1. A. Representative results of alterations at a selected tetra-nucleotide repeating region (D9S303). PCR products were separated by polyacrylamide gel electrophoresis and visualized by silver stain. Case 5 (right panel) exhibits elevated microsatellite alternations at selected tetra-nucleotide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

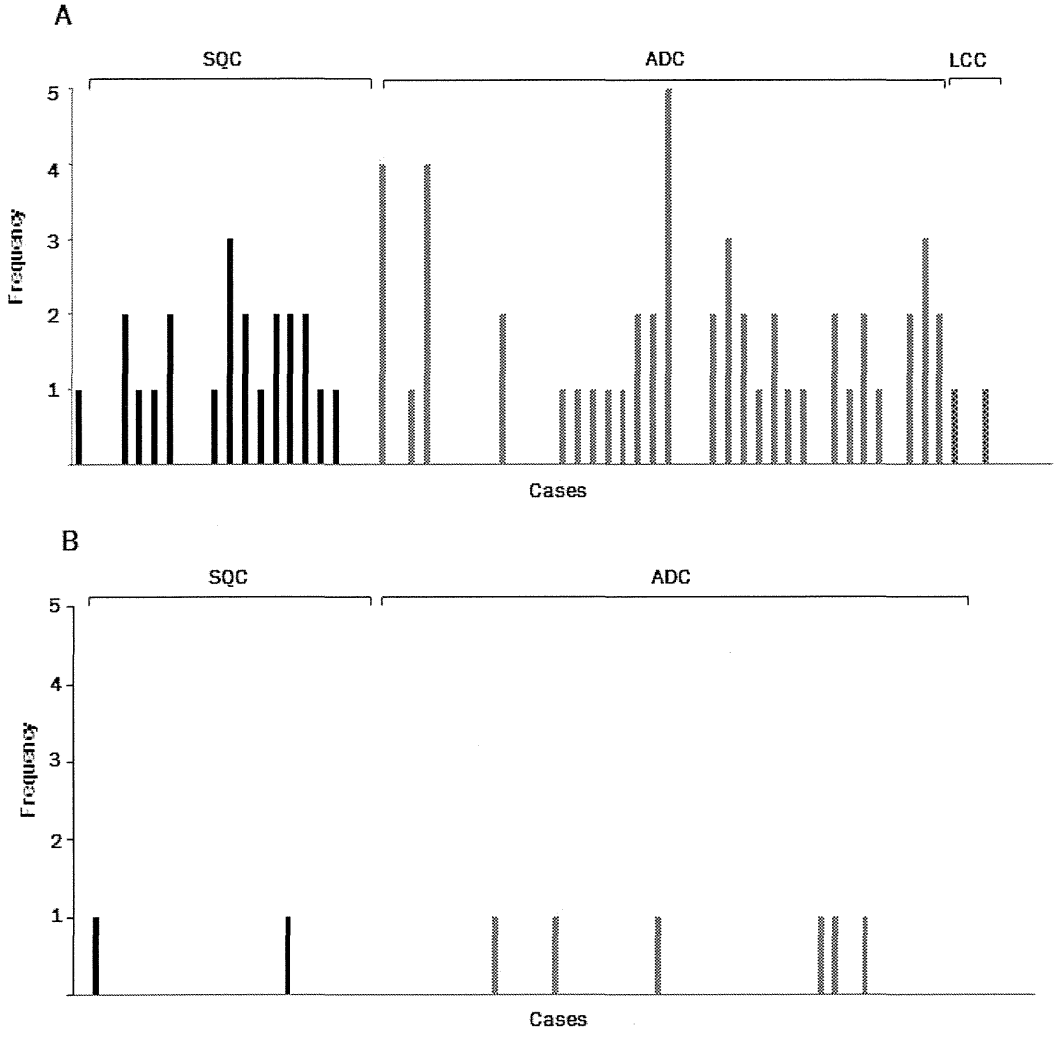

Figure 2. A. Frequency of EMAST (the number of regions where instability occurred among the ten selected tetranucleotide-repeating regions) in each case. B. Frequency of traditional microsatellite instability (MSI) (number of regions where instability occurred among the five regions of the Bethesda panel)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

