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Original Article

Characteristics of loss of heterozygosity in large cell neuroendocrine carcinomas of the lung and small cell lung carcinomas

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Large cell neuroendocrine carcinoma (LCNEC) of the lung is a new entity. Besides morphological characteristics, its molecular biological features have been investigated by many researchers and compared to those of other neuroendocrine carcinomas, small cell lung carcinoma (SCLC) and carcinoid tumor (CT). However, there are few reports that show the significantly different genetic characteristics between them. The purpose of the present paper was to study the frequency of loss of heterozygosity (LOH) at chromosome 3p (3p14.2) in 38 neuroendocrine carcinomas of the lung (13 LCNEC, 11 SCLC and 14 CT) and 10 large cell carcinomas (LCC). The frequencies of LOH at 3p14.2 were 69.2% in LCNEC, 81.8% in SCLC, 50.0% in LCC and 7.14% in CT. Those at 22q13.3 were 30.8% in LCNEC, 72.7% in SCLC, 45.5% in LCC and 7.14% in CT. In particular, the frequency of SCLC with LOH at both 3p14.2 and 22q13.3 (63.6%) was significantly higher than that of LCNEC (15.4%). LCNEC and SCLC had different characteristics of LOH patterns at 3p14.2 and 22q13.3. The combined analysis of the LOH at 3p14.2 and 22q13.3 is thought to be useful for differential diagnosis between LCNEC and SCLC.

Key words: 22q13.3, 3p14.2, large cell neuroendocrine carcinoma, loss of heterozygosity, small cell lung carcinoma

Neuroendocrine tumors of the lung have been divided into two categories, namely small cell lung carcinomas (SCLC) with high-grade malignancy and carcinoid tumors (CT) with low-grade malignancy. Recently the World Health Organization (WHO) added a new tumor entity, large cell neuroendocrine carcinoma (LCNEC), which also has neuroendocrine features.[1] The clinicopathological and immunohistochemical characteristics of LCNEC have been extensively investigated and many reports indicated that the LCNEC has similar clinicopathological characteristics to the SCLC.[2–4] Concerning genetic alterations in neuroendocrine carcinomas of the lung, losses of chromosome 3p are frequently reported in SCLC. In the frequent losses of chromosome 3p, chromosome 3p14, 3p21 and 3p25 have been well-documented as the common genetic aberrant regions, and tumor suppressor genes are expected to be located in these regions.[5–7] In particular, the *FHIT* gene isolated at 3p14.2 has attracted attention as a candidate for a tumor suppressor gene.[8,9]

Besides the 3p region, frequent losses of other regions such as 5q, 11q, 13q, 17p and 22q have been also reported.[10] For example, Onuki *et al*. demonstrated that the frequency of loss of heterozygosity (LOH) at 3p, 5q, 11q, 13q and 17p increased in typical carcinoids, atypical carcinoids, LCNEC and SCLC, in that order.[2] The LOH in the 5q21 region, which is located between the *MCC* and *APC* genes, in particular, indicated a poor prognostic character in neuroendocrine carcinomas, including SCLC and LCNEC. Losses of 3p, 4q, 5q, and 13q and gain of 5p have also been documented as characteristics of the SCLC and LCNEC. These results indicate that the genetic alterations in SCLC and LCNEC are very similar and that their genetic backgrounds are also closely associated.

In contrast, our group reported the results of amplotyping of lung carcinoma by arbitrary primed polymerase chain reaction (PCR). In the report, the SCLC characteristically lost the telomeric region of 22q (22q13.3).[11] Chromosome 22 has been reported to show a frequent LOH in many primary carcinomas where the *neurofibromatosis 2* (*NF2*) gene is located. The region 22q13.3 is located at a more telomeric site than the *NF2* gene.[12,13] The LOH of the region has a high frequency in SCLC (84.6%) compared to non-small cell carcinomas (25–27.3%). In bronchioloalveolar adenocarcino-

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mas, the frequency of LOH at 22q13.3 is increasing according to sequential progression.[14] However, the LOH analysis for neuroendocrine carcinomas of the lung has not been reported.

