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-

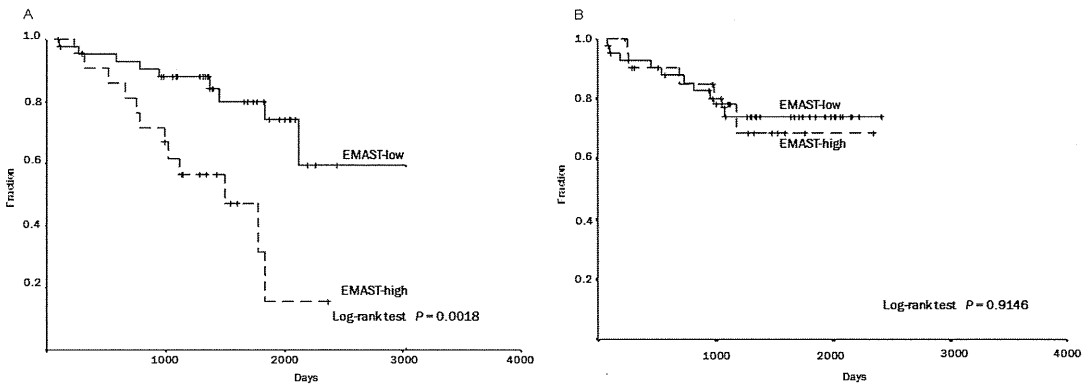

Figure 6. Association between EMAST and 5-year overall survival (A) and 5-year disease-free survival (B). Kaplan-Meier survival curves are shown. EMAST-high: tumors exhibiting EMAST in two or more of the tetra-nucleotide-repeating regions. EMAST-low: tumors exhibiting the EMAST in one or none of the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

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 tetra-nucleotide-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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Large Cell Neuroendocrine Carcinoma of the Lung: Is it Possible to Diagnose from Biopsy Specimens?

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Objective: We have recently proposed new diagnostic criteria for high-grade non-small cell neuroendocrine carcinoma, i.e. possible large cell neuroendocrine carcinoma, in biopsy specimens and have started a clinicopathological comparative study of high-grade neuroendocrine carcinomas in an advanced stage. This study aimed to elucidate the usefulness of our diagnostic criteria for inoperable advanced large cell neuroendocrine carcinoma and to know the true incidence of large cell neuroendocrine carcinoma among lung cancers.
Methods: We reviewed all cancer lesions (1040 specimens) obtained by transbronchial lung biopsies in our hospital from 2002 to 2009 and selected 38 biopsy specimens that satisfied our diagnostic criteria for high-grade non-small cell neuroendocrine carcinoma. All 38 cases were clinicopathologically investigated and all biopsy specimens were precisely studied for their morphological characteristics.
Results: Clinicopathological information about the selected 38 cases was very similar to the clinicopathological characteristics of large cell neuroendocrine carcinoma reported. Of 38 cases, six were at Stage I, II or IIIA, underwent surgery, and the diagnosis was confirmed to be large cell neuroendocrine carcinoma using surgical tumor specimens. In the 38 biopsy specimens, features of neuroendocrine morphology such as organoid nesting, peripheral palisading and rosette formation were not frequent histological features and the majority of tumor cells contained nuclei with a fine chromatin pattern. Mitoses were difficult to find; however, immunohistochemical Ki-67/MIB1 labeling indices were quite useful for evaluating proliferative activity, which ranged from 43.4 to 99.0%.
Conclusions: Our study showed the diagnostic potential of using biopsy specimens for large cell neuroendocrine carcinoma, and we herein proposed more simplified diagnostic criteria for possible large cell neuroendocrine carcinoma in practical diagnostic use.

Key words: large cell neuroendocrine carcinoma – biopsy diagnosis – Ki-67 – neuroendocrine markers – small cell carcinoma

INTRODUCTION

Lung carcinoma is clinically classified into two categories, i.e. small cell lung carcinoma (SCLC) and non-SCLC (NSCLC), with regard to their response to chemoradiotherapy. In 1991, Travis et al. (1) separated a group of high-grade neuroendocrine carcinoma from NSCLC and proposed the new histological category of large cell neuroendocrine carcinoma (LCNEC), which was adopted into the WHO classification in 1999 (2) and retained in the 2004 WHO classification (3). The main criteria for diagnosing LCNEC are: (1) large

cell morphology; (2) high mitotic rate of more than 11 or greater/10 high power fields (HPFs); and (3) the detection of neuroendocrine morphology and immunohistochemical markers (1–3). However, it became clear that the clinical features of LCNEC are similar to those of SCLC, and the differential diagnosis between LCNEC and SCLC is quite difficult in some cases (4,5). The morphological and clinical similarities of these high-grade neuroendocrine carcinomas are problematic for clinicians and pathologists (1,4,5).

Although the role of chemotherapy in LCNEC is uncertain, Iyoda et al. (6) have reported that patients who received adjuvant chemotherapy had a better prognosis than patients who did not receive adjuvant chemotherapy. Moreover, recent advances in chemotherapy have elucidated that platinum-based adjuvant chemotherapy is effective and significantly improves the survival of patients with LCNEC compared with non-platinum-based adjuvant chemotherapy, suggesting that the initial treatment response of LCNEC to chemotherapy might be similar to that of SCLC (7–10). The results of chemotherapy in previous LCNEC studies were obtained by adjuvant chemotherapy after surgery and the diagnosis of LCNEC was made by using resected surgical materials. Not only for resectable LCNEC, but also for non-resectable LCNEC the pathological diagnosis should be made for selecting an appropriate chemotherapy. However, for the diagnosis of LCNEC in a biopsy specimen, the criteria of LCNEC cannot fit because, for example, the mitotic rate cannot necessarily be counted in 10 HPFs in a small necrosis-rich and/or crushed specimen. Thus, for the tumors that can possibly be LCNEC, another criterion for the diagnosis of possible LCNEC is required. Therefore, we focused attention on unresectable high-grade neuroendocrine carcinoma of the lung and aimed to perform a retrospective comparative study in order to know the chemotherapy response of LCNEC and SCLC (9). This comparative study used biopsy specimens instead of surgical specimens to diagnose LCNEC correctly and to differentiate LCNEC from SCLC. For this purpose, we modified the diagnostic criteria of LCNEC proposed by Travis et al. (1) and proposed new diagnostic criteria of high-grade non-small cell neuroendocrine carcinoma (HNSCNEC), which likely includes most LCNEC (9).

