npg

Malignancy-associated X chromosome allelic losses in foregut endocrine neoplasms: further evidence from lung tumors

Tiziana D'Adda[1], Lorena Bottarelli[1], Cinzia Azzoni[1], Silvia Pizzi[1], Massimo Bongiovanni[2], Mauro Papotti[2], Giuseppe Pelosi[3], Patrick Maisonneuve[4], Tommaso Antonetti[1], Guido Rindi[1] and Cesare Bordi[1]

[1]Department of Pathology and Laboratory Medicine, Section of Pathological Anatomy, University of Parma, Parma, Italy; [2]Department of Pathology, University of Turin, Turin, Italy; [3]Division of Pathology, European Institute of Oncology, University of Milan, Milan, Italy and [4]Division of Epidemiology and Biostatistics, Epidemiology Unit, European Institute of Oncology, University of Milan, Milan, Italy

Association of X chromosome allelic losses with tumor malignancy has been identified in foregut but not in midgut endocrine neoplasms. The aim of this study was to investigate the association of deletions on X chromosome with malignancy in lung neuroendocrine tumors, another family of foregut neoplasms comprising four categories with increased malignancy: typical and atypical carcinoids, large cell neuroendocrine and small cell lung carcinomas. To evaluate loss of heterozygosity, DNA extracted from nine typical carcinoids, 17 atypical carcinoids, six large cell neuroendocrine carcinomas and five small cell lung carcinomas was PCR-amplified for 18 microsatellite markers spanning the whole X chromosome. All tissue samples were formalin-fixed and paraffin-embedded. X chromosome losses were absent in typical carcinoids, whereas they were found in nine out of 17 atypical carcinoids and in five out of six large cell neuroendocrine carcinomas (involving 28 and 70% of informative loci, respectively). On the contrary, deletions on X chromosome were an extremely rare event in small cell lung carcinomas. In atypical carcinoids, the presence of losses was associated with larger tumor size, higher pT status and advanced stage. No death occurred in atypical carcinoid patients without deletions on X chromosome, whereas all atypical carcinoid patients who had died from disease showed allelic losses. In conclusion, X chromosome allelic losses, absent in benign 'typical' carcinoids, progressively increased in frequency from intermediate-grade 'atypical' carcinoids to high-grade large cell neuroendocrine carcinomas. These results extend the association of deletions on X chromosome with malignancy, already demonstrated in other foregut endocrine neoplasms, to lung neuroendocrine tumors. The absence of X chromosome allelic losses in small cell lung carcinomas underlines a striking difference from large cell neuroendocrine carcinomas, possibly linked to different pathogenetic mechanisms of these two highly aggressive neuroendocrine lung tumors.
Modern Pathology (2005) **18**, 795–805, advance online publication, 3 December 2004; doi:10.1038/modpathol.3800353

Neuroendocrine tumors of the lung represent a heterogeneous group of neoplasms sharing a common neuroendocrine phenotypic differentiation but differing from each other in their biological behavior and clinical aggressiveness. According to the WHO classification,[1] four distinct pathological entities are recognized: typical and atypical carcinoids, characterized by low and intermediate grades of malignancy, respectively; large cell neuroendocrine and small cell lung carcinomas, both of high-grade malignancy. Typical carcinoids are associated with an excellent prognosis and an overall 10-year survival rate of 90%,[2,3] even if 5–10% of cases may develop metastases to regional lymph nodes.[4] Atypical carcinoids are morphologically similar to typical carcinoids; however, they are larger in size, display a higher mitotic activity and show a less favorable prognosis, with a 10-year survival rate ranging from 25 to 60%.[2,4] Large cell

neuroendocrine carcinomas and small cell lung carcinomas are poorly differentiated neoplasms, both characterized by a poor prognosis, with a 10-year survival rate of 5 and 11%, respectively.[4]

In their embryologic classification of carcinoid tumors, supported by histological and clinical features, Williams and Sandler[5] subdivided these neoplasms according to the embryonic divisions of the gut from which they derive. Accordingly, tumors of the bronchus, stomach and pancreas, which are all foregut derivatives, were separated from those of the midgut (small intestine, caecum and right to mid colon) and of the hindgut (descending colon and rectum). Previous studies from our laboratory showed a correlation between loss of heterozygosity (LOH) on X chromosome and malignancy in gastric and pancreatic endocrine tumors, of foregut derivation, but not in carcinoids of midgut and hindgut origin.[6,7]

The aim of the present study was to extend the analysis of the relationship between X chromosome LOH and malignancy to another group of foregut-derived endocrine neoplasms, the lung neuroendo-crine tumors. To this end, a fine allelotyping of X chromosome was performed on the four types of tumors encompassing the whole spectrum of neuroendocrine neoplasms of the lung. With the present detailed analysis of both chromosomal arms, we have attempted to provide more information on the X chromosome loci and related oncosuppressor genes potentially involved in the clinical behavior of foregut endocrine tumors.

Materials and methods

Tumors

A total number of 37 neuroendocrine lung tumors from 37 female patients were collected from the files of the Departments of Pathology of the University of Turin and of the European Institute of Oncology in Milan. These included nine typical carcinoids, 17 atypical carcinoids, six large cell neuroendocrine carcinomas and five small cell lung carcinomas (Table 1). One poorly differentiated tumor (#32) was of a combined type and composed of large (#32a)

Table 1 Clinicopathological data of 37 female patients with neuroendocrine tumors of the lung

Case #	Tumor type	Age	Tumor size (cm)	pT	pN	Stage	Survival (months)	Vital status	Distal metastasis
1	TC	48	3.6	2	0	1B	68	Alive	No
2	TC	42	5	2	2	3A	55	Alive	No
3	TC	51	3.5	2	0	1B	54	Alive	No
4	TC	68	4.5	2	0	1B	52	Alive	No
5	TC	72	4.6	2	0	1B	55	Alive	No
6	TC	69	2.8	1	0	1A	45	Alive	No
7	TC	71	3	1	0	1A	46	Alive	No
8	TC	51	3.6	2	0	1B	36	Alive	No
9	TC	53	2.5	1	0	1A	35	Alive	No
10	AC	53	6	4	1	3B	54	Alive	Thyroid
11	AC	75	3.5	2	0	1B	49	Dead	NA
12	AC	34	2.5	1	0	1A	120	Alive	No
13	AC	77	3.5	2	0	1B	54	Alive	No
14	AC	57	1.5	1	x	—	90	Alive	No
15	AC	73	5	2	0	1B	20	Dead	NA
16	AC	42	1.2	1	2	3A	10	Alive	No
17	AC	34	3	2	2	3A	6	Alive	No
18	AC	72	1	1	0	1A	13	Alive	No
19	AC	30	5	2	0	1B	17	Alive	No
20	AC	40	2	1	1	2A	20	Alive	No
21	AC	63	1.3	1	1	2A	19	Alive	No
22	AC	64	3.5	2	2	3A	30	Alive	No
23	AC	43	3.3	2	2	3A	25	Alive	No
24	AC	26	3.2	2	2	3A	12	Dead	Lung
25	AC	64	3.5	2	2	3A	24	Alive	No
26	AC	79	1.2	1	1	2A	48	Alive	No
27	LCNEC	66	5.5	2	x	—	2	Dead	NA
28	LCNEC	45	8	2	1	2A	11	Dead	Lung, liver
29	LCNEC	54	5	2	2	3A	24	Dead	Lung, liver
30	LCNEC	70	1.2	1	1	2A	37	Dead	Bone, liver
31	LCNEC	35	2	2	2	3A	6	Alive	No
32	LC/SC	66	2.5	4	2	3B	5	Alive	No
33	SCLC	68	5	2	1	3B	56	Alive	Liver
34	SCLC	51	2.5	1	2	3A	16	Alive	Lung
35	SCLC	62	3	2	2	3B	20	Alive	Brain
36	SCLC	70	2.5	1	1	2A	23	Dead	Bone
37	SCLC	55	1.7	1	0	2A	20	Alive	Liver