In the present study we focused on two regions, 3p14.2 (FHIT region) and 22q13.3, and examined their losses among SCLC, LCNEC, CT and large cell carcinomas (LCC).

MATERIALS AND METHODS

Materials

A total of 73 lung tumors were used in the present study (23 LCNEC, 19 SCLC, 13 LCC and 18 CT). They were surgically resected at the National Cancer Center Hospital, National Cancer Center Hospital East, Saiseikai Central Hospital, Tochigi Cancer Center Hospital and University Hospital of Tsukuba. Formalin- or methanol-fixed and paraffin-embedded tissue blocks were cut into 5 μm-thick sections. After deparaffinization, the sections were stained with HE, and six pathologists (Pathology panel of the Japanese LCNEC study group including TK and MN) diagnosed these cases. All 73 tumors were examined immunohistochemically using three neuroendocrine makers (chromogranin A, synaptophysin and CD56) and, among the 73 tumors, 48 tumors could be examined for LOH using PCR.

Immunohistochemical study

Polyclonal antibodies against synaptophysin and chromogranin A (Dako, Glostrup, Denmark) and a monoclonal antibody against CD56 (Nippon Kayaku, Tokyo, Japan) were used for immunohistochemistry. Immunohistochemistry was performed using the standard streptavidin–biotin–peroxidase complex (Dako LSAB Kit, Dako Japan, Kyoto, Japan) following the manufacturer's instructions. We termed the results positive when we found any tumor cells that were stained clearly.[15]

DNA extraction

From other 5 μm sections, tumor cells and non-tumor cells were independently collected by a microdissection method using a toothpick. The dissected cells were dissolved in DNA extraction buffer (20 mmol/L Tris pH 8.0, 1 mmol/L EDTA, 0.5% Tween 20, 0.2 mg/mL proteinase K) and incubated overnight at 37°C. After heat denaturation at 90°C for 7 min, the genomic DNA was extracted using phenol and chloroform.

PCR analysis

To evaluate LOH, we used microsatellite markers, D3S1300, D3S1312 and D3S1313 for 3p14.2 and D22S1140, D22S1161 and D22S1170 for 22q13.3. Each maker was labeled with fluorochromes 6-carboxyfluorescein, hexachloro-fluorescein, or tetrachloro-fluorescein. Multiplex PCR reactions were performed in 20 μL total volumes with three labeled makers containing 5–20 ng of template DNA, 5–10 pmol of each primer, 62.5 μmol/L each deoxyribonucleotide triphosphate, 2 μL 10× Ex Taq buffer containing 20 mmol/L $MgCl_2$, 5% of dimethyl sulphoxide, and 1.5 units TaKaRa Ex Taq (Takara Bio, Otsu Japan). After initial denaturation at 95°C for 2 min, reaction products were subjected to 35 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min, with a final extension at 72°C for 7 min. The products were mixed with internal standard size marker Tamra 500 (PE Applied Biosystems, Foster City, CA, USA), denatured at 95°C for 5 min, and loaded on a 5% denaturing polyacrylamide gel on an Abi Prism 377 automatic sequencer (PE Applied Biosystems). The gel image was analyzed using Genescan software (PE Applied Biosystems).

