

Genetic alterations in combined neuroendocrine neoplasms of the lung

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Large-cell neuroendocrine and small-cell lung carcinomas are highly aggressive neuroendocrine tumors that can be associated in a variant of 'small-cell lung carcinoma combined with large-cell neuroendocrine carcinoma'. Little is known about this rare tumor type with biphenotypic neuroendocrine differentiation. The aim of the present study was to genetically characterize each component of a series of combined small-cell/large-cell neuroendocrine carcinomas, to gain information on their histogenesis and to compare the alterations observed with those found in their respective pure forms. To this end, 22 formalin-fixed, paraffin-embedded lung neuroendocrine tumors obtained from surgical resections were investigated: six combined small-cell/large-cell carcinomas, eight pure large-cell carcinomas and eight pure small-cell carcinomas. For the combined neuroendocrine neoplasms, DNA was extracted separately from each of the two cytologically different populations. Allelic imbalance was investigated by PCR amplification of 30 highly polymorphic microsatellite markers located at 11 different chromosomal regions. A common background of genetic alterations, similar in both components of the combined neoplasms, was demonstrated at 17p13.1, 3p14.2–3p21.2, 4q12–4q24, 5q21 and 9p21. In fact, the two components appeared to be more similar to each other than to their respective pure forms. In addition, allelic imbalances preferentially involving one of the two components were found. These alterations often appeared to be specific for this histological variant, as compared with those observed in pure forms or in the literature. In conclusion, this is the first report in which a molecular characterization of the variant of small-cell lung carcinoma combined with large-cell neuroendocrine carcinoma was performed. The finding of common alterations in the two phenotypically different neuroendocrine cell components suggests a close genetic relationship and supports the hypothesis of a monoclonal origin from a common ancestor. The genetic differences observed provide the basis for the divergent differentiation and parallel the morphological differences in the two components of these combined neuroendocrine neoplasms.

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Neuroendocrine tumors of the lung are a family of neoplasms arising from the embryological foregut, which share common neuroendocrine markers plus the presence of neuroendocrine granules at electron microscopy, but which differ greatly in their biological behavior. They include typical and atypical carcinoids, with respectively low and intermediate malignant potential, and large-cell neuroendocrine and small-cell lung carcinomas, with a highly malignant clinical course and a dismal prognosis.^{1,2}

The distinction between large- and small-cell carcinomas is based substantially on cytological features, including larger vs smaller cell size, abundant vs scant cytoplasm, the presence vs absence of prominent nucleoli, coarsely vs finely granular chromatin, and polygonal vs round to fusiform shapes, respectively.³ However, a histological differential diagnosis between these two aggressive forms of neuroendocrine tumors can be difficult in some cases, due mostly to a frequent overlapping of cell size.^{4,5} On a molecular level, both similarities and differences have been described.^{6–8} While some authors have emphasized the distinctive features separating large- and small-cell carcinomas,^{6,7} others have underlined similarities, and called for their inclusion within the single group of high-grade neuroendocrine tumors.^{4,8}

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The only variant of small-cell lung carcinoma described in the latest WHO classifications of lung neoplasms is the 'combined small-cell lung carcinoma', in which small-cell carcinoma is associated with an additional non-small-cell component.⁹ This category also includes small-cell carcinomas combined with large-cell neuroendocrine carcinomas, but this is a rare variant that has not been extensively studied, particularly as regards the genetic alterations occurring in each of the two neuroendocrine components.

The aim of the present study was to separately analyze the genetic alterations characterizing each tumor component in a series of combined small-cell/large-cell neuroendocrine carcinomas of the lung. Both chromosomal regions harboring genes commonly involved in the pathogenesis of lung carcinomas, and regions reported to be more selectively involved in large-cell neuroendocrine or small-cell lung carcinoma were investigated. For comparison, the same analysis was also performed in a series of pure high-grade neuroendocrine lung tumors.

Materials and methods

Tumors

A total of 22 formalin-fixed and paraffin-embedded pulmonary neuroendocrine tumors obtained from surgical resections were investigated: six combined small-cell/large-cell neuroendocrine carcinomas, eight pure large-cell neuroendocrine carcinomas

and eight pure small-cell lung carcinomas (Table 1). Informed consent was obtained from all patients and/or guardians, in accordance with Italian law. All tumors were examined independently by three pathologists who are expert in the field (G Pelosi, C Bordi, and G Rindi), classified according to the histopathological criteria proposed by the WHO classification⁹ (Figure 1), and staged according to the latest TNM system (TNM, 6th edition, UICC, 2002). In the event of disagreement, a final consensus diagnosis was reached.

The combined neuroendocrine neoplasms were characterized by a clear-cut separation of the two cell populations (Figure 1c), thus allowing a molecular analysis of each neoplastic component to be performed. Both tumor and non-tumor tissues (ie, adjacent histologically normal lung parenchyma or uninvolved regional lymph nodes) were available.

