Molecular markers for reinforcement of histological subclassification of neuroendocrine lung tumors

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The degree of malignancy of neuroendocrine lung tumors (NEs) increases in this order: from typical carcinoids (TCs) through atypical carcinoids (ACs) to large cell neuroendocrine carcinomas (LCNECs) and small cell lung carcinomas (SCLCs). However, histological classification has sometimes proved difficult. We here investigated loss of heterozygosity (LOH) using eight microsatellite markers and expression of p53, Bcl-2 and Bax proteins using immunohistochemical methods in 57 NEs (19 TCs, 5 ACs, 14 LCNECs and 19 SCLCs), looking for objective genetic markers to distinguish between subtypes. The frequencies of LOHs on D3S1300, RBi2 and TP53, the combinations of LOH status for RBi2 and TP53, and the immunohistochemically demonstrated Bcl-2/Bax ratios and p53-positive rates significantly differed among histopathologically diagnosed NEs. Differentiation between TC and AC was possible with reference to LOH on D3S1300, RBi2 and TP53, and the combined LOH status on RBi2 and TP53 (i.e., both LOH(–) versus one LOH(+)). For comparison between AC and LCNEC+SCLC, LOH on TP53 or the combination of two markers—one LOH(+) versus both LOH(+)—was applied. Furthermore, in three discordant cases of diagnoses based on histology and LOH markers, diagnoses using the latter were considered to be more probable by survival analysis. The present study indicated that assessment of LOHs using microsatellite markers could provide objective markers that can distinguish subtypes of NEs, for which histological assessment may commonly result in disagreement. (Cancer Sci 2004; 95: 334–341)

Neuroendocrine lung tumors (NEs) include four histological types: typical carcinoid (TC), atypical carcinoid (AC), large cell neuroendocrine carcinoma (LCNEC) and small cell lung carcinoma (SCLC). These show diverse clinical behaviors, but have morphological and functional features in common.[1)] TC grows slowly and has a low malignant potential and excellent prognosis. AC is more aggressive with survival intermediate between TC, and LCNEC and SCLC.[2)] The latter two are highly malignant diseases marked by rapid and disseminated tumor growth in the majority of patients. Since the degree of malignancy of NEs correlates well with the histological type, it is essential to be able to distinguish between them. At present, subclassification of NEs depends on evaluating histologic criteria: carcinoid or neuroendocrine morphology, cytologic features, number of mitoses, tumor necrosis and positive immunohistochemical staining. Among these, the number of mitoses and necroses are most important for separating atypical from typical carcinoids, large cell neuroendocrine and small cell carcinomas.[1,3)] However, these parameters are influenced by tissue sampling, and if the sample is a biopsy specimen, it may be impossible to make a reliable count number of mitoses per 2 mm^2 or to find necrosis. Even with surgical material, the number of mitoses in a histological specimen taken from one part of a tumor may not reflect the true number of mitoses in the tumor. Therefore, there is often difficulty in making a specific diagnosis based on the available criteria: even among experienced lung pathologists, disagreement commonly occurs in distinguishing between TC and AC, AC and LCNEC, and LCNEC and SCLC.[4–6)] Thus, it is clear that alternative objective markers are needed to assist in classification.

Recently, analyses of gene expression profiles using cDNA arrays for small cell carcinoma and carcinoid of the lung, along with the creation of a hierarchical clustering dendrogram with comparative genomic hybridization (CGH) data for carcinoids —including atypical carcinoid, other neuroendocrine carcinomas and non-small cell lung carcinomas—have been reported.[7,8)] The results suggest that the carcinoid belongs to a group discrete from small cell and non-small cell lung carcinoma, which suggests the possibility that gene abnormalities can be objective markers for the classification of NEs. Genetic alterations of SCLCs have been well studied. Activation of dominant proto-oncogenes[9)] and inactivation of recessive tumor suppressor genes associated with chromosomal deletion, such as the *FHIT* (3p), *MCC* (5q), *APC* (5q), *RB* (13q) and *p53* (17p), have been implicated in the genesis of human small cell lung cancer.[10–12)] While genetic analysis of TC, AC and LCNEC has been conducted only very recently, alterations of several chromosome arms (*p53*, *RB* and *MEN 1*) have been reported in numerous studies.[8,13–18)] Overall, however, the literature provides limited comparative information about molecular alterations among the four lung NEs. More extensive analysis of NEs at the DNA level is thus warranted to correlate genetic alterations with histological types.

Genetic regulation of apoptosis is of critical importance during tumorigenesis,[19,20)] with the Bcl-2 protein suppressing, and Bax promoting, this form of cell death. Expression levels of these proteins are regulated by wild-type, but not mutant, p53, and the ratio of suppressor to promoter helps determine a cell's likelihood of undergoing apoptosis.[21,22)] The relationships of these proteins in NEs were recently examined using immunohistochemistry[23)]: A significant inversion of the Bcl-2/Bax ratio between low-grade NEs (TC and AC) and high-grade NEs (LCNEC and SCLC), with predominant Bax expression in the former and predominant Bcl-2 expression in the latter, was found. However, differences in ratios between TC and AC, AC and LCNEC, and LCNEC and SCLC, have not yet been analyzed.