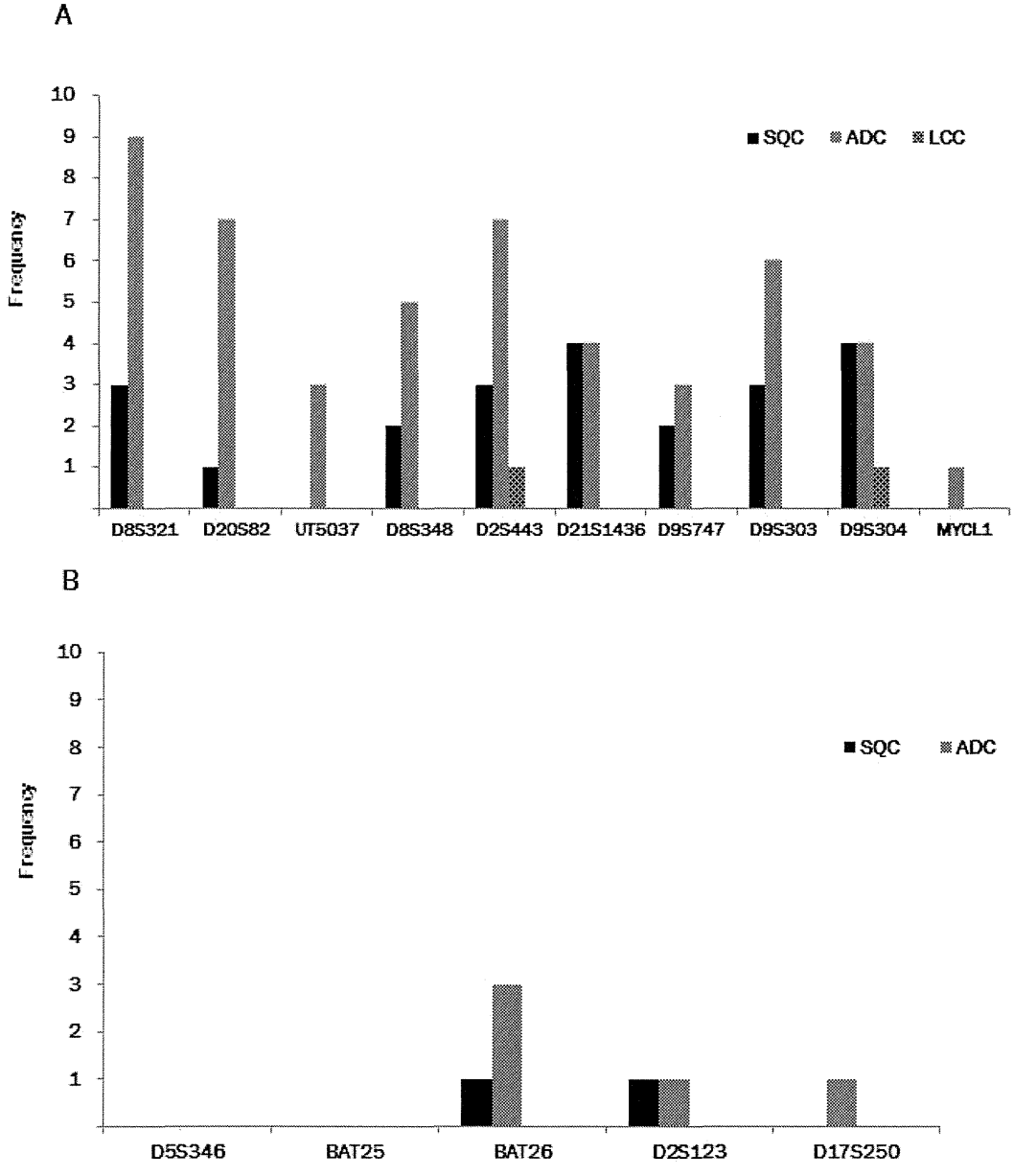

Figure 3. A. Frequency of EMAST (number of tumors exhibiting instability in tumors that could be examined) in each region. B. Frequency of MSI (number of tumors exhibiting instability in tumors that could be examined) in each region SQC, squamous cell carcinoma; ADC, adenocarcinoma; LCC, large cell carcinoma.