DNA Extraction

Normal and tumor areas were manually microdissected from serial 4- μ m-thick histological sections stained with hematoxylin. The two cell populations of combined neuroendocrine neoplasms were microdissected separately in order to extract DNA from each single tumoral component. A neoplastic cellularity of at least 80% was obtained for all tumor samples. DNA extraction and purification were performed using a commercial kit (DNeasy Tissue kit; QIAGEN Inc., Valencia, CA, USA).

Table 1 Clinicopathological data of 22 patients affected by neuroendocrine tumors of the lung

Case number	Tumor type	Sex	Age at onset	Tumor size (cm)	pT	pN	Stage	Survival (months)	Vital status	Distal metastasis
1	Combined	F	66	2.5	4	2	3B	4	Dead	Lost to follow-up
2	Combined	M	81	5	2	2	3A	1	Dead	No
3	Combined	M	73	10.5	2	2	3A	15	Dead	Liver, bone
4	Combined	M	64	1.2	1	1	2A	14	Dead	Lung, liver, CNS
5	Combined	M	66	2.4	1	0	1A	2	AW	No
6	Combined	M	45	3.1	2	0	1B	9	AWD	Mediastinal lymph node
7	LCNEC	M	73	5	3	x	—	5	Dead	Lost to follow-up
8	LCNEC	F	66	5.5	2	x	—	2	Dead	Lost to follow-up
9	LCNEC	F	44	8	2	1	2B	11	Dead	Lung, liver
10	LCNEC	F	53	5	2	2	3A	24	Dead	Lung, liver
11	LCNEC	F	69	1.2	1	1	2A	37	Dead	Bone, liver
12	LCNEC	F	35	2	2	2	3A	3	Dead	Bone, CNS
13	LCNEC	M	45	7	4	1	3B	30	AW	No
14	LCNEC	M	71	5	2	1	2B	2	Dead	Lost to follow-up
15	SCLC	F	68	5	2	1	3B	43	Dead	Lung, liver
16	SCLC	M	35	1.5	1	x	—	—	—	Lost to follow-up
17	SCLC	F	51	2.5	1	2	3A	23	Dead	Lung, liver, thoracic wall
18	SCLC	F	62	3	1	2	3A	35	Dead	Brain, CNS, axilla
19	SCLC	F	70	2.5	1	1	2A	48	Dead	CNS, skin
20	SCLC	F	55	1.7	1	0	2A	72	AWD	Liver
21	SCLC	M	75	2	4	1	3B	13	AWD	CNS, mediastinum
22	SCLC	M	67	3.5	2	0	1B	28	AW	No

—, not available; AW, alive and well; AWD, alive with disease; CNS, central nervous system; Combined, small-cell lung carcinoma combined with large-cell neuroendocrine carcinoma; LCNEC, large-cell neuroendocrine carcinoma; SCLC, small-cell lung carcinoma.