Using our criteria of HNSCNEC, at least two papers have been published so far and it was concluded that the efficacy of chemotherapy for unresectable LCNEC is comparable with that of SCLC (9,10). In this study, we morphologically reviewed all lung biopsy specimens obtained from our hospital from 2002 to 2009 and aimed to improve the criteria of HNSCNEC for better practice with using biopsy specimens and to estimate the true frequency of LCNEC in lung cancer.

PATIENTS AND METHODS

Biopsy Samples

From September 2002 to December 2009, transbronchial lung biopsy was performed on 1566 patients with lung tumor at Shizuoka Cancer Center Hospital. Using hematoxylin and eosin (H&E)-stained paraffin sections of these biopsy specimens, we histologically reviewed their diagnosis. Of 1566 biopsy cases, 1040 were evaluated to have carcinoma tissue, with a diagnosis of adenocarcinoma (518 cases), squamous cell carcinoma (318 cases), adenosquamous carcinoma (6 cases), SCLC (121 cases), large cell carcinoma (24 cases) and pleomorphic carcinoma (15 cases). Finally, we selected 38 HNSCNECs according to our criteria (9). Selected from the criteria shown in Table 1 (9), we used the following as essential conditions: no differentiation to squamous cell carcinoma or adenocarcinoma, positive immunostaining for at least one of neuroendocrine markers, large nuclear size with moderate or marked nuclear atypia, a Ki-67/MIB1 labeling index higher than 40%, and nuclear features (fine chromatin and/or prominent nucleoli) or one of the features of neuroendocrine morphology such as organoid nesting, peripheral palisading, rosettes and/or trabecular architecture.

We excluded poorly differentiated adenocarcinomas by positive periodic acid-schiff-alcian blue mucin staining after diastase digestion. We also excluded squamous cell carcinomas by p63 and keratin 5/6 immunopositivity. The immunohistochemical Ki-67 labeling index was the indicator of high-grade malignancy. Neuroendocrine carcinoma was defined by at least one positive neuroendocrine marker of chromogranin A, synaptophysin and neural cell adhesion molecule (NCAM) and by the neuroendocrine morphology.

Clinical information of these 38 patients was obtained from patients' records (Table 2).

Table 1. Applied criteria of high-grade non-small cell neuroendocrine carcinoma (HNSCNEC)

1. Solid tumor nesting without either acinar or squamous differentiation
2. Moderate or marked cellular atypia
3. Large cell size with low nuclear/cytoplasmic ratio or abundant cytoplasm
4. Vesicular and/or fine nuclear chromatin
5. Frequent nucleoli
6. Positive immunostaining for one or more neuroendocrine markers (NCAM, chromogranin A and synaptophysin)
7. Ki-67/MIB1 labeling index >40%
8. Frequent mitosis
9. Frequent massive necrosis
10. Intercellular space (cleft) with loose intercellular adhesion
11. Organoid nesting, basal palisading, rosettes and/or trabecular architecture

Proposed criteria for diagnosis of HNSCNEC using biopsy specimens (Table 2 of ref. (9)).

Immunohistochemistry

For immunostaining, 3 μm-thick sections were prepared from the formalin-fixed and paraffin-embedded tumor specimens. After deparaffinization and blocking of endogenous

Table 2. Clinical features of 38 biopsy cases used in this study

Case number	Age	Gender	Smoking	Tumor size on CT (cm)	Tumor margin on CT	Tumor location	Site of biopsy	T	N	M	Stage	Initial pathological diagnosis	Surgery	Status of patients (months)
1	74	Male	Yes	3.6 × 2.0	Non-lob	p	rt M	1	0	0	IA	LCNEC	Yes	NED (73)
2	70	Male	Yes	3.1 × 2.8	lob	p	lt U	2	0	0	IB	LCNEC	Yes	DOD (68)
3	63	Male	Yes	4.0 × 3.0	Non-lob	p	rt M	2	0	0	IB	Poorly diff. carcinoma	Yes	NED (5)
4	82	Male	Yes	3.8 × 2.8	lob	p	lt U	2	0	0	IB	High-grade NE carcinoma	Yes	AWD (17)
5	56	Male	Yes	3.8 × 3.6	lob	p	lt U	2	1	0	IIB	High-grade NE carcinoma	Yes	NED (14)
6	64	Male	Yes	2.4 × 1.9	lob	p	lt L	3	2	0	IIIA	High-grade NE carcinoma	Yes	NED (8)
7	57	Male	Yes	3.5 × 3.5	Non-lob	c	rt U	4	3	0	IIIB	Adenocarcinoma	No	NED (61)
8	72	Male	Yes	2.5 × 1.8	lob	p	rt L	1	3	0	IIIB	Poorly diff. adenocarcinoma	No	DOD (9)
9	67	Male	Yes	4.5 × 4.5	Non-lob	p	lt L	4	3	1	IV	Combined SCLC	No	DOD (8)
10[a]	71	Male	Yes	7 × 6 × 4.5	lob	p	lt L	2	1	1	IV	Non-small cell NE carcinoma	No	DOD (11)
11[a]	74	Male	Yes	3.9 × 2.7	Non-lob	c	rt U	2	3	1	IV	High-grade NE carcinoma	No	DOD (4)
12	75	Male	Yes	2.0 × 1.8	Non-lob	p	lt U	1	2	1	IV	LCNEC	No	NED (62)
13[a]	75	Male	Yes	4.5 × 3.6	lob	c	lt U	2	3	1	IV	LCNEC	No	DOD (3)
14	66	Male	Yes	4.8 × 2.6	Non-lob	p	lt L	2	2	1	IV	High-grade NE carcinoma	No	DOD (7)
15[a]	63	Male	Yes	2.5 × 2.1	Non-lob	p	rt U	1	3	1	IV	SCLC	No	DOD (17)
16	84	Male	Yes	8.7 × 5.0	lob	p	rt L	2	1	1	IV	LCNEC	No	LTF (3)
17	67	Male	Yes	2.6	Non-lob	p	rt U	4	0	1	IV	LCNEC	No	DOD (2)
18[a]	74	Male	Yes	6.5 × 6.0	lob	p	rt U	4	3	1	IV	High-grade NE carcinoma	No	DOD (9)
19	74	Male	Yes	7.5 × 6.0	lob	p	rt U	2	2	1	IV	LCNEC	No	DOD (3)
20	62	Female	Yes	5.3 × 4.3	Non-lob	p	rt L	2	3	1	IV	Large cell carcinoma	No	DOD (7)
21	70	Male	Yes	7.0 × 3.5	lob	p	rt L	4	3	1	IV	Large cell carcinoma	No	DOD (7)
22	76	Male	No	3.0 × 2.3	Non-lob	c	lt L	1	3	1	IV	Combined SCLC	No	DOD (11)
23	59	Male	Yes	8.0 × 5.5	Non-lob	c	rt U	4	3	1	IV	High-grade NE carcinoma	No	DOD (12)
24	73	Male	Yes	2.3 × 2.0	lob	p	rt U	4	0	0	IV	Poorly diff. adenocarcinoma	No	LTF (22)
25	64	Male	Yes	3.5 × 3.4	lob	c	lt L	4	3	1	IV	LCNEC	No	DOD (11)
26	77	Female	Yes	3.8 × 3.0	lob	p	lt L	3	1	1	IV	Large cell carcinoma	No	DOD (20)
27	62	Male	Yes	Unknown	Non-lob	c	rt MB	TX	NX	1	IV	High-grade NE carcinoma	No	LTF (1)
28	89	Male	Yes	4.7 × 3.7	Non-lob	p	rt M	2	3	1	IV	Large cell carcinoma	No	LTF (1)
29	67	Male	Yes	6.2 × 3.6	Non-lob	c	rt U	4	3	1	IV	High-grade NE carcinoma	No	DOD (3)
30	81	Male	No	Unknown	Non-lob	c	lt MB	1	0	0	IV	Large cell carcinoma	No[b]	DOD (16)
31	59	Female	Yes	1.5 × 1.3	lob	c	lt U	1	3	1	IV	LCNEC	No	DOD (7)
32	63	Male	Yes	5.2 × 5.0	lob	p	rt U	4	3	1	IV	Carcinoma	No	DOD (16)
33	69	Male	No	5.5 × 3.2	lob	p	lt U	4	2	1	IV	Large cell carcinoma	No	AWD (31)
34	65	Male	Yes	4.7 × 4.1	lob	p	lt U	4	1	1	IV	LCNEC	No	LTF (17)
35	76	Male	Yes	14.4 × 8.5	Non-lob	c	lt U	4	3	1	IV	High-grade NE carcinoma	No	DOD (2)
36	64	Male	Yes	9.6 × 4.6	Non-lob	c	lt L	4	3	1	IV	Poorly diff. carcinoma	No	DOD (22)
37	65	Female	Yes	2.4 × 1.5	lob	p	lt U	1	3	1	IV	LCNEC	No	DOD (17)
38	67	Male	Yes	6.1 × 4.9	lob	p	rt L	4	3	1	IV	High-grade NE carcinoma	No	DOD (2)