TC: typical carcinoid; AC: atypical carcinoid; LCNEC: large cell neuroendocrine carcinoma; SCLC: small cell lung carcinoma; NA: not available.
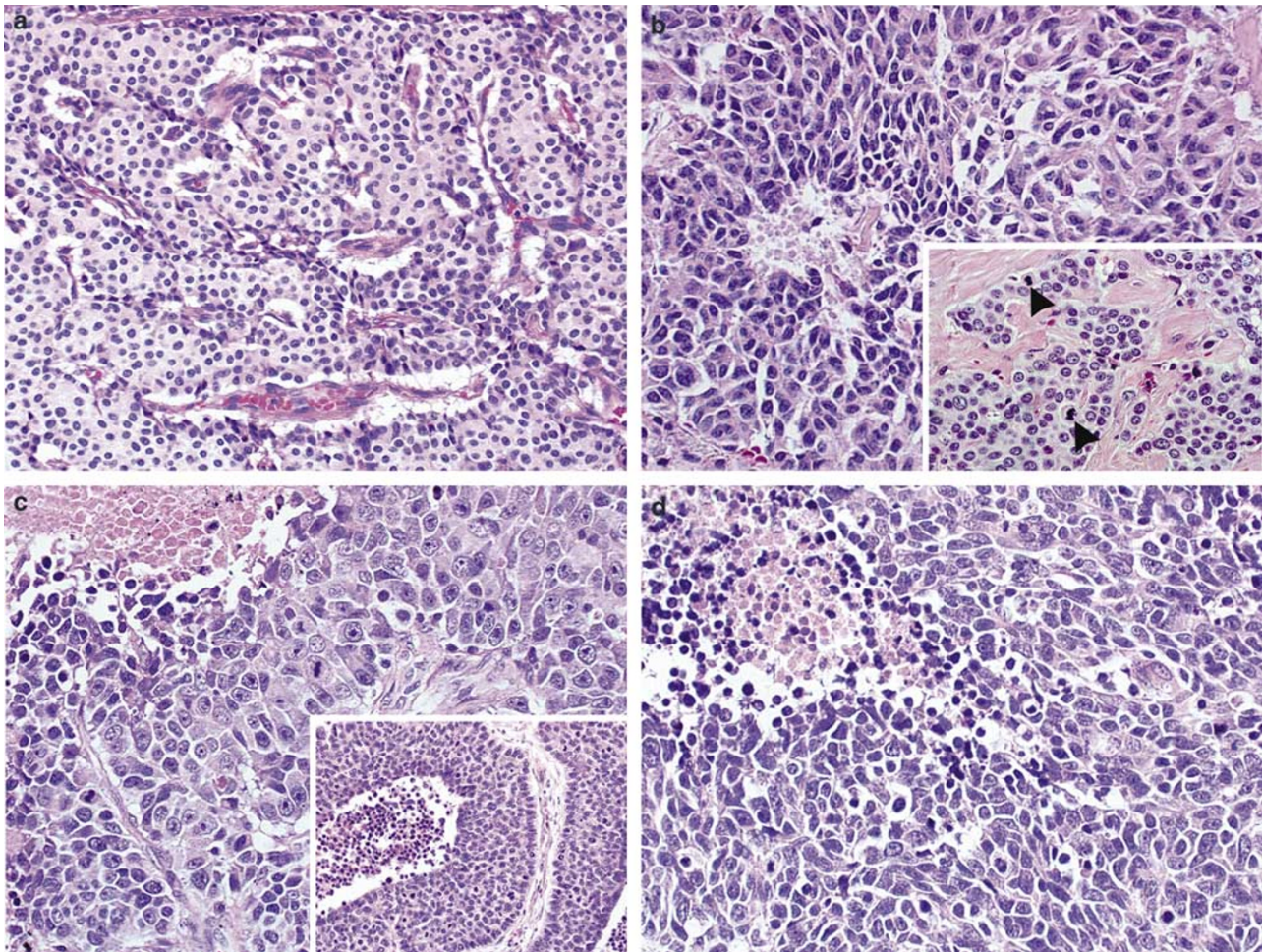
Figure 1 Representative histologic features of neuroendocrine tumors of the lung: (**a**) typical carcinoid tumor showing nested and trabecular aggregates of bland-appearing, uniform neoplastic cells, with numerous thin blood vessels but no necrosis nor mitotic figures; (**b**) atypical carcinoid with small foci of punctate tumor necrosis and/or increased mitotic activity (arrow-heads in the inset); (**c**) large cell neuroendocrine carcinoma characterized by large tumor cells with prominent nucleoli and coarse chromatin, confluent necrotic areas and peripheral palisading of tumor aggregates (inset); (**d**) small cell lung carcinoma showing oval to spindle, small tumor cells with hyperchromatic and molding nuclei, high nucleus/cytoplasmic ratio, evenly dispersed chromatin, inconspicuous nucleoli, and extensive geographic necrosis.

and small cell components (#32b), both available for the study. Informed consent was obtained from all patients and/or guardians, according to Italian law.