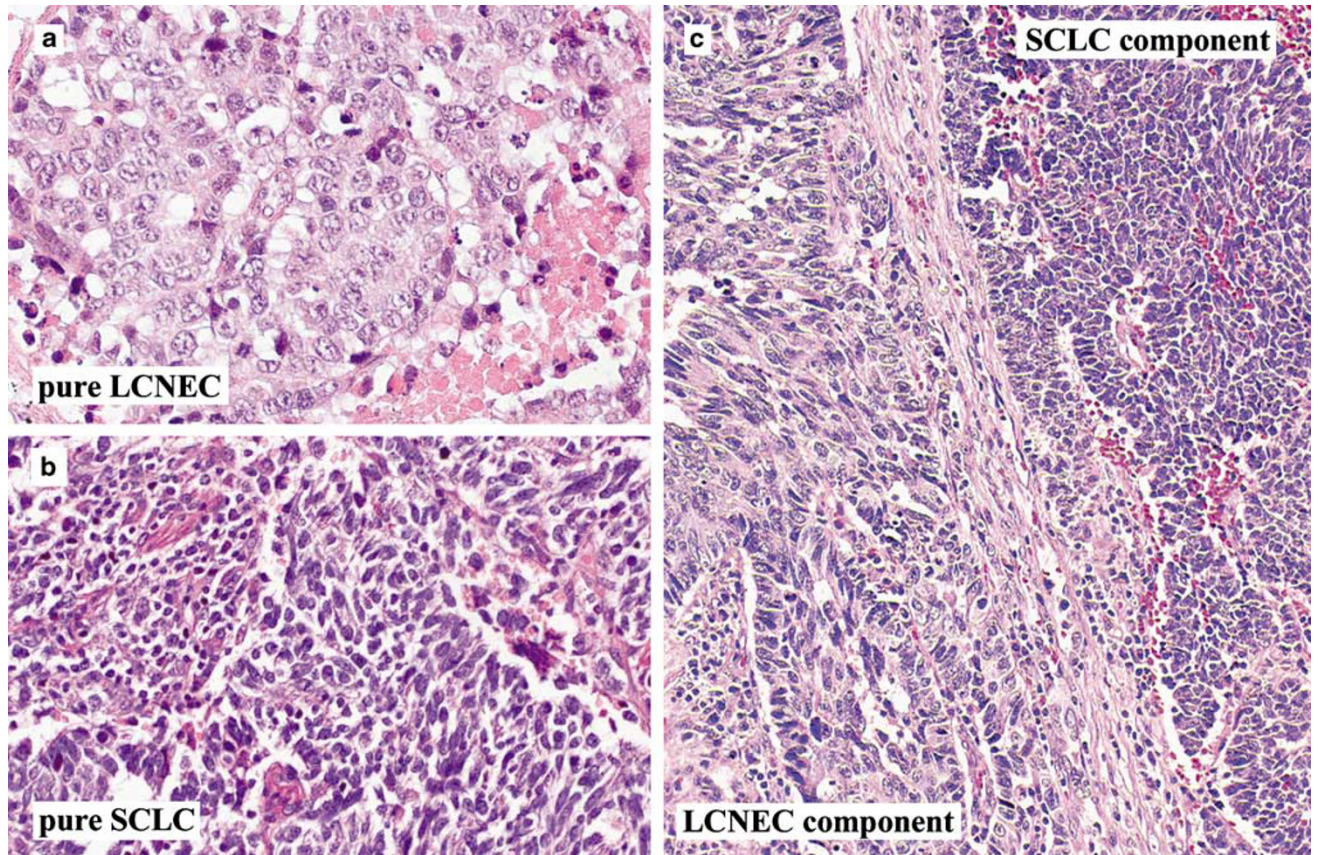


Figure 1 (a) Pure large-cell neuroendocrine carcinoma (LCNEC) of the lung, with cells characterized by abundant cytoplasm and prominent nucleoli (H&E, $\times 200$). (b) Pure small-cell lung carcinoma (SCLC), with cells characterized by scant cytoplasm and small, round to oval, nuclei with inconspicuous nucleoli (H&E, $\times 200$). (c) Combined small-cell/large-cell neuroendocrine carcinoma characterized by clear-cut separation of the two tumor-cell components (H&E, $\times 150$).

PCR Amplification and Fragment Analysis

Allelic imbalance was investigated with 30 highly polymorphic microsatellite markers (Table 2) located at 11 chromosomal regions (3p, 4q, 5q, 6p, 6q, 9p, 10q, 16q, 17p, X–Y pseudoautosomal regions 1 and 2). Microsatellite markers were chosen according to two different criteria: some located in chromosomal regions commonly altered in lung carcinomas (3p, 5q, 9p, 16q, 17p), while others in regions reported to be potentially discriminating between small-cell and large-cell neuroendocrine carcinoma (4q, 5q33, 6p, 10q, X).^{6,7,10,11} All tumors from female patients had previously been investigated for X chromosome loss of heterozygosity.¹¹ To verify the involvement of X chromosome in male patients, microsatellites located in the homologous regions at both ends of sex chromosomes (X–Y pseudoautosomal regions, X–Y PAR 1 and 2) were investigated. Microsatellite markers were PCR-amplified from tumor and control DNA using primers labelled with Beckman Coulter WellRED fluorescent dyes D3 or D4 (Beckman Coulter, Fullerton, CA, USA). For PCR amplification, 2 μ l of DNA were combined in a 25- μ l reaction mixture

containing 10 mM Tris-HCl (pH 9), 50 mM KCl, 0.1% Triton X-100, 200 μ M of each dNTP (Promega, Madison, WI, USA), 0.4 μ M of each primer, 1.5–2.0 mM MgCl₂ and 1.25 U *Taq* polymerase (Promega). PCR amplifications were performed with an AB 2700 (Applied Biosystems, Foster City, CA, USA) thermal cycler for 35 cycles. Individual PCR products were run on an eight-capillary CEQ™8000 DNA sequencer (Beckman Coulter), as previously described.¹¹ CEQ8000 Fragment Analysis software (Beckman Coulter) generated electrophoretic profiles in which alleles appeared as peaks, with area and height proportional to the concentration of PCR fragments.

Evaluation of Allelic Imbalance

An asymmetric allelic ratio in the tumor DNA at a microsatellite locus, as compared with the ratio observed in normal DNA, was described as allelic imbalance. Heterozygosity, that is, the presence of two distinct alleles in the normal tissue, was the prerequisite for evaluation of allelic imbalance. Peak height values produced by the Fragment Analysis

Table 2 Results of microallelotype analysis from six combined neuroendocrine lung carcinomas