In the present paper, we document findings on loss of heterozygosity (LOH) using many microsatellite markers for DNA and expression levels of p53, Bcl-2 and Bax proteins assessed

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immunohistochemically in NEs, with emphasis on objective genetic markers for distinguishing between histological types.

Materials and Methods

Tissue specimens. A total of 57 surgically resected primary lung NEs and corresponding normal tissue samples were obtained, 33 from the Saitama Cancer Center, 19 from the Cancer Institute Hospital and 5 from the Antituberculosis Association Fukujuji Hospital. All were formalin-fixed and paraffin-embedded. Blocks of each tumor were cut to yield nine serial sections. The first and the last, cut at 4 μm, were used for hematoxylin-eosin (HE) staining, and another six, cut at the same thickness, were mounted on silanized slide glasses for immunohistochemical staining. The remaining one, 10 μm thick, was used for microdissection to extract DNA.

Slides stained with HE were reviewed by one of the authors (E.T.) for histological diagnosis based on the 1999 WHO classification.[3] Pathological stages (p-stage) were determined using the International Union Against Cancer (UICC) TNM staging system.[24]

DNA extraction and LOH analysis. We performed microdisection under direct observation with a stereo microscope using an 18G or 22G needle, and DNA was extracted as previously described.[25] The DNA concentration was adjusted to about 50 cell equivalents per microliter. To assess the quality of ex-

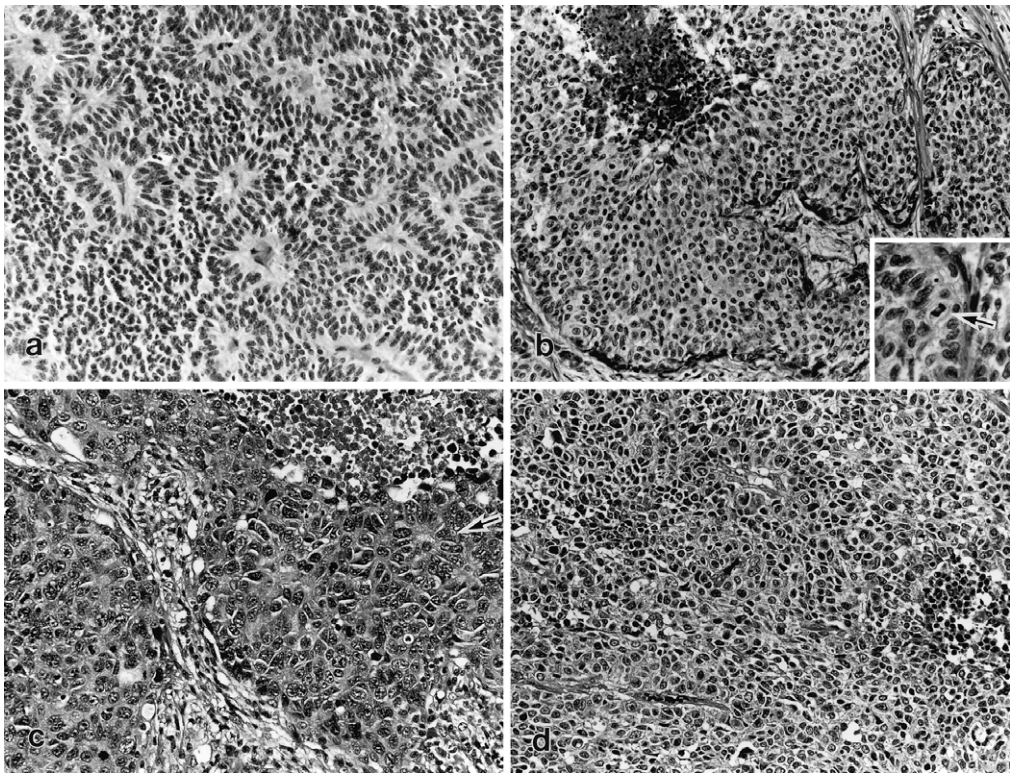

Fig. 1. Histological types of neuroendocrine lung tumors. a) Typical carcinoid. Tumor cells having uniform cytologic features with oval nuclei and moderate cytoplasm are growing in a palisading or pseudo-rosette pattern. b) Atypical carcinoid. Necroses are present within the nest of tumor cells, which show anaplastic features including increased mitoses (inset, arrow) and nuclear atypia. c) Large cell neuroendocrine carcinoma. Rosette-like formation (arrow) is present along with necrosis at the right upper corner. Mitoses are numerous. d) Small cell lung carcinoma. The tumor cells are densely packed and small, with scant cytoplasm and finely granular nuclear chromatin. Hematoxylin-eosin staining. Original magnification, ×200.