LOH band peak of tumor tissues was compared with that of normal tissues and the result was confirmed in duplicate. The incidence of LOH was calculated as: total number of cases with LOH at any chromosome region/total number of cases examined.[14]

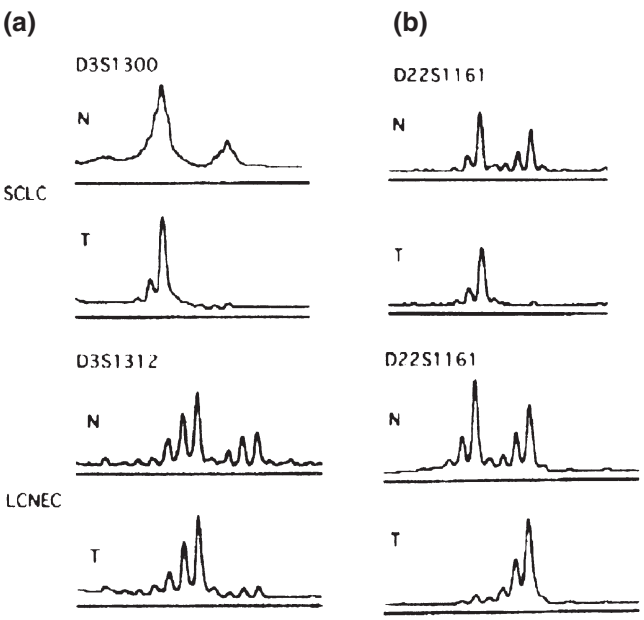

Figure 1 Representative results of loss of heterozygosity analysis at (**a**) 3p14.2 (D3S1300 and D3S1312) and (**b**) 22q13.3 (D22S1161) in SCLC and LCNEC. LCNEC, large cell neuroendocrine carcinoma; N, non-tumor tissue; SCLC, small cell lung carcinoma; T, tumor tissue.