Microsatellite marker ^a / cytogenetic localization/ main associated gene		Combined small-cell/large-cell neuroendocrine carcinomas (n = 6)											
		No. 1		No. 2		No. 3		No. 4		No. 5		No. 6	
		LC	SC	LC	SC	LC	SC	LC	SC	LC	SC	LC	SC
D3S1100	3p22–3p21.3	●	●	●	●	■	■	■	●	○	○	●	●
D3S1478	3p21.3–21.2	■	■	●	●	■	■	■	■	○	○	■	■
D3S1621	3p21.2–14.2	●	●	●	●	●	●	★	★	○	○	★	★
D3S1481	3p14.2	●	●	■	■	●	●	■	●	○	○	■	■
D4S2690	4q12–4q21	○	○	●	■	■	■	■	●	●	●	■	■
D4S411	4q24	○	○	■	■	●	●	■	●	○	○	★	★
D5S299	5q15–5q22	●	●	○	○	■	■	■M	○	○	○	★	★
D5S1965	5q22.2	■	■	○	○	★	★	○	○	★	★	★	★
D5S346	5q21–5q22	★	★	○	○	★	★	○	○	○	○	○	○
D5S422	5q32–5q33	■	■	○	○	★	★	○M	○	○	○	○	●
D6S470	6p25–6p23	★	★	★	★	■	■	○M	○	★	★	○	○
D6S1610	6p21.3	○	○	★	★	○	○	○M	○	○	●M	○	○
D6S87	6q22.3–23.1	■	■	★	★	○	○	■M	○	■	○	●	●
D6S976	6q23.2	★	★	★	★	★	★	■M	○	●	○	■	■
D9S157	9p23–9p22	○	○	○	○	■	■	○	○	○	○	■	■
D9S171	9p21	■	■	○	○	●	●	○M	○M	○	○	■	■
D10S569	10q22.3	○	○	■	○	●	●	★M	★	○	○	■	■
D10S1765	10q23–10q24	○	○	○	○	■	■	■	○	○	○	○	○
D10S1671	10q24	○	○	■	○	★	★	■	○	●	●	●	●
D10S209	10q25.3–26.1	○	○	■	○	■	■	■	○	○	○	○	○
D10S169	10q26.3	○	○	★	★	★	★	■	○	○	○	★M	★M
D16S301	16q22.1	●	●	★	★	○	○	○	○	■	■	●	●
D16S421	16q22.1	★	★	○	○	○	○	■	○	●	●	■	■
D16S496	16q22.1	■	■	★	★	○	○	■	★M	★	■	■	■
D16S507	16q23.2	●	●	○	○	○	○	○M	○	●	●	★	★
D16S422	16q24.2	●	●	○	○	●	●	●	○	■	■	●	●
TP53	17p13.1	●	●	■	■	●	●	■	■	●	●	●	●
DXYS233	X–Y PAR1	■	■	○	○	○	○	○	■	●	●	○	○
SHOX		■	■	○	○	○	○	○	●	●	●	○	○
DXYS154	X–Y PAR2	■	■	○	○	○	○	○	●	●	●	●	●
Fractional allelic imbalance index		0.73	0.73	0.44	0.33	0.73	0.73	0.64	0.45	0.64	0.64	0.73	0.82

^aPrimer sequences and microsatellite marker localizations available at the Genome Database (www.gdb.org) and PubMed UniSTS (http://www.ncbi.nlm.nih.gov) web sites.
LC, large-cell neuroendocrine carcinoma component; SC, small-cell lung carcinoma component; ●, AI with relative reduction of the smaller allele; ■, AI with relative reduction of the larger allele; ○, allelic retention; ★, not informative; M, suspected microsatellite instability.

software were used to calculate the following ratio: (lower allele/higher allele)_{TUM}/(lower allele/higher allele)_{NORM}. Allelic imbalance, scored when the ratio values were <0.6 or >1.67, may reflect the complete loss of one allele masked by the presence of a background of normal cells, by tumor heterogeneity or by non-clonal loss, or it may reflect an increased DNA copy number.¹² For each tumor case, a fractional allelic imbalance index was calculated as follows: number of chromosomal regions harboring at least one allelic imbalance/number of chromosomal regions with at least one informative marker.

Genetic Concordance in Combined Neuroendocrine Neoplasms

Genetic concordance was defined as the finding of an absence of alterations or the presence of

imbalances involving the same allele in both components of each combined neuroendocrine tumor.

Fluorescent *In Situ* Hybridization

Six male patients' tumors displaying allelic imbalance at X–Y PAR regions (combined tumor nos. 4 and 5, large-cell neuroendocrine carcinoma nos. 13 and 14, small-cell lung carcinoma nos. 21 and 22) were investigated by fluorescent *in situ* hybridization (FISH) as described elsewhere,¹³ in order to understand which sex chromosome was affected by the imbalance. The chromosome enumeration probe (CEP) used was a mixture of a spectrum orange-labelled CEP X probe specific for the α -satellite centromeric region of chromosome X, and a spectrum green-labelled CEP Y probe specific for the satellite III (Yq12) region of chromosome

Y (Vysis Inc., Downers Grove, IL, USA). The absence of orange or green spots in the tumors was indicative of loss of chromosome X or Y, respectively.