All tumors were examined independently by three pathologists expert in the field (MP, GP, CB) and classified according to the histopathological criteria proposed by the WHO classification[1] (Figure 1), using the TNM staging system (TNM, 6th Edition, UICC, 2002). In cases of disagreement, the tumors were reviewed by all the experts together, until agreement was reached. According to WHO classification, typical carcinoids are characterized by a number of mitotic figures less than 2 per 10 high-power fields (HPFs), and by the absence of necrosis. Atypical carcinoids are identified by a number of mitotic figures ranging from 2 to 10 per 10 HPFs and/or by the presence of necrosis. Large cell neuroendocrine carcinomas and small cell lung carcinomas share a high mitotic rate (more than 10 mitotic figures per 10 HPFs) and the extensive necrosis, while they differ for the cytological characteristics of tumor cells, particularly cell size (nonsmall in large cell neuroendocrine carcinomas), nucleus/cytoplasmic ratio (low in large cell neuroendocrine carcinomas, high in small cell lung carcinomas), chromatin pattern (coarsely granular in large cell neuroendocrine carcinomas, finely and evenly dispersed in small cell lung carcinomas), and presence/absence of nucleoli (obvious in large cell neuroendocrine carcinomas, absent or inconspicuous in small cell lung carcinomas). In all the cases investigated, both tumoral and nontumoral tissue (ie, adjacent histologically normal lung parenchima and/or uninvolved regional lymph nodes from the same resected surgical specimens) were available. The study was performed on archival, routinely formalin-fixed and paraffin-embedded material.

DNA Extraction

Serial 4-μm-thick histological sections stained with hematoxylin were examined under a stereomicroscope: normal and tumoral areas were manually microdissected using sterile scalpels. A neoplastic cellularity of at least 80% was obtained for each tumor sample. Microdissected tissues were suspended in an optimized buffer for tissue lysis, incubated overnight with Proteinase K, then DNA extraction and purification were performed using a commercial kit (Dneasy Tissue kit, QIAGEN Inc., Valencia, CA, USA), following the manufacturer's protocol. Quality and amplificability of DNA extracted from formalin-fixed, paraffin-embedded material were assessed by PCR for the human β-globin gene.

PCR Amplification

To evaluate the presence of LOH, 18 highly polymorphic microsatellite markers spanning the whole X chromosome (Table 2) were PCR amplified from tumor and control DNA using primers labeled with Beckman Coulter WellRED fluorescent dyes D2, D3, D4 (Beckman Coulter, Fullerton, CA, USA). All the microsatellite markers contained dinucleotide repeats. For PCR amplification, 2 μl of suitably diluted DNA were combined in a 25 μl reaction mixture containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 200 μM of each dNTP (Promega, Madison, WI, USA), 0.4 μM/L of each primer, 1.5–

2.0 mM $MgCl_2$ and 1.25 U Taq polymerase (Promega, Madison, WI, USA). Data on annealing temperature and $MgCl_2$ concentration for each microsatellite marker investigated are listed in Table 2. PCR amplifications consisted of 35 cycles and were performed in a Primus 25 (MWG-Biotech, Ebersberg, Germany) or in an AB 2700 (Applied Biosystems, Foster City, CA, USA) thermal cycler. The presence and correct size of PCR amplimers was checked by electrophoresis on a 2% agarose gel (Qbiogene Inc., Carlsbad, CA, USA).

Polyacrylamide Gel Electrophoresis and Fragment Analysis

To identify and precisely size the amplified DNA fragments, individual PCR products were run on a CEQ™2000XL capillary DNA sequencer (Beckman Coulter, Fullerton, CA, USA), with a linear polyacrylamide denaturing gel filling the capillary array (eight 33-cm-long silica capillaries) representing the separation medium. For each sample, 0.5–3 μl of PCR-amplified mixture and 0.3 μl of CEQ DNA size standard-400 (Beckman Coulter WellRED dye D1-coupled) were added to 25 μl of sample loading solution containing deionized formamide and loaded into a 96-well sample microplate. After automatic denaturation at 90°C for 120 s, samples were run under the following electrophoretic conditions: capillary temperature 40°C, injection voltage 2.0 kV for 30 s, separation voltage 7.5 kV for a run time of 35 min. Excitation of the WellRED dyes

Table 2 Panel of 18 microsatellite markers spanning the whole X chromosome: cytogenetic localization, informativity, PCR amplification characteristics and LOH frequencies

Microsatellite marker[a]	Cytogenetic localization[a]	Informativity (%)	Annealing T (°C)	MgCl₂ (mM)	LOH cases/informative cases[b]	
					N	%
DXS996	Xp22.3–Xp22.3	89	57	1.5	9/21	43
DXS207	Xp22.2–Xp22.2	78	59	1.5	8/19	42
DXS989	Xp22–Xp22	68	55	1.5	5/14	36
DXS1237	Xp21.3–Xp21.2	81	59	2.0	6/20	30
MAOA.PCR1	Xp11.4–Xp11.3	70	57	2.0	8/19	42
DXS1367	Xp11.3–Xp11.23	84	64	1.5	7/20	35
DXS1003	Xp11.23	78	55	1.5	7/19	37
DXS1111	Xq12–Xq12	62	57	2.0	5/15	33
DXS56	Xq13.2–Xq13.3	89	57	1.5	9/22	41
DXS738	Xq21.1–Xq21.1	59.5	57	1.5	3/12	25
DXS990	Xq21.33	78	57	1.5	7/20	35
DXS1153	Xq22.1–Xq22.3	54	60	1.5	1/10	10
DXS1220	Xq23–Xq23	68	59	1.5	5/15	33
DXS1001	Xq24–Xq24	68	53	1.5	5/15	33
DXS1047	Xq25–Xq25	81	59	1.5	7/19	37
DXS1192	Xq26–Xq26	78	55	1.5	9/19	47
DXS102	Xq26	68	61	2.0	9/18	50
DXS731	Xq27–Xq28	68	55	2.0	4/14	29

[a]Primer sequences and microsatellite marker localizations are available at the Genome Database (www.gdb.org) and Genatlas (www.genatlas.org) web sites.
[b]Frequencies of LOH are calculated for atypical carcinoids and large cell neuroendocrine carcinomas.

was induced by 650 and 750 nm diode lasers, and fluorescence was emitted in the infrared region. CEQ2000XL Fragment Analysis software (Beckman Coulter Inc., Fullerton, CA, USA) converted the fluorescent signals into electrophoretic profiles (electropherograms), in which alleles appeared as peaks. The area and height of the peaks calculated by the software were proportional to the concentration of the PCR fragments in the sample.

Evaluation of LOH

Heterozygosity, that is, the presence of two distinct alleles in normal tissue, is the essential requisite for evaluation of LOH. Peak height data produced by the CEQ2000XL Fragment Analysis software were used to calculate the following ratio:

$$(\text{lower allele/higher allele})_{tum}/$$
$$(\text{lower allele/higher allele})_{norm}$$

Ratio values below 0.6, reflecting an allelic imbalance of 40% or more, were considered as indicators of allelic losses.

Evaluation of LOH at Each Microsatellite Marker

For each microsatellite marker investigated, the frequency of LOH was obtained by calculating the ratio between the number of cases with allelic losses and the number of informative cases (Table 2).