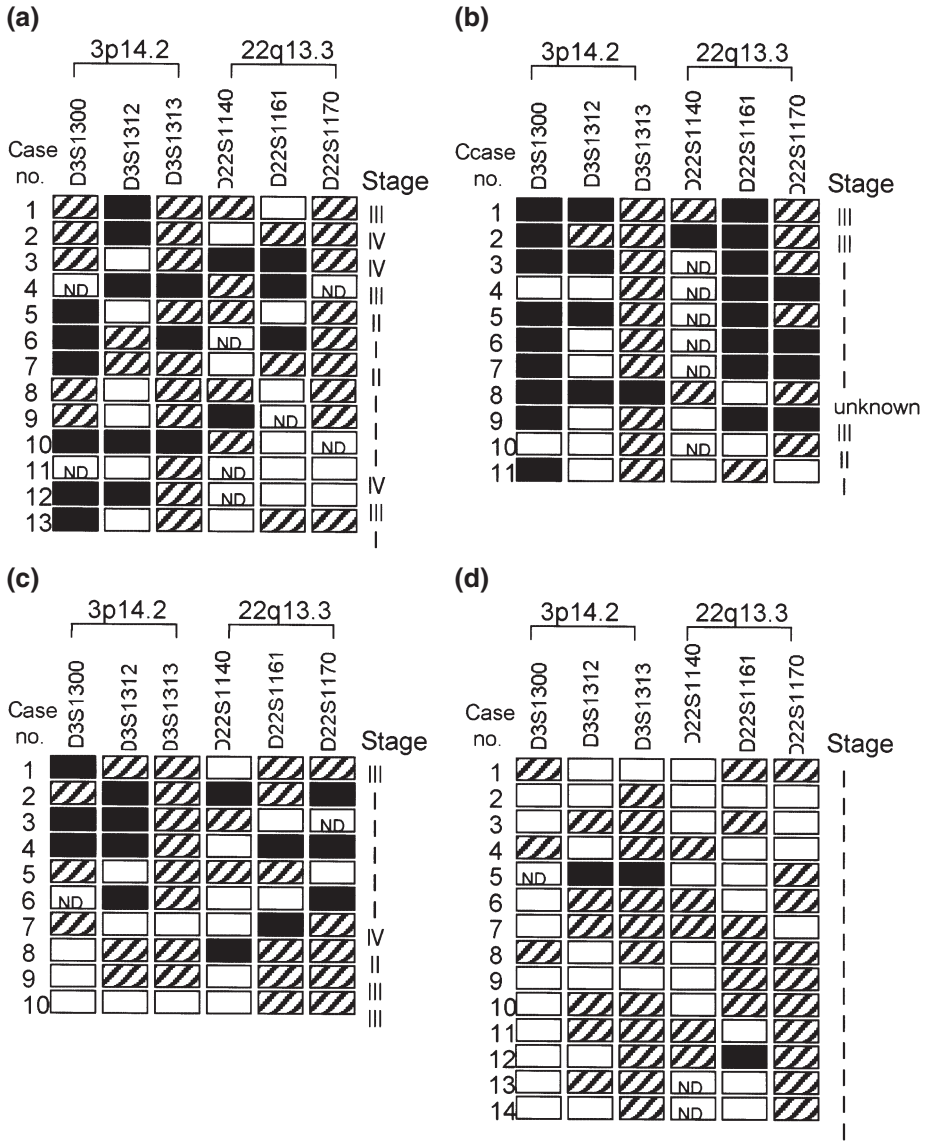

Figure 2 Summary of the loss of heterozygosity (LOH) analysis at chromosome 3p and 22q in four histological subtypes of lung carcinoma. (**a**) Large cell neuroendocrine carcinoma; (**b**) small cell lung carcinoma; (**c**) large cell carcinoma; (**d**) carcinoid tumor. (■) LOH; (□) heterozygous; (▨) homozygous; ND, not able to be determined.

The statistical analysis of the correlation between the diagnosis and the frequency of LOH was performed using *t*-test. $P < 0.05$ was considered to denote statistical significance.

Results

Six different pathologists including T. K. and M. N. examined the HE stained sections histologically and made a consensual diagnosis for each case. In the immunohistochemical analysis, the stainability of all three antibodies could be judged for 64 tumors, and that of synaptophysin could be judged for four additional SCLC. The results of the immunohistochemistry are summarized in Table 1. Not only CT but also SCLC had a high frequency of expression for synaptophysin, chromogranin A and CD56. All the LCNEC cases expressed at least one of the neuroendocrine makers and met the criteria of the LCNEC as defined by the WHO classification. However, expression of the neuroendocrine makers in LCC were significantly lower than in CT, LCNEC and SCLC.

Table 1 Immunohistochemistry

	Chromogranin A *n* (%)	Synaptophysin *n* (%)	CD 56 *n* (%)
LCNEC	10/22 (45.5)	15/22 (68.2)	18/22 (81.8)
SCLC	10/11 (90.9)	15/15 (100)	9/11 (81.8)
LCC	2/13 (15.4)	5/13 (38.5)	0/13 (0)
CT	18/18 (100)	18/18 (100)	18/18 (100)

CT, carcinoid tumor; LCC, large cell carcinoma; LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma.

In LOH analysis, we succeeded in PCR amplification of the DNA samples extracted from 48 tumors (13 LCNEC, 11 SCLC, 10 LCC and 14 CT). Representative results of the LOH analysis are given in Figure 1 and a summary of the

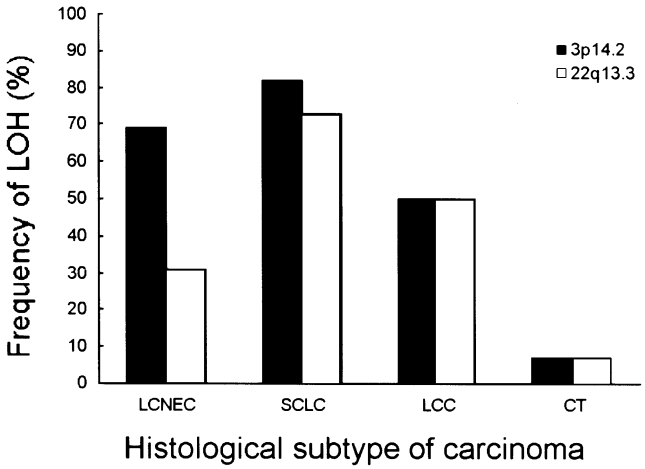

Figure 3 Frequency of loss of heterozygosity at (■) chromosome 3p (3p14.2) and (□) 22q (22q13.3) in four histological subtypes of lung carcinoma. CT, carcinoid tumor; LCC, large cell carcinoma; LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma.