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

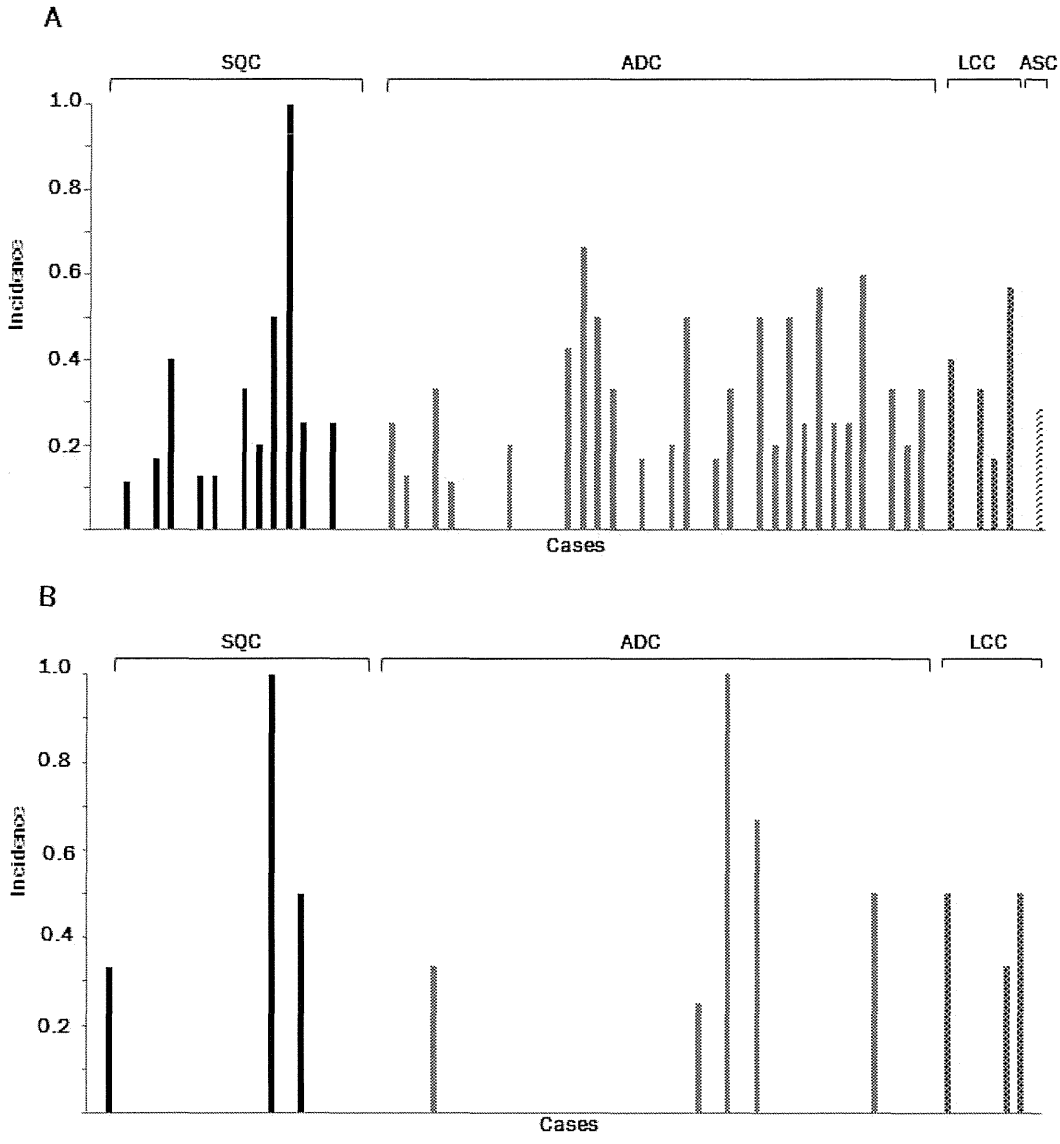

Figure 4. A. Incidence of loss of heterozygosity (LOH) in the selected tetra-nucleotide-repeating regions informative in each case. B. Incidence of LOH in the regions informative of the Bethesda panel in each case. SQC, squamous cell carcinoma; ADC, adenocarcinoma; LCC, large cell carcinoma.