Statistical Analysis

Data were presented as frequencies and/or percentages and compared by using the Mantel–Haenszel χ^2 test for trend or the Fisher's exact test and the t test. Estimates for overall survival were calculated by the Kaplan–Meier method and compared using the log-rank test. All analyses were carried out using the SAS statistical software (SAS Institute, Inc., Cary, NC, USA). All *P*-values were based on two-sided testing.

Results

Typical Carcinoids (Table 3)

No allelic losses on X chromosome were found in any of the 126 informative microsatellite loci investigated in the nine typical carcinoids under examination.

Aypical Carcinoids (Table 3)

Of the 17 atypical carcinoids studied, LOH on X chromosome for at least one microsatellite marker was found in nine, accounting for 61 out of 221 informative microsatellite loci (28%). Five tumors showed loss of most (#11, 15, 22) or all (#10, 23)

informative markers. Four atypical carcinoids showed partial (#13b, 24) or interstitial (#17, 25) losses. Nine tumors showed retention of heterozygosity for all markers investigated. In two cases (#17 and 24), microsatellite instability was found with the marker DXS1047, as indicated by the appearance in the tumor tissue of a novel allele, not present in normal tissue. Tumor #13 was characterized by the presence of two histologically different areas, one with trabecular (#13a) and the other with solid structure (#13b). The trabecular area showed retention of heterozygosity with all the markers investigated, while the solid area showed LOH with the telomeric markers DXS1192 and DXS102 (Figure 2). Primary tumor and a lymph node metastasis were investigated in case #26, and retention of heterozygosity for all informative markers was found in both.

Large Cell Neuroendocrine Carcinomas (Table 3)

Of the six large cell neuroendocrine carcinomas examined, LOH on X chromosome was found in five, accounting for 53 out of 76 informative microsatellite loci (70%). In four cases, loss of most (#30, 32a) or all (#27, 28) of the informative markers investigated was found. One case (#31) showed partial losses concentrated in the long arm of X chromosome. One case (#29) showed retention of heterozygosity with all markers investigated. In the single case of poorly differentiated tumor with combined large cell and small cell carcinoma (#32), the large cell component (#32a) showed loss of all but two informative markers examined, while the small cell component (#32b) showed loss of all. Whenever LOH was found in both components, the same allele was affected. However, the extent of reduction of the allele height in the small cell component (#32b) was always much more pronounced than in the large cell component (#32a), often leading to the complete disappearance of allele (Figure 3).

Small Cell Lung Carcinomas (Table 3)

Of the five pure small cell lung carcinomas investigated, LOH on X chromosome was found only in one out of 66 informative microsatellite loci (1.5%). Three tumors (#34, 35, 37) showed complete retention of heterozygosity, whereas one (#36) showed loss at a single microsatellite locus (DXS1192). The remaining tumor (#33) showed microsatellite instability at DXS990 locus.

Frequency of LOH in the Different Groups of Lung Neuroendocrine Tumors and Clinico-Pathological Correlations

The number of LOH events was significantly different in the four groups of lung neuroendocrine
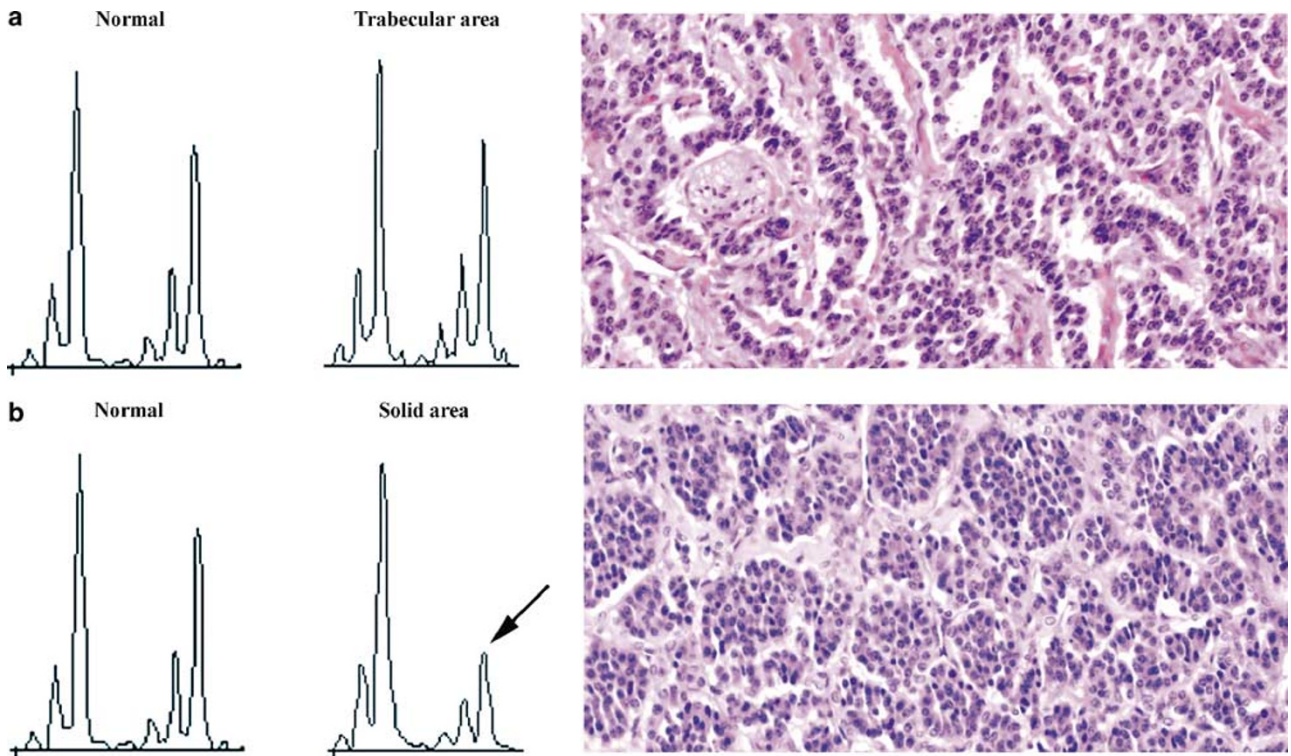
Table 3 LOH analysis on X chromosome in 37 neuroendocrine tumors of the lung