Statistical Analysis

Frequencies of allelic imbalance were compared using Fisher's exact test. Fractional allelic imbalance indexes were compared with the non-parametric Mann–Whitney test. All analyses were carried out using GraphPad InStat version 3.06 for Windows (GraphPad Software, San Diego, CA, USA). All *P*-values were based on two-sided testing. *P*-values less than 0.05 were considered statistically significant.

Results

Allelic Imbalances in Combined Neuroendocrine Carcinomas (*n* = 6)

Allelic imbalances involving both components of combined small-cell/large-cell neuroendocrine carcinomas with the same frequency were found at 17p (p53) in 100% of cases (6/6); at 3p (RASSF1A, FHIT) and 4q in 83% of cases (5/6) and at 5q (APC) and 9p (CDKN2A and CDKN2B) in 50% of cases (3/6) (Figure 2a; Table 2). Allelic imbalances preferentially involving one of the two components were also found, although these differences did not reach statistical significance. The large-cell component was more frequently altered than the small-cell component at 10q (83 vs 50%), 16q (83 vs 67%) and 6q (80 vs 40%). The small-cell component was more frequently altered at X–Y PAR2 (67 vs 50%), X–Y PAR1 (50 vs 33%) and 6p (40 vs 20%). Median fractional allelic imbalance indexes did not disclose any statistically significant difference, being 0.69 in both components (range 0.44–0.73 in large-cell and 0.33–0.82 in small-cell components). At FISH analysis, the combined neuroendocrine carcinoma no. 4 showed the presence of both signals in the large-cell component, in agreement with an absence of allelic imbalance at PAR regions, whereas the presence of X and an absence of Y signals was found in the small-cell component, indicating a complete loss of Y chromosome. The combined tumor no. 5 showed the presence of X and an absence of Y signal, indicative of a loss of Y chromosome in both components.

Genetic Concordance Between the Two Components of Combined Neuroendocrine Carcinomas

A high degree of genetic concordance between the two components was found in five out of six combined neuroendocrine carcinomas, ranging from 82 to 100% of all informative chromosomal regions investigated (Figure 2b; Table 2). Only tumor no. 4 showed a very low level of concordance (18%)

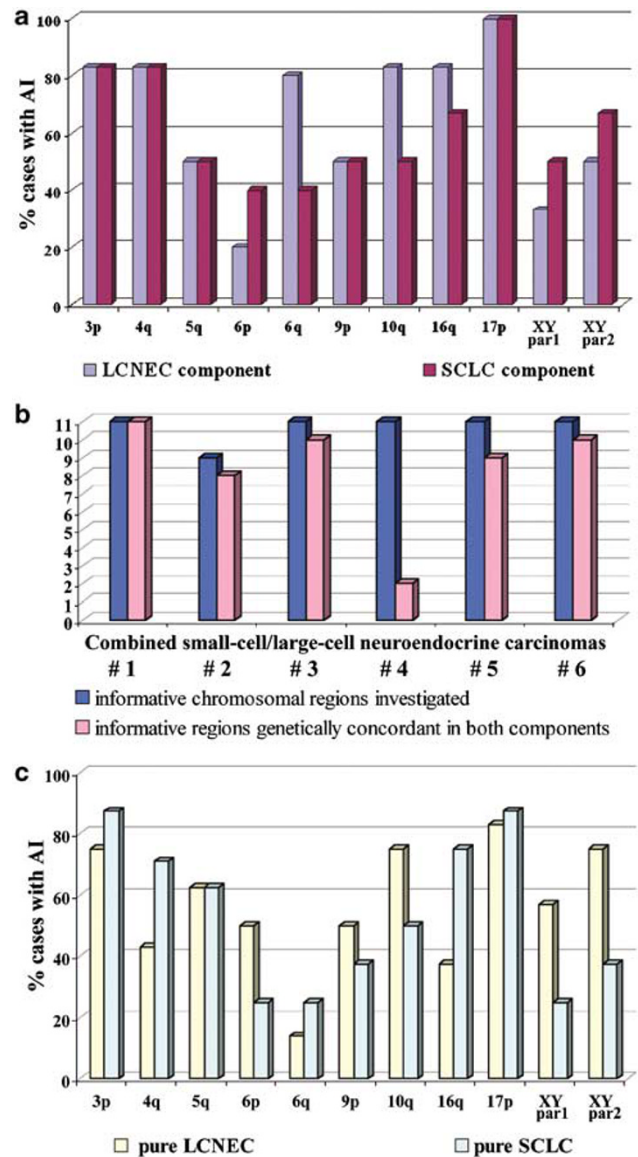


Figure 2 (a) Frequency of allelic imbalances at 11 chromosomal regions in the two tumor-cell components of six combined neuroendocrine carcinomas of the lung. (b) Genetic concordance between components in each combined carcinoma investigated. (c) Frequency of allelic imbalances at 11 chromosomal regions in eight pure large-cell neuroendocrine carcinomas (LCNEC) and eight pure small-cell lung carcinomas (SCLC).

between the two components. This case displayed some microsatellite instability in the large-cell component, although amplification of markers commonly used for instability testing (BAT26, BAT40 and L-MYC) failed to demonstrate a classic unstable pattern.