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-

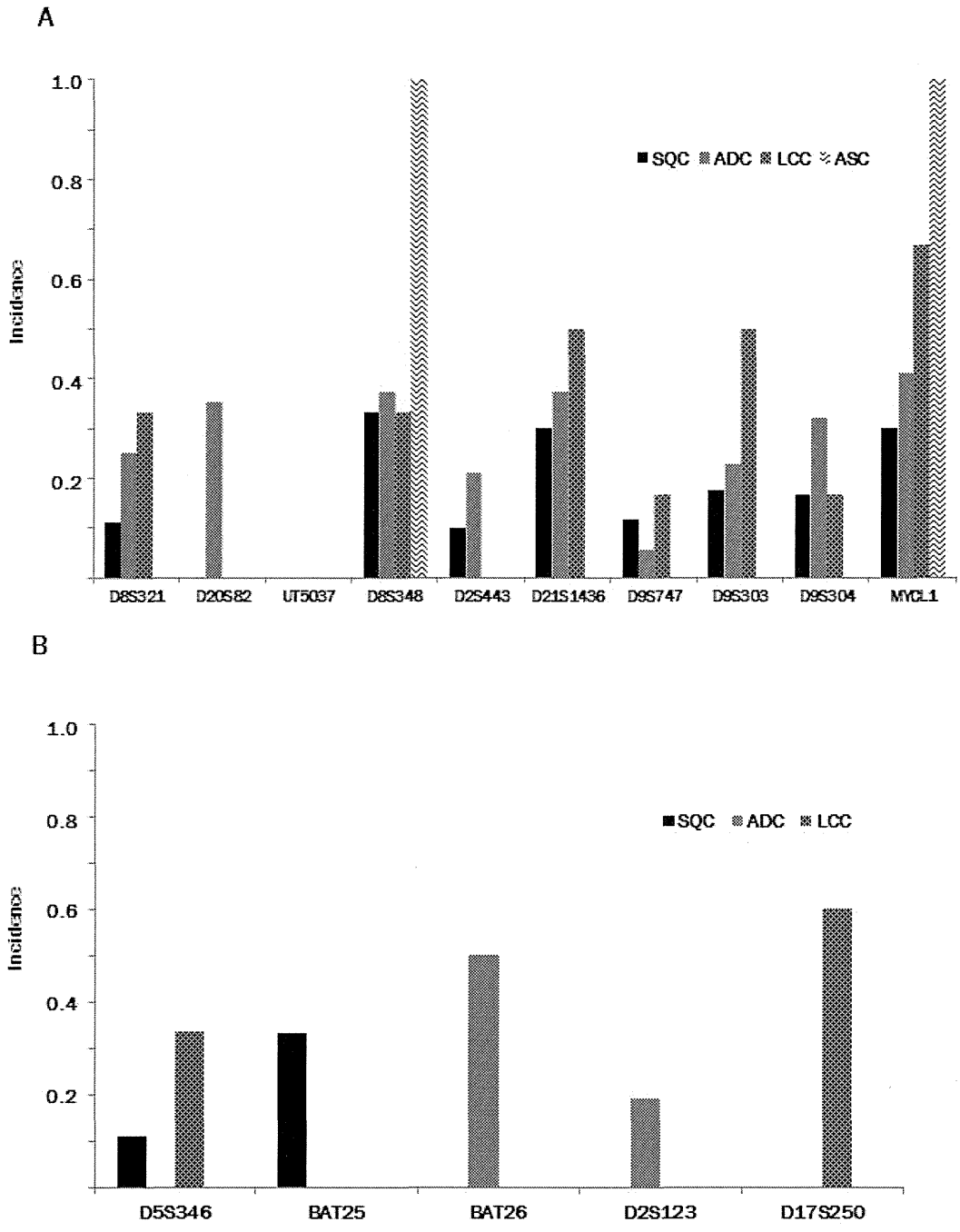

Figure 5. A. Incidence of tumors exhibiting LOH in the selected tetra-nucleotide-repeating regions informative in each region. B. Incidence of tumors exhibiting LOH in the regions informative of the Bethesda panel in each region. SQC, squamous cell carcinoma; ADC, adenocarcinoma; LCC, large cell carcinoma.

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-

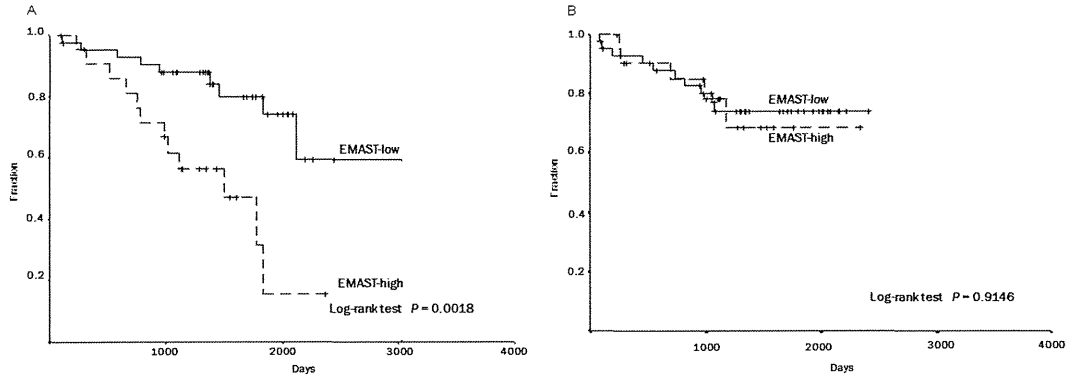

Figure 6. Association between EMAST and 5-year overall survival (A) and 5-year disease-free survival (B). Kaplan-Meier survival curves are shown. EMAST-high: tumors exhibiting EMAST in two or more of the tetra-nucleotide-repeating regions. EMAST-low: tumors exhibiting the EMAST in one or none of the 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 clinico-pathologic 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