CT, computed tomography; non-lob, non-lobulated margin; p, peripherally located; rt, right; M, middle lobe; LCNEC, large cell neuroendocrine carcinoma; NED, no evidence of disease; lob, lobulated margin; lt, left; U, upper lobe; DOD, dead of disease; NE, neuroendocrine; AWD, alive with disease; L, lower lobe; c, centrally located; SCLC, small cell lung cancer; LTF, lost to follow-up; MB, main bronchus; TX, primary tumor cannot be assessed by imaging; NX, regional lymph nodes cannot be assessed.

[a]The case was examined in ref. (9).

[b]Patient with a history of lung cancer resection 7 years before.

peroxidase activity by 0.3% hydrogen peroxide in methanol, antigen retrieval was carried out using the conventional autoclave method (for 10 min at 121°C) with 0.01 M sodium citrate buffer (pH 6.0), if necessary. The details of the primary antibodies were as follows: NCAM (clone NCC-Lu-243; Nippon Kayaku, Tokyo, ×200), chromogranin A (code No. A0430; DAKO, Glostrup, Denmark, ×5000), synaptophysin (Cat No. 261-01; SIGNET, Dedham, MA, USA, ×100), Ki-67/MIB1 (clone MIB1, code No. M7240; DAKO, ×100), p63 (clone 4A4, Cat No. MS-1081-P; Lab Vision, Kalamazoo, MI, USA, ×500) and keratin 5/6 (code No. M7237; DAKO, ×100). The sections were incubated with the primary antibody for 30 min at 37°C and followed by the Dako EnVision+® detection system (code K4001; Dako Cytomation North America, Inc., CA, USA) and diaminobenzidine as the chromogen to visualize the antigens according to the manufacture's instructions. Negative control slides of each case without first antibody reaction and positive control slides of other normal organ tissue for each antibody were always used and stained simultaneously.

Histological Comparison Between Biopsy and Resected Specimens of Six Resected Cases

Six cases were resected after biopsy because they were in the early stages. We compared the histological findings of these six pairs of biopsy and surgical specimens.

RESULTS

Clinicopathological Findings

Clinicopathological information about the 38 HNSCNEC cases used in this study is shown in Table 2. By computed tomography (CT) imaging of these tumors, 20 tumors (52.6%) showed solid masses with a sharply lobulated margin and 26 tumors (68.4%) were located in the periphery of the lung. The patients' average age was 69.4 (range 56–89 years). Men predominated (M:F = 34:4) and 35 out of 38 patients were smokers (92.1%). Thirty patients (78.9%) were clinically at Stage IV at diagnosis and 32 (84.2%) were unresectable. Sixteen patients (42.1%) had intrapulmonary metastasis and 23 (60.5%) had distant metastasis at the time of diagnosis, in the brain (13 patients), bone (12 patients), liver (8 patients) and adrenal gland (6 patients). Six tumors at clinical TNM Stage I, II or IIIA were surgically resected and pathologically diagnosed as LCNEC from surgical specimens.

In 38 HNSCNEC cases, serum pro-gastrin releasing peptide (pro-GRP; normal value <46.0 pg/ml), carcinoembryonic antigen (normal value <5.0 ng/ml) and neuron-specific enolase (normal value <10.0 ng/ml) levels were elevated in 13 cases (13/37, 35.1%), 24 cases (24/37, 64.9%) and 27 cases (27/38, 71.1%), respectively.

Table 3. Morphological analysis of biopsies in 38 patients, of which 6 were resected and a diagnosis of LCNEC was made

Histological findings	Number of biopsies (%)	Number of biopsies in operated cases (%)
Fine nuclear chromatin	30 (78.9)	6 (100)
Intercellular clefts	21 (55.3)	4 (66.7)
Prominent nucleoli	18 (47.4)	2 (33.3)
Massive necrosis	17 (44.7)	2 (33.3)
Organoid nesting	14 (36.8)	1 (16.7)
Peripheral palisading	6 (15.8)	1 (16.7)
Rosette formation	6 (15.8)	1 (16.7)
Trabecular arrangement	5 (13.2)	0 (0)

Histological and Immunohistochemical Analyses

Initial pathological diagnoses of 38 HNSCNEC biopsy specimens were shown in Table 2. Eleven cases were finally diagnosed as LCNEC, and non-small cell neuroendocrine carcinoma (case No. 10) was most likely to be LCNEC. Eleven cases were given the diagnosis of high-grade neuroendocrine carcinoma, which seemed to be difficult to differentiate from SCLC. On the other hand, only three cases were diagnosed as SCLC including the combined type. Eleven cases were poorly differentiated carcinoma including large cell carcinoma and adenocarcinoma. These results indicated that two-thirds of HNSCNEC biopsy cases suggested to the pathologists the diagnosis of high-grade neuroendocrine carcinoma and one-third was histologically beyond the scope of neuroendocrine carcinoma.