X Chromosome microsatellites	Typical carcinoids (n = 9)									Atypical carcinoids (n = 17)																		Large cell neuroendocrine carcinomas (n = 6)							Small cell lung carcinomas (n = 5)				
	1	2	3	4	5	6	7	8	9	10	11	12	13a	13b	14	15	16	17	18	19	20	21	22	23	24	25	26a	26b	27	28	29	30	31	32a	32b	33	34	35	36	37
DXS996	○	○	★	○	○	○	○	○	○	●	●	○	○	○	★	●	○	○	★	○	○	○	●	●	★	○	○	○	●	●	○	●	○	●	●	○	○	○	○	○
DXS207	○	○	★	★	○	★	○	○	○	●	●	○	○	○	○	★	○	★	○	○	○	★	●	●	★	○	○	○	●	●	○	●	★	●	●	○	○	○	○	○
DXS989	○	○	○	★	○	○	★	○	○	★	★	○	○	○	○	○	●	★	★	○	○	★	○	★	●	○	○	○	●	★	★	●	★	●	●	○	○	○	○	★
DXS1237	○	○	○	○	○	○	○	○	○	●	★	○	○	○	○	○	○	○	○	○	●	●	○	○	○	○	○	○	★	●	★	●	○	●	●	○	○	★	★	★
MAOA	○	○	★	★	○	★	○	○	○	●	●	○	●	○	○	●	○	★	○	○	★	●	○	●	★	★	○	○	●	●	★	●	○	●	○	○	★	★	★	○
DXS1367	○	○	○	★	○	○	★	○	○	●	●	○	○	★	★	○	●	★	★	○	○	○	●	●	○	○	○	○	●	●	○	○	★	○	●	○	★	○	○	○
DXS1003	○	★	○	○	○	★	○	○	★	●	●	○	○	○	○	○	○	●	★	○	○	○	○	●	★	○	○	○	●	●	★	★	○	★	★	○	○	○	○	○
DXS1111	○	○	★	○	★	★	○	○	○	★	★	○	★	★	○	●	★	★	○	○	○	○	★	●	★	○	○	○	★	●	○	●	★	●	●	○	★	○	★	○
DXS56	○	★	○	○	○	○	○	○	○	●	●	○	○	○	○	○	★	○	○	○	●	○	★	○	●	○	○	○	●	●	○	○	●	●	○	○	○	★	○	○
DXS738	○	○	○	○	★	○	○	★	○	★	●	○	○	○	★	★	★	○	○	★	○	○	★	★	○	★	★	★	★	○	○	★	○	●	○	●	○	★	○	○
DXS990	★	○	○	○	○	★	★	○	○	★	●	○	○	○	○	●	○	○	○	○	○	○	●	○	★	○	★	○	●	●	○	○	○	●	●	MI	★	○	○	★
DXS1153	★	○	○	○	○	○	★	○	★	★	★	○	★	○	★	○	○	★	★	○	★	○	●	○	★	○	○	○	★	○	★	★	★	★	★	○	○	○	○	○
DXS1220	○	○	○	○	○	○	★	○	○	●	★	○	●	○	○	○	●	○	○	○	★	★	★	●	○	○	★	○	○	★	★	★	○	★	★	★	○	★	○	★
DXS1001	○	○	○	○	○	○	★	○	○	●	●	○	●	○	○	★	★	○	★	○	★	★	●	●	★	○	○	○	○	★	★	○	○	★	★	★	○	★	○	★
DXS1047	○	○	★	○	★	○	○	○	○	●	●	○	★	★	○	★	○	MI	○	○	○	○	●	★	MI	○	★	★	●	●	○	★	●	●	●	○	○	○	○	○
DXS1192	○	○	○	★	★	○	○	○	○	●	●	○	○	○	●	★	★	○	○	○	★	○	●	●	○	○	★	★	●	●	○	●	●	★	★	○	★	○	●	○
DXS102	○	○	★	★	○	○	○	★	★	★	●	○	○	○	●	★	★	○	○	○	★	○	●	●	○	★	★	★	●	●	○	●	●	●	●	○	★	★	○	○
DXS731	○	○	○	○	○	○	○	○	○	★	●	○	★	★	★	○	○	○	○	○	★	○	●	★	○	★	★	★	★	★	○	●	●	★	★	★	○	○	★	○
LOH %	0	0	0	0	0	0	0	0	0	100	93	0	0	14	0	82	0	11	0	0	0	0	92	100	17	8	0	0	100	100	0	79	50	83	100	0	0	0	7	0

○: retention of heterozygosity; ●: loss of heterozygosity; ★: not informative; MI: microsatellite instability.

Figure 2 Relation between LOH and histological structure in an atypical carcinoid (#13). (**a**) No allelic losses at DXS102 microsatellite marker in an area with trabecular arrangement. (**b**) LOH with the same marker in an area with solid arrangement (lost allele indicated by an arrow).
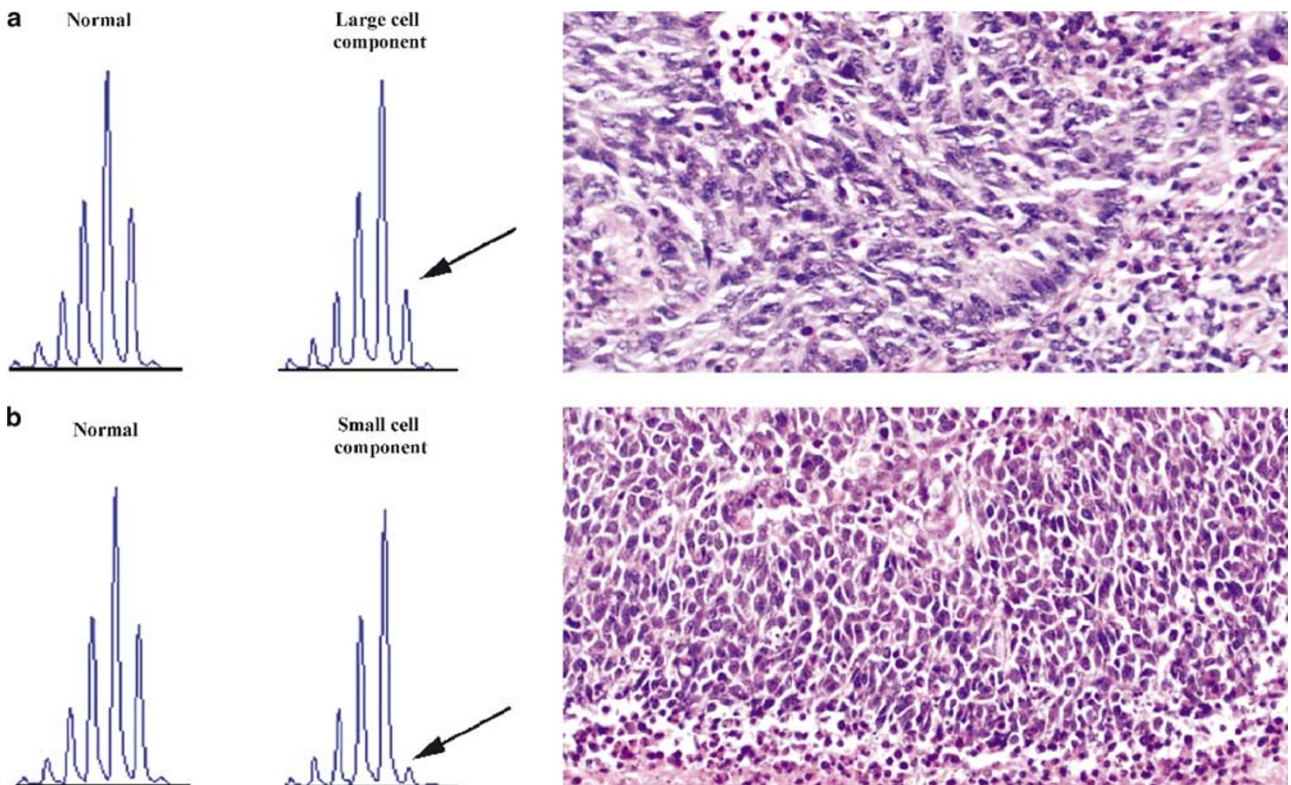