Table 1. Clincopathological parameters for lung NE tumors

Histological type	No. of patients	Age (years)	Sex	Tumor size (mm)	Mitotic count[1)]	Necrosis	Pathological stage			Chemotherapy or radiotherapy before surgery	Smoking index[2)]
		Mean±SD (range)	Male/ female	Mean±SD (range)	Mean±SD (range)	+/–	I	II	III	+/–	Mean±SD (range)
TC	19	49.2±14.8 (76–22)	12/7	24.8±11.1 (50–8)	0.5±0.6 (1–0)	0/19	17	2	0	1/18	348.8±405.5 (1480–0)
AC	5	48.2±7.6 (62–40)	3/2	33.2±10.3 (50–20)	5.0±3.3 (9–2)	2/3	3	1	1	2/3	408.0±413.5 (4130–225)
LCNEC	14	69.9±7.7 (82–55)	14/0	38.2±18.1 (80–17)	40.5±14.7 (71–14)	14/0	7	2	5	1/13	1410.7±1077.2 (4130–225)
SCLC	19	63.6±8.2 (73–47)	15/4	28.1±11.8 (46–10)	75.7±43.9 (162–15)	19/0	11	3	5	4/15	1048.6±727.3 (2500–0)

1) Number of mitotic cells per 2 mm^2 (ten high-power fields).
2) Number of cigarettes smoked per day×duration of smoking (years).

tracted DNA, taking into account degradation by formalin fixation, we used PCR amplification of GAPDH with an expected product size of about 120 bases. Samples in which the product was not observed or was present in only small amounts were not analyzed.

LOH was examined using a PCR-based approach as follows. Eight microsatellite markers were used for amplification of tumor and normal DNA samples: CI31107(3p),[26] D3S1300(3p),[25] D5S644(5q),[27] D9S171(9p),[28] mfd220(9q),[29] D11S4938(11q),[30] RBi2(13q)[31] and TP53(17p).[32] PCR amplification reactions were performed in a 10.0-μl final volume, with 2 μl (about 100 cells) of template DNA, 20 ng of forward and reverse primers (forward primer end-labeled with deoxyadenosine triphosphate tagged with γ-phosphorus 32 by T4 polynucleotide kinase), 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 0.1 mg/ml gelatin, 1.5 m*M* $MgCl_2$, 0.1 m*M* each dNTP and 1 unit of *Taq* polymerase. The amplification reactions were performed by means of 35 cycles in a GeneAmp PCR System 9600 (Perkin-Elmer Corp.) with appropriate annealing temperature (55–62°C) for each primer. PCR products were mixed with sequencing stop buffer and denatured at 75°C for 10 min. For LOH analysis, 3 μl aliquots were loaded onto 6% acrylamide:bis (19:1), 7 *M* urea and 32% formamide gels for electrophoresis at 2000 V for 1–2 h. The gels were dried and placed in contact with X-ray film overnight. For informative cases, allelic loss was determined when the intensity of one allele in the tumor DNA was judged by two independent observers (E.T. and Y.K.) to be obviously (visually) reduced compared with that for the corresponding normal allele.

Immunohistochemical analysis of p53, Bcl-2 and Bax protein. Immunohistochemical staining was performed by the standard method as reported previously.[33] In brief, sections were deparaffinized with xylene and hydrated through a graded series of ethanol. To enhance antigenicity, they were immersed in 10 m*M* citrate buffer (pH 6.0) and autoclaved for 15 min. Next, to inhibit endogenous peroxide activity, the sections were treated with 0.3% hydrogen peroxide in methanol. Exposure to primary antibodies was done overnight at 4°C. For p53, the DO-7 monoclonal antibody (1/50; Dako, Glostrup, Denmark), was used. Bcl-2 was detected with the clone 124 monoclonal antibody from Dako (1/20), and Bax was detected with the N-20 rabbit polyclonal antibody (1/200; Santa Cruz Biotechnology, Santa Cruz, CA). Incubation with the secondary antibody—peroxidase-labeled ENVISION polymer reagent (Dako)—was done for 45 min at room temperature. After visualization of reaction complexes with 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.03% hydrogen peroxide in 50 m*M* Tris-HCl buffer (pH 7.6), sections were observed under a light microscope. For controls, the antibody was replaced with normal rabbit or mouse serum.

A total of 500 or more tumor cells was examined at an original magnification of ×400 by light microscopy, and the percentages of positive cells, along with the intensity of staining, were recorded for each immunostained specimen. At the time, observers were blind as to case numbers. Immunohistochemical detection of p53 protein was considered positive when at least 15% of the cells had stained nuclei, a cut-off inferred from a previous comparison of complete sequence analysis of the *p53* gene and immunohistochemical staining in 107 cases of adenocarcinoma of the lung. Positive immunostaining, so defined, was 76% concordant with a missense mutation, with the wild type showing 27% positive staining.[34] For Bcl-2 and Bax, immunostaining scores were calculated by multiplying the percentage of labeled cells by the intensity (1+, 2+ or 3+) of staining compared with the 3+ Bcl-2 or 3+ Bax intensity of the background lymphocytes as described by Brambilla.[23]

Statistical analysis. Fisher's exact test and the Student's *t* test were used for statistical analysis of differences.

Results

Review of histology. Two of the 4 ACs from Saitama Cancer Center were changed to LCNEC and SCLC, and two of the 11 TCs and one SCLC collected from other hospitals were changed to ACs and a LCNEC, after review. The resultant distribution of subtypes was: 19 TCs, 5 ACs, 14 LCNEC and 19 SCLCs (Fig. 1). Data for other clinicopathological parameters—sex, age, tumor size, mitotic counts, necrosis, pathological stages, therapy and patient's smoking status—are presented in Table 1.

Allelic loss. Representative results for LOH, with allelic loss in cases with eight markers, are shown in Fig. 2. NE cells usually grow in large cell nests with small numbers of normal cells in the surrounding tissue, but since only nests were collected by microdissection for the present analysis of LOH, there was no problem in judgment of band loss in tumor compared with normal tissue in almost all informative cases. The findings are summarized in Table 2. With TCs, most cases showed no, or only one, LOH, except for case 2, with four LOHs and the frequency of allelic loss for each marker was low (ranging from 0 to 27%). With ACs, the number of LOH for each case with all markers was variable (0–5), and the frequency for each marker was 33 to 75%, except for one with 0%. On the other hand, with LCNECs and SCLCs, frequencies of alteration were greater than 50% except for D9S171, with the two types showing similar allelic loss patterns. These were therefore combined in one group (LCNSEs+SCLCs:L/S). Before comparison of LOH frequencies among the three groups, TCs, ACs and L/S, we compared the frequencies between p-stage I and II, and p-stage I and II+III in each group. Results showed that the frequencies on all markers except CI31107 and D3S1300 (3p

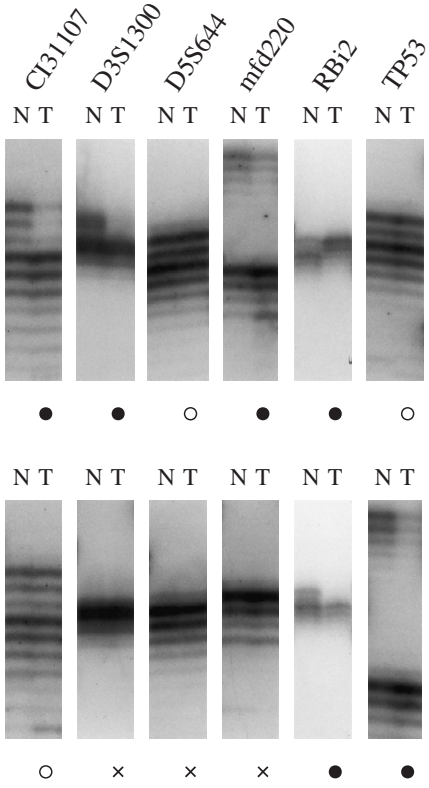

Fig. 2. Typical results of loss of heterozygosity analysis of an atypical carcinoid (upper column, case 20) and a large cell neuroendocrine carcinoma (lower column, case 26) are shown for six microsatellite markers. N, normal tissue; T, tumor tissue; ●, LOH; ○, retention of constitutional heterozygosity; ×, not informative.

Table 2. Summary of results for LOH and immunoreactivity of lung NE tumors

Case no.	Histological type	LOH								Immunoreactivity				p-stage
		3p		5q	9p	9q	11q	13q	17p	p53[1]	Bcl-2 score[2]	Bax score[2]	Bcl-2/ Bax	
		CI31107	D3S1300	D5S644	D9S171	mfd220	D11S4938	RBi2	TP53					
1	TC	○	○	×	○	×	○	○	×	−	0	126	<1	IA
2	TC	●	●	×	●	●	○	○	○	−	0	126	<1	IA
3	TC	○	×	×	×	○	○	×	○	−	0	212	<1	IA
4	TC	●	○	×	×	×	×	○	○	−	0	208	<1	IA
5	TC	○	×	×	○	×	○	○	○	−	0	108	<1	IB
6	TC	×	○	×	×	○	●	×	×	−	0	139	<1	IA
7	TC	●	○	×	×	○	×	×	×	−	0	145	<1	IB
8	TC	○	○	○	×	×	×	○	×	−	0	204	<1	IA
9	TC	○	×	×	×	○	×	○	×	−	0	180	<1	IA
10	TC	○	×	×	×	○	×	×	×	−	14	160	<1	IA
11	TC	○	○	×	○	○	○	×	○	−	10	165	<1	IA
12	TC	○	○	○	○	○	×	○	○	−	0	210	<1	IB
13	TC	○	×	○	○	○	○	○	○	−	0	181	<1	IA
14	TC	○	○	○	×	○	●	○	○	−	0	174	<1	IA
15	TC	○	○	○	×	○	●	○	○	−	0	125	<1	IA
16	TC	×	○	×	×	×	○	×	○	−	0	121	<1	IIB
17	TC	○	○	×	○	×	○	○	○	−	0	94	<1	IIA
18	TC	○	×	×	○	×	×	○	○	−	0	80	<1	IB
19	TC	○	×	×	●	×	×	○	○	−	0	90	<1	I
20	AC	×	○	×	×	○	×	○	×	−	155	74	>1	IIIA
21	AC	●	●	○	●	●	○	●	○	−	165	136	>1	IIB
22	AC	○	×	○	○	●	○	●	●	−	248	187	>1	IA
23	AC	●	●	×	×	×	○	○	●	+	28	218	<1	IA
24	AC	●	●	●	×	×	×	×	○	−	198	180	>1	IA
25	LCNEC	●	○	●	○	●	×	●	×	+	215	0	>1	IA
26	LCNEC	○	×	×	●	×	○	●	●	+	209	0	>1	IA
27	LCNEC	●	×	×	×	●	●	●	●	+	181	94	>1	IIIA
28	LCNEC	×	●	○	×	×	×	×	×	+	183	0	>1	IIIA
29	LCNEC	×	×	×	×	●	×	×	×	−	211	49	>1	IIIA
30	LCNEC	●	●	●	×	●	○	●	×	+	192	154	>1	IIIA
31	LCNEC	○	×	×	×	○	●	×	●	+	198	163	>1	IB
32	LCNEC	●	○	×	○	×	×	●	●	+	248	92	>1	IA
33	LCNEC	●	●	×	×	×	×	×	×	+	210	81	>1	IB
34	LCNEC	●	×	×	×	×	×	×	●	−	240	0	>1	IIIB
35	LCNEC	●	×	●	×	●	○	●	●	+	173	38	>1	IIA
36	LCNEC	×	○	×	○	●	×	●	●	+	171	85	>1	IIB
37	LCNEC	×	●	×	×	○	●	×	●	+	217	140	>1	IB
38	LCNEC	×	×	×	×	●	○	○	●	+	269	180	>1	IA
39	SCLC	●	●	×	○	○	●	●	●	+	103	0	>1	IIIA
40	SCLC	×	●	●	×	●	●	●	●	−	264	14	>1	IIB
41	SCLC	●	●	×	×	○	×	●	●	−	202	0	>1	IA
42	SCLC	●	●	×	×	●	●	●	●	−	275	189	>1	IA
43	SCLC	●	×	●	×	●	●	●	×	+	125	58	>1	IIIA
44	SCLC	×	●	×	×	×	●	●	●	+	138	32	>1	IIB
45	SCLC	○	○	×	○	●	○	●	●	−	219	186	>1	IA
46	SCLC	○	×	×	×	○	○	●	●	−	99	118	<1	IIIA
47	SCLC	●	●	×	×	○	○	●	●	−	267	0	>1	IIB
48	SCLC	●	×	×	×	×	×	×	●	+	240	18	>1	IIIA
49	SCLC	×	●	●	×	●	×	×	×	−	279	102	>1	IIA
50	SCLC	×	●	×	×	×	×	×	●	+	284	159	>1	IB
51	SCLC	●	○	○	×	×	×	×	●	+	264	0	>1	IA
52	SCLC	○	○	×	×	●	×	×	●	−	158	130	>1	IA
53	SCLC	●	○	●	●	●	×	×	●	−	172	133	>1	IB
54	SCLC	●	○	×	×	×	×	×	×	+	285	107	>1	IB
55	SCLC	×	●	●	×	●	○	●	×	+	0	183	<1	IB
56	SCLC	●	○	×	×	○	○	●	○	−	124	66	>1	IA
57	SCLC	×	●	×	○	×	●	●	×	−	90	93	<1	I

●, LOH; ○, retention of constitutional heterozygosity; ×, not informative.
1) p53 was considered as positive when at least 15% of cells were stained.
2) Score=percentage of stained cells×intensity (1 to 3).

markers) were almost the same between stages. However, on 3p markers the frequencies in L/S were different both between p-stage I and II, and p-stage I and II+III (P=0.20000 and 0.0247, respectively, on CI31107; P=0.2213 and 0.0691, respectively, on D3S1300). Therefore, on the markers except 3ps we compared the LOH frequencies among the three groups without dividing the p-stages, while on 3p markers, comparison of the frequencies was performed between those of p-stage I (Table 3).

In comparing between TCs and L/S, five markers—CI31107, D5S644, mfd220, RBi2 and TP53—showed statistically significant differences, with lower LOH frequency in the former. For three markers out of these five (D5S644, RBi2 and TP53) the frequencies gradually increased from TCs to ACs to L/S, and the differences between TCs and ACs for RBi2 and TP53, and between ACs and L/S for TP53, were statistically significant. With D3S1300, the frequency of alteration in TCs was also lower than in ACs with statistical significance, although this was confined to comparisons between markers of p-stage I.

In addition, we looked for other markers which might show differences in LOH status among the three histological types. As shown in Table 4, when the case distribution was compared between TC and AC by LOH status for the combination of RBi2 and TP53—with retention of both markers versus one allelic loss of either—all TC cases, but none of the ACs, showed the former status, and the difference was statistically significant (P=0.0152, Fisher's exact test). The same combination, with LOH status of loss with both markers versus retention of one marker, also showed a borderline significant difference (P=0.1078, Fisher's exact test) between ACs and L/S.

Expression of p53, Bcl-2 and Bax. The results of immunohistochemical staining are shown in Fig. 3 and summarized in Tables 2 and 5. The frequency of p53 protein expression and case distributions of Bcl-2 and Bax scores did not show any differences among p-stages for each histology. Therefore, in the following examination, all cases for each histology were analyzed. The frequency of p53 protein expression was low for TCs and ACs (0% and 20%, respectively), and medium for SCLCs (42%), whereas for LCNECs it was detected in 86% of the cases. Differences between LCNECs, and TCs, ACs or SCLCs were all statistically significant (P<0.0001 and P=0.0173 and 0.0151, respectively, Fisher's exact test) (Table 5).