All HNSCNECs were composed of NSCLC with large nuclei, three times larger than resting lymphocytes and abundant cytoplasm or a low nuclear/ cytoplasmic ratio. As shown in Table 3, morphological architectural characteristics of LCNEC proposed by Travis et al. (1) were reviewed in the 38 biopsy specimens used in this study. With regard to neuroendocrine morphology, organoid nesting (Fig. 1a) was the most frequently observed structure, but its frequency was only 36.8% of all specimens. Peripheral palisading, rosette formation (Fig. 1b) and trabecular arrangement were observed in only 6 (15.8%), 6 (15.8%) and 5 (13.2%) specimens, respectively. In other morphological characteristics of LCNEC, massive necrosis (Fig. 1c) was seen in 17 biopsy specimens (44.7%). Although most tumor cells contained nuclei with a fine chromatin pattern (Fig. 1d) and faint or visible nucleoli, prominent nucleoli (Fig. 1d, arrows) were observed in about half of all specimens (47.4%). Again, in about half of all biopsy specimens (55.3%), tumor cells had a distinct cell border with intercellular clefts and were often discohesive (Fig. 1b and d).

We counted mitotic figures in all 38 tumor specimens. Among them, 11 tumors lacked enough area for mitotic counting, that is to say, 9 HPFs or less. There were 7 tumors

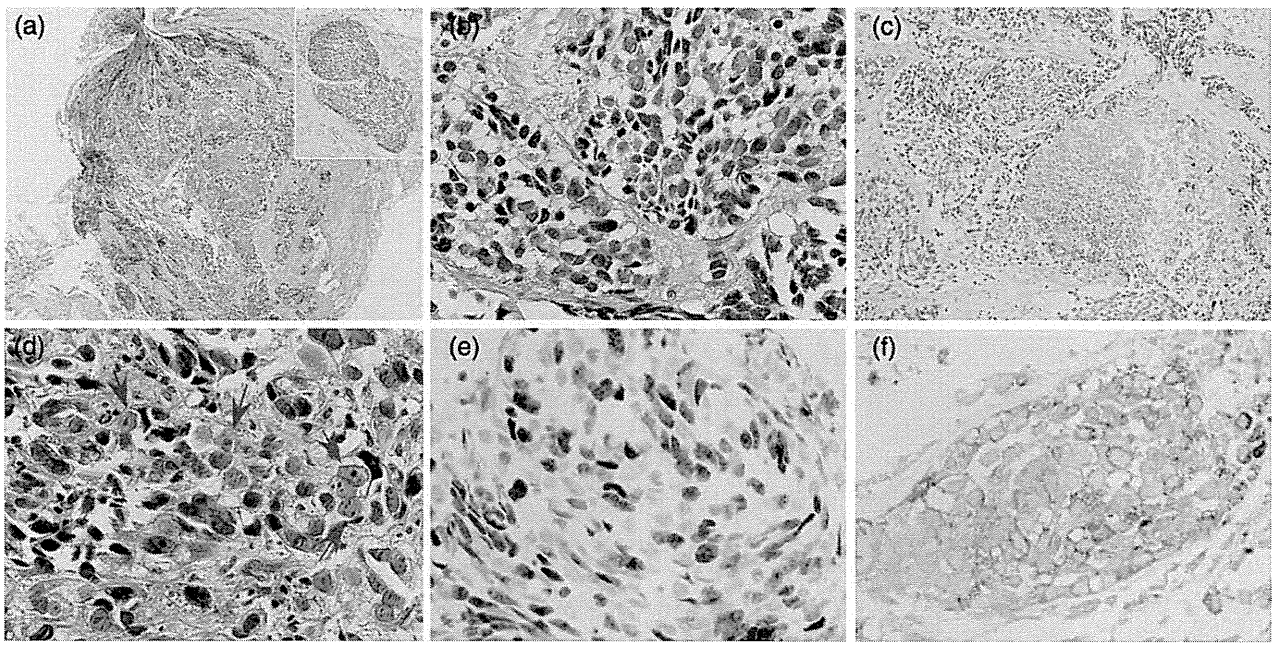

Figure 1. Characteristic morphological features of 38 biopsy specimens used in this study. In the low-powered view [a, hematoxylin and eosin (H&E), ×4], organoid nesting (a, inset) and massive necrosis (c, H&E, ×10) are recognized structure features in the biopsy specimen. In the high-powered view, tumor cells reveal special arrangements such as rosette formation (b, H&E, ×40), intercellular cleft (b) as well as nuclear characteristics such as a fine chromatin pattern (d, H&E, ×40) and prominent nucleoli (d, arrows, H&E, ×40). The immunohistochemistry shows high nuclear positivity of Ki-67 (e, ×40) and membrane positivity of the neural cell adhesion molecule (NCAM) (f, ×40) in the majority of biopsy specimens.

in which no mitotic figure was found, and another 7 tumors which had a high mitotic rate consistent with 11 or greater per 10 HPFs. In contrast to the mitotic counting, Ki-67/MIB1 labeling indices could be evaluated in all 38 tumor specimens (Fig. 1e), which ranged from 41.9 to 99.0% (median: 80.5%). The 7 biopsy specimens with 11 or more mitoses/10 HPFs showed high Ki-67/MIB1 labeling indices from 64.5 to 98.0% (median: 87.3%).

The results of the neuroendocrine immunophenotype in 38 HNSCNECs are shown in Table 4. NCAM (Fig. 1f) was the most frequently observed neuroendocrine marker (78.9%) among them. There were 12 (31.6%) triple marker-positive cases. No tumor was positive for chromogranin A alone.