Figure 3 Representative patterns of LOH in different components of a combined large cell/small cell poorly differentiated carcinoma (#32). (**a**) LOH at DXS207 in the large cell tumor component (lost allele indicated by an arrow). (**b**) More extensive loss of the same allele in the small cell component (lost allele indicated by an arrow).
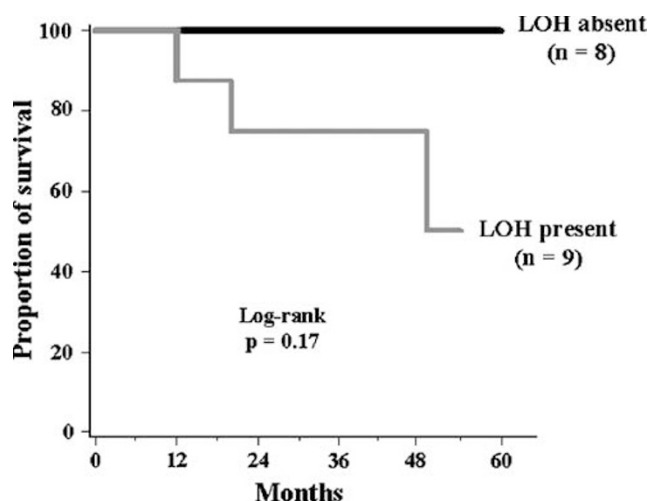
Figure 4 Survival curves of 17 patients with atypical carcinoid tumors of the lung according to their X chromosome LOH status.

tumors ($P < 0.0001$), except for the comparison between typical carcinoids and small cell lung carcinomas ($P = 0.2881$). In atypical carcinoids, presence of LOH was significantly associated with tumor size ($P = 0.043$) and pT status ($P = 0.003$), with no allelic losses found in tumors less than 3 cm of diameter. A trend toward association was found between LOH status and tumor stage ($P = 0.063$), with allelic losses most frequently found in atypical carcinoid patients with stage 3 of the disease. The survival curve of atypical carcinoid patients shown in Figure 4 reveals a shorter survival rate in patients with LOH than in those without LOH, although a statistical significance was not reached. No association was found with age ($P = 0.83$) or lymph node involvement ($P = 0.166$). In large cell neuroendocrine carcinomas, no significant association was found between LOH status and any of the clinicopathologic parameters investigated.

Frequency of LOH: Comparison at Different Microsatellite Loci (Table 2)

When the tumor groups with significant occurrence of X chromosome LOH were considered (ie atypical carcinoids and large cell neuroendocrine carcinomas), four chromosomal regions more frequently deleted could be identified. LOH ratios above 40% were found at the following microsatellite markers: DXS996 (Xp22.3, 43%) – DXS207 (Xp22.2, 42%); MAOA (Xp11.4–Xp11.3, 42%); DXS56 (Xq13.2–Xq13.3, 41%); DXS1192 (Xq26, 47%) – DXS102 (Xq26, 50%).

Discussion

Lung neuroendocrine tumors comprise a heterogeneous group of tumor types characterized by different degrees of clinical aggressiveness. Thus, they represent a useful model for the study of

genetic alterations associated with malignancy. In this regard, our study showed a progressive increase in the frequency of X chromosome LOH in lung neuroendocrine tumors, ranging from low-grade typical carcinoids to intermediate-grade atypical carcinoids to highly aggressive large cell neuroendocrine carcinomas. In contrast, small cell lung carcinomas presented only one exceptional loss. These results are in keeping with previous observations of a progressive accumulation of genetic defects from low- to high-grade neuroendocrine lung neoplasms.[8,9] Although a transition of either typical or atypical carcinoids to high-grade neuroendocrine carcinomas is currently deemed to be unlikely,[10] we herein indicate that carcinoids and large cell neuroendocrine carcinomas may share common genetic alterations on X chromosome, suggesting a critical role for these chromosomal regions in the pathogenesis of both tumors. Moreover, the finding of several X chromosome LOH in large cell neuroendocrine carcinomas but not in small cell lung carcinomas supports the view that these highly aggressive lung neuroendocrine carcinomas are genetically different.[10–12] Among atypical carcinoids, the correlation between LOH status and clinicopathological parameters such as tumor size, pN and stage, as well as the separation between survival curves of patients with and without losses, showing a shorter survival time for the former, both suggest a correlation of X chromosome LOH with a more aggressive phenotype. Additional associations between LOH and other clinicopathological parameters may have been hidden by the relatively low number of cases investigated, in particular for large cell neuroendocrine carcinomas, due to the overall low frequency of these neoplasms coupled with their prevalence in male rather than in female patients.[13]

The X chromosome has been implicated in many hereditary and sporadic diseases and LOH has been reported in different human tumors, especially in cancers of reproductive and urologic organs (breast, ovary, prostate, bladder; for a review see Liao et al[14]), with a few candidate oncosuppressor genes proposed. In the present study, the LOH status of X chromosome was assessed through a detailed allelotyping using 18 microsatellite markers distributed on both chromosomal arms, in an attempt to define minimal common deletion regions, that most likely harbor tumor suppressor genes. In this regard, although a statistical significance was not reached, it may be interesting to note that four chromosomal regions were found to include microsatellite markers with LOH rate above 40%: Xp22.3–22.2, Xp11.4–11.3, Xq13.2–13.3 and Xq26.