Table 5. Essential clincopathologic information and EMAST among cases with multiple malignant neoplasms

					alternation in the ten selected tetra-nucleotide-repeating regions												
No	Sex	Age	Histology	EMAST level	D8S321	D20S82	UT5037	D8S348	D2S443	D21S1436	D9S747	D9S303	D9S304	MYCL1	Overlapped neoplasms	Outcome	Cause of death
1	M	66	SQC	H	-	Ins	-	-	-	-	-	Ins	-	-	GC+RCC	Dead	RCC
2	M	68	SQC	H	-	-	-	-	Ins	-	-	Ins	-	-	LC+RC	Dead	unknown
3	M	79	SQC	H	-	-	-	-	Ins	Ins	-	-	LOH	-	GC	Dead	AMI
4	M	74	ADC	H	-	Ins	-	-	Ins	-	-	-	-	-	PC	Dead	pneumonia
5	M	60	SQC	H	-	-	-	Ins	-	-	Ins	LOH	-	LOH	RC	Dead	RC
6	M	58	SQC	H	Ins	-	-	LOH	-	Ins	-	-	LOH	-	BC	Dead	BC
7	F	77	ADC	H	Ins	Ins	-	-	LOH	-	-	Ins	-	LOH	MSC	Alive	-
8	M	53	ADC	H	Ins	-	-	-	Ins	-	-	-	-	LOH	GC	Alive	-
9	M	64	ADC	H	Ins	Ins	-	-	-	-	-	-	-	LOH	GC	Alive	-
10	M	82	SQC	L	-	-	-	-	-	LOH	Ins	-	-	-	GC	Dead	NSCLC
11	F	76	ADC	L	-	-	-	-	-	-	-	-	-	-	UC	Alive	-
12	M	55	ADC	L	-	LOH	-	NA	-	Ins	-	-	-	LOH	ML	Dead	ML
13	M	75	ADC	L	-	-	-	-	-	-	-	-	LOH	-	SS	Alive	-
14	M	78	ADC	L	-	-	-	-	-	-	-	-	-	-	PC	Alive	-
15	M	77	SQC	L	LOH	-	-	NA	Ins	-	-	-	NA	-	SCLC	Dead	SCLC
16	M	73	SQC	L	-	-	-	-	-	-	-	-	-	-	GC	Alive	-

EMAST, elevated alterations of selected tetra-nucleotide; SQC, squamous cell carcinoma; ADC, adenocarcinoma; M, male; F, female; H, high; L, low; Ins, instable; LOH, loss of heterozygosity; NA, Not available; -, no alteration; NSCLC, Non-small cell lung carcioma; SCLC, small cell lung carcinoma; GC, gastric cancer; RCC, renal cell cancer; LC, laryngeal cancer; RC, rectal cancer; UC, uteirne cancer; ML, malignant lymphoma; PC, prostate cancer; SS, synovial sarcoma; BC, bladder cancer; MSC, maxillary sinus cancer; AMI, acute myocardial infarction.

MSI and the clinicopathologic parameters examined (data not shown).

Association between LOH and clinicopathologic parameters

Tumors exhibiting LOH in two or more tetranucleotide-repeating regions were defined as LOH/selected tetra-nucleotide (ST)-high (20/65, 30.8%), and all other tumors were defined as LOH/ST-low (45/65, 69.2%). There were no significant correlations between the level of LOH/ST and the clinicopathologic parameters (**Table 4**).

Similarly, tumors exhibiting LOH in two or more regions of the Bethesda panel were defined as LOH/Bethesda panel (BP)-high (2/58, 3.4%), and all other tumors were defined as LOH/BP-low (56/58, 96.6%). There were no significant correlations between the level of LOH/BP and the clinicopathologic parameters (data not shown).

Association between EMAST and clinical outcome

The EMAST-high group showed a poorer postoperative overall survival than the EMAST-low group (mean survival time was 1394 days for the EMAST-high group and 2396 days for the EMAST-low group; log-rank test, $P = 0.0018$) (**Figure 6A**). Of the 22 patients with EMAST-high tumors, 12 died; 3 died of NSCLCs (the primary cause), 3 other malignant neoplasms (i.e., renal cell cancer, rectal cancer, bladder cancer), and 6 non-neoplastic diseases (i.e., acute myocardial infarction, cardiac failure, and pneumonia). Of the 43 patients with EMAST-low tumors, 10 died; 6 died of NSCLCs (the primary cause), 2 other malignant neoplasms (i.e., small cell lung cancer and malignant lymphoma), and 2 non-neoplastic diseases (i.e., cardiac failure and decrepitude).

There was no significant difference in the disease-free survival (mean survival time was 1844 days for the EMAST-high group and 1947 days for the EMAST-low group; log-rank test, $P = 0.9146$) (**Figure 6B**), and no association between the level of EMAST and disease recurrence (recurrent rate, 5/22 in the EMAST-high group versus 11/43 in the EMAST-low group, Chi-square test, $P = 0.962$). Moreover, no significant associations were found between the level of LOH/ST, LOH/BP, or MSI and any of the clinicopathologic parameters examined (data not shown).

