Typical and Atypical Carcinoid Tumors of the Lung Are Characterized by 11q Deletions as Detected by Comparative Genomic Hybridization

Axel K. Walch,* Horst F. Zitzelsberger,† Michaela M. Aubele,* Anita E. Mattis,§ Manfred Bauchinger,† Sonja Candidus,§ Heinz W. Präuer,§ Martin Werner,§ and Heinz Höfler*§

From the Institutes of Pathology and Radiobiology;† GSF-National Research Center for Environment and Health, Neuherberg, Institute of Radiation Biology;¶ Ludwig-Maximilians-University Munich, Munich, and Institute of Pathology and Department of Surgery;§ Technical University Munich, Munich, Germany

Neuroendocrine tumors of the lung represent a wide spectrum of phenotypically distinct entities with different biological characteristics such as typical carcinoid tumor (TC), atypical carcinoid tumor (AC), large-cell neuroendocrine carcinoma (LCNEC), and small-cell lung carcinoma (SCLC). The histogenetic relationships between TC, AC, LCNEC, and SCLC are still unclear. This study was carried out to provide cytogenetic data about pulmonary neuroendocrine tumors and to evaluate their characteristic alterations and histogenetic relations for an improved understanding of the mechanisms of tumor development. Twenty-nine paraffin-embedded tumor samples of TC (n = 17), AC (n = 6), LCNEC (n = 3), and SCLC (n = 3) were selected for isolation of tumor DNA and subsequent comparative genomic hybridization (CGH) analysis. To confirm the comparative genomic hybridization results for specific chromosomal imbalances, selected cases were additionally investigated by loss of heterozygosity analysis. For statistical evaluation, we also used comparative genomic hybridization data from 45 published SCLC cases. DNA underrepresentations of 11q were the most frequent findings in TC (8 of 17) and AC (4 of 6), whereas these aberrations were rare in LCNEC (1 of 3) and SCLC (0 of 3). Furthermore, AC showed DNA underrepresentation of 10q (3 of 6) and 13q (3 of 6). In contrast, SCLC and LCNEC were characterized by a different pattern of DNA losses (5p−, 4q−, 5q−, 13q−, and 15q−) and gains (5p+, 17p+, and +20). Statistical analysis revealed significantly different occurrences of 11q deletions in TC/AC versus SCLC (45 published cases of SCLC and our 3 cases; P = 0.002; Fisher’s exact test). Thus, TC and AC display frequent loss of 11q material including the MEN1 gene locus, which represents a characteristic genetic alteration in these tumors. Losses of 10q and 13q sequences allow a further cytogenetic differentiation between TC and AC. These additional changes might be responsible for the more aggressive behavior of AC. Three cases of LCNEC, the first to be analyzed by comparative genomic hybridization, exhibited similar complex abnormal patterns (4q−, 5q−, 10q−, 13q−, 15q−) to those of SCLC. Although neuroendocrine tumors of the lung share common phenotypic features, suggesting a genotypic relationship, they differ remarkably in their cytogenetic characteristics, highlighting an early fundamental molecular divergence during the development of these tumors.

The histogenetic relationship between the wide spectrum of phenotypically and biologically distinct neuroendocrine tumors (NETs) of the lung is still unclear and remains a subject of controversy. Although these tumors have well-characterized cytomorphological and immunophenotypic features,1–9 there is little known about the cytogenetic and molecular genetic changes underlying their tumorigenesis.

In 1991, Travis et al8 proposed a four-category scheme for classification of NETs including typical carcinoid tumor (TC), atypical carcinoid tumor (AC), large-cell neuroendocrine carcinoma (LCNEC), and small-cell lung carcinoma (SCLC). Although this classification system is based on light microscopic, electron microscopic, immunohistochemical, and clinical aspects of these four tumor types, investigation of genetic abnormalities in NETs may reveal additional characteristics that might be helpful in improving the reliability of prognosis and in classification of these tumors. In particular, the classification of NETs of the lung is a complex and controversial problem.10 The lack of uniform acceptance of a classification scheme has led to the proposal of several approaches.2,9,11

Several cytogenetic and molecular genetic alterations associated with SCLC have been reported. Recently,
cytogenetic analysis. These studies, however, were insufficient cytogenetic data available for all entities.