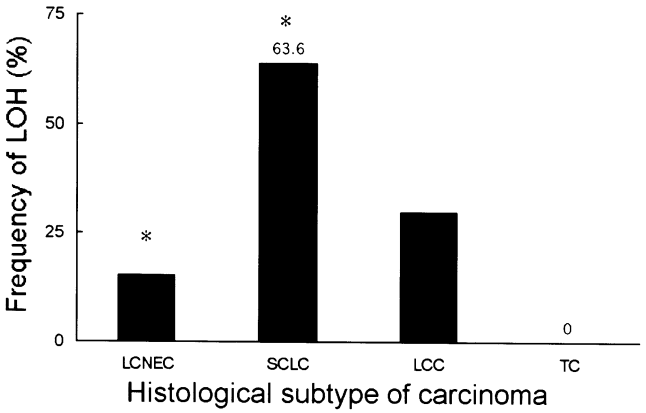

Figure 4 Frequency of loss of heterozygosity (LOH) at (■) both 3p14.2 and 22q13.3 in four histological subtypes of lung carcinoma. CT, carcinoid tumor; LCC, large cell carcinoma; LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma. $^*P < 0.05$.

results is given in Fig. 2. As Fig. 3 shows, the incidence of LOH at 3p14.2 was 69.2% (9/13 cases) in LCNEC, 81.8% (9/11 cases) in SCLC, 50.0% (5/10 cases) in LCC and 7.14% (1/14 cases) in CT. The incidence of LOH at 22q13.3 was 30.8% (4/13 cases) in LCNEC, 72.7% (8/11 cases) in SCLC, 50.0% (5/10 cases) in LCC and 7.14% (1/14 cases) in CT. At 3p14.2, LOH was frequently detected in both LCNEC and SCLC but the incidence in CT was very low. In contrast, at 22q13.3, the LOH incidence in LCNEC was lower than that of SCLC and even LCC. Because the two markers of 3p14.4 and 22q13.3 were frequently detected in SCLC, we compared the incidence of LOH at both 3p14.4 and 22q13.3. The incidence of LOH in both 3p14.2 and 22q13.3 in SCLC (63.6%, 7/11 cases) was significantly higher than that in LCNEC (15.4%, 2/13 cases; $P < 0.05$), LCC (30%, 3/10 cases) and CT (0%, 0/14 cases; Fig. 4).

In the 11 cases of SCLC, seven of them had LOH both at 3p14.2 and 22q13.3, but the other cases had LOH only at 3p14.2 (cases 8, 11), only at 22q13.3 (case 4), or did not have LOH at any region (case 10). In contrast, in the 13 cases of LCNEC, two cases (4 and 6) had LOH both at 3p14.2 and 22q13.3 (Fig. 2). These six cases stained positive for some of the antibodies against chromogranin A, synaptophysin and CD56 and showed neuroendocrine features. We then reexamined these cases histologically. The histology of the SCLC cases without LOH at 3p14.2 or 22q13.3 (cases 4, 8, 10 and 11) was similar to those with the LOH. However, the size of the nuclei in the LCNEC cells of cases 4 and 6 that had LOH at both sites was found to be slightly smaller than those of other LCNEC, and their nucleoli tended to be inconspicuous compared to the other LCNEC (Fig. 5).