Discussion

The present study demonstrated that a considerable fraction of NSCLCs was unstable in the ten tetra-nucleotide-repeating regions and that the incidence of EMAST is unequivocally higher than that of traditional MSI. These findings are comparable to those of previous studies which showed an incidence of EMAST in NSCLC of 35-51% [21-23]. The incidence of EMAST differs among the types of malignant neoplasms, 5% in prostate cancer [6], 13% in ovarian cancer [29], 75% in skin cancer [27], and 43.9-45% in bladder cancer [27, 28]. These findings suggest a potential molecular basis for the unique properties in different types of malignant neoplasms.

The most interesting finding of the present study is that patients with EMAST-high NSCLC were affected by additional malignant neoplasms including gastric cancer and renal cell cancer at a significantly higher incidence (42.9% [9/21] in the EMAST-high group versus 16.3% [7/43] in the EMAST-low group). For the 16 patients who were affected by multiple neoplasms, essential information of their clinicopathologic characteristics and alterations in the tetra-nucleotide-repeating markers are described in **Table 5**. Similarly, patients with HNPCC (Lynch syndrome) are also often affected by additional neoplasms, such as endometrial and gastric cancer [14, 15, 31, 32]. HNPCC is an autosomal dominant disease with germ line mutations in the mismatch repair genes (i.e., *h*MSH2, *h*MLH1, and *h*MSH6) [10, 11, 14, 15, 32]. Defects in DNA mismatch repair due to mutations cause traditional MSI and manifest as frame-shift mutations in mono- or di-nucleotide-repeating regions [10, 11, 14, 15, 31, 32]. Traditional MSI has been found in 85-95% of HNPCC (and in 10-15% of sporadic colorectal cancers, in which the mismatch repair genes are silenced by the acquired epigenetic modification) and is well accepted to be an important molecular basis for promoting carcinogenesis of certain types of malignant neoplasms [7-12]. On the other hand, EMAST, distinct from traditional MSI, is not associated with defects in mismatch repair [23, 28]. Although the actual

molecular mechanism of EMAST remains unclear, previous studies of some AAAG-type tetra-nucleotide repeating regions suggest that p53 alterations could be involved [22]. One recent study demonstrated an association between the heterogeneous nuclear expression of hMSH3 and EMAST in colorectal cancer cells, suggesting that an acquired hMSH3 alteration could be its molecular mechanism [36]. The present study investigated the involvement of p53 (LOH of p53 gene and its potential mutations evaluated by immunohistochemistry) in EMAST, but failed to obtain a result that supports previous findings [16, 21-24, 27]. The difference in the tetra-nucleotide repeating regions examined might be responsible for this discrepancy. Thus, establishment of universal markers to evaluate EMAST, like the Bethesda panel, is necessary to verify its clinicopathologic significance. Moreover, a comprehensive search for potential alterations of DNA replication/repair molecules like hMSH3 may lead to elucidation of the molecular mechanism of EMAST.

As for the clinical outcome, a pronounced difference in the overall survival was found between the EMAST high- and low-groups. However, no significant difference was found in the disease-free survival and the recurrent rate, or in histological grade and proliferating activity of neoplastic cells. Notably, 3 of 22 (13.6%) patients with EMAST-high tumor died of other malignant neoplasms, while 2 of 43 (4.7%) patients with EMAST-low tumor died of other neoplasms. These findings suggest that the poorer overall survival in the EMAST-high group might be due to a high susceptibility to malignant neoplasm, and EMAST itself does not promote the progression process of their carcinogenesis (that is, it does not promote the acquisition of highly malignant activity of neoplastic cells).

In conclusion, impairment of molecular machinery that maintains stable replication of the tetra-nucleotide-repeated regions may elevate susceptibility to NSCLCs and certain neoplastic diseases. Elucidation of the potential molecular mechanism of EMAST could lead to discovery of a novel genetic background determining susceptibility to NSCLCs and establishment of a novel disease susceptible for multiple neoplasms including NSCLCs.

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Declaration of Conflicts of interest

None declared.

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References

[1] Hoffman PC, Mauer AM, Vokes EE. Lung cancer. Lancet 2000; 335: 479-85.

[2] Spira A, Ettinger DS. Multidisciplinary Management of Lung Cancer. N Engl J Med 2004; 350: 379-92.

[3] Yang P, Allen MS, Aubry MC, Aubry MC, Wampfler JA, Marks RS, Edell ES, Thibodeau S, Adjei AA, Jett J, Deschamps C. Clinical features of 5,628 primary lung cancer patients: Experience at Mayo Clinic From 1997 to 2003. Chest 2005; 128: 452-62.