In our study, we investigated 29 pulmonary NETs for chromosomal imbalances by CGH. For the first time we perform CGH on TCs, ACs, and LCNECs with confirmation of CGH findings by loss of heterozygosity (LOH) analysis of selected microsatellite loci.

The aims of this study were 1) to provide cytogenetic data on the rarely analyzed entities TC, AC, and LCNEC; 2) to identify possible genotypic relations within the entities of pulmonary NETs; and 3) to evaluate characteristic aberrations for each entity for an improved understanding of the mechanisms of tumor development.

### Materials and Methods

#### Tissue Samples

The study was carried out on 29 NETs of lung from specimens obtained from 29 patients. The histopathological classification of the tumors was based on previously established criteria. The histopathological and clinical data from each case are summarized in Table 2. Follow-up information was available on 24 cases (mean follow-up, 51 months; range, 1 to 101 months). All samples investigated were derived from formalin-fixed and paraffin-embedded tissues. Tissue sections (10 μm) for DNA extraction were prepared on glass slides, deparaffinized, and rehydrated. Hematoxylin and eosin-stained sections were used to evaluate the tumor area of the samples, which were selectively trimmed to enrich the tumor cell content to a minimum of 80%. DNA was extracted according to previously published protocols.

#### Table 1. Overview of Previous Reports of Cytogenetic and Molecular Genetic Findings in Pulmonary Carcinoid Tumors

<table>
<thead>
<tr>
<th>References</th>
<th>Tumor type</th>
<th>n</th>
<th>Material</th>
<th>Cytogenetic analysis*</th>
<th>Molecular genetic analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Teyssier et al (1985)23</td>
<td>Lung carcinoid tumor</td>
<td>1</td>
<td>Cell line</td>
<td>Trisomy 7</td>
<td></td>
</tr>
<tr>
<td>Hurr et al (1996)29</td>
<td>TC, AC</td>
<td>17</td>
<td>Frozen tissue</td>
<td></td>
<td>No LOH at 3p loci</td>
</tr>
<tr>
<td>Debelenko et al (1997)42</td>
<td>TC, AC</td>
<td>11</td>
<td>Frozen tissue</td>
<td></td>
<td>LOH on 11q13 (4/11); mutation of MEN1 gene (4/11)</td>
</tr>
</tbody>
</table>

*Rearranged (abnormal) chromosomes are indicated if deletions, translocations, inversions or isochromosomes were detected.
Six hundred nanograms of biotin-16-dUTP were labeled with biotin-16-dUTP using standard nick DNAs and DNAs from formalin-fixed normal lung tissue modifications. Isolated whole genomic DNAs (tumor Inc., Downers Grove, IL), as well as 25 normal female or male total human genomic DNA (Vysis, labeled DNA and 600 ng of SpectrumRed direct-labeled according to Kallioniemi et al32 and DuManoir et al33 with CGH of labeled tumor and normal DNA was performed of peripheral blood lymphocytes of healthy female and male donors. For each CGH experiment, biotinylated normal male or female DNA was hybridized against normal male or female reference DNA (SpectrumRed) as a control. Additionally, DNA was isolated from normal lung tissue of six donors.

### Metaphase Preparation

Metaphase spreads were prepared according to standard protocols from phytohemagglutinin-stimulated peripheral blood lymphocytes of healthy female and male donors.