As shown in Table 2, six patients underwent surgery after biopsy, and all resected tumors were pathologically examined and diagnosed as LCNEC. In case No. 1 (Table 2), for example, the biopsied specimen showed organoid nesting (Fig. 2a) and rosette formation, and tumor cells had abundant cytoplasm and crushed nuclei with a fine chromatin pattern (Fig. 2b). The immunohistochemistry elucidated NCAM (Fig. 2c) in addition to a high Ki-67/MIB1 labeling index (Fig. 2d). This biopsied tumor was pathologically diagnosed as strongly suggestive of LCNEC. As this tumor was peripherally located and clinically showed T1N0M0 (Stage IA), a right middle lobectomy was carried out after biopsy. Morphologically, the resected tumor was composed of large tumor cells with abundant cytoplasm, and showed fine or vesicular nuclei with prominent nucleoli and frequent mitoses (Fig. 3a and b). There was a necrotic area and tumor cells formed characteristic intercellular clefts and rosettes. Immunohistochemical results were almost identical to those of the biopsy specimen. Finally, the diagnosis of LCNEC was confirmed for this tumor.

Table 4. Distribution of immunohistochemical results of neuroendocrine markers in 38 biopsy specimens

Positively stained markers	Number of positive biopsy specimens (%)
NCAM	30 (78.9)
NCAM, SYN, CGA	12 (31.6)
NCAM, SYN	3 (7.9)
NCAM, CGA	1 (2.6)
NCAM alone	14 (36.8)
SYN	23 (60.5)
SYN, CGA	5 (13.2)
SYN alone	3 (7.9)
CGA	18 (47.4)
CGA alone	0 (0)

NCAM, neural cell adhesion molecule; SYN, syanptophysin; CGA, chromogranin A.

Histological details of six biopsy specimens with a confirmed diagnosis of LCNEC are shown in Tables 3 and 5. Although the sample size was small, morphological characteristics showed a similar tendency to those of other

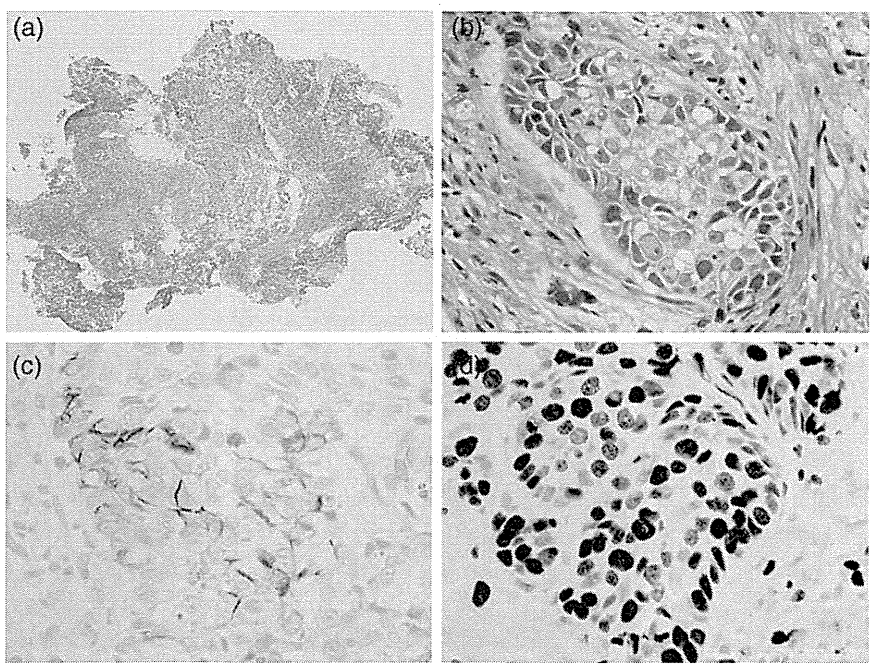

Figure 2. Morphological and immunohistochemical features of a biopsy specimen (Case 1). The low-powered view shows an irregular organoid nesting structure (a, H&E, ×4). In the high-powered view, the tumor consists of two types of cells, cells with nuclei with a fine chromatin pattern and larger cells with prominent nucleoli, and tumor cells show incomplete rosette and intercellular cleft formation (b, H&E, ×40). Immunohistochemically, NCAM (c, ×40) is positive and the Ki-67 labeling index is high (d, ×40).

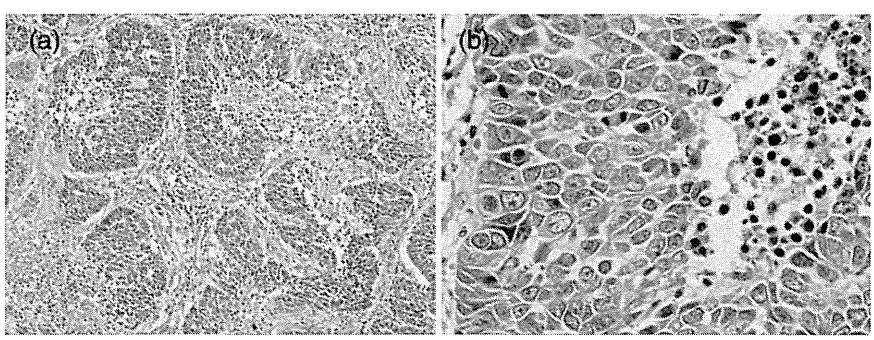

Figure 3. The morphology of a surgically resected tumor of Case 1. Tumor cells form an organoid nesting structure with necrosis (a, H&E, ×4) and show the features of non-small cell lung cancer with large nuclei with prominent nucleoli (b, H&E, ×40).

HNSCNECs. Among them, a fine nuclear chromatin pattern was the most frequent finding. These six biopsy specimens had a Ki-67/MIB1 labeling index from 56.6 to 98.7% (median: 70.6%).

The comparisons between histological findings of the biopsy specimen and the resected one in each case are shown in Fig. 4 and Table 5. Fine chromatin in biopsy specimens generally has changed to be rather course chromatin in resected specimens, and peripheral palisading, rosette formation, trabecular arrangement and organoid pattern have become clearly visible in four cases out of six.

DISCUSSION

Using our HNSCNEC criteria (9), we selected 38 cases from all lung biopsy specimens obtained in our hospital from 2002 to 2009. As shown in Table 2, various clinical characteristics of the 38 cases corresponded well with those of LCNEC cases, which have been already reported by many previous papers using surgically resected LCNEC (1,11–18). For example, CT findings have already revealed that LCNEC is peripherally located and has a solid mass with a lobulated margin (19). In this study, the serum pro-GRP level was elevated in 13 (35.1%) of 37 cases, but no report about the frequency of elevated serum pro-GRP in LCNEC has appeared in the literature. Previous investigations reported that the frequency of the elevated serum pro-GRP level is 68% in SCLC and 4.2% in NSCLC (20). The frequency of the elevated serum pro-GRP level might be low compared with that of SCLC.