DISCUSSION

Since the new histological entity of high-grade neuroendocrine carcinoma, LCNEC, was established in the histological typing of WHO classification (3rd edition), several studies have been reported that have tried to characterize the LCNEC clinicopathologically and genetically. However, most reports indicated that SCLC and LCNEC are similar neuroendocrine carcinomas with poor prognosis and contain similar genetic alterations. For example, high frequency of LOH at 3p and 5q in LCNEC and SCLC was reported. Although it was described that genetic alterations of LCNEC were similar to SCLC, the abnormality of the *p16* gene was observed in LCNEC frequently but not in SCLC.[16] We found frequent loss of chromosome 22q sequences in SCLC by arbitrarily primed PCR and determined the sequence localized to chromosome 22q13. However, there are no reports that examined LOH at 22q13.3 for LCNEC. In the present study we applied a new LOH marker, 22q13.3, which is reported to be specific for SCLC, and examined the difference of LOH pattern between SCLC and LCNEC.

As Fig. 3 shows, the frequency of LOH at 3p14.2 is similarly high both in SCLC and LCNEC, but the loss at 22q13.3 is relatively low in LCNEC (30.8%) compared with SCLC (72.7%). Because the two markers of 3p14.4 and 22q13.3 were frequently detected in SCLC, we compared the incidence of LOH at both 3p14.4 and 22q13.3. The number of cases with both LOH is significantly higher in SCLC (63.6%) than in LCNEC (15.4%; $P < 0.05$). As we described before, the LOH at 22q13.3 is a very limited characteristics in SCLC and the present study confirmed these results.[11] However, the feature is not correlated with neuroendocrine features of the tumor because not only CT, but also LCNEC did not have