[4] Ignatius Ou SH, Zell JA, Ziogas A, Anton-Culver H. Prognostic Factors for Survival of Stage I Nonsmall Cell Lung Cancer Patients: A Population-based Analysis of 19,702 Stage I Patients in the California Cancer Registry From 1989 to 2003. Cancer 2007; 110: 1532-41.

[5] Asamura H, Goya T, Koshiishi Y, Sohara Y, Eguchi K, Mori M, Nakanishi Y, Tsuchiya R, Shimokata K, Inoue H, Nukiwa T, Miyaoka E; Japanese Joint Committee of Lung Cancer Registry. A Japanese Lung Cancer Registry study: prognosis of 13,010 resected lung cancers. J Thorac Oncol 2008; 3: 46-52.

[6] Burger M, Denzinger S, Hammerschmied CG, Tannapfel A, Obermann EC, Wieland WF, Hartmann A, Stoehr R. Elevated microsatellite alternations at selected tetranucleotides (EMAST) and mismatch repair gene expression in prostate cancer. J Mol Med 2006; 84: 833-41.

[7] Mao L, Lee DJ, Tockman MS, Erozan YS, Askin F, Sidransky D. Microsatellite alterations as clonal markers for the detection of human cancer. Proc Natl Acad Sci USA 1994; 91: 9871-5.
[8] Schlegel J, Bocker T, Zirngibl H, Hofstädter F, Rüschoff J. Detection of microsatellite instability in human colorectal carcinomas using a non-radioactive PCR-based screening technique. Virchows Arch 1995; 426: 223-7.
[9] Cheng KC, Loeb LA. Genomic instability and tumor progression: mechanistic considerations. Adv Cancer Res 1993; 60: 121-56.
[10] Lengauer C, Kinzler KW, Vogelstein B. Genetic instabilities in human cancers. Nature 1998; 396: 643-9.
[11] Lothe RA. Microsatellite instability in human solid tumors. Mol Med Today 1997; 3: 61-8.
[12] Horii A, Han HJ, Shimada M, Yanagisawa A, Kato Y, Ohta H, Yasui W, Tahara E, Nakamura Y. Frequent replication errors at microsatellite loci in tumors of patients with multiple primary cancers. Cancer Res 1994; 54: 3373-5.
[13] Zhou X, Kemp BL, Khuri FR, Liu D, Lee JJ, Wu W, Hong WK, Mago L. Prognostic implication of microsatellite alternation profiles in early-stage non-small Cell lung Cancer. Clin Cancer Res 2000; 6: 559-65.
[14] Lynch HT, de la Chapelle A. Genetic susceptibility to non-polyposis colorectal cancer. J Med Genet 1999; 36: 801-18.
[15] Umar A, Boland CR, Terdiman JP, Syngal S, de la Chapelle A, Rüschoff J, Fishel R, Lidor NM, Burgart LJ, Hamelin R, Hamilton SR, Hiatt RA, Jass J, Lindblom A, Lynch HT, Peltomaki P, Ramsey SD, Rodriguez-Bigas MA, Vasen HF, Hawk ET, Barrett JC, Freedman AN, Srivastava S. Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch Syndrome) and microsatellite instability. J Natl Cancer Inst 2004; 96: 261-8.
[16] Fong KM, Zimmerman PV, Smith PJ. Microsatellite instability and other molecular abnormalities in non-small cell lung cancer. Cancer Res 1995; 55: 28-30.
[17] Ninomiya H, Nomura K, Satoh Y, Okumura S, Nakagawa K, Fujiwara M, Tsuchiya E, Ishikawa Y. Genetic instability in lung cancer: concurrent analysis of chromosomal, mini- and microsatellite instability and loss of heterozygosity. Br J Cancer 2006; 94: 1485-91.
[18] Pylkkänen L, Karjalainen A, Anttila S, Vainio H, Husgafvel-Pursiainen K. No evidence of microsatellite instability but frequent loss of heterozygosity in primary resected lung cancer. Environ Mol Mutagen 1997; 30: 217-23.
[19] Sekido Y, Fong KM, Minna JD. Molecular Genetics of Lung Cancer. Annu Rev Med 2003; 54: 73-87.
[20] Schayek H, Krupsky M, Yaron P, Yellin A, Simansky DA, Friedman E. Genetic analyses of non-small cell lung cancer in Jewish Israeli patients. Isr Med Assoc J 2006; 8: 159-63.
[21] Woenckhaus M, Stoehr R, Dietmaier W, Wild PJ, Zieglmeier U, Foerster J, Merk J, Blaszyk H, Pfeifer M, Hofstaedter F, Hartmann A. Microsatellite insatability at chromosome 8p in non-small cell lung cancer is associated with lymphnode metastasis and squamous differentiation. Int J Oncol 2003; 23: 1357-63.
[22] Xu L, Chow J, Bonacum J, Eisenberger C, Ahrendt SA, Spafford M, Wu L, Lee SM, Piantadosi S, Tockman MS, Sidransky D, Jen J. Microsatellite instability at AAAG repeat sequence in respiratoty tract cancers. Int J Cancer 2001; 91: 200-4.
[23] Ahrendt SA, Decker PA, Doffek K, Wang B, Xu L, Demeure MJ, Jen J, Sidransky D. Microsatellite instability at selected tetranucleotide repeats is associated with p53 mutations in non-small cell lung cancer. Cancer Res 2000; 60: 2488-91.
[24] Chang JW, Chen YC, Chen CY, Chen JT, Chen SK, Wang YC. Correlation of genetic insatiability with mismatch repair protein expression and p53 mutations in non-small cell lung cancer. Clin Cancer Res 2000; 6: 1639-46.
[25] Shridhar V, Siegfried J, Hunt J, del Mar Alonso M, Smith DI. Genetic instability of microsatellite sequence in many non-small cell lung carcinomas. Cancer Res 1994; 54: 2084-7.
[26] Miozzo M, Sozzi G, Musso K, Pilotti S, Incarbone M, Pastorino U, Pierotti MA. Microsatellite alterations in bronchial and sputum specimens of lung cancer patients. Cancer Res 1996; 56: 2285-8.
[27] Danaee H, Nelson HH, Karagas MR, Schned AR, Ashok TD, Hirao T, Perry AE, Kelsey KT. Microsatellite instability at tetranucleotide repeats in skin and bladder cancer. Oncogene 2002; 21: 4894-9.
[28] Catto JW, Azzouzi AR, Amira N, Rehman I, Feeley KM, Cross SS, Fromont G, Sibony M, Hamdy FC, Cussenot O, Meuth M. Distinct patterns of microsatellite instability are seen in tumours of the urinary tract. Oncogene 2003; 22: 8699-706.
[29] Singer G, Kallinowski T, Hartmann A, Dietmaier W, Wild PJ, Schraml P, Sauter G, Mihatsch MJ, Moch H. Different types of microsatellite instability in ovarian carcinoma. Int J Cancer 2004; 112: 643-6.
[30] Goldstraw P, Groome P. TNM Classification of Malignant Tumors, 7th Edition. Edited by Sobin LH, Gospodarowicz MK, Wittekind C. Oxford, Wiley-Blackwell, 2009, pp: 136-146.
[31] Peltomäki P, Lothe RA, Aaltonen LA, Pylkkänen L, Nyström-Lahti M, Seruca R, David L, Holm R,