The majority of the 38 cases had pathologically Stage IV disease and many patients died within 1 year after the lung

Table 5. Comparison of histological findings between biopsy specimen and resected ones (biopsy/resection) in Cases 1–6 in Table 2

Finding	Cytoplasm[a]	Nucleus[a]		Structure[a]					
	Wide	Fine chromatin	Nucleolar prominence	Peripheral palisading	Rosettes	Trabecular	Organoid	Discohesive	Necrosis
Case 1*	2/1	2/0[b]	2/2	2/1	2/2	0/0	3/3	1/3	0/2
Case 2	1/1	2/1	1/2	1/3	1/1	0/3	1/3	1/1	0/0
Case 3**	1/2	2/0	2/2	0/0	0/3	0/1	0/1	0/1	0/1
Case 4	2/2	2/1	1/2	0/0	1/2	0/0	1/3	1/0	2/1
Case 5**	2/3	3/1	0/2	0/2	0/1	0/1	0/2	3/1	2/2
Case 6	3/3	3/1	0/1	0/0	0/0	0/0	1/2	1/0	0/1

[a]Each numeral indicates: [b]0, not observed; 1, partially or focally observed; 2, easily and/or widely observed; 3, remarkably observed. Underline indicates that a difference of 2 or 3 degrees exists between evaluation numerals of biopsy specimen and resected one.
*Findings shown in Figs 2 and 3.
**Findings shown in Fig. 4.

biopsy in this study. LCNEC is a highly malignant neoplasm and previous comparative studies using surgically resected high-grade neuroendocrine carcinoma cases revealed that the prognosis of LCNEC is similar to that of SCLC (16,18,21). Of the 38 cases in this study, 6 were clinical TNM Stage I, II or IIIA, which then underwent surgery after biopsy and the diagnosis was pathologically confirmed as LCNEC using surgically resected tumor specimens. These findings suggest that our HNSCNEC criteria are applicable in practical use for the diagnosis of LCNEC using biopsy specimens.

Among high-grade neuroendocrine carcinoma of the lung, the overall clinicopathological features of LCNEC, including unresectable cases, is still uncertain compared with those of SCLC (22). As it is extremely difficult to make a final diagnosis of LCNEC using only biopsy and/or cytology specimens, the true frequency of LCNEC is undetermined. As the results of a study using surgically resected LCNEC cases, the frequency has been reported to be 1.6–3.1% (7,11,13). If all 38 cases in our study were compatible with LCNEC, the true frequency of LCNEC would be 3.7% (38 out of 1040 biopsies). In the Japanese lung cancer registry study using a large number of surgical and non-surgical cases in 2002, the proportions of adenocarcinoma, squamous cell carcinoma and small cell carcinoma were 56.7, 25.7 and 9.2%, respectively (23). In our biopsy series, these proportions were 49.8, 30.6 and 11.6%, which showed a similar tendency to those of the Japanese lung cancer registry study, although the number is small; therefore, the real frequency of LCNEC might be over 3.7%.

In 1991, Travis et al. (1) proposed a category of LCNEC that was adopted in the WHO classification of lung cancer in 1999 and 2004 (2,3); however, these criteria are applicable only to surgically resected specimens, not to biopsy specimens. Biopsy specimens are too small to have enough morphological information and to count the number of mitoses; therefore, we modified the WHO criteria of LCNEC and proposed new criteria for diagnosing LCNEC using biopsy specimens (9). In our criteria, the immunohistochemical Ki-67/MIB1 labeling index was used instead of the mitotic count. Our study clearly showed that it was very difficult or impossible to count mitoses in small biopsy specimens, but immunohistochemical Ki-67/MIB1 labeling indices could be useful for evaluating the proliferation activity. Fortunately, for 7 of 38 biopsy specimens the number of mitoses could be counted and these specimens were elucidated to have more proliferative activity than biopsy specimens with an impossible mitosis count. In Stage I NSCLCs, the mean Ki-67/MIB1 labeling index is 19.3% (24), and the prognosis of NSCLC patients is reported to differ between the '20% or higher' group and the 'lower than 20%' group of the Ki67/MIB1 labeling index (25). When compared with the positivity of the Ki-67/MIB1 labeling index of NSCLC, the labeling index of our biopsy specimens in this study was quite high, which suggests that high proliferative activity is one of the characteristic features of LCNEC (26).

In the WHO criteria, large cell morphology is one of the most important criteria for LCNEC, such as large tumor cell size with abundant cytoplasm as well as vesicular nuclei and prominent nucleoli (2,3). Our criteria of HNSCNEC (9) also followed the WHO criteria; however, the recognition of this large cell morphology is problematic among pathologists. In previously published papers discussing the interobserver variability of SCLC and LCNEC, the cytologic features of a nuclear/cytoplasmic ratio may be recognized arbitrarily or not quantitatively among diagnostic pathologists (4,5,17,27). It is also reported that there are borderline cases between LCNEC and SCLC in high-grade neuroendocrine carcinoma (17). In this study, about one-third of cases were diagnosed as high-grade neuroendocrine carcinoma, but there was a hesitation to diagnose them as LCNEC, because of the cytological similarity to SCLC. Previous studies using surgically resected tumors revealed that LCNEC and SCLC have distinct characteristic morphological features; namely, a large tumor cell size, peripheral palisading and prominent nucleoli