Xq26, investigated with the markers DSX1192 and DXS102 and appearing as the most frequently deleted region in the present study, was reported to show high frequency of LOH in several types of cancers, especially those of the ovary and breast.[14–17] A potential oncosuppressor gene located in this

region is the *MEF* (myeloid elf-1-like factor) gene, an ETS transcription factor located at Xq26.1.[18] In particular, *MEF* suppresses the tumorigenesis of human nonsmall cell lung carcinoma cell lines A549 both *in vitro* and *in vivo*, most likely through the inhibition of matrix metalloproteinases and interleukin-8 gene transcription. Also the glypican 3 (*GPC3*) gene, encoding for a membrane-bound heparan sulfate proteoglycan, maps at Xq26.[15] Mutations of *GPC3* are implicated in the pathogenesis of the Simpson–Golabi–Behmel syndrome, an X-linked disorder characterized by overgrowth, dysplasia and multiple congenital anomalies.[15] *GPC3* has been found to be silenced in breast and lung cancers, malignant mesotheliomas and ovarian cancer cell line.[14] Moreover, recent studies have shown that *GPC3* expression is decreased in lung adenocarcinoma and in nonmalignant lung tissue of smokers. These data suggest that *GPC3* may be a lung tumor suppressor gene involved in cigarette smoke-associated lung carcinogenesis.[19] In our study, Xq26 was the most frequently deleted region; however, a relationship between this LOH and cigarette smoke in atypical carcinoids and large cell neuroendocrine carcinomas seems to be unlikely because the same region was unaffected in small cell lung carcinoma, a lung cancer strongly associated with smoke.[20]

Interestingly, in a single case of atypical carcinoid (#13), a relation of LOH restricted to the two markers at Xq26 with tumor histological arrangement was found, with the losses occurring only in the solid but not in the trabecular component of the tumor. This finding could explain on the one hand the reported genetic heterogeneity of atypical carcinoids as a function of tumor sampling,[21] and on the other, could support the transition of some atypical carcinoids directly from a pre-existing typical carcinoid. Analysis of further cases is warranted to substantiate this observation and to evaluate its biological significance.

LOH at Xp22.3–22.2, investigated in our study with the markers DXS996 and DXS207, was found in ovarian cancer, with allelic losses twice as frequent in patient carriers of germ-line *BRCA1* mutations: these data suggest that a tumor suppressor gene important for *BRCA1*-associated carcinogenesis may be located at that region.[22] Moreover, LOH at pseudoautosomal regions at the end of Xp (Xp22.3) and Xq (Xq28) arms was found in sporadic breast cancers, in association with negative hormonal receptor phenotype, an indicator of poorer prognosis and more aggressive behavior in these neoplasms.[23]

Microsatellite MAOA at Xp11.4–Xp11.3 is a dinucleotide repeat included in the *monoamine oxidase A* gene, which encodes for a mitochondrial outer membrane protein implicated in the catabolism of neuroactive and vasoactive amines in the central nervous system and in peripheral tissues. *MAOA* gene product preferentially catalyzes the oxidative deamination of biogenic amines such as 5-hydroxytryptamine, norepinephrine and epinephrine.[24] To our knowledge, no data on cancer-associated allelic losses involving the microsatellite MAOA, as well as DXS56 at Xq13.2–Xq13.3, have been reported in the literature so far.

In our study, a striking difference was found between the two types of high-grade neuroendocrine lung tumors, with the highest frequency of X chromosome losses observed in large cell neuroendocrine carcinomas and the virtual absence of such losses seen in small cell lung carcinomas. This result contributes to the current debate on the histogenetic relationship between these two neuroendocrine tumor types of the lung, with particular regard to their origin from either common or independent progenitor cells. Indeed, both genetic similarities[8,9,25–27] and striking genetic differences[11,12] between large cell neuroendocrine carcinomas and small cell lung carcinomas have been reported. Both tumor types have been found to show a high number of chromosomal aberrations, sharing losses of chromosome 3p, 4, 5q, 13 and gains of 5p, all changes commonly observed also in nonneuroendocrine lung carcinomas.[11] In contrast, if only aberrations peculiar to neuroendocrine tumors are considered, some of them (6p gains) have been found to be specific for large cell neuroendocrine carcinomas and others (loss of 10 and 16q, gain of 3q) for small cell lung carcinomas.[11] Moreover, although frequent abnormalities in the tumor suppressor gene *Rb* pathway have been shown in both types of high-grade lung neuroendocrine tumors, gene inactivation was found to be achieved through different mechanisms, mostly represented by Rb protein loss in small cell lung carcinomas, and by p16 protein loss and cyclin D1 overexpression in large cell neuroendocrine carcinomas.[12] Our results on X chromosome LOH introduce further evidence for different genetic events between large cell neuroendocrine carcinomas and small cell lung carcinomas.

Although combined small cell carcinoma is a well-recognized morphological variant of this tumor,[1] our findings of common genetic losses in either large- or small cell component point to a different molecular pathogenesis of this tumor from the pure forms of small cell lung carcinomas. Further studies on a larger number of combined lesions are clearly warranted to elucidate this important point.

Taken together with previous data from other tumor types, our results allow us to assume that X chromosome LOH is a common and specific feature of malignant neuroendocrine tumors of foregut-derivative organs. Indeed, the association between X chromosome allelic losses and malignancy has been clearly documented in endocrine tumors of the stomach[7] and pancreas.[6,28] Moreover, X chromosome LOH has recently been found to correlate with aggressive post operative tumor

804

growth in pancreatic gastrinomas.[29] In contrast, endocrine tumors of midgut and hindgut origin, even if malignant, did not present a significant occurrence of X chromosome losses.[6] In this regard, it is worth noting that neuroendocrine tumors of foregut and midgut origin differ also in the genetic mechanisms involved in tumor induction. The inactivation of the *MEN1* gene, in fact, appears to be restricted to neoplasms of foregut derivative organs, as shown by both LOH studies at 11q13[30,31] and by the pattern of tumor involvement in the *MEN1* syndrome.[32] In midgut tumors, in contrast, evidence for the involvement of *SDHD* (succinate-ubiquinone oxidoreductase subunit D) gene at 11q23[33] and/or of a still unidentified tumor suppressor gene located on chromosome 18q, potentially responsible for early tumor development, support different mechanisms of tumor induction.[34,35]

In conclusion, these results provide a genetic basis for the classification of Williams and Sandler[5] and support the assumption that at least two genetically distinct families of neoplasms (of foregut and midgut origin, respectively) may be identified among neuroendocrine tumors arising in the lung and gastroenteropancreatic tract.

Acknowledgements

References

1 Travis WD, Colby TV, Corrin B, *et al.* The spectrum of neuroendocrine tumours. In: Sobin LH (ed). WHO International Histological Classification of Tumours. Histological Typing of Lung and Pleural Tumors. Springer-Verlag: Berlin, 1999, pp 7–9.

2 Mezzetti M, Raveglia F, Panigalli T, *et al.* Assessment of outcomes in typical and atypical carcinoids according to latest WHO classification. Ann Thorac Surg 2003;76:1838–1842.

3 Kovatich A, Friedland DM, Druck T, *et al.* Molecular alterations to human chromosome 3p loci in neuroendocrine lung tumors. Cancer 1998;83:1109–1117.

4 Colby TV, Koss MN, Travis WD. Carcinoids and other neuroendocrine tumors. In: Rosai J, Sobin LH (eds). Atlas of tumor pathology. Tumors of the Lower Respiratory Tract. Vol. 13, Armed Forces Institute of Pathology: Washington, 1995, pp 287–317.

5 Williams ED, Sandler M. The classification of gastro-intestinal carcinoid tumors. Lancet 1963;1:238–239.

6 Pizzi S, D'Adda T, Azzoni C, *et al.* Malignancy-associated allelic losses on the X-chromosome in foregut but not in mid-gut endocrine tumours. J Pathol 2002;196:401–407.

7 D'Adda T, Candidus S, Denk H, *et al.* Gastric neuroendocrine neoplasms: tumour clonality and malignancy-associated large X-chromosomal deletions. J Pathol 1999;189:394–401.

8 Onuki N, Wistuba II, Travis WD, *et al.* Genetic changes in the spectrum of neuroendocrine lung tumors. Cancer 1999;85:600–607.

9 Gugger M, Burckhardt E, Kappeler A, *et al.* Quantitative expansion of structural genomic alterations in the spectrum of neuroendocrine lung carcinomas. J Pathol 2002;196:408–415.

10 Ullmann R, Petzmann S, Klemen H, *et al.* The position of pulmonary carcinoids within the spectrum of neuroendocrine tumors of the lung and other tissues. Genes Chromosomes Cancer 2002;34:78–85.

11 Ullmann R, Petzmann S, Sharma A, *et al.* Chromosomal aberrations in a series of large-cell neuroendocrine carcinomas: unexpected divergence from small-cell carcinoma of the lung. Hum Pathol 2001;32:1059–1063.

12 Beasley MB, Lantuejoul S, Abbondanzo S, *et al.* The P16/cyclin D1/Rb pathway in neuroendocrine tumors of the lung. Hum Pathol 2003;34:136–142.

13 Corrin B (ed). Pathology of the lungs. Churchill Livingstone: London, 2000, 668pp.

14 Liao DJ, Du QQ, Yu BW, *et al.* Novel perspective: focusing on the X chromosome in reproductive cancers. Cancer Invest 2003;21:641–658.

15 Liu Y, Ganesan TS. Tumour suppressor genes in sporadic epithelial ovarian cancer. Reproduction 2002;123:341–353.

16 Choi C, Kim MH, Juhng SW. Loss of heterozygosity on chromosome Xp22.2–p22.13 and Xq26.1–q27.1 in human breast carcinomas. J Korean Med Sci 1998;13:311–316.

17 Choi C, Cho S, Horikawa I, *et al.* Loss of heterozygosity at chromosome segment Xq25–26.1 in advanced human ovarian carcinomas. Genes Chromosomes Cancer 1997;20:234–242.

18 Seki Y, Suico MA, Uto A, *et al.* The ETS transcription factor MEF is a candidate tumor suppressor gene on the X chromosome. Cancer Res 2002;62:6579–6586.

19 Kim H, Xu GL, Borczuk AC, *et al.* The heparan sulfate proteoglycan GPC3 is a potential lung tumor suppressor. Am J Respir Cell Mol Biol 2003;29:694–701.

20 Stewart BW, Kleihues P (eds). World cancer report. IARC Press: Lyon, 2003, 352pp.

21 Ullmann R, Schwendel A, Klemen H, *et al.* Unbalanced chromosomal aberrations in neuroendocrine lung tumors as detected by comparative genomic hybridization. Hum Pathol 1998;29:1145–1149.

22 Buekers TE, Lallas TA, Buller RE. Xp22.2–3 loss of heterozygosity is associated with germline BRCA1 mutation in ovarian cancer. Gynecol Oncol 2000;76:418–422.

23 Roncuzzi L, Brognara I, Cocchi S, *et al.* Loss of heterozygosity at pseudoautosomal regions in human breast cancer and association with negative hormonal phenotype. Cancer Genet Cytogenet 2002;135:173–176.

24 Shih JC, Thompson RF. Monoamine oxidase in neuropsychiatry and behavior. Am J Hum Genet 1999;65:593–598.

25 Walch AK, Zitzelsberger HF, Aubele MM, *et al.* Typical and atypical carcinoid tumors of the lung are characterized by 11q deletions as detected by comparative genomic hybridization. Am J Pathol 1998;153: 1089–1098.

26 Przygodzki RM, Finkelstein SD, Langer JC, *et al.* Analysis of p53, K-ras-2, and C-raf-1 in pulmonary neuroendocrine tumors. Correlation with histological subtype and clinical outcome. Am J Pathol 1996;148: 1531–1541.

27 Araki K, Ishii G, Yokose T, *et al.* Frequent overexpression of the c-kit protein in large cell neuroendocrine carcinoma of the lung. Lung Cancer 2003;40: 173–180.

28 Missiaglia E, Moore PS, Williamson J, *et al.* Sex chromosome anomalies in pancreatic endocrine tumors. Int J Cancer 2002;98:532–538.

29 Chen YJ, Vortmeyer A, Zhuang Z, *et al.* X-chromosome loss of heterozygosity frequently occurs in gastrinomas and is correlated with aggressive tumor growth. Cancer 2004;100:1379–1387.

30 D'Adda T, Pizzi S, Azzoni C, *et al.* Different patterns of 11q allelic losses in digestive endocrine tumors. Hum Pathol 2002;33:322–329.

31 Leotlela PD, Jauch A, Holtgreve-Grez H, *et al.* Genetics of neuroendocrine and carcinoid tumours. Endocr Relat Cancer 2003;10:437–450.

32 Brandi ML, Bordi C, Tonelli F, *et al.* Multiple endocrine neoplasia type 1. In: Bilezikian JP, Raisz LG, Rodan GA (eds). Principles of Bone Biology, Vol. 2, 2nd edn. Academic Press: San Diego, 2002, pp 1047–1066.

33 Kytölä S, Nord B, Elder EE, *et al.* Alterations of the SDHD gene locus in midgut carcinoids, Merkel cell carcinomas, pheochromocytomas, and abdominal paragangliomas. Genes Chromosomes Cancer 2002;34: 325–332.

34 Löllgen RM, Hessman O, Szabo E, *et al.* Chromosome 18 deletions are common events in classical midgut carcinoid tumors. Int J Cancer 2001;92:812–815.

35 Kytölä S, Höög A, Nord B, *et al.* Comparative genomic hybridization identifies loss of 18q22–qter as an early and specific event in tumorigenesis of midgut carcinoids. Am J Pathol 2001;158:1803–1808.