Few cases of TC showed staining with Bcl-2. Conversely, all cases were positive for Bax, with a mean score of 150±41 (Table 5). For ACs, both proteins were positive, and the mean Bcl-2 score (159±73) was the same as that for Bax (159±50). For LCNECs and SCLCs, most cases were positive for Bcl-2, with mean scores of 208±28 and 189±82, respectively, but for Bax only 70% of cases (10/14, 15/19, respectively) were positive, with mean scores being 77±62 and 84±67, respectively. The Bcl-2/Bax ratio was less than 1 for all TCs, but for other histological types more than 80% had values higher than 1, and the differences between TCs and each other type (ACs, LCNECs and SCLCs) were statistically significant (P=0.0005, <0.0001 and <0.0001, respectively, Fisher's exact test) (Table 5).

Subclassification of NEs by LOH and immunostaining markers. We mixed the histologically diagnosed TCs and ACs, ACs and L/Ss, and LCNECs and SCLCs, respectively, and reclassified these three groups using all the molecular markers, except D3S1300, which showed significant (or mostly significant) differences between TC, AC, LCNEC and SCLC. D3S1300 was used to differentiate the mixed group of TCs and ACs in p-stage I. Concordance rates of the diagnoses performed by both methods are shown in Table 6. In the group diagnosed as AC and TC by histology, the concordance rate was highest for the immunohistochemical marker Bcl-2/Bax (96%), medium for the combination of LOH markers, RBi2+TP53 and D3S1300 (92%), and lowest for RBi2 and TP53 (88%). For AC versus L/S, the rate was higher for TP53 (89%) than the combination of two markers (83%). As for LCNEC and SCLC, p53 immunostaining showed a concordance rate of 70%. When all the NEs were mixed and classified by combinations of two markers, the concordance rate among TC, AC and L/S was 89% (Table 6).

Prognosis of discordant cases between histological and LOH diagnosis and ACs. Discordant cases between histological diagnosis and LOH diagnosis with the four markers, D3S1300, RBi2, TP53 and the combination of Rbi2 and TP53, are shown in Table 7. When both RBi2 and TP53 were informative in a case, diagnosis with the combination of the two markers was adopted instead of either alone. The discordance was observed in one each of the histological TCs, LCNECs and SCLCs, and three (case no. 20, 22 and 24) of five ACs. In three (case no. 2, 24 and 56) of these six discordant cases, different diagnoses between the LOH markers used were observed. The discordant LCNEC and SCLC were the cases for which histological diagnoses had been changed from ACs after review.

The outcomes of the discordant cases and ACs are shown in Table 7. Only one patient had died with all others surviving. The follow-up periods were more than 36 months after the operation for five cases. Of these, case 56 (histological diagnosis of SCLC, but molecular diagnosis of AC or TC) and case 20 (AC by histology, but possible TC by molecular markers) were still surviving at 99 and 80 months, respectively, when the latter died of a non-tumor-related disease. Case 21 (AC with concordant diagnoses by two methods) and case 24 (AC by histology, but not TC or TC by the markers) were also alive after more than 53 months. Case 22 (AC by histology, but L/S by a molecular marker) was still alive after 43 months.

Table 4. Comparison of LOH status between ACs and other subtypes for combinations of two specific markers

Histological type	No. of cases		
	RBi2 (13q) and TP53 (17p)		
	Both LOH−	One LOH+	Both LOH+
TC	10	0 *2)*	
AC	0	2 *2)*	1 *3)*
L/S[1)]		2	13 *3)*

1) LCNEC+SCLC. *P=2)* 0.0152 and *3)* 0.1078, by Fisher's exact test.

Table 3. LOH frequencies for lung NE tumors

Histological type	No. with LOH/informative cases (%)							
	3p[1)]		5q	9p	9q	11q	13q	17p
	CI31107	D3S1300	D5S644	D9S171	mfd220	D11S4938	RBi2	TP53
TC	3/16 (19) *3)*	1/10 (10) *7)*	0/5 (0) *4)*	2/9 (22)	1/11 (9) *5)*	3/11 (27)	0/13 (0) *8)* *6)*	0/13 (0) *8)* *6)*
AC	2/3 (67)	2/2 (100)	1/3 (33)	1/2 (50)	2/3 (67)	0/3 (0)	2/4 (50)	2/4 (50) *9)*
L/S[2)]	9/13 (69)	7/15 (47)	8/10 (80)	2/8 (25)	15/22 (68)	9/18 (50)	19/20 (95)	22/23 (96)

1) Only stage I cases are examined. *2)* LCNEC+SCLC, *P=3)* 0.0084, *4)* 0.0070, *5)* 0.0024, *6)* <0.0001, *7)* 0.0454, *8)* 0.0441 and *9)* 0.0485, by Fisher's exact test.

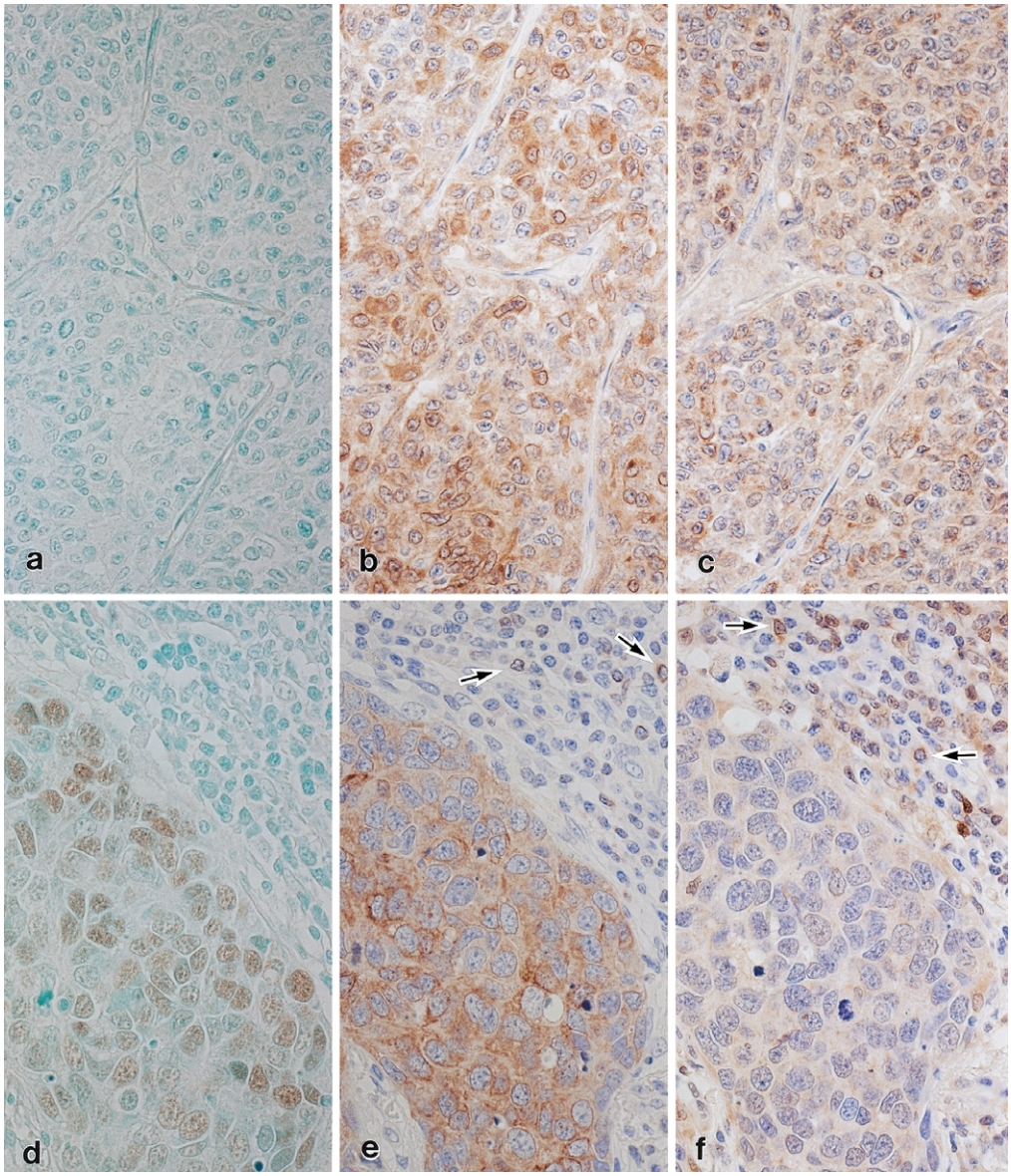

Fig. 3. Immunohistochemical staining for p53, Bcl-2 and Bax in an atypical carcinoid (a, b and c, respectively; case 20) and a large cell neuroendocrine carcinoma (d, e and f, respectively; case 26). Immunoreactivity for p53 (a and d) is apparent in nuclei of tumor cells in d, and Bcl-2 (b and e) and Bax (c and f) are positive in cytoplasm of tumor cells in b, c and e and lymphocytes (e and f, arrows). The atypical carcinoid is p53-negative with a Bcl-2/Bax ratio >1. The large cell neuroendocrine carcinoma is p53-positive with a Bcl-2/Bax ratio >1. Original magnification, ×400.

Discussion

Histologically, NEs consist of four subtypes, TC, AC, LCNEC and SCLC. In a review of NEs collected from three hospitals where many lung cancer patients undergo surgery, the discordance rate of diagnoses performed by one of the authors (E.T.) and the pathologists of other hospitals was 12.5%, but a discordance of 6% was also observed between the previous and the present diagnoses of the same NE series in the author's hospital. Most of these discordant cases involved diagnosis of AC versus other histologies.

The frequency of AC is the lowest among NE subtypes, with five cases being the largest number so far reported in Japan.[35, 36] The five cases of AC in our study thus constitute a rather large series.

As for immunohistochemical staining, our results for p53 positivity in SCLCs (42%) are in line with the literature (42–50%),[20, 23, 37, 38] but findings for LCNECs have varied (69%, 58% and 40%), with our figure being the highest (86%).[23, 39, 40] With regard to Bcl-2 and Bax staining, mean scores in our study were almost the same as those of Brambilla, except for the Bcl-2 score for ACs. Our Bcl-2 score for ACs was 159, placing it between the values for TCs and LCNECs/SCLCs, and a higher positive rate of Bcl-2 staining for ACs than TCs was also reported earlier.[20] Brambilla found a value of only 10, the lowest for studied NEs.[23]

On the basis of these LOH and immunostaining results, we looked for molecular markers. Since differentiations between TCs and ACs, ACs and L/S, and LCNECs and SCLCs have proved most difficult on histological grounds, we concentrated our attention on these pairs. For TCs versus ACs, five markers —D3S1300, RBi2, TP53, the combination of RBi2 and TP53, and the Bcl-2/Bax ratio—showed statistically significant differences in the frequency of LOHs or positive immunostaining. The concordance rates between diagnoses based on these markers and histology were more than 88% for all markers. These

Table 5. p53, Bcl-2 and Bax immunoreactivity for the four histological types of lung NE tumors

Histological type	No. of cases		Bcl-2 Mean score±SD	Bax Mean score±SD	No. of cases	
	Examined	p53-positive (%)			Ratio of Bcl-2/Bax	
					<1	>1
TC	19	0 (0)	1±4	150±41	19	0
AC	5	1 (20)	159±73	159±50	1	4
LCNEC	14	12 (86)	208±28	77±62	0	14
SCLC	19	8 (42)	189±82	84±67	3	16

P=*1*) 0.0173, *2*) 0.0151, *3*) <0.0001 and *4*) 0.0005, by Fisher's exact test.

Table 6. Concordance rate between diagnoses using molecular markers and histology

Histology	No. of concordant cases/examined cases (%)					
	LOH markers				Immunostaining	
	D3S1300[2)]	RBi2	TP53	RBi2+TP53	p53	Bcl-2/Bax
TC versus AC	11/12 (92)	15/17 (88)	15/17 (88)	12/13 (92)	—	23/24 (96)
AC versus L/S[1)]	—	—	24/27 (89)	15/18 (83)	—	—
LCNEC versus SCLC	—	—	—	—	23/33 (70)	—
TC versus AC versus L/S[1)]	—	—	—	25/28 (89)	—	—

1) LCNEC+SCLC. *2*) Only stage I cases examined.

Table 7. Prognosis of ACs and cases of discordance between histological and LOH diagnosis

Case no.	Histological diagnosis	LOH diagnosis				Months after surgery	Outcome
		Name of markers					
		D3S1300[1)]	RBi2	TP53	RBi2+TP53		
2	TC	not TC	—	—	TC	13	alive
20	AC	—	TC	—	—	80	dead[2)]
21	AC	—	—	—	AC	53	alive
22	AC	—	—	—	L/S	43	alive
23	AC	not TC	—	—	AC	4	alive
24	AC	not TC	—	TC	—	68	alive
38	LCNEC	—	—	—	AC	19	alive
56	SCLC	TC	—	—	AC	99	alive

1) Only stage I cases examined.
2) Not tumor related.

rates were considered high enough to justify the use of these markers for reinforcement of the subclassification. Among LOH markers, combinations of two markers and D3S1300 (3p)—which would apply only in p-stage I cases—proved most precise in diagnosis. The effectiveness of 3p deletion for differentiation has also been reported in CGH analysis.[8)] RBi2 and TP53 are generally precise and simple for practical use, since only one marker is used. The CGH analysis further suggested a loss of 11q as a genetic marker for differentiation, but our LOH data on 11q did not indicate any difference between them.[8)] More analyses on 11q are warranted. Regarding the Bcl-2/Bax ratio, TCs were <1 but ACs >1, the ratio most effective in differentiation. However, in Brambilla's report, TCs and ACs were both <1, even though the same method was used for immunoreactivity scoring.[23)] Further analyses are also warranted for this ratio.

For the differentiation of ACs from L/S, two markers—TP53 and the combinations of two markers—could be used. The concordance rates with these markers were high (more than 83%). Although a clear separation of carcinoids (ACs+TCs) from LCNECs+SCLCs has been reported with the creation of a hierarchical clustering dendrogram, no individual genetic markers useful for differentiation of the two groups have been noted.[8)]

Regarding LCNECs versus SCLCs, only p53 immunostaining showed a difference in positive frequency, but the concordance rate (70%) was not particularly high. In addition, there have been reports of positive rates of p53 immunostaining that were lower than ours for LCNECs, and few analyses of staining of this tumor type have so far been performed, probably because of the very recent histological definition by the WHO.[23, 39, 40)] Further analyses using p53 immunostaining for differentiation are thus needed.

Furthermore, even among NEs with no further subtype information, one marker, the combination of RBi2 and TP53, showed very high concordance rates (more than 89%) among TC, AC and L/S.

The 5-year survivals of TC, AC, LCNEC and SCLC have been reported as 87, 56, 27 and 9%, respectively.[1)] We compared these prognoses with those of the three cases (cases no. 20, 24 and 56) which showed discordance between histological and LOH diagnosis, to determine which diagnostic method is more appropriate from the biological standpoint. For case 56, the LOH diagnosis of AC or TC was more appropriate than the histological diagnosis of SCLC, and in cases 20 and 24, TC by one of the LOH markers seemed more appropriate than AC by histology.

In conclusion, we propose that LOH analysis—allelic loss of D3S1300, RBi2, TP53, and the combinations of RBi2 and TP53 for TC versus AC; TP53 and the combinations of two markers for AC versus L/S; and the combinations of two markers for TC versus AC versus L/S—is effective as a diagnostic aid for distinguishing between histological types.

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