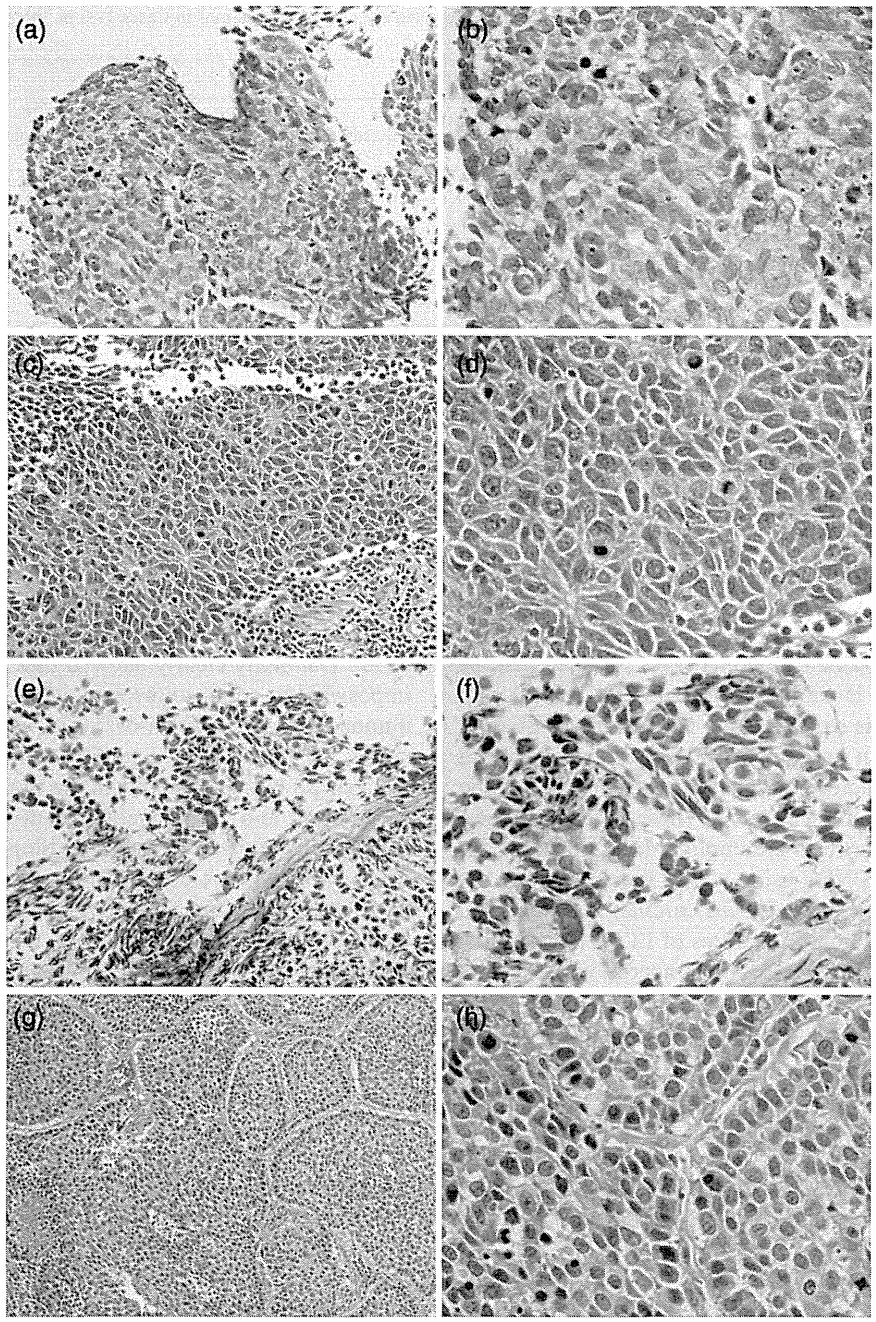

Figure 4. Histological comparison between biopsy and resected specimens in two of the resected six cases. In Case 3 (a–d), the biopsy specimen shows fine chromatin architecture with visible nucleoli and an obscure rosette pattern (a, H&E, ×20; b, H&E, ×40). However, in the resected specimen, a large number of rosettes are observed (c, H&E, ×20; d, H&E, ×40). The chromatin is coarse and the cytoplasm is abundant. In Case 5 (e–h), the biopsy specimen is crushed and neither the neuroendocrine structure nor nucleoli can be recognized (e, H&E, ×20; f, H&E, ×40). However, in the resected specimen, peripheral palisading, organoid nesting and nucleoli are clearly observed (g, H&E, ×10; h, H&E, ×40).

are characteristic of LCNEC, and a small cell size, high nuclear/cytoplasmic ratio and fine chromatin pattern are characteristic findings of SCLC (1–3,17,18); however, these morphological features are known to overlap among LCNEC and SCLC (1,17,18). As shown in Table 3, morphological analysis of 38 biopsy specimens revealed that the incidence of the specimen with a fine chromatin pattern was the highest, and those with intracellular cleft and prominent nucleoli followed in a decreasing order. Therefore, the morphological fine chromatin pattern is not only seen in surgically resected SCLC, but is also a characteristic finding of LCNEC in biopsy specimens as shown in Figs 2 and 4, Tables 3 and 5. Even in surgically resected LCNEC, the appearance of tumor cells with fine nuclear chromatin has been

Table 6. Comparison among diagnostic criteria of WHO (3), our former proposal (9) and the present study for LCNEC

Findings	WHO 2004 (3)	Igawa et al. (9)	Present study
Applied specimen	Resected	Biopsy	Biopsy
Differentiation			
Histological differentiation without SCLC morphology, glandular and squamous differentiation	EA, Ess	EA, Ess	EA, Ess
Cell size			
Large cell size and low nuclear/cytoplasmic ratio	EA, Ess	EA, Ess	EA, Ess
Neuroendocrine differentiation			
Immunostain positive for at least one of neuroendocrine markers	EA, Ess	EA, Ess	EA, Ess
Proliferating activity			
Mitosis (11 or greater/mm^2)	EA, Ess	DA	DA, nd
Ki67/MiB-1 labeling index (>40%)	nd	EA, Ess	EA, Ess
Nuclear findings			
Nucleoli (frequent and prominent)	EA, LS	EA, LS	EA, LS
Chromatin pattern (vesicular, coarse or fine)[a]	EA, Ess (vesicular or coarse chromatin pattern is common)	EA, Ess	EA (fine chromatin pattern is common)
Neuroendocrine morphology			
Organoid nesting, palisading and rosettes, trabecular	EA, Ess	CA, SF	CA, SF
Others			
Necrosis	EA, common	CA, SF	CA, SF
Cellular atypia	nd	Moderate to severe, Ess	nd
Intercellular space (cleft)	nd	EA, SF	EA, SF

LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma; EA, easily assessed; Ess, essential; DA, difficult to be assessed; nd, not determined, LS, lacking sometimes; CA, can be assessed sometimes; SF, supportive findings.
[a]Ref. (2) and Table 1.08 in ref. (3).

noted (1,18). As our resected cases were found to show a chromatin pattern changed from the fine pattern in the biopsy specimen to a coarse or vesicular pattern (Fig. 4 and Table 5), it is supposed that the different fixative conditions might have some effects on the chromatin morphology of tumor cells. The greatest difference might be due to a rapid start and full immersion in formalin soon after taking a biopsy specimen, in contrast to a delayed start and immersion in a relatively small ratio of fixative amount/specimen size for a resected specimen, resulting in fine chromatin morphology and a coarse or vesicular one, respectively. Furthermore, as another histological finding, intercellular cleft might reflect poor intercellular connection and might be one of the characteristics of LCNEC.