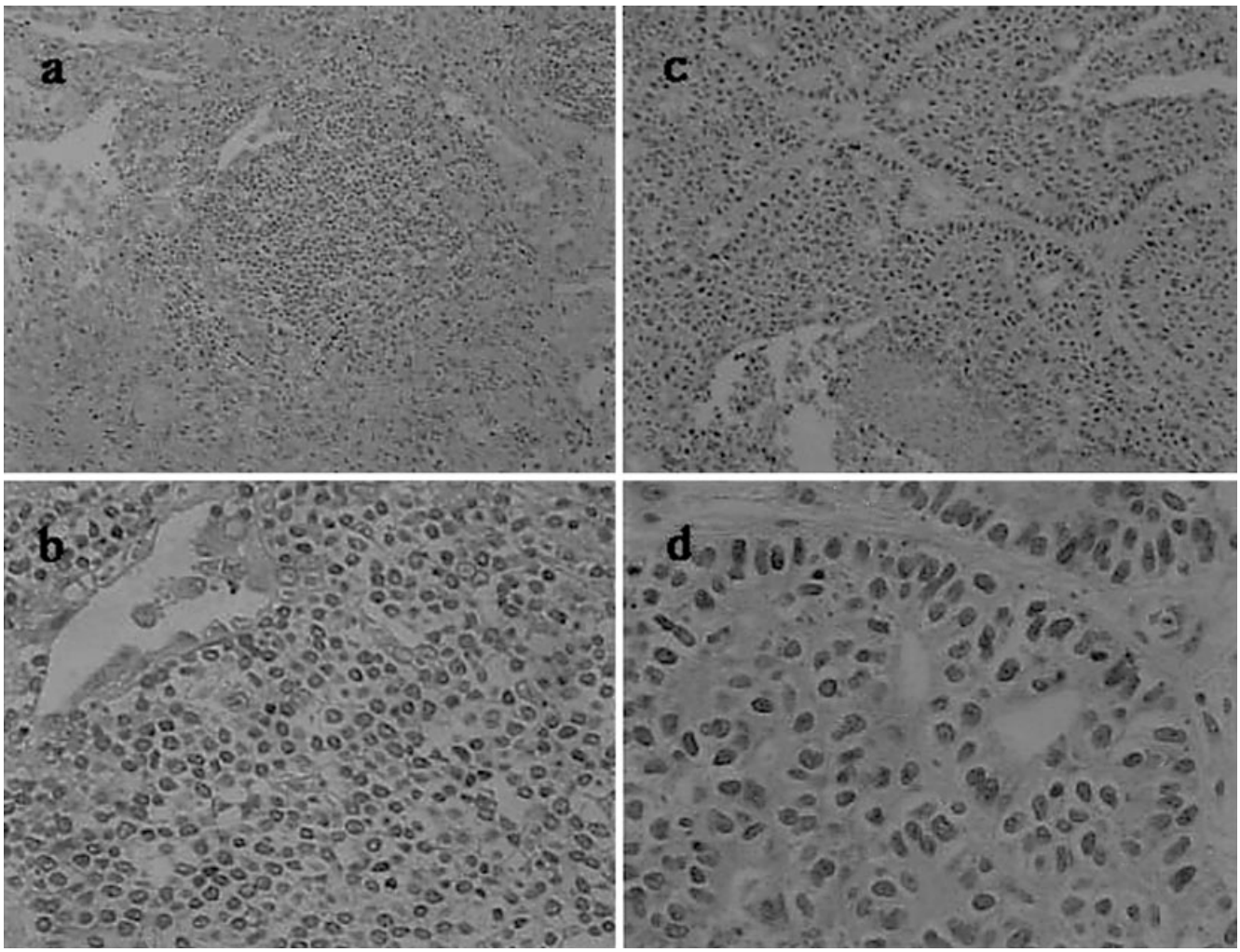

Figure 5 Histological features of large cell neuroendocrine carcinomas (LCNEC). (**a**,**b**) LCNEC (case 6) was positive for LOH at 3p14.2 and 22q13.3; (**c**,**d**) LCNEC (case 1) was positive for LOH at 3p14.2 and negative for LOH at 22q13.3. Compared to the representative LCNEC (case 1), the nuclei of the LCNEC (case 6) are small and the nucleoli are inconspicuous. They are similar to those of small cell lung carcinoma. (**a**,**c**) HE stain, original magnification ×20; (**b**,**d**) HE stain, original magnification ×60.

a high frequency of LOH at 22q13.3. These results indicated that the loss of 22q13.3 is not one of the neuroendocrine makers, but that there might be an important and specific antioncogene at 22q13.3 in carcinogenesis of SCLC.

LCNEC is a special variant of LCC that has special morphological characters and neuroendocrine features. LCC is an undifferentiated malignant epithelial tumor and is a diagnosis of exclusion made after ruling out the presence of a component of squamous cell carcinoma, adenocarcinoma or small cell carcinoma. LCNEC has a poor prognosis compared with LCC without neuroendocrine features. Even for early stage cancer, the prognosis was poorer than that for the same stage of other non-small cell carcinomas.[17,18] In addition it was reported that the proliferative activity of LCNEC was higher than for LCC.[19] The LCC in the present study had a relatively lower incidence of LOH at 3p14.2 and a higher incidence of LOH at 22q13.3, than those of LCNEC. Compared to SCLC and LCC, the difference in the LOH frequency between 3p14.2 and 22q13.3 in LCNEC was characteristic. A high frequency of LOH at 3p14.2 and a low frequency of LOH at 22q13.3 is thought to be an important character of LCNEC.

There were two cases of LCNEC (cases 4, 6) that had LOH at both 3p14.2 and 22q13.3 in the same way as SCLC. They met the histological and immunohistochemical criteria of LCNEC (palisading arrangement, rosette formation, and expression of neuroendocrine markers such as CD56, synaptophysin and chromogranin A). However, their nuclei tended to be smaller than those of the other typical LCNEC, and their nucleoli were not conspicuous (Fig. 5). Therefore, it is thought that discrimination between these cases and SCLC is not easy. The histological similarity to SCLC in the

LCNEC may be linked with LOH at both 3p14.2 and 22q13.3. The histological characteristics and LOH pattern in LCNEC should be examined extensively in large-scale studies.

In the present study we demonstrated the different characteristics of the LOH pattern between LCNEC and SCLC. The status of the LOH at 3p14.2 and 22q13.3 could be useful for differentiating LCNEC from high-grade neuroendocrine carcinomas.

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