Ryberg D, Haugen A, Brøgger A, Børresen AL, de la Chapelle A. Microsatellite instability is associated with tumors that characterize the hereditary non-polyposis colorectal carcinoma syndrome. Cancer Res 1993; 53: 5853-5.

[32] Rodriguez-Bigas MA, Boland CR, Hamilton SR, Henson DE, Jass JR, Khan PM, Lynch H, Perucho M, Smyrk T, Sobin L, Srivastava S. A National Cancer Institute Workshop on Hereditary Nonpolyposis Colorectal Cancer Syndrome: Meeting Highlights and Bethesda Guidelines. J Natl Cancer Inst 1997; 89: 1758-62.

[33] Futreal PA, Barrett JC, Wieseman RW. An Alu polymorphism intragenic to the TP53 gene. Nucleic Acids Res 1991; 19: 6977.

[34] Okudela K, Woo T, Mitsui H, Yazawa T, Shimoyamada H, Tajiri M, Ogawa N, Masuda M, Kitamura H. Morphometric profiling of lung cancers-its association with clinicopathologic, biologic, and molecular genetic features. Am J Surg Pathol 2010; 34: 243-55.

[35] Woo T, Okudela K, Yazawa T, Wada N, Ogawa N, Ishiwa N, Tajiri T, Rino Y, Kitamura H, Masuda M. Prognostic value of KRAS mutations and Ki-67 expression in stage I lung adenocarcinomas. Lung Cancer 2009; 65: 355-62.

[36] Lee SY, Chung H, Devaraj B, Iwaizumi M, Han HS, Hwang DY, Seong MK, Jung BH, Carethers JM. Microsatellite Alterations at Selected Tetranucleotide Repeats Are Associated with Morphologies of Colorectal Neoplasias. Gastroenterology 2010; 139: 1519-25.