Allelic Imbalances in Pure Forms (*n* = 8)

In both types of pure neoplasms, the frequency of allelic imbalance was found to be identical at region 5q (63% of cases, 5/8) and similar at 17p (83% in large-cell vs 88% in small-cell carcinomas) and 3p

(75 vs 88%) (Figure 2c; Table 3). Allelic imbalances more frequently involving pure large-cell neuroendocrine carcinomas were found at 10q (75 vs 50%), X-Y PAR2 (75 vs 38%), X-Y PAR1 (57 vs 25%), 6p (50 vs 25%) and 9p (50 vs 38%). Allelic imbalances more frequently involving pure small-cell carcinomas were found at 16q (75 vs 38%), 4q (71 vs 43%) and 6q (25 vs 14%). Although remarkable at some chromosomal regions, none of these differences reached statistical significance. Median fractional allelic imbalance index was 0.60 in pure large-cell neuroendocrine (range 0.2–1.0) and 0.55 in pure small-cell carcinomas (range 0.09–0.80), a difference not significant.

FISH data showed the presence of orange signal for the CEP X probe and an absence of the green signal for the CEP Y probe, indicating a complete loss of Y chromosome in both cases of pure large-cell carcinomas from male patients with multiple imbalances at PAR regions (nos. 13 and 14). FISH data showed the presence of both X and Y signals in the two pure small-cell carcinomas from male patients with multiple imbalances at PAR regions (no. 21 and 22). Case no. 21 was characterized by

an absence of Y signal in 30% of cells, whereas case no. 22 revealed a polysomic pattern, with an over-representation of both sex chromosomes (average number of signals per cell: 2.9 for X and 1.4 for Y). Thus, in this case, the X chromosome increased copy number most likely accounted for the allelic imbalances at PAR region observed at microallelotyping.

Comparison of Alterations in Combined and Pure Forms

The comparison of large-cell neuroendocrine components of combined neoplasms vs pure large-cell neuroendocrine carcinomas disclosed similar frequencies of allelic imbalance, with differences below 25%, at 3p, 5q, 9p, 10q, 17p and X-Y PAR1 regions. Wider differences, above 25%, were found at the following regions: 4q (83% combined vs 43% pure), 6p (20 vs 50%), 6q (80 vs 14%; $P=0.072$), 16q (83 vs 38%) and X-Y PAR2 (50 vs 75%). Only the differences at 6q regions approached statistical significance (Figure 2a and c).

Table 3 Results of microallelotyping analysis from 16 pure high-grade neuroendocrine lung carcinomas

Microsatellite marker ^a	Large-cell neuroendocrine carcinomas (n = 8)								Small-cell lung carcinomas (n = 8)								
	No. 7	No. 8	No. 9	No. 10	No. 11	No. 12	No. 13	No. 14	No. 15	No. 16	No. 17	No. 18	No. 19	No. 20	No. 21	No. 22	
D3S1100	●	★	★	○	●	○	●	●	3p	■	★	○	●	★	●	■	●
D3S1478	●	○	●	○	●	○	●	★		●	●	○	●	★	●	■	●
D3S1621	●	★	★	○	■	○	●	●		■	●	★	●	○	●	★	●
D3S1481	■	○	■	○	★	■	★	■		●	★	★	●	●	■	★	●
D4S2690	■	○	●	★	○	○	■	○	4q	●	○	○	●	■	■	●	★
D4S411	★	★	○	★	★	○	■	○		●	○	★	★	■	■	★	★
D5S346	●	○	■	○	■	○	■	■	5q	●	○	○	●	○	■	●	■
D5S422	■	○	★	○	■	○	★	●		●	○	○	■	○	■	●	●
D6S470	○	○	○	★	○	●	○	●	6p	○	●	○	★	●	○	○	★
D6S1610	●	○	○	○	○	★	●	○		○	○	○	○	●	○	○	○
D6S87	■	○	★	○	★	★	★	○	6q	○	○	★	○	●	■	○	○
D6S976	★	★	○	○	○	○	★	★		○	○	○	○	★	★	○	○
D9S157	○	○	●	○	○	★	■	★	9p	●	○	★	○	○	●	○	■
D9S171	○	○	★	○	★	●	○	●		●	★	○	★	○	★	○	●
D10S569	●	○	●	●	●	○	●	★	10q	■	○	★	■	○	○	■	●
D10S1765	■	○	○	★	■	○	●	■		■	○	★	●	★	★	●	■
D10S1671	★	○	○	●	●	★	★	●		●	○	○	●	○	★	★	■
D10S209	■	○	○	●	■	○	■	★		★	★	○	○	○	○	■	●
D10S169	●	★	★	★	■	○	■	★		★	★	★	○	★	★	●	★
D16S301	○	★	★	○	■	○	★	○	16q	■	○	○	●	■	●	★	○
D16S421	★	★	★	★	■	★	■	○		★	★	○	★	○	●	■	○
D16S496	○	■	★	○	●	○	■	○		■	○	★	●	○	■	★	○
D16S507	★	●	○	○	★	○	●	○		■	○	○	●	○	■	★	●
D16S422	○	■	★	★	■	○	■	★		■	○	○	■	○	○	■	★
TP53	●	■	○	■	★	■	★	■	17p	●	■	■	■	●	○	●	●
DXYS233	○	■	★	○	●	★	●	●	X-Y PAR1	○	★	○	○	○	○	■	★
SHOX	○	■	★	○	■	○	■	■		○	○	○	★	○	○	○	●
DXYS154	○	■	●	○	■	○	■	■	X-Y PAR2	○	■	○	○	○	○	■	●
FAI	0.64	0.36	0.60	0.20	0.60	0.45	1.0	0.73	0.64	0.36	0.09	0.55	0.55	0.55	0.73	0.80	