### CGH

CGH of labeled tumor and normal DNA was performed according to Kallioniemi et al32 and DuManoir et al33 with modifications. Isolated whole genomic DNAs (tumor DNAs and DNAs from formalin-fixed normal lung tissue) were labeled with biotin-16-dUTP using standard nick translation.64 Six hundred nanograms of biotin-16-dUTP-labeled DNA and 600 ng of SpectrumRed direct-labeled normal female or male total human genomic DNA (Vysis, Inc., Downers Grove, IL), as well as 25 μg of unlabeled Cot-1 DNA (Life Technologies, Inc., Grand Island, NY), were hybridized to denatured normal lymphocyte metaphase spreads. CGH images were captured by a black/white video charge-coupled device camera using chip integration. The three colors were digitized consecutively with specific single-color filter combinations that were automatically changed on a Zeiss Axioplan2 microscope (Zeiss, Jena, Germany). For processing of captured images, an image analysis software from MetaSystems (Altussheim, Germany) was used. For one CGH analysis, at least 10 to 15 homologues of each chromosome were measured after 4′-6-diamidino-2-phenylindole karyotyping of 5 to 10 metaphases. Average ratio profiles were then calculated after automatically scaling the profiles of individual homologous chromosomes of the same length. Average ratio profiles were interpreted according to published criteria32,35 using statistical confidence limits based on F-statistics.

### Controls

For each CGH experiment, biotinylated normal male or female DNA was hybridized against normal male or female reference DNA (SpectrumRed) as a control. Additionally, DNA was isolated from normal lung tissue of six cases and hybridized against normal reference DNA. No chromosomal changes were detected in these specimens. Three selected aberrant tumor cases were hybridized a second time by reverse labeling.

### LOH Analysis

Eight polymorphic microsatellite markers along chromosome 11q were chosen to analyze 11 lung tumors and...
their corresponding normal tissue. These microsatellite markers consisted of D11S4936 (11q13), D11S4933 (11q13), D11S987 (11q13), D11S901 (11q14), D11S1356 (11q23.3), D11S925 (11q23.3), D11S934 (11q23 to 11q24), and D11S968 (11q25). Markers D11S4933 and D11S4936 are tightly linked to the \textit{MEN1} gene locus. Primer sequences were obtained from the genome database (http://gdbwww.gdb.org). The sense primer of each primer pair was fluorescent labeled. Microsatellite polymerase chain reaction was carried out in a total volume of 25 \(\mu\)L with 75 ng of isolated genomic DNA, 10 pmol of each primer, 100 \(\mu\)mol/L of each deoxynucleotide triphosphate, 1.5 mmol/L of MgCl\(_2\), and 1.25 U AmpliTaq Gold polymerase (Perkin-Elmer Corp., Norwalk, CT) using a Perkin-Elmer thermal cycler (system 9600). After a "hot start" (94°C for 10 minutes), polymerase chain reaction consisting of 35 cycles was performed as follows: 94°C for 1 minute; 55°C, 57°C, 60°C, and 62°C for 30 seconds each; and 72°C for 30 seconds, followed by a final extension of 72°C for 7 minutes. After visualizing the resulting DNA products on a 3% agarose gel, an appropriate dilution of each sample was loaded on a 6% polyacrylamide denaturing gel and analyzed with an automated fluorescent ABI 377 sequencing apparatus (Perkin-Elmer). Evaluation of LOH was performed as described elsewhere.\(^{36}\) A tumor was considered to be LOH positive, if the allele peak ratio was equal to or less than 0.6, indicating an allelic signal reduction of at least 40%. To exclude the possibility of contaminations or technical artifacts, samples were reanalyzed by independent polymerase chain reactions and gel loadings.

### Results

Chromosomal imbalances were detected in 27 of 29 cases of NET of the lung (Table 3). They occurred with a frequency of 4.2 aberrations per case on average (range, 0 to 11 aberrations per case). Two cases of TC (cases 18 and 21) revealed no DNA copy number changes. The average number of aberrations increased from 3.4 in TC (range, 0 to 9) and 4.8 in AC (range: 2 to 7) to 6.3 in SCLC/LCNEC (range, 2 to 11). In total, 71 DNA losses and 53 DNA gains were observed in all tumors analyzed, reflecting a predominance of DNA losses. Moreover, no high-level amplifications were diagnosed. These are defined by red/green ratios \(>2\) or diagnostic profiles for chromosomal gain detectable also by visual inspection.\(^{35}\) Representative profiles for typical chromosomal imbalances detected in each entity are demonstrated (see Figure 2).

