CD44 and OTP Are Strong Prognostic Markers for Pulmonary Carcinoids

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Abstract

Purpose: Pulmonary carcinoids are well-differentiated neuroendocrine tumors showing usually a favorable prognosis. However, there is a risk for late recurrence and/or distant metastasis. Because histologic classification in typical and atypical carcinoids is difficult and its reliability to predict disease outcome varies, we evaluated three genes as potential prognostic markers, that is, orthopedia homeobox (OTP), CD44, and rearranged during transfection (RET).

Experimental Design: These genes were analyzed in 56 frozen carcinoids by quantitative real-time PCR (qRT-PCR). RET was further studied by methylation and mutation analysis. Immunohistochemistry for CD44 and OTP protein expression was conducted on 292 carcinoids.

Results: Low mRNA expression levels of CD44 ($P = 1.8 \times 10^{-3}$) and OTP ($P = 0.00054$), and high levels of RET ($P = 0.025$), were strongly associated with a low 20-year survival of carcinoid patients. High RET expression was not related to promoter hypomethylation or gene mutations. A direct link between gene expression and protein levels was confirmed for CD44 and OTP but not for RET. Within all carcinoids as well as atypical carcinoids, absence of CD44 protein was significantly associated with low 20-year survival ($P = 0.00014$ and $0.00013$, respectively). The absence of nuclear OTP followed by complete loss of expression was also significantly associated with unfavorable disease outcome in all carcinoids ($P = 5.2 \times 10^{-5}$). Multivariate analyses revealed that age at diagnosis, histopathology, stage, and cytoplasmic OTP immunoreactivity were independent predictors of prognosis.

Conclusions: Our study indicates that CD44 and OTP are strong indicators of poor outcome. We therefore argue for implementation of these markers in routine diagnostics in addition to histopathology to improve subclassification of pulmonary carcinoids into prognostically relevant categories. Clin Cancer Res; 19(8); 2197–207. ©2013 AACR.

Introduction

Pulmonary carcinoids comprise a group of well-differentiated neuroendocrine tumors (NET) with little relation to cigarette smoking (1). In contrast to high-grade lung carcinomas, such as small-cell lung cancer (SCLC) and large-cell neuroendocrine carcinoma (LCNEC), carcinoids are characterized by a lower metastatic rate and a relatively favorable prognosis. According to the World Health Organization (WHO) classification, lung carcinoids are subclassified as typical carcinoids or atypical carcinoids (2). Atypical carcinoids are more often characterized by malignant behavior and have a lower 5-year survival rate as compared with typical carcinoids (61%–88% vs. 92%–100%, respectively; ref. 3). Metastases will develop in 4% to 64% of carcinoid patients (typical carcinoids, 4%–14%; atypical carcinoids, 35%–64%), usually in regional lymph nodes, but also at distant sites including liver, bones, brain, subcutaneous tissue, and breast (3, 4). The only curative treatment of pulmonary carcinoids is surgical resection, whereas the use of chemotherapy or radiotherapy for patients with metastatic disease has limited curative potential (1). In the inherited multiple endocrine neoplasia type I (MEN1) syndrome, approximately 5% of patients develop bronchial carcinoids (5). The MEN1 gene is mutated in approximately 18% of sporadic lung carcinoids (6).
Pulmonary carcinoids are well-differentiated neuroendocrine tumors, which however show a risk for late recurrence and/or distant metastasis. Histologic classification in typical and atypical carcinoids can be difficult and may complicate prediction of disease outcome. Alternatives to subdivide carcinoids into prognostically relevant categories are therefore desired. The current study examines the expression of 3 genes in relation to patient survival, that is, CD44, orthopedia homeobox (OTP), and rearranged during transfection (RET). Our data reveal that a combination of CD44 and OTP immunostaining with histopathology reliably predicts patient outcome, even within carcinoid subgroups. We therefore argue for the implementation of these markers in routine diagnostics to identify patients at risk for tumor relapse. These patients may be offered an intensified surveillance.