^aPrimer sequences and microsatellite marker localizations available at the Genome Database (www.gdb.org) and PubMed UniSTS (<http://www.ncbi.nlm.nih.gov>) web sites. FAI, fractional allelic imbalance index; ●, AI with relative reduction of the smaller allele; ■, AI with relative reduction of the larger allele; ○, allelic retention; ★, not informative.

The comparison of small-cell components of combined neoplasms vs pure small-cell carcinomas disclosed similar frequencies of allelic imbalance at 3p, 4q, 5q, 6p, 6q, 9p, 10q, 16q and 17p regions, with differences below 25%. Differences above 25%, although not statistically significant, were found only at X–Y PAR1 (50% combined vs 25% pure) and X–Y PAR2 (67 vs 38%).

Discussion

The combined small-cell/large-cell neuroendocrine carcinoma variant is a pulmonary neoplasm with biphenotypic neuroendocrine differentiation that has never been extensively studied. To our knowledge, this is the first study in which a separate genetic characterization of the two components of this combined neoplasm was performed. Ullmann *et al*⁶ performed a comparative genomic hybridization (CGH) study on five mixed small-cell/large-cell neuroendocrine carcinomas of the lung, but no discrimination between the two phenotypic components was reported. In the present investigation, only combined neoplasms with physically separated large-cell and small-cell areas were taken into account to allow separate microdissection, DNA extraction and genetic analysis through microallelotyping of the two components. Microallelotype analysis is a useful tool to disclose allelic imbalances, mostly reflecting the loss of tumor-suppressor genes, and to define the polyclonal or monoclonal origin of mixed, collision and multiple tumors, by comparison of the respective molecular patterns.^{14–16}

In the present series of combined neuroendocrine neoplasms, a common background of allelic imbalances similarly involving both components was demonstrated. These genetic alterations mostly targeted chromosomal regions known to harbor tumor-suppressor genes frequently involved in lung carcinogenesis, namely 17p13.1, 3p14.2–3p21.2, 5q21 and 9p21.^{7,17–19} Alterations of chromosomes 3p, 9p21 and 17p13 are frequently found even in precursor lesions and, therefore, are considered as critical steps in early tumorigenesis.¹⁷ The report of common genetic alterations, especially in chromosomal regions involved in early carcinogenesis, suggests a close genetic relationship between the two phenotypically different components of these combined neoplasms. When the genetic relationship between the distinct components of each biphenotypic neuroendocrine neoplasm was considered, more similarities emerged. In fact, in the majority of patients the two components showed a high degree of genetic concordance, represented by either lack of alterations or presence of imbalances involving the very same allele. These similarities support the hypothesis of a monoclonal carcinogenesis mechanism, with tumor cells of the two

components deriving from a common precursor undergoing divergent differentiation.

In keeping with this interpretation is our observation of 4q12–4q24 imbalances occurring with the same high frequency (83%) in both components of our neuroendocrine combined cancers. On the contrary, the literature on pure forms has reported that losses at 4q21 and 4q24 occur exclusively in small-cell lung carcinomas, being observed in 40% of these, whereas absent in large-cell neuroendocrine carcinomas.⁷ Our data on pure neoplasms, although indicating a certain degree of 4q alterations also in large-cell carcinomas (43%), are in agreement with the literature, confirming their prevalence in small-cell lung carcinomas (71%). Therefore, the distinct neuroendocrine components of combined neoplasms appear to be more similar to each other than to their respective pure forms, another clue supporting the close genetic relationship between the two components.

Besides a common background of genetic alterations in both components, differences were also observed in combined neoplasms, suggesting divergent differentiation and paralleling morphological differences, even though no statistically significant differences were found, possibly due to the limited number of combined cases investigated.

The chromosomal regions more differentially altered in combined neoplasms were 6q, 10q and 16q in the large-cell component, and 6p and X–Y PAR regions in the small-cell component. Allelic losses on chromosome 10q are frequently reported in human neoplasms, and, in lung carcinomas, have been suggested as being associated with tumor progression and metastatic phenotype.^{7,20} Recently, losses at 10q22.1–26.3 have been found to significantly correlate with poor prognosis in large-cell neuroendocrine carcinomas, thereby suggesting that tumor-suppressor gene(s) responsible for an aggressive phenotype may be harbored in that chromosomal region.⁷ In the present study, imbalances at 10q were more frequently observed in large-cell than in small-cell carcinomas, in both combined and pure tumors.

Recently, a high-resolution CGH analysis has reported losses at 16q21–24 as one of the newly identified chromosomal alterations common to both small and large-cell neuroendocrine carcinomas.⁷ Losses at 16q21–24 seem to be a specific neuroendocrine alteration, probably playing a key role in the pathogenesis of high-grade neuroendocrine tumors, being infrequently reported in non-small-cell lung carcinomas.⁷ In our study, the involvement of the 16q region in high-grade neuroendocrine tumors was confirmed. In combined neoplasms, allelic imbalances at 16q appeared to be slightly more frequent in the large- than in the small-cell component, whereas in pure forms this alteration was more prevalent in small-cell carcinomas.

Losses at 6q have frequently been reported in various human neoplasms. Among these, sporadic

endocrine pancreatic tumors (sharing with neuroendocrine lung neoplasms a common origin from the embryonal foregut) present two hot spots at 6q22.1 and 6q23–q24, potentially harboring putative tumor-suppressor genes involved in their oncogenesis and malignant progression.²¹ In the present study, 6q imbalances had occurred in the large-cell component of combined neoplasms, with a frequency double that of the small-cell component (80 vs 40%). It is notable that this region was not substantially affected in the pure forms (below 30%), including the large-cell neuroendocrine carcinomas. Therefore, a marked difference in the frequency of 6q allelic imbalances was observed in pure large-cell neuroendocrine carcinomas vs the large-cell component of combined neoplasms, a difference approaching statistical significance despite the limited number of cases investigated.

In our combined neoplasms, imbalances at 6p25–21.3 occurred more frequently in the small- rather than in the large-cell component. Similar to region 4q, these data do not agree with those reported in the literature in the corresponding pure tumors. In fact, losses at 6p21.3, harboring the tumor necrosis factor- α , were reported more often in large- than in small-cell neuroendocrine carcinomas of the lung,⁷ a finding in agreement with our own data on pure forms, which show double the frequency of imbalances in pure large-cell- as compared with small-cell carcinomas (50 vs 25%).

A similar discrepancy between pure and combined high-grade neuroendocrine carcinomas of the lung was observed also at X–Y PAR regions, that is, those regions of sex chromosomes sharing X–Y homologies, pairing regularly at male meiosis and undergoing recombination.¹³ In previous studies by our laboratory, allelic losses on X chromosome were found to correlate with malignancy in foregut endocrine neoplasms^{11,22} and colorectal cancers.¹³ In lung neuroendocrine tumors from female patients, X chromosome deletions proved to be a frequent event in large-cell carcinomas, but very rare in small-cell carcinomas.¹¹ To verify this potentially discriminating marker also in male patients, we amplified microsatellite markers located in the X–Y PAR regions. Indeed, pure neuroendocrine lung neoplasms confirmed previous data,¹¹ with a frequency of alterations higher in large- than in small-cell carcinomas. Conversely, in the two components of combined neoplasms, all cases showed a concordant genetic pattern, with the exception of case no. 4, which was characterized by allelic imbalances confined to the small-cell component alone. Interestingly, FISH analysis performed in male patients with imbalances at PAR regions demonstrated the complete absence of Y signal in pure large-cell carcinomas and both components of the combined tumors investigated. In these cases, therefore, Y chromosome appears to be the sex chromosome targeted by the deletions, suggesting the presence of putative tumor-suppressor

gene(s) on it, as already suspected in sporadic colorectal cancers.¹³ Again, the mechanism of sex chromosome involvement would seem to be different in small-cell lung carcinomas, in which the reduction of Y signal is only relative, and in one case is due to over-representation of X signal rather than to Y allelic loss.

In conclusion, this is the first report, which describes a molecular characterization of the rare variant of small-cell lung carcinoma combined with large-cell neuroendocrine carcinoma. Overall, the phenotypically different components of these combined neoplasms appear to be closely related, and the high degree of concordance between them strongly suggests a common origin. The distinct components of these combined neoplasms appear to be more similar to each other than to their respective pure forms. On the other hand, when differences did emerge, they often seemed quite specific for this histological variant as compared with pure forms. All these findings support the view that combined small-cell/large-cell neuroendocrine carcinomas do not represent a mere combination of different neuroendocrine growth patterns, but instead constitute transition carcinomas in the spectrum of high-grade neuroendocrine pulmonary tumors, where the two extremes are represented by the corresponding pure forms.

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Conflict of interest

None declared.

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