#### TCs

Chromosomal imbalances observed in 17 cases of TC are shown in Figure 1 and Table 3. This entity exhibited losses on 19 chromosomes. Most frequently affected was chromosome 11, with losses in 8 of 17 cases (47%).
of eight cases exhibited a deletion on 11q involving a common region on 11q13, whereas 3 of 8 cases showed the loss of one homologue of chromosome 11. Deletions on chromosome 6q occurred in 4 of 17 cases, which represent the second most frequent finding of DNA losses in TCs.

Gains of DNA sequences or whole chromosomes were additionally detected on chromosomes 19 (6 of 17), 17 (6 of 17), 16 (3 of 17), and 20 (3 of 17).

ACs

Chromosomal imbalances in six cases of AC are shown in Figure 1 and Table 3. Representative profiles for typical chromosomal imbalances detected in AC are demonstrated in Figure 2.

Chromosomal losses were observed on 15 chromosomes. Chromosome 11 was affected in four of six cases (66%). Again, the q arm was most frequently affected, with losses in two of six cases including the consensus region 11q13 as detected in TC. Deletions of 10q (including monosomy 10 in one case) and 13q occurred frequently in three cases of AC. The pattern of chromosomal gains was similar to the overrepresentations observed in TC.

SCLC and LCNEC

In contrast to TC and AC, a different pattern of DNA losses was observed in 3 SCLC and 3 LCNEC cases (Table 3). Although 11q was affected only in one case, 5q losses were observed in all cases. Additional frequent DNA losses became apparent on 13q (four of six cases), 4q (four of six cases), 3p (two of six cases), and 15q (two of six cases). In SCLC and LCNEC, gains were detected on chromosome 19 (four of six cases), chromosome 20 (three of six cases), chromosome 5p (two of six), and chromosome 17 (two of six cases).

LOH Analysis

We investigated eight different microsatellite loci along chromosome 11q in 11 cases (Table 4). The LOH analysis of these eight markers confirmed that this is a commonly deleted region in TC and AC. Eight of 11 cases (73%) displayed LOH along chromosome 11q. LOH on 11q13, affecting at least one polymorphic marker near the MEN1 gene, was detected in 7 of 11 cases (63%) of TC and AC. Localization of microsatellite markers, results of LOH analysis, and comparison with CGH results for chromosome 11q are shown in Table 4.
Figure 2. Averaging ratio profiles for selected chromosomes exhibiting characteristic changes in TC, AC, and SCLC. For averaging profiles (white line), 95% statistical confidence limits are indicated to diagnose losses (red line) or gains (green line) of DNA sequences. Profiles of characteristic changes in TC (11q-), AC (11q-, 10q-, 13q-) and SCLC are given.
groups, allowing a cytogenetic discrimination between first CGH study on these entities of pulmonary NET. Chromosomal aberrations. To our knowledge, this is the AC and LCNEC have only rarely been investigated for in each case (Table 3). In contrast to SCLC, TC as well as somes 19 and 20. Additional gains and losses on other found losses on 5q, 4q, and 3p and gains on chromo- In accordance with these studies, we most frequently malities could be detected as those previously reported. of SCLC were investigated in this study to ensure that with our CGH procedure a comparable pattern of abnor- malties in histologically distinct subgroups comprising TC/AC cases and 48 SCLC were analyzed using Fisher’s exact test. The cytogenetic findings did not correlate with metastasis, death caused by disease, sex, or smoking history (P > 0.05 for each analysis).

SCLC/LCNEC and TC/AC differ significantly for 4q (P = 0.006) and 5q (P = 0.008) deletions. A further significant difference (P = 0.002) could be observed for 11q deletions between our 23 TC/AC cases and 48 SCLC cases from the literature.