Histologic classification of lung carcinoids is difficult and its reliability to predict disease outcome is variable (compare refs. 7–9). Furthermore, although most patients remain cancer-free within 5 years after surgery, there is a risk for late recurrence and/or distant metastasis, the occurrence of which cannot be sufficiently predicted by the present WHO classification. Alternatives to subclassify carcinoids into prognostically relevant categories are therefore desired.

A few studies have reported clinical and molecular parameters associated with a higher risk of developing metastases and a poor disease outcome of carcinoid patients, such as size 3.5 cm or more, high Ki-67 and Bcl-2 expression, deletions of chromosome 11q22.3-q25, and in typical carcinoids low membranous expression of the standard splice variant of CD44 (7, 10–14). To analyze novel and more reliable prognostic markers for pulmonary carcinoids, we have selected 3 genes from a gene expression profiling screen of pulmonary carcinoids, comparing cases with a favorable and a poor disease outcome. In this comparison, the OTP gene, was most strongly downregulated in tumors from patients with a poor disease outcome with a median fold-change of 845. CD44, for which protein expression has been reported earlier to be associated with a favorable prognosis in typical carcinoid tumors (11), was also among the 15 strongest downregulated genes with a median fold-change of 29. The RET proto-oncogene showed the highest upregulation in the cases with a poor disease outcome with a median fold-change of 60.

**Collection of tumor material, clinical data, and cell lines**

We collected frozen material of 56 carcinoid tumors and 16 high-grade neuroendocrine carcinomas (Table 1A), as well as cell lines derived from NETs and RNA from normal tissues. Furthermore, we acquired a large series of formalin-fixed paraffin-embedded (FFPE) tissue, including 227 typical carcinoids, 64 atypical carcinoids, 1 not further subclassified carcinoid, 24 large cell neuroendocrine carcinomas, 35 SCLCs, and 1 not further subclassified high-grade neuroendocrine carcinoma.

Clinicopathologic data from the majority of patients could be collected, including survival data up to 20 years.

**Selection of genes from gene expression profiling**

Three genes were selected from a gene expression profiling screen of pulmonary carcinoids (our unpublished data; available at http://www.ebi.ac.uk/arrayexpress/ under accession number E-MEXP-3790), comparing cases with a favorable and a poor disease outcome. In this comparison, the OTP gene, was most strongly downregulated in tumors from patients with a poor disease outcome with a median fold-change of 845. CD44, for which protein expression has been reported earlier to be associated with a favorable prognosis in typical carcinoid tumors (11), was also among the 15 strongest downregulated genes with a median fold-change of 29. The RET proto-oncogene showed the highest upregulation in the cases with a poor disease outcome with a median fold-change of 60.

**Quantitative real-time PCR**

RNA isolation from frozen tissue was conducted using the RNeasy Mini Kit (Qiagen GmbH). After conversion of RNA into cDNA using the iScript cDNA Synthesis Kit (BioRad), qRT-PCR was conducted to assess mRNA expression of CD44, OTP, and RET, as well as of 4 housekeeping genes (ACTB, CYPA, GUSB, and HPRT) using primers listed in Supplementary Table S1A.

**RET mutation and methylation analysis**

RET mutation analysis was conducted for exons 10, 11, and 16, known hotspot regions of mutation, using primers listed in Supplementary Table S1B. These primers were M13-tailed to facilitate sequencing.

Promoter hypermethylation of the RET proto-oncogene was assessed using nested methylation-specific PCR (MSP), as described previously (15). All primer sequences are provided in Supplementary Table S1C. Methylation and unmethylation-specific primers were described previously (16).

**Immunohistochemistry**

Immunohistochemistry on FFPE tissue sections was conducted using the following primary antibodies: (i) mouse anti-CD44 (standard variant) monoclonal antibody, clone DF1485 (Dako); (ii) rabbit anti-OTP polyclonal antibody (HPA039365; Atlas Antibodies); and (iii) rabbit anti-RET polyclonal antibody (HPA008356; Atlas Antibodies).
Antibodies were detected by Bright Vision Poly-HRP-anti-mouse/rabbit/rat immunoglobulin G (IgG; Immunologic) followed by peroxidase-DAB (3,3'-diaminobenzidine) visualization. All stained tumor sections were independently scored by 2 of the authors (D.R.A. Swarts and R.-J. van Suylen) who were blinded for patient outcome; in case of disagreement, a consensus was reached after analysis by a third observer (E.-J.M. Speel). Scoring results were categorized into 5 different groups (0–4; see Supplementary Data). Positive cases were defined as having a scoring index >1 and negative cases displayed scoring indices ≤1. For OTP, a distinction was made between nuclear and cytoplasmic staining, which was scored separately.

Statistical analysis

Possible correlations between clinical data, qRT-PCR, and immunohistochemistry results were determined using the $\chi^2$ test, the Fisher exact test, the Student $t$ test, and Pearson’s correlation, when appropriate. Survival curves were created using the Kaplan–Meier method and the log-rank test was used to test for differences between subgroups. Cox-regression was used for multivariate analyses.

Results

Lung carcinoids with a poor disease outcome show downregulation of OTP and CD44 and upregulation of RET mRNA transcripts

The expression levels of 3 genes selected from an expression profiling study, that is, CD44, OTP, and RET, were analyzed by qRT-PCR on frozen material of 56 carcinoid tumors and 16 high-grade lung neuroendocrine carcinomas (see Table 1A), 4 normal tissues and 9 neuroendocrine cell lines. In Fig. 1A–C, the qRT-PCR results are provided as

Table 1. Clinicopathologic characteristics of the FFPE and frozen tissue series of pulmonary carcinoids and high-grade neuroendocrine carcinomas

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Carcinoids</th>
<th>Carcinomas</th>
<th>Carcinoids</th>
<th>Carcinomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis (mean)</td>
<td>53 (range, 21–83)</td>
<td>67 (range, 48–79)</td>
<td>53 (range, 16–83)</td>
<td>64 (range, 41–79)</td>
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<td>Sex</td>
<td>Male</td>
<td>20</td>
<td>13</td>
<td>130</td>
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<tr>
<td></td>
<td>Female</td>
<td>36</td>
<td>2</td>
<td>159</td>
</tr>
<tr>
<td></td>
<td>Unknown</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Smoking history</td>
<td>Current or former smoker</td>
<td>21</td>
<td>9</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>Never-smoker</td>
<td>19</td>
<td>0</td>
<td>59</td>
</tr>
<tr>
<td>Smoking status unknown</td>
<td>16</td>
<td>7</td>
<td>146</td>
<td>36</td>
</tr>
<tr>
<td>Analyzed tissue</td>
<td>Biopsy</td>
<td>0</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Resection</td>
<td>56</td>
<td>16</td>
<td>280</td>
</tr>
<tr>
<td></td>
<td>Autopsy</td>
<td>0</td>
<td>0</td>
<td>1</td>
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<tr>
<td>Histopathologic classification</td>
<td>TC</td>
<td>31</td>
<td>227</td>
<td></td>
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<td>AC</td>
<td>23</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LCNEC</td>
<td>7</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SCLC</td>
<td>8</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Not further subclassified</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Lymph node metastasis at diagnosis</td>
<td>Present</td>
<td>10</td>
<td>5</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>36</td>
<td>6</td>
<td>212</td>
</tr>
<tr>
<td></td>
<td>Unknown</td>
<td>10</td>
<td>5</td>
<td>43</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td>At diagnosis</td>
<td>3</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>During follow-up</td>
<td>3</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>46</td>
<td>8</td>
<td>271</td>
</tr>
<tr>
<td></td>
<td>Unknown</td>
<td>4</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Stage at diagnosis</td>
<td>IA</td>
<td>26</td>
<td>2</td>
<td>158</td>
</tr>
<tr>
<td></td>
<td>IB</td>
<td>10</td>
<td>3</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>IIA</td>
<td>6</td>
<td>2</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>IIB</td>
<td>1</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>IIIA</td>
<td>3</td>
<td>4</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>IIIB</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>3</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Unknown</td>
<td>7</td>
<td>1</td>
<td>38</td>
</tr>
<tr>
<td>Total number of cases</td>
<td>56</td>
<td>16</td>
<td>292</td>
<td>60</td>
</tr>
</tbody>
</table>

Abbreviations: AC, atypical carcinoid; TC, typical carcinoid.
scatterplots. The gene expression levels of CD44 and OTP in the different tumor subtypes were highly correlated \((P = 3.15 \times 10^{-7})\). The mean relative gene expression levels of CD44 and OTP, normalized to the levels of the housekeeping genes ACTB and CYPA, showed a large variation within the group of carcinoid tumors but were close to 0 in high-grade neuroendocrine carcinomas (Fig. 1A, B, D, and E). RET expression did not differ significantly between these 2 groups, but was slightly lower in high-grade lung NETs as compared with carcinoids (Fig. 1C and F). Relative expression values of CD44 (Fig. 1D) and OTP (Fig. 1E) were significantly decreased for carcinoid patients with a poor prognosis (defined as having distant metastasis and/or deceased within 20 years after initial diagnosis), whereas
the values for RET (Fig. 1F) were increased. Cutoff values for survival analyses were determined using time-dependent area under the receiver operating characteristic (ROC) curve examination and were chosen to maximize sensitivity and specificity for adverse disease outcome (Fig. 1G–I). Downregulation of CD44 and OTP, and upregulation of RET had a large negative impact on 20-year overall survival, both within the complete group of lung NETs (CD44: \( P = 1.1 \times 10^{-7} \); OTP: \( P = 6.7 \times 10^{-5} \); RET: \( P = 0.011 \)) and within the group of carcinoids (Fig. 1J–L). Within the qRT-PCR series, the differential expression levels for all 3 genes outperformed histologic subclassification into typical carcinoids and atypical carcinoids (\( P = 0.029 \); not shown) as indicators of patient outcome (see \( P \) values in Fig. 1J–L).

**RET is not activated by gene mutation or hypomethylation of its promoter region in lung carcinoids**

We decided to further investigate the RET proto-oncogene, as this gene is mutated in the multiple endocrine neoplasia type II (MEN2) syndrome and in other sporadic neuroendocrine neoplasms (17). We investigated the possibility that either a mutation or promoter hypomethylation of the RET oncogene is responsible for the increase in its gene expression levels. Therefore, we conducted mutation and methylation analyses of 5 carcinoid cases with high RET gene expression levels and a poor prognosis and 5 cases with low expression levels and a favorable disease outcome. However, we did not identify mutations in exons 10, 11, and 16, known to be the hotspot regions of RET mutations (18). Furthermore, we were unable to detect methylation of the RET promoter in these 10 tumors (data not shown).

### Establishment of CD44 and OTP as strong prognostic indicators for pulmonary carcinoids using immunohistochemistry

To further validate CD44, OTP, and RET as prognostic markers for lung carcinoids, we conducted immunohistochemistry for their encoded proteins on a series of 292 lung carcinoids and 60 high-grade pulmonary neuroendocrine carcinomas (see Table 1B), including 60 of 72 cases included in the qRT-PCR analyses (Supplementary Table S2). A preselection was made for extra atypical carcinoids to enhance the number of carcinoids with an unfavorable prognosis to increase the power of the study. The results of the immunohistochemical studies are summarized in Table 2. Representative images are provided for carcinoid tumors in Fig. 2, for normal (lung) tissue in Supplementary Fig. S1, and for high-grade lung neuroendocrine carcinomas in Supplementary Fig. S2.

The immunohistochemistry results were correlated with clinical follow-up data, the outcome of which is shown in Fig. 3. Also, histopathologic subclassification into typical carcinoids and atypical carcinoids was correlated to disease outcome. Atypical carcinoid histology was strongly associated with poor disease outcome (\( P = 8.2 \times 10^{-7} \); Fig. 3A).

### CD44

CD44 displayed a strong membranous staining pattern for most positive carcinoid cases (Fig. 2A), whereas a small number of the CD44-negative tumors (Fig. 2B) showed a weak nuclear staining in dispersed cells. Most cases showed a homogenous staining pattern for CD44.

### Table 2. Immunohistochemistry results of CD44 and OTP protein expression in neuroendocrine lung tumors

<table>
<thead>
<tr>
<th>CD44</th>
<th>( n )</th>
<th>Membranous staining</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinoids</td>
<td>282</td>
<td>180 (64%)</td>
<td>102 (36%)</td>
</tr>
<tr>
<td>TC</td>
<td>220</td>
<td>155 (70%)</td>
<td>65 (30%)</td>
</tr>
<tr>
<td>AC</td>
<td>61</td>
<td>37 (61%)</td>
<td>24 (39%)</td>
</tr>
<tr>
<td>Carcinomas</td>
<td>53</td>
<td>3 (6%)</td>
<td>50 (94%)</td>
</tr>
<tr>
<td>LCNEC</td>
<td>23</td>
<td>2 (9%)</td>
<td>21 (91%)</td>
</tr>
<tr>
<td>SCLC</td>
<td>30</td>
<td>1 (3%)</td>
<td>29 (97%)</td>
</tr>
<tr>
<td>Total</td>
<td>335</td>
<td>183 (55%)</td>
<td>152 (45%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>OTP</th>
<th>( n )</th>
<th>Exclusive nuclear staining</th>
<th>Nuclear and cytoplasmic staining</th>
<th>Exclusive cytoplasmic staining</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinoids</td>
<td>289</td>
<td>13 (5%)</td>
<td>194 (67%)</td>
<td>32 (11%)</td>
<td>50 (17%)</td>
</tr>
<tr>
<td>TC</td>
<td>225</td>
<td>10 (4%)</td>
<td>165 (73%)</td>
<td>17 (8%)</td>
<td>33 (15%)</td>
</tr>
<tr>
<td>AC</td>
<td>63</td>
<td>3 (5%)</td>
<td>28 (44%)</td>
<td>15 (24%)</td>
<td>17 (27%)</td>
</tr>
<tr>
<td>Carcinomas</td>
<td>59</td>
<td>1 (2%)</td>
<td>4 (7%)</td>
<td>8 (14%)</td>
<td>46 (78%)</td>
</tr>
<tr>
<td>LCNEC</td>
<td>24</td>
<td>0 (0%)</td>
<td>1 (4%)</td>
<td>2 (8%)</td>
<td>21 (88%)</td>
</tr>
<tr>
<td>SCLC</td>
<td>34</td>
<td>1 (3%)</td>
<td>3 (9%)</td>
<td>6 (18%)</td>
<td>24 (71%)</td>
</tr>
<tr>
<td>Total</td>
<td>348</td>
<td>14 (4%)</td>
<td>198 (57%)</td>
<td>40 (12%)</td>
<td>96 (28%)</td>
</tr>
</tbody>
</table>

Abbreviations: AC, atypical carcinoids; \( n \), number of cases; TC, typical carcinoids.
CD44 was also strongly correlated with distant metastasis. Within the total carcinoid tumor group, absence of CD44 as well as within the subgroup of atypical carcinoids (Fig. 3C), the patients with nuclear OTP reactivity had the best outcome, patients with cytoplasmic staining displaying intermediate survival and negative tumors having the worst disease outcome.

High-grade neuroendocrine lung carcinomas most often did not exhibit nuclear OTP staining (Table 2). Only 5 cases (8.5%) exhibited nuclear reactivity (Supplementary Fig. S2B). 8 cases (13.6%) retained cytoplasmic expression, and 46 cases (78.0%) were negative (Supplementary Fig. S2C). Interestingly, 2 OTP-positive, limited-stage SCLCs (Supplementary Fig. S2B) were disease-free 5 and 10 years after surgery, respectively. Also, within the combined group of NETs, absence of nuclear (P = 3.6e−11, 195 positive cases and 117 negative cases) or cytoplasmic (P = 5.0e−11, 219 positive cases and 93 negative cases) OTP reactivity was associated with low 20-year survival. Absence of nuclear OTP immunostaining was also related to distant metastasis (P = 1.8e−3). Again, the subdivision into 3 OTP staining categories (Supplementary Fig. S3) showed a strong relation to prognosis.

CD44 combined with OTP. Positive immunostaining for membranous CD44 and nuclear OTP reactivity were found to be highly correlated (P = 3.3e−3). When

Figure 2. CD44, OTP, and RET immunohistochemistry of pulmonary carcinoids. Representative immunohistochemical staining patterns for CD44 (A and B), OTP (C–E) and RET (F). A, typical carcinoid of a patient with no evidence of disease (NED) 7 years after diagnosis. A very strong membranous CD44 staining is observed in all cells. B, atypical carcinoid of a patient that died from metastatic disease 12 years after initial diagnosis. In this case membranous CD44 staining is absent. However, in a subset of cells (5%–10%) faint nuclear staining is seen. C, typical carcinoid of a patient with NED 25 years after initial diagnosis. This tumor shows both strong nuclear and cytoplasmic OTP staining. When nuclear staining was prominent cytoplasmic staining could be either strong as in the depicted case, or faint to negative. D, atypical carcinoid of a patient alive 10 years after diagnosis. This case has lost nuclear OTP staining but retains strong cytoplasmic localization. E, typical carcinoid of a patient that died 5 years after initial diagnosis, which lost OTP expression completely. F, liver metastasis of an atypical carcinoid of a patient that died of the disease 13 years after initial diagnosis. Note the punctate cytoplasmic RET staining. Immunohistochemistry results for normal (lung) tissues are provided in Supplementary Fig. S1 and for high-grade lung NETs in Supplementary Fig. S2.

whereas a small minority of cases had somewhat more heterogenous immunoreactivity pattern. Normal lung displayed positivity of basal cells in the bronchial epithelium and in seromucous glands (Supplementary Fig. S1A). From the data shown in Supplementary Table S2, it is obvious that the gene and protein expression levels of CD44 are directly correlated (P = 4.3e−10). Of the carcinoid tumors, 102 cases (36.2%) were negative for CD44, whereas 180 cases (63.8%) were scored CD44-positive (Table 2). Absence of CD44 staining was strongly correlated with low 20-year overall survival in the complete group of carcinoid tumors (Fig. 3B), as well as within the subgroup of atypical carcinoids (Fig. 3C). Within the total carcinoid tumor group, absence of CD44 was also strongly correlated with distant metastasis (P = 7.0e−7). There was also a correlation between CD44-negativity and Ki-67 proliferative index (P = 0.017, using a Ki-67 cutoff point of 2%; our unpublished data).

The tumor cells of high-grade lung NETs were usually CD44-negative (50 cases, 94.3%), although strong mem-

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combining OTP and CD44 results, no gain in survival rate was seen when both were found positive, but in case of a negative reaction for 1 of 2, negativity for the other was of strong additive value in predicting poor outcome within the total group of carcinoids. The period is indicated in months. Censored cases are indicated by an asterisk. A, histopathologic classification for the 281 cases that could be subclassified into typical carcinoids and atypical carcinoids and for which follow-up data were available. The solid line indicates typical carcinoids and the dotted line refers to atypical carcinoids. B and C, correlation between survival and CD44 immunostaining. Dotted lines indicate staining intensities ≤ 1, whereas solid lines depict cases with staining intensities > 1. Overall survival is depicted for the 264 of 282 cases with available follow-up from the complete group of carcinoids (B) or for the 59 of 61 atypical carcinoids with available follow-up only (C). D–F, correlation between survival and OTP immunostaining. Overall survival for nuclear OTP reactivity for the 269 of 289 cases with available follow-up from the complete group of carcinoids (D) or for the 207 of 225 typical carcinoids with available follow-up only (E). Dotted lines indicate staining intensities ≤ 1, whereas solid lines depict cases with staining intensities > 1. F, gradient of OTP immunostaining for the 269 of 289 carcinoids with available follow-up. The solid lines indicate cases with nuclear staining which may or may not display cytoplasmic immunoreactivity, the dashed lines indicate tumors which have lost nuclear staining but retain cytoplasmic positivity, and the dotted lines indicate negative tumors.

**RET.** In lung carcinoids, RET did not show the typical membranous staining pattern as described for normal ileum and colon (ref. 19; Supplementary Fig. S1C), but was located cytoplasmically, often showing a punctate staining pattern (Fig. 2F) and sometimes a weak nuclear staining in a subset of cells. In normal lung, RET expression was found in the cytoplasm of the bronchial epithelial cells and was seen in mucous glands with a membranous staining with occasionally a punctate pattern (Supplementary Fig. S1D). Because the RET immunohistochemistry results could not be correlated to the qRT-PCR results (Supplementary Table S2), and because our initial immunostaining results could not be associated with patient outcome (data not shown), we did not proceed with the analysis of RET in the complete series.
Univariate and multivariate analysis. Univariate analyses were conducted on tumor subgroups using clinicopathologic parameters (age at diagnosis, histopathologic distinction between typical carcinoids and atypical carcinoids, sex, stage, and tumor diameter) and molecular parameters (CD44 and OTP immunohistochemistry results, Ki-67 proliferation index; Supplementary Table S3). In multivariate analysis, age at diagnosis, histopathology, stage, and absence of cytoplasmic reactivity for OTP were significantly associated with decreased 20-year overall survival within the group of carcinoids (Supplementary Table S3). Within the group of atypical carcinoids, only stage was significant in multivariate analysis, although CD44 immunostaining showed a trend toward significance ($P = 0.069$). Within the group of typical carcinoids, age at diagnosis and nuclear OTP status were significantly correlated with disease outcome (Supplementary Table S3).

Discussion

So far, only few prognostic parameters have been described for pulmonary carcinoids, including histology (7, 10–14). In this report, we evaluated the value of CD44, OTP, and RET expression to improve the prediction of pulmonary carcinoid prognosis. By analyzing a series of almost 300 carcinoids (227 typical carcinoids, 64 atypical carcinoids), we could show that loss of CD44 or OTP expression is a strong indicator of adverse patient outcome. The immunohistochemical staining patterns are easy to interpret and their detectability in paraffin-embedded tissue sections makes them readily applicable in routine diagnostics.

With respect to CD44, we observed a strong positive effect on patient outcome when membranous staining was present. This is the case for both typical carcinoids and atypical carcinoids, the latter being largely neglected in the literature. Because the standard variant of CD44 was shown to be the most potent prognostic marker in the study by Granberg and colleagues (11), we chose to analyze only this variant in the underlying study. Our results are in agreement with the literature, where in smaller series of neuroendocrine lung tumors the presence of CD44 expression has been associated with a favorable disease outcome. Carcinoids have been reported to be frequently positive for both the standard and variant CD44 forms (11, 20–23). In the study by Granberg and colleagues (11), the presence of the standard variant, as well as the CD44 variant 9, was associated with a positive outcome within 43 typical carcinoids. In a study of Sun and colleagues (23) on carcinoids, including 20 cases of pulmonary origin, CD44 negativity was associated with metastasis. SCLCs are most often negative for CD44 (20, 22), which is confirmed by our data. These combined data point to a tumor suppressive role for CD44. Indeed, CD44-null fibroblasts are tumorigenic in nude mice, and CD44 has been suggested to promote apoptosis (24, 25). However, on the other hand, CD44 is involved in epithelial-to-mesenchymal transition and in promoting cell survival (24, 26). It is therefore not surprising that within human cancers CD44 expression has been described as an indicator of both a favorable (27–29) as well as a poor prognosis (30–32).

From the underlying study, using almost 300 cases of carcinoids with variable disease outcome, it is evident that also OTP downregulation is undeniably related to tumor progression. Our data indicate a gradual loss of OTP protein expression in correlation with prognosis. Nuclear OTP expression was strongly correlated to CD44 protein expression. OTP gene expression levels were close to 0 in neuroendocrine tumors.
cancer cell lines and in most high-grade neuroendocrine carcinomas, where the typical nuclear staining pattern was very rare. Because nuclear OTP was also present in 2 limited stage SCLC cases (both CD44-negative) with good outcome, OTP might also be a prognostic indicator in high-grade neuroendocrine lung carcinomas. For OTP, an evolutionary conserved homeodomain-containing transcription factor, currently only functions within the central nervous system have been described (33–35). OTP has important roles in the development of the neurosecretory system in the hypothalamus and in terminal differentiation of neuroblasts (33, 35). Especially differentiation, proliferation, and migration of Otp-expressing cells were severely abrogated in Otp−/− mice (33). Amir-Zilberstein and colleagues (34) reported that Otp can be induced by stress, leading to transcriptional activation of, for example, corticotropin-releasing hormone. Only recently, Kim and colleagues (36) have described OTP for the first time in the context of cancer, showing OTP methylation in breast cancer using CpG microarray analysis. Therefore, the need for a study into the function of OTP within neuroendocrine tissues and neuroendocrine (lung) tumors is obvious.

We noticed that the combination of CD44 and OTP results allows an even better separation of tumors into prognostic categories, because CD44 is a strong prognostic marker within the group of atypical carcinoids, whereas the loss of nuclear OTP immunoreactivity has a strong prognostic value within the group of typical carcinoids. In addition, they partially compensate each other’s shortcomings from a methodologic point of view. CD44 shows occasionally a heterogeneous staining pattern and the antibody strongly reacts with near-necrotic cells, particularly in high-grade lung NETs. This might cause problems when analyzing small biopsy samples. For OTP no monoclonal antibody is currently available, which impedes standardization of the staining procedure, and the cytoplasmic and nuclear immunoreactivity of OTP may make evaluation slightly more complicated.

Besides CD44 and OTP immunostaining, also other parameters such as histopathology were significantly related to disease outcome. Multivariate analysis showed that histopathologic distinction between typical carcinoids and atypical carcinoids, stage and OTP immunostaining are independent predictors of disease outcome within carcinoids. In addition, after carcinoid classification, OTP is the most optimal prognostic indicator in typical carcinoids. In the group of atypical carcinoids, CD44 showed a trend toward significance, next to stage. Although histopathology alone is a strong prognostic indicator in the underlying study, classification of lung carcinoids using the WHO criteria may be difficult and its ability to predict the outcome of especially atypical carcinoids varied in previous studies (7–9). Furthermore, counting mitoses is time consuming and may be difficult, because for example pyknotic apoptotic nuclei and mitoses are hard to distinguish (37, 38). Only 2 small studies showed interobserver variation to be present and more prominent in atypical carcinoids than in typical carcinoids (37, 39). Taken together, we argue for the combined use of histopathology and CD44 and OTP immunostaining.

Staining for both CD44, which is usually negative in high-grade lung NETs, and OTP, which is negative or confined to the cytoplasm in these tumors, may aid in the differential diagnosis between SCLC and carcinoid tumors, which is known to be difficult in the case of small biopsies (40). The absence of membranous CD44 and nuclear OTP expression in most high-grade lung NETs provides also further evidence for separate tumorigenesis pathways of these tumors and carcinoids (1).

Expression of the RET proto-oncogene, a tyrosine kinase receptor (17), was also correlated at the transcriptional level with patient outcome. Unfortunately, this could not be confirmed at the protein level, and RET expression was therefore not tested in the complete series. However, the transcriptional upregulation of RET in aggressive carcinoids made it worthwhile to study the methylation status of its promoter region. Methylation of the RET promoter has been shown in colorectal cancer by Mokarram and colleagues (16), who did not study its effect on gene expression levels. We did not identify RET methylation in 10 pulmonary carcinoid cases, and we therefore concluded that hypomethylation of the promoter region does not underly its activation in pulmonary carcinoids, at least not in our studied cases.

Both gain and loss of function of RET is implicated in disease and can lead to MEN2 and Hirschsprung disease, respectively (17). In contrast to the MEN1-syndrome, caused by mutations in the MEN1 gene, pulmonary carcinoids have not been associated with the MEN2-syndrome. In this study, we exclude mutation as the cause for RET upregulation in the ten cases that were studied for mutations in exons 10, 11, and 16. Few other studies have assessed RET mutations in sporadic lung NETs. Komminoth and colleagues (41) did not find mutations in 11 lung carcinoids and 7 SCLCs. Futami and colleagues (42) identified 2 identical mutations in 2 of 6 SCLC cell lines and their respective primary tumors, but not in an additional 12 cases (43). No RET mutations were identified in a separate study of 54 SCLC cell lines (44).

Alternatively, investigation of RET translocations has become relevant because of the recent reports that such translocations can take place in lung adenocarcinomas (45–47).

Conclusion

Using a series of almost 300 cases, we present CD44 and OTP as powerful prognostic markers for pulmonary carcinoids. In lung NETