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Deletions of 11q22.3-q25 Are Associated with Atypical Lung Carcinoids and Poor Clinical Outcome

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Carcinoids are slow-growing neuroendocrine tumors that, in the lung, can be subclassified as typical (TC) or atypical (AC). To identify genetic alterations that improve the prediction of prognosis, we investigated 34 carcinoid tumors of the lung (18 TCs, 15 ACs, and 1 unclassified) by using array comparative genomic hybridization (array CGH) on 3700 genomic bacterial artificial chromosome arrays (resolution ≤1 Mb). When comparing ACs with TCs, the data revealed: i) a significant difference in the average number of chromosome arms altered (9.6 versus 4.2, respectively; $P = 0.036$), with one subgroup of five ACs having more than 15 chromosome arms altered; ii) chromosomal changes in 30% of ACs or more with additions at 9q (≥1 Mb) and losses at 1p, 2q, 10q, and 11q; and iii) 11q deletions in 8 of 15 ACs versus 1 of 18 TCs ($P = 0.004$), which was confirmed via fluorescence *in situ* hybridization. The four critical regions of interest in 45% ACs or more comprised 11q14.1, 11q22.1-q22.3, 11q22.3-q23.2, and 11q24.2-q25, all telomeric of *MEN1* at 11q13. Results were correlated with patient clinical data and long-term follow-up. Thus, there is a strong association of 11q22.3-q25 loss with poorer prognosis, alone or in combination with absence of 9q34.11 alterations ($P = 0.0022$ and $P = 0.00026$, respectively). ***(Am J Pathol 2011, 179:1129–1137; DOI: 10.1016/j.ajpath.2011.05.028)***

Pulmonary carcinoids comprise a group of usually smoking-unrelated neuroendocrine tumors. Compared with poorly differentiated neuroendocrine tumors of the lung, ie, large-cell neuroendocrine carcinoma and small-cell lung cancer, carcinoids are well-differentiated and characterized by a low metastatic rate and a relatively favorable prognosis. On the basis of histopathologic features (number of mitoses and presence of necrosis), lung carcinoid tumors are classified as typical carcinoids (TCs) or atypical carcinoids (ACs), although classification is sometimes difficult and its reliability to predict disease outcome is variable.[1] Compared with TCs, in general, ACs more often exhibit malignant behavior and are associated with a lower 5-year survival rate (61% to 88% and 92% to 100%, respectively).[2] Metastases will develop in 4% to 64% of patients with carcinoids (TCs, 4% to 14%, and ACs, 35% to 64%), usually in regional lymph nodes but also at distant sites including liver, bone, brain, subcutaneous tissue, and breast.[2,3] Although most patients remain cancer-free within 5 years after surgery, there is no curative treatment available for metastatic disease.

A few studies have reported clinical and molecular factors associated with higher risk of developing metastases or with poor disease outcome. Clinical factors with prognostic value include size 3.5 cm or larger, mitotic index, degree of differentiation, presence of necrosis, co-secretion of peptides, and metastasis.[4,5] Immunohis-

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tochemistry on TC samples revealed that a high Ki-67 labeling index or up-regulation of the anti-apoptotic proteins Bcl-2 and p53 were associated with metastatic disease and shorter survival time, whereas immunostaining for the adhesion molecule CD44 was associated with localized disease and lower mortality.[6] An additional study of 121 pulmonary neuroendocrine tumors including 21 carcinoids demonstrated a shift to low Bax and high Bcl-2 expression in association with ACs, resulting in an unfavorable prognosis.[7] The Rb pathway is more often modified in ACs than in TCs. P16 negativity was observed in 23% of ACs, compared with 9% of TCs, and absent staining for pRb in 21% of ACs and no TCs.[8]

Genomic alterations contribute to carcinogenesis by changing the expression levels of critical oncogenes and tumor-suppressor genes. In lung carcinoid tumorigenesis, few *p53* and no *EGFR* or *KRAS* gene mutations have been detected, although the percentage of lung carcinoids expressing *EGFR* is higher in TCs than in ACs.[9,10] Previous studies have primarily demonstrated multiple endocrine neoplasia type 1 (*MEN1*) gene mutations and/or chromosome 11q deletions.[11–17] MEN1 is a syndrome in which an inherited mutation in the *MEN1* gene, located at 11q13, predisposes to formation of multiple neuroendocrine tumors. Although formation of bronchial carcinoid tumors has been observed in only 2% of patients with MEN1,[18] functional inactivation of menin, the *MEN1* gene product, has been implicated in the tumorigenesis of sporadic lung carcinoids. In these bronchial carcinoids not associated with MEN1 syndrome, the frequency of loss of heterozygosity at 11q (36%) is higher than the somatic *MEN1* mutation rate (18%), pointing to the presence of other tumor-suppressor genes at this chromosome arm and/or involvement of epigenetic silencing mechanisms.[12]

To improve the discrimination between pulmonary carcinoid tumors with a favorable or poor prognosis and to identify critical genetic events in lung carcinoid tumorigenesis, we investigated 34 reclassified bronchial carcinoids by using array-based comparative genomic hybridization (array CGH) with a resolution of ≤1 Mb (megabase). Fluorescence *in situ* hybridization (FISH) was used to determine chromosome copy numbers and to validate array CGH data. Furthermore, the array CGH data were correlated with available histopathologic data and long-term clinical follow-up.

Materials and Methods

Tumor Material, Histopathologic Analysis, and Clinical Data Collection

Both frozen and formalin-fixed, paraffin-embedded (FFPE) tumor material were collected from 34 patients [14 male and 20 female; mean age, 50 years (age range, 16 to 83 years)] with lung carcinoid tumors from the archives of the Departments of Pathology of our institutions. Patient material was used according to the Code for Proper Secondary Use of Human Tissue (Federation of Medical Scientific Societies, The Netherlands; 2003). All material was reclassified by two experienced pathologists (R.-J.v.S. and F.B.J.M.T.) on the basis of histopathologic features according to the most recent World Health Organization classification,[1] resulting in 18 TCs [no necrosis and mitotic index less than 2 per 10 high-power fields; mean patient age, 47 years (age range, 19 to 68 years)] and 15 ACs (necrosis and/or mitotic index between 2 and 10 per 10 high-power fields; mean patient age, 56 years (age range, 22 to 83 years)]. One carcinoid tumor could not be reliably classified. In earlier studies, eight patients underwent conventional CGH[17] and *MEN1* mutation analysis.[12] Follow-up data were collected from 33 of 34 patients, and ranged from 3 to 25 years (median, 101 months). Clinical data and tumor characteristics are given in Supplemental Table S1 (available at *http://ajp.amjpathol.org*).

Array CGH Analysis

DNA was extracted from the frozen and FFPE tissues with at least 70% tumor cells using the DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany). Array CGH was performed on microarrays containing 3700 FISH-verified bacterial artificial chromosome (BAC) clones at a resolution of ≤1 Mb.[19] Genomic DNA labeling procedures were performed as described elsewhere, using 500 ng tumor and reference DNA each in a randomly primed labeling procedure.[20] These Cy3-labeled tumor and Cy5-labeled normal reference DNA samples were mixed together with 120 μg Cot-1 DNA (F. Hoffman-La Roche AG, Basel Switzerland) and co-precipitated for at least 30 minutes in ethanol at −80°C. Pellets were air-dried and resolved in a total volume of 130 μL hybridization mixture containing 50% formamide, 10% dextran sulfate, 4% SDS, 100 μg yeast transfer RNA, and 2X saline sodium citrate. This probe mixture was denatured for 5 minutes at 80°C and pre-annealed for 30 minutes at 37°C. After pre-annealing, the sample was applied to the BAC array using the HS4800 hybridization station (Tecan Group Ltd., Männedorf, Switzerland). After hybridization for 23 hours, the arrays were rinsed with 40% formamide in 2X saline sodium citrate at 47°C, followed by 2X saline sodium citrate containing 0.1% SDS at 47°C and 0.1X saline sodium citrate at 30°C. The slides were dried using liquid nitrogen.

Fluorescence images of the arrays were acquired using a Scan Array Express scanner (PerkinElmer Life and Analytical Sciences BV, Groningen, The Netherlands) and analyzed using GenePix Pro 6.0 (Axon Instruments, Inc, Foster City, CA) as described previously.[20] To obtain a genomic copy number ratio for each spot, the median local background was subtracted from the median pixel intensity of both dyes. Data normalization was performed for each microarray subgrid, and median fluorescence values per clone were determined. All data were $\log_2$-transformed and interpreted as follows: the fluorescence signal intensity of the hybridized DNA at a certain BAC clone was considered significantly altered when demonstrating a change in the $\log_2$ ratio ≥0.2 (frozen tissue) or ≥0.3 (paraffin) when comparing the tumor with the normal reference tissue. For each corresponding profile, alterations in chromosomal regions were determined us-

ing two different procedures. First, regions of gain and/or loss ≥10 Mb were listed. These correspond to alterations that can also be detected by using conventional CGH (see Supplemental Table S1 at http://ajp.amjpathol.org).[21] Second, to analyze smaller regions of interest, altered regions ≥1 Mb were determined. These comprise at least three adjacent BAC clones with significantly altered fluorescence signals, allowing a maximum of two not significantly changed signals in between two of these altered regions.[20] Chromosomal instability (CIN) was defined as the presence of at least one alteration ≥10 Mb in a minimum of eight chromosome arms, as described previously.[22] Amplifications were defined as regions containing two or more adjacent signals demonstrating a change of $\log_2$ chromosome copy number ratio ≥1.0 (≥1.5 for paraffin). All mapping information about clone locations, cytogenetic bands, and genomic content was retrieved from the University of California at Santa Cruz Genome Browser (version hg19; UCSC Genome Bioinformatics Group, Santa Cruz, CA).[23] All raw array CGH data are available in the ArrayExpress database (*http://www.ebi.ac.uk/arrayexpress*) under accession number E-MEXP-3145.

DNA Copy Number Analysis Using FISH

FISH with centromere-specific probes for chromosomes 1, 3, 7, and 11 was performed on 4-μm tumor sections to obtain an indication of the ploidy at $\log_2 = 0$ and to validate chromosome copy number alterations detected by using array CGH. In addition, deletions of 11q were validated in five cases using FISH-mapped cosmid probes specific for the *MEN1* gene locus at 11q13[24] and loci telomeric of *MEN1* at 11q13 (clone cK034[25]), 11q13.4-q21 (clone cCI11-270), 11q22.2 (clone U836), and 11qter (clone cCI11-314[26]), in all cases together with a centromere 11–specific probe. (Probes were kindly provided by J. Hoovers, Academic Medical Center Amsterdam, and A. Geurts van Kessel, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands). Amplification of 8q24.21 was analyzed using the Vysis LSI MYC Dual Color, Break Apart Rearrangement Probe (Abbott Molecular, Abbott Laboratories, Abbott Park, IL). FISH on paraffin-embedded tissue sections using centromere probes and touch preparations using both centromere and cosmid probes was performed as described previously,[17] with modification of the 85% formic acid/3% H_2O_2 step from 20 to 5 minutes for the paraffin sections. Probe visualization, nuclear counterstaining, signal scoring, and evaluation were performed as described previously.[17] For each tumor hybridization, signals of 100 interphase nuclei were scored.

Immunohistochemistry

Immunohistochemical protein staining on 4-μm thick FFPE and frozen sections was performed for Bcl-2 (clone 124; Dako A/S, Glostrup, Denmark), p53 (clone DO-7; Dako A/S), and pRb (clone LM95.1; Oncogene Research Products, La Jolla, CA) as follows. To retrieve epitopes, FFPE sections were first deparaffinized and subsequently microwave heated for 3 × 5 minutes and cooled down in between for 5 minutes at room temperature in 0.01 mmol/L citrate buffer (pH 6.0) for p53 or for 20 minutes in 0.1 mmol/L Tris-EDTA buffer (pH 9.0) for Bcl-2 and pRb. Endogenous peroxidase activity of the tissue was inhibited by incubation with 0.3% to 2% H_2O_2 in methanol for 30 minutes. For Bcl-2 and p53 staining, frozen sections were fixed for 10 minutes in methanol (−20°C). For pRb staining, frozen sections were fixed for 15 minutes in 4% formaldehyde/0.1% Triton X-100 in PBS. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in PBS for 20 minutes. Both frozen and FFPE sections were blocked with 3% bovine serum albumin in PBS before incubation with the primary antibody. The antibodies against p53 and Bcl-2 were used at a dilution of 1:50, and the pRb antibody was diluted to 1:33. Antibodies were diluted in 1% bovine serum albumin in PBS, and incubation was performed overnight at 4°C. As a secondary antibody, PowerVision Poly-HRP goat anti-mouse/anti-rabbit/anti-rat IgG (Immunologic, Duiven, The Netherlands) was used. Peroxidase activity was detected using 0.5 mg/mL diaminobenzidine/2% H_2O_2. Sections were counterstained with hematoxylin and mounted in Entellan (Merck KGaA, Darmstadt, Germany). Staining intensities were graded as follows: for Bcl-2, < 1% positive staining; +, 1% to 5% positive staining; ++, 5% to 20% positive staining; and +++, > 20% positive staining; for p53, 0% positive staining; +, < 5% positive staining; ++, 5% to 20% positive staining; and +++, > 20% positive staining; and for pRb, < 1% positive staining; +, 1% to 10% positive staining; ++, 10% to 20% positive staining; and +++, > 20% positive staining.

Statistical Analysis

Possible correlations between clinical data and chromosomal alterations were determined using SPSS for Windows (version 15.0; SPSS Inc., Chicago, IL). Associations between the number of genomic alterations and histopathologic classification of ACs or TCs were analyzed using Student's *t*-test. Associations between sex, diameter, classification, protein expression, and specific chromosomal alterations, and follow-up were analyzed using the χ^2 test or Fisher's exact test, when appropriate. Correlation of age or number of alterations with clinical follow-up were determined using log regression analysis. Survival curves were created using the Kaplan-Meier method. The log-rank test was used to test for differences between subgroups. $P \leq 0.05$ (two-sided) was considered statistically significant.

Results

Subsets of ACs Show Either Low or High Numbers of Chromosomal Alterations

Genomic DNA isolated from 34 bronchial carcinoid tumors (15 ACs, 18 TCs, and 1 unclassified) was analyzed by using array CGH for the presence of chromosomal copy number changes. Representative array CGH pro-

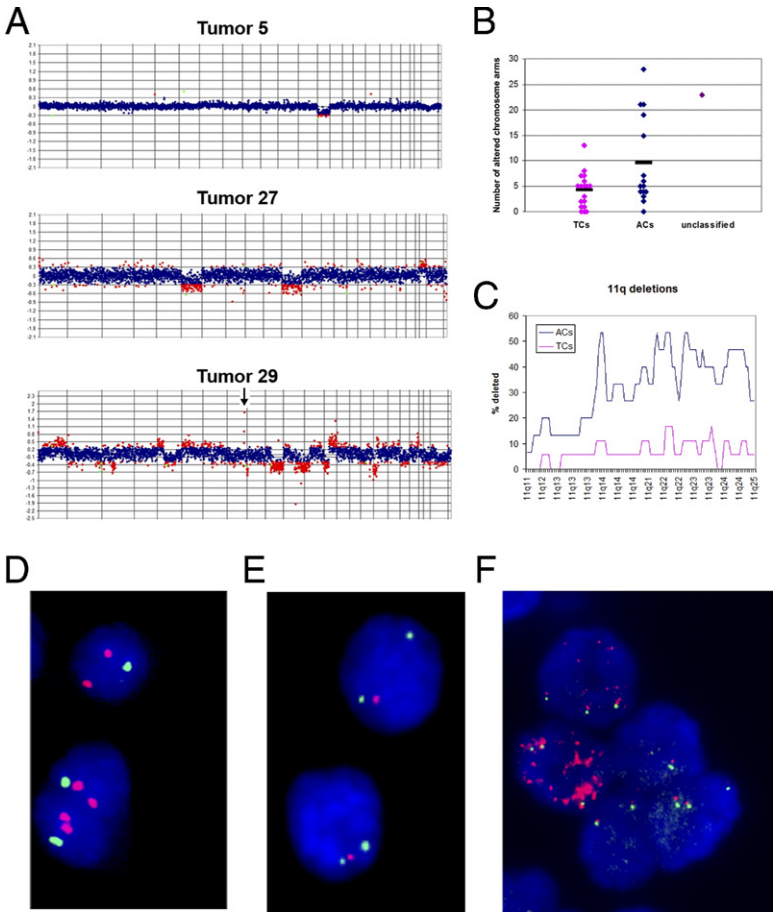

Figure 1. Analysis of chromosomal alterations in lung carcinoid tumors. **A:** Representative array CGH profiles of a typical carcinoid (TC) and two atypical carcinoids (ACs). Case 5 (TC; see Supplemental Table S1 at *http://ajp.amjpathol.org*) demonstrates deletion of chromosome 13q; case 27 (AC), deletion (≥10 Mb) of chromosomes 6pq and 11pq and gain of chromosome 22pq; and case 29 (AC), gain of chromosomes 1q, 5p, 6p, 13q, 18q, and 20q and loss of chromosomes 3p, 5q, 8p, 9q, 10q, 11q, 13p, 17p, and Xq. Furthermore, case 29 exhibits amplification at 8q24, the region containing the oncogene *MYC* (**arrow**). **B:** Difference in number of chromosome arms containing at least one altered region ≥10 Mb per tumor between TCs and ACs (4.2 ± 3.4 versus 9.5 ± 8.7, respectively; $P = 0.036$). Black bar indicates the mean number of altered chromosome arms. **C:** Graph shows frequency of deletion of specific regions at chromosome arm 11q comparing ACs (blue) with TCs (red). **D–F:** Fluorescence *in situ* hybridization (original magnification ×630) of (**D**) centromere 1 (green) and centromere 7 (red) in case 13 (see Supplemental Table S1 at *http://ajp.amjpathol.org*) exhibiting two copies of chromosome 7 and one copy of chromosome 1. **E:** An 11q probe located at the *MEN1* locus at 11q13 (green) and a probe located at 11q13.4–21 (red) in case 23 (see Supplemental Table S1 at *http://ajp.amjpathol.org*), showing loss at 11q telomeric of the *MEN1* gene. **F:** Vysis LSI MYC Dual Color, Break Apart Rearrangement Probe in case 29 (see Supplemental Table S1 at *http://ajp.amjpathol.org*) shows three copies for the Spectrum Green–labeled probe target located 1.6 Mb telomeric of *MYC* (green) and three copies for the Spectrum Orange–labeled probe target 120 kb centromeric of *MYC* in approximately 75% of the nuclei (red). Multiple copies of the Spectrum Orange–labeled probe target were observed in the remaining 25% of nuclei, indicating amplification of this region.

files of one TC and two ACs are shown in Figure 1A. Chromosomal alterations ≥10 Mb were observed in 14 of 18 TCs and 14 of 15 ACs (see Supplemental Table S1 at *http://ajp.amjpathol.org*). A maximum of 28 altered chromosome arms (with at least one region that was ≥10 Mb) was identified, with a mean ± SD of 7.1 ± 6.9 for the entire group of carcinoids. ACs exhibited a mean number of altered chromosome arms of 9.6 ± 8.7, whereas TCs demonstrated significantly fewer alterations, ie, 4.2 ± 3.4 ($P = 0.036$, Student's *t*-test; Figure 1B). Of the group of ACs, 8 of 15 harbored few or no chromosome arms with copy number changes (3.7 ± 2.4), similar to TCs, whereas the remaining cases exhibited higher numbers of altered arms (20.0 ± 5.0), indicating CIN.

11q Deletions Are the Most Frequent Aberrations in Pulmonary Carcinoids and Are Strongly Associated with ACs

Insofar as alterations ≥10 Mb, 8 of 15 ACs (53%) exhibited a deletion in chromosome 11q, whereas a deletion of this region was observed in only 1 of 18 TCs ($P = 0.004$; Fisher's exact test; Figure 1C). Alterations in other chromosome arms were not present significantly more often in ACs than in TCs (data not shown). Significant chromosomal alterations (≥1 Mb, three or more subsequent clones) detected in greater than 30% of ACs are given in Table 1. The size of altered regions ranged from 2.80 to 57.91 Mb, and the most frequent alteration in ACs was deletion of 11q13.5-qter. Not the region harboring the tumor-suppressor gene *MEN1* but four regions located telomeric of 11q13 were most often deleted: 11q14, 11q22.1-q22.3, 11q22.3-q23.2, and 11q24.2-q25 (Figure 1C; Table 2). Only 2 of 34 lung carcinoids demonstrated loss of the 11q13 region harboring *MEN1*, in both cases together with a *MEN1* mutation [case 21 (see Supplemental Table S1 at *http://ajp.amjpathol.org*), D172V, exon 3; and case 24 (see Supplemental Table S1 at *http://ajp.amjpathol.org*), 434ins29, exon 2], as reported previously.[12] Also, five additional cases (10, 17, 18, 20, and 23) in which no *MEN1* mutations were observed did not exhibit deletion of the *MEN1* locus at 11q13. Other frequent alterations in ACs included gains on chromosomes 8, 9q, and 17 and deletions on chromosomes 1p, 2q, 10q, and 22q13.2 (Table 1; see also Supplemental Table S2 at *http://ajp.amjpathol.org*). Gains on chromosomes 8 and 9 and losses on chromosome 22q were most often observed in ACs without deletion of 11q (data not shown).

Amplifications were observed in two ACs. Case 30 (see Supplemental Table S1 at *http://ajp.amjpathol.org*) demonstrated amplifications at chromosomes 8q21.13 and 17q23.23-q24.2, and case 29 at 8q24.21 (Figure 1A). Of note, both patients had an aggressive tumor with

Table 1. Chromosomal Alterations (≥1 Mb Regions in at Least 30% of ACs)

Cytogenetic region	Type	Size (Mb)	Frequency (%)	Survival analysis (Log rank *P* value)
1p31.1	Loss	3.75	33	NS
1p21.2-p21.3	Loss	2.80	38	NS
2q22.1-q22.3	Loss	9.22	33	NS
9q33.3-q34.13	Gain	5.71	33	0.044 (↑)
10q21.2-q21.3	Loss	6.59	33	NS
11q13.5-q25	Loss	57.91	42	
11q14.1*	Loss	3.27	48	NS
11q22.1-q22.3*	Loss	5.34	50	0.0070 (↓)
11q22.3-q23.2*	Loss	7.10	49	0.0022 (↓)
11q24.2-q25*	Loss	9.02	47	0.0028 (↓)

*These losses occurred in 45% or more of ACs.
↑, positive effect on survival; ↓, negative effect on survival; AC, atypical carcinoid; NS, not significant.

11q loss, and died within 3 years after diagnosis. Alterations in TCs were randomly distributed over the genome, and reached a threshold of only 25% or greater in five chromosomal regions (see Supplemental Table S2 at *http://ajp.amjpathol.org*).

Bronchial Carcinoids Show (Near) Diploid DNA Content

To assess the chromosome copy number at $\log_2 = 0$ in the array CGH profiles of lung carcinoid tumors, FISH was performed using probes for chromosomes 1, 3, 7, and 11 centromeres. All carcinoids were disomic for at least three chromosome targets, indicating a (near) diploid DNA index. FISH was also used for validation of the array CGH results given in Supplemental Table S1 (available at *http://ajp.amjpathol.org*), and confirmed loss of centromere 1 in cases 12 and 32 (Figure 1D), loss of centromere 11 in case 27, and gains of centromere 7 in cases 15, 24, and 34. In five cases, 11q deletion was confirmed using FISH with cosmid probes (Table 2; Figure 1E).

Amplification of the 8q24.21 region in case 29 (see Supplemental Table S1 at *http://ajp.amjpathol.org*) was confirmed using an *MYC* dual-color translocation probe. All nuclei exhibited three copies of the Spectrum Green–labeled probe target located 1.6 Mb telomeric of *MYC* (alias c-*myc*). In approximately 75% of nuclei, three copies were also observed with the Spectrum Orange–labeled probe target 120 kb centromeric of *MYC*. However, the remaining 25% of nuclei exhibited multiple copies of this target, indicating amplification (Figure 1F). This indicates that *MYC* was involved in the amplification, with the breakpoint of this amplification telomeric of the gene.

Deletion of 11q22.3-q25 Is Associated with Poor Clinical Outcome

Analysis of 10-year overall survival demonstrated a significant difference between TCs and ACs (Figure 2), in agreement with the literature.[1,2] The subgroup of ACs harboring CIN demonstrated no correlation with favorable or poor prognosis (data not shown). Patients with loss of 11q22.3-q23.2 (Figure 3A) or 11q24.2-q25 had a significantly worse prognosis than did patients without loss of 11q, both in the total group of carcinoids (P = 0.0022 for 11q22.3-q23.2, and P = 0.0028 for 11q24.2-q25) and within ACs (P = 0.033 for both deletions) (Figure 3B). Deletion of the two more centromeric regions demonstrated a weaker (11q22.1-q22.3; P = 0.007) or no (11q14.1) correlation with poor prognosis. Gain of 9q34.11 was associated with a favorable prognosis, both in the total group of carcinoids (P = 0.044; Figure 3C) and within ACs (P = 0.023; Figure 3D). A combination of loss of 11q22.3-q23.2 or 11q24.2-q25 in the absence of alterations in chromosome 9q34.11 was the best predictor of poorer outcome, both in the total group of carcinoids (P = 0.00026; Figure 3E) and within the group of ACs (P = 0.018; Figure 3F). Deletion of 11q22.3-q23.2 or 11q24.2-q25 without 9q34.11 gain (P = 0.026) was also correlated with a higher risk of distant metastases. In addition, larger tumor diameter (≥3.5 cm; P = 0.024) correlated with metastases, in agreement with earlier studies.[4,5] There was a trend toward an association be-

Table 2. Copy Number Evaluation of 11q-Specific Targets in Lung Carcinoid Tumors Using FISH with Cosmid Probes

Tumor	11C*	11q13 (*MEN1*)	11q13	11q13.4-21	11q22.2	11q23.3-24.1
12	2†	2	2	1	1	1
22	2	2	2	NA	NA	NA
23	2	1	1	1	1	1
25	2	2	2	1	1	1
26	2	1	1	1	1	1

*Centromere of chromosome 11.
†Maximal number of centromere copies per nucleus in 20% or more of nuclei in the tumor.
NA, not analyzed.

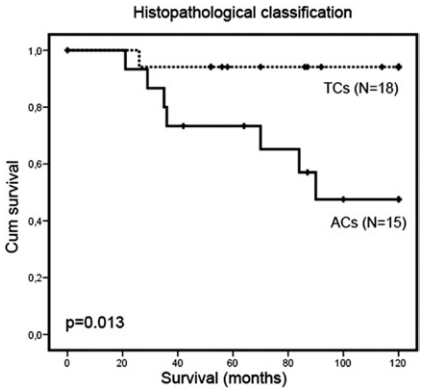

Figure 2. Survival analysis based on histopathologic classification. Kaplan-Meier curve comparing overall 10-year survival of typical carcinoids (TCs; dotted line) and atypical carcinoids (ACs; solid line). +, Censored cases.

tween deletion of 11q and larger diameter ($P = 0.058$). No relation between smoking and our clinical or array CGH data was observed.

Presence of Tumor Marker Proteins in Carcinoids

Immunohistochemistry was performed for Bcl-2, p53, and pRb, markers described as having potential prognostic significance in neuroendocrine lung tumors.[6–8] Positivity for Bcl-2 was observed in 23 of 30 cases, for p53 in 5 of 31 cases, and for pRb in 23 of 29 cases (see Supplemental Table S1 at *http://ajp.amjpathol.org*). Positive staining for these markers was not associated with histopathologic findings, survival, or metastasis, although low pRb expression (<10% positive nuclei) was correlated with deletions of 11q14 ($P = 0.029$), 11q22.1-q22.3 ($P = 0.0084$), and 11q22.3-q23.2 ($P = 0.018$).

Discussion

This is the first report to provide a genome wide array CGH analysis of chromosomal alterations in TCs and ACs at a resolution of ≤1 Mb. Furthermore, the most frequently observed genomic alterations were correlated with long-term clinical follow-up data. We observed that deletion of 11q22.3-q25 is associated with ACs, and more particularly with a poor clinical outcome in this subgroup of carcinoid tumors. The combination of this deleted region with absence of 9q34.11 alterations further improves the predictive value of this assay. No correlations with clinical outcome were observed for the frequently observed deletion of 11q14, a region with few annotated functional genes; presence of CIN; or protein expression of Bcl-2, p53, or pRb.

*11q22.3-q25 versus 11q13 (*MEN1*) in Carcinogenesis of Pulmonary Carcinoids*

In the present study, deletions of 11q14, 11q22.1-q22.3, 11q22.3-q23.2, and 11q24.2-q25 were the most frequently (≥48%) observed alterations in ACs. These regions are located telomeric of the *MEN1* gene positioned at 11q13, which was previously reported to be the most critical region of loss in bronchial carcinoids and several other neuroendocrine tumors.[27] Inactivation of *MEN1* has been proposed as an early genetic event in the carcinogenesis of lung carcinoids.[11,12] Deletion of 11q13 was observed in only two cases, both with a mutation of the *MEN1* gene. Much higher frequency of 11q13 loss has been published previously,[28] and we cannot exclude a microdeletion of this locus because *MEN1* was not covered by the probe targets on our CGH array. However, the absence of *MEN1* gene mutations in cases without 11q13 deletion makes this possibility unlikely. Together, these observations imply that other mechanisms of tumorigenesis exist in pulmonary carcinoids not associated with *MEN1* gene aberrations, including involvement of candidate genes in the 11q22.3-q25 region.

Conflicting results have been published on the frequency of 11q loss in lung carcinoids in conventional CGH or loss of heterozygosity studies.[13,14,16,29,30] Frequency ranged from 0% to 56% (mean, 34%) in TCs, and from 10% to 73% (mean, 50%) in ACs. These differences are most likely due to either the use of older classification

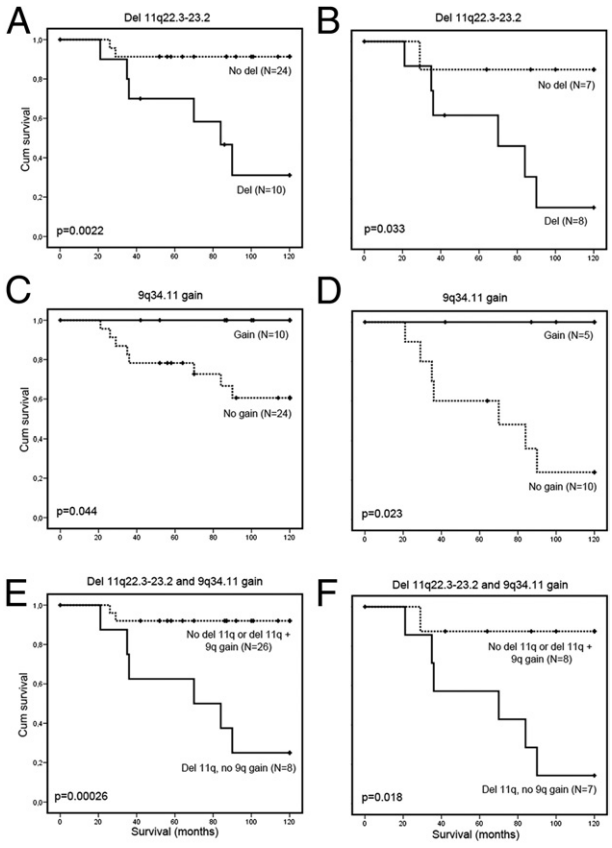

Figure 3. Survival analysis based on array CGH data. Kaplan-Meier curve comparing overall 10-year survival of all carcinoids (**A**) and atypical carcinoids (ACs) (**B**) with (solid line) or without (dotted line) 11q22.3-q23.2 deletion; all carcinoids (**C**) and ACs (**D**) with (solid line) or without (dotted line) 9q34.11 gain; all carcinoids (**E**) and ACs (**F**) with 11q22.3-q23.2 deletion but no 9q34.11 gain (solid line) compared with cases without 11q22.3-q23.2 deletion or with 11q22.3-q23.2 deletion in the presence of 9q34.11 gain (dotted line). +, Censored cases.

systems[31] or differences in the ratio between TCs and ACs included in the respective studies. In two previous array CGH studies of 16 lung carcinoids[15] and 19 lung carcinoids,[32] respectively, a critical region of loss on chromosome 11q could not be specified and correlation with patient survival could not be provided.

Deletion of 11q22.3-q25 Rather Than CIN Is Correlated with Poor Prognosis

In the present study, deletion of the telomeric part of chromosome 11q, ie, 11q22.3-q25, was strongly correlated with poor prognosis. Gain of chromosome 9q34.11 was associated with a favorable outcome. Deletion of 11q has also been identified in other neuroendocrine tumors in association with metastatic disease including neuroblastomas (11q23)[33,34] and insulinomas (11q24.1).[20] Loss of 11q22.3-q23.2 or 11q24.2-q25 in the absence of 9q34.11 alterations was the best predictor of poor prognosis, both for the total group of tumors and within ACs. Protein expression of three well-described genetic markers (Bcl-2, p53, and pRb) was not correlated with disease outcome in our tumor series, which underlines the prognostic importance of 11q deletion.

Prognosis in patients with ACs was significantly worse than in patients with TCs, consistent with the literature,[2] although the discrimination of lung carcinoids with a favorable and poor prognosis could be strongly improved using the above-mentioned genetic biomarkers.

We observed that a subset of ACs demonstrated CIN, an accumulation of chromosomal gains and losses, which is often induced by shortening of telomeres to a critical length. Two of our cases with CIN demonstrated loss of more than 10 telomeric regions, established because six consecutive telomeric BAC clones exhibited loss. In addition, CIN may be present more frequently in ACs and metastasized carcinoids than in TCs.[30] However, the tumors that demonstrated CIN described by those authors exhibited lower numbers of alterations than did those in the underlying study, in which a cutoff of at least one alteration ≥10 Mb in 8 or more chromosome arms was used to indicate CIN. Nevertheless, their results support our finding that ACs are associated with a higher number of chromosomal alterations. Furthermore, none of the tumors included in their study demonstrated distant metastases, and the two tumors with the most extensive lymph node metastases both exhibited deletion of 11q. Thus, not the total number of chromosomal alterations but 11q22.3-q25 status is strongly correlated with the malignant potential of lung carcinoids.

Candidate Genes on 11q22.3-q25

Putative tumor-suppressor genes or suppressors of tumor progression are located at 11q22.3-q23.3 and 11q24.2-q25. Candidates comprise genes that are frequently hypermethylated and silenced in other lung tumors, eg, the angiogenesis inhibitor *ADAMTS-8*[35] and the cell adhesion molecule *OPCML*[36–38] in non–small cell lung cancer, both located at 11q25. Furthermore, genes involved in the carcinogenesis of other neuroendocrine tumors might have a role. For example, there may be involvement of genes located at 11q23 such as *SDHD,*[39] reported to be mutated in sporadic parasympathetic paragangliomas; *CADM1,*[40] down-regulated in neuroblastomas and significantly associated with an unfavorable outcome; and *ATM*[41] and the menin interacter *MLL,* respectively reported to be mutated or translocated in leukemia. *MLL* encodes a protein that, together with menin, resides in a complex that methylates histone 3 lysine 4, an epigenetic mark associated with gene activation.[42] The menin-MLL complex activates transcription of the cyclin-dependent kinase inhibitors *p18*, *p21*, and *p27* and several homeobox genes, eg, *Hoxa9*, *Hoxc6*, and *Hoxc8*.[42–45] Because bronchial carcinoids are characterized by low mitotic activity, escape from apoptosis rather than promotion of proliferation might have an important role in their carcinogenesis. A cluster of caspases and genes encoding other caspase activation and recruitment domain proteins are located at 11q22.2-q23.[46–50] Although their exact function is currently unknown, their presence at this region may indicate involvement in suppressing apoptosis in pulmonary carcinoids in addition to the previously reported higher expression of anti-apoptotic Bcl-2 and lower expression of pro-apoptotic Bax protein in (atypical) carcinoids.[7,51]

Mechanisms of Carcinogenesis in Pulmonary Carcinoids

Apart from MEN1-related tumorigenesis in inherited and a subset of sporadic carcinoids, our array CGH data suggest several mechanisms of tumorigenesis for the nonfamiliar pulmonary carcinoids.

First, a group of tumors independent of CIN and 11q deletion demonstrates a favorable prognosis and comprised the TCs and a subset of ACs. We demonstrated that fewer alterations are present in TCs than in ACs. Combined results of the underlying and earlier studies indicate that alterations in TCs are infrequent and randomly distributed over the genome. They comprise gains of 19q (10%), 20p (10%), 17q (9%), and 19p (9%) and losses of 11q (22%), 11p (10%), 13q (8%), and 6q (8%).[13,16,29,30,52] In these cases, one might suggest involvement of processes such as epigenetic silencing of tumor-suppressor genes or deregulation of apoptosis.[51,53]

Second, a subset of carcinoids with highly malignant potential demonstrates deletion of 11q22.3-q25 as the major genetic event during carcinogenesis. In these tumors, it is likely that one or more tumor-suppressor genes are inactivated on the retained arm. Two of our cases in this group, with (large) 11q deletions, also demonstrated somatic *MEN1* gene mutations. In contrast to familiar MEN1-related tumors, which usually are associated with a good prognosis, these two cases demonstrated a poor disease outcome as a result of 11q22.3-q25 loss.

Third, carcinoids that exhibit extensive CIN demonstrate a variable clinical outcome. Most of these cases also harbor deletion of chromosome 11q. Most of these

exhibit 9q34 gain and are associated with a relatively good prognosis. Here, 11q may be deleted as a consequence of CIN, with genes on the retained 11q arm likely intact, resulting in a less malignant phenotype. Two patients with lung carcinoids with extensive CIN exhibited both 11q deletion and amplifications at other chromosomal regions, and died within 3 years after diagnosis. Our data indicate that in one of these patients, the amplification involved the proto-oncogene *MYC*. Amplification of *MYC* has not previously been reported in lung carcinoids, although they are common in high-grade neuroendocrine carcinomas such as small-cell lung cancer and large-cell neuroendocrine carcinoma.[54] Because this patient demonstrated a relatively high mitotic count and the array CGH profile resembled that of large-cell neuroendocrine carcinoma,[13] it is tempting to speculate that this tumor represented a borderline lesion between AC and large-cell neuroendocrine carcinoma.

In conclusion, deletion of 11q22.3-q25 rather than 11q13 is involved in pulmonary carcinoid tumors. This deletion is associated with ACs and lower 10-year survival, indicating the presence of genes that potentially suppress carcinoid progression in this chromosomal region. Gain in 9q34.11 was associated with a better prognosis, and a combination of 11q22.3-q25 loss and absence of 9q34.11 gain seems to be the best predictor of unfavorable outcome with bronchial carcinoids. On the basis of these genomic alterations in combination with clinical outcome, we postulate three different mechanisms of tumorigenesis other than MEN1-related pathogenesis. In particular, assessment of 11q22.3-q25 loss may be useful in diagnosis of lung carcinoid as an indicator of poor prognosis, which may have consequences for patient management.

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