In LCNEC, the appearance of neuroendocrine morphology such as organoid nesting, palisading, rosette and trabeculae has been frequently observed and their frequencies are reported to be 90.9, 59.1, 72.7 and 31.8%, respectively (18). In contrast, Figs 2 and 4 revealed that the neuroendocrine morphology was relatively difficult to recognize in biopsy specimens. This may be attributed to the small size of specimens. Therefore, immunohistochemical detection of a neuroendocrine marker is a more reliable method to identify the neuroendocrine features of biopsy specimens. In a previous report, synaptophysin was frequently positive at the rate of 77% in LCNEC, and chromogranin A and CD56 followed (17). In our study, NCAM was the most frequently immunopositive neuroendocrine marker and chromogranin A followed after synaptophysin. This may be explained by the difference of the pattern of immunostaining because NCAM tends to stain tumor cells diffusely compared with chromogranin A or synaptophysin. For immunohistochemical evaluation of neuroendocrine features in both surgically resected or biopsy samples, immunohistochemistry using three neuroendocrine markers is necessary.

Although the cases in the early stage can be resected and histologically confirmed, the cases in an advanced stage should receive chemotherapy without histologically examining resected specimens. Then the criteria of HNSCNEC may give the chance for lesions that have the possibility of being LCNEC to be treated as inoperable advanced probable LCNEC cases. For this reason some papers have reported that the clinical efficacy of chemotherapy for LCNEC is comparable with that for SCLC (7–10); the diagnosis of

Table 7. Proposed diagnostic criteria for possible LCNEC using biopsy specimens

Major points
1. Poorly differentiated NSCLC with neither acinar nor squamous differentiation
(a) Tumor cell contains a nucleus larger than the size of three small resting lymphocytes, and low nuclear/cytoplasmic ratio or abundant cytoplasm
(b) Tumor nucleus with a fine chromatin pattern and/or prominent nucleoli
2. Ki-67/MIB1 labeling index >40%
3. Positive immunostaining for one or more neuroendocrine markers (NCAM, chromogranin A and synaptophysin)
Minor points
1. Neuroendocrine morphology (organoid nesting, peripheral palisading[a], rosettes, trabecular architecture)
2. Frequent massive necrosis
3. Intercellular space (cleft) or discohesiveness

[a]Peripheral palisading is mentioned as 'basal palisading' in Table 1; both stand for a similar finding.

HNSCNEC as a surrogate of LCNEC or as a probable LCNEC would help these lesions to receive more appropriate therapy. When the criteria of HNSCNEC work, the number of patients who receive chemotherapy for LCNEC, comparable to that for SCLC, might increase by 0.6–2.1% of the number of all lung carcinomas. This estimation is according to the comparison between the reported frequency (1.6–3.1%) of LCNEC (7,11,13) and the incidence (3.7%, 38 out of 1040 lung biopsies positive for cancer) of HNSCNEC in our hospital, which has already been discussed.

WHO criteria (3), our previous criteria (9) and our new criteria have three common essential points as indicated in the upper one-third of Table 6; and there is one more essential point on proliferating activity. In our new criteria, the Ki67/MIB1 labeling index is adopted instead of mitotic counting. Then our new criteria can be shown as in Table 7. Summarizing the data obtained in this study using biopsy specimens, we simplified our previous criteria for HNSCNEC and proposed new diagnostic criteria suitable for possible LCNEC (28). In the new criteria (Table 7), the major points are essential for diagnosing LCNEC using biopsy specimens. In addition to the major points, the findings due to additional minor points would increase the possibility of an LCNEC diagnosis. We expect these new criteria to be used in routine surgical pathology and to facilitate the clinicopathological study of high-grade neuroendocrine carcinoma, especially LCNEC.

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Conflict of interest statement

None declared.

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ORIGINAL ARTICLE

Efficacy of bevacizumab-containing chemotherapy for non-squamous non-small cell lung cancer with bone metastases

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Abstract
Purpose Skeletal-related events (SREs) negatively affect the quality of life of patients with cancer. Vascular endothelial growth factor receptor (VEGFR)-targeted therapy is effective against bone metastasis in animal models, but the clinical efficacy of anti-VEGFR inhibitors against bone metastases remains unclear. Therefore, we aimed to investigate the efficacy of chemotherapy with bevacizumab, an anti-VEGF antibody, against bone metastases.
Methods We retrospectively reviewed consecutive patients with non-squamous non-small cell lung cancer who received first-line platinum-based chemotherapy with zoledronic acid at Shizuoka Cancer Center between 2007 and 2011.
Results Of 25 patients, 13 received bevacizumab-based chemotherapy (BEV group) and 12 received chemotherapy without bevacizumab (non-BEV group). The overall response (54 vs. 8 %, $p = 0.01$) and disease control (100 vs. 50 %, $p = 0.01$) rates were higher in the BEV group than in the non-BEV group. The bone-specific response (23 vs. 0 %, $p = 0.038$) and disease control (100 vs. 67 %, $p = 0.01$) rates were also higher in the BEV group. The median time to progression (TTP) for bone metastases was higher in the BEV group (13.7 vs. 4.3 months, $p = 0.06$), whereas that for overall disease was similar between the groups (5.7 vs. 2.6 months, $p = 0.17$). The proportions of patients with SREs were 23 and 50 % in the BEV and non-BEV groups, respectively ($p = 0.16$).
Conclusion Bevacizumab might potentiate the antitumor activity of chemotherapy against systemic disease and bone metastases, prolonging bone-specific TTP and reducing the incidence of SRE.

Keywords Bone metastases · Skeletal-related event · Bevacizumab · Chemotherapy

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Introduction

The incidence of bone metastases in patients with lung cancer is approximately 30–40 %, and the median survival time of patients with such metastases is 7 months [1]. A more recent retrospective review of 435 patients with non-small cell lung cancer (NSCLC) indicated an incidence of 24 % for skeletal metastases. In this review, most instances of skeletal metastases (66 %) were detected at the time of initial staging [2].

Patients with metastatic bone disease frequently experience osteoclast-mediated bone destruction, resulting in clinically important complications such as a fracture, the need for bone radiation or surgical therapy, spinal cord compression, or hypercalcemia [3, 4]. These complications, collectively known as skeletal-related events (SREs) [5–7], lead to pain and decreased quality of life [8]. Thus, SREs have a negative impact on the quality of life, performance status, and functioning of patients with cancer. In a Japanese retrospective review of 259 patients with NSCLC [9], 30 % of patients were found to have skeletal metastases during their clinical course, and 50 % of these patients had SREs. Among 135 stage IV patients, 41 % had