Discussion

In the present study, 29 cases of NETs of the lung have been investigated for characteristic chromosomal abnor- malties in histologically distinct subgroups comprising TC, AC, LCNEC, and SCLC. SCLC is a cytogenetically well characterized tumor type with typical underrepresenta- tion of 3p, 4q, 5q, 10q, 13q, and 17p and overrepresenta- tion of 3q, 5p, 8q, and 17q detected by CGH12–16 and cytogenetic studies.17,18 Therefore, only three cases of SCLC were investigated in this study to ensure that with our CGH procedure a comparable pattern of abnor- malties could be detected as those previously reported. In accordance with these studies, we most frequently found losses on 5q, 4q, and 3p and gains on chromo- somes 19 and 20. Additional gains and losses on other chromosomes reflected a complex pattern of aberrations in each case (Table 3). In contrast to SCLC, TC as well as AC and LCNEC have only rarely been investigated for chromosomal aberrations. To our knowledge, this is the first CGH study on these entities of pulmonary NET.

Our CGH findings strongly suggest that distinctive chromosomal imbalances occur in the various sub- groups, allowing a cytogenetic discrimination between TC/AC and SCLC/LCNEC. The most striking difference are underrepresentations on chromosome 11q, which are frequent in TC (8 of 17) and AC (4 of 6) but rare in SCLC (0 of 3) and LCNEC (1 of 3). For statistical analysis we used published CGH data on 45 cases of SCLC13–15 in addition to our own CGH results on three cases of SCLC. Statistical analysis reveals a significant difference (P = 0.002; Fisher’s exact test) in the occurrence of 11q losses involving chromosomal region 11q13 between TC/AC (10 of 23; our cases) and SCLC (4/48; published cases and our cases). Thus, TCs and ACs are both characterized by underrepresentation of 11q, but ACs show further losses on 10q and 13q.

A first indication of 11q aberrations in pulmonary car- cinoid tumors came from recent LOH studies.30,37 Based on these LOH studies, it was hypothesized that chromo- somal losses in the MEN1 gene-containing region might be significant in the pathogenesis of TC and AC, both associated and not associated with the multiple endo- crine neoplasia type 1 (MEN1) syndrome. The MEN1 locus was previously localized to chromosome 11q1338–41 and was recently cloned.42 A recent study of sporadic lung carcinoid tumors showed two inactivated copies of the MEN1 gene in 4 of 11 cases.43 These findings are now supported by our CGH data, which implicate DNA losses on chromosome 11q with the pathogenesis of sporadic lung NETs, representing a characteristic cytogenetic alteration in these tumors.

To date, 10q deletions have been reported for endo- metrial carcinomas,44 malignant meningiomas,45 gliomas,46 and prostate carcinomas47 and have been asso- ciated with tumor progression. For the much more aggressive SCLC, 10q losses have also recently been reported.13–16 These finding in SCLC provide a clue for the MX1 gene located on 10q24–25 as a potentially affected tumor suppressor gene, given that its function as a negative regulator of myc oncoproteins coincides with frequent amplification and overexpression of myc onco- genes in advanced SCLC.19,20 MX1 has also been sug- gested to act as a tumor suppressor in prostate cancer.47

13q losses in AC include the RB1 locus, which is a well

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<th>Table 4. Data on Allelic Deletions on Chromosome 11q Detected by CGH and LOH Analysis in Eight Cases of TC and Three Cases of AC</th>
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<tr>
<td>Case</td>
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*Polymorphic markers from the left to right are listed from the telomeric to centromeric direction. Markers D11S4933 and D11S4936 tightly linked to the MEN1 gene locus. ○, LOH; ●, retention of constitutional heterozygosity; ×, not informative; –, not analyzed.
known tumor suppressor gene, loss of which is associated with tumor progression and poor prognosis in several tumor types. LOH in the RB1 locus has previously been detected in 92% of SCLCs, 80% of other neuroendocrine carcinomas, and 33% of carcinoid tumors.26

Our three cases of LCNEC, representing a rare pulmonary tumor, exhibit similar chromosomal changes to our three SCLC cases. The chromosomal imbalances include preferential losses of chromosomes 4q, 5q, 13q, and 15q, which is in accordance with previously published CGH data from SCLC. We did not observe loss of chromosome 17p in any of the SCLC and LCNEC cases reported in this study. This possibly reflects the rather early tumor stages of our cases (pT1 and pT2). 17p losses were also absent in our subset of TC and AC, which is in good accordance with recent p53 studies of typical and atypical carcinoids, which did not show any p53 mutations.27 However, Lohmann et al26 reported mutations of the p53 gene in pulmonary carcinoid tumors at a low frequency (4/25), whereas they occurred in more than 80% of the 27 SCLC cases they investigated.48

Loss of the chromosomal region 5q13–21 has frequently been described in SCLC,12–16,49 and candidate tumor suppressor genes APC and MCC have been mapped to this region. 3p deletions are also very common in SCLC and have been demonstrated in several cytogenetic and molecular genetic studies identifying distinct deleted subregions on 3p.17,18,12,13,15,16,50,51 They co-localize in our SCLC cases with chromosomal regions potentially harboring tumor suppressor genes such as the FHIT gene on 3p14.2,52 which was reported to exhibit deletions on several exons in 80% of SCLCs.21

Our CGH results on TC and AC suggest that 3p deletions are rare events in these tumors (1 of 17 TCs and 1 of 6 ACs). Previous cytogenetic studies of a few pulmonary carcinoid tumors did not detect any 3p deletions,23,24 except for that of Lai et al.,25 who reported chromosomal abnormalities of 3p in four of four pulmonary NETs.

We confirmed our CGH results by LOH studies of microsatellite loci along 11q in 11 TC/AC (7 cases with 11q deletion and 4 cases without 11q deletion for controls). LOH findings on 11q loci are in good accordance with our CGH results (Table 4), except for two cases (cases 18 and 27, Table 4) with LOH in D11S4933 and D11S901, which exhibit either DNA loss in another chromosomal region (case 27) or no DNA loss on 11q (case 18). This can be explained by the extent of the deleted segment, which is possibly below the detection limit of CGH.

In this investigation we found no relationship between chromosomal DNA changes and clinical parameters as survival, metastasis, sex, or smoking history. This may be attributed to the number of cases in each tumor group, which is likely too small to show clear statistical correlations between clinical and CGH findings.

Our CGH results from TC, AC, and LCNEC together with published CGH data on SCLC characterize chromosomal aberration patterns for each subgroup with a predominant occurrence of 11q losses in TC and AC and different aberration patterns in SCLC and LCNEC. Therefore, it appears that carcinoid tumors and the high-grade tumors have substantially different chromosomal changes that could be explained either by different progenitor cells or differences in carcinogen exposure, such as cigarette smoking. Strong epidemiological differences between lung carcinoid tumors and SCLCs indicate a markedly different process of carcinogenesis, which casts doubt on the hypothesis of a common cell precursor.53,54

Although NETs of the lung share common histomorphological features, they differ greatly in their cytogenetic characteristics, highlighting a fundamental molecular divergence between these tumors.

Acknowledgments

We thank D. Angermeier, S. Holthaus, S. Osswald, and S. Schulte-Overberg for their excellent technical assistance in processing the tumor specimens, and H. Braselmann for statistical analysis of the data.

References


19. Brennan J, O’Connor T, Makuch RW, Simmons AM, Russel E, Linnoila M. myc family DNA amplification in 107 tumors and tumor cell lines from patients with small-cell lung cancer treated with different combination chemothera-


23. Teyssier JR, Sadrin R, Nou JM, Buraeu G, Adnet JJ, Bagolle F, Pigeon F. Trisomy 7 in a lung carcinoid tumor: precocious index of malignant transfor-


33. Leenstra S, Bijsma EK, Troost D, Osting J, Westerveld A, Bos JA, Hulsbos TJ. Allele loss on chromosomes 10 and 17p and epidermal growth factor receptor gene amplification in human malignant astrocy-


35. Samishima Y, Matsuno Y, Hirohashi S, Shimozato Y, Moriguchi H, Sugimura T, Terada M, Yokota J. Alterations of the p53 gene are common and critical events for the maintenance of malignant pheno-


37. Yokota J, Wada M, Shimozato Y, Terada M, Sugimura T. Loss of
heterozygosity on chromosome 3, 13 and 17 in small cell carcinoma and on chromosome 3 in adenocarcinoma of the lung. Proc Natl Acad Sci USA 1987, 84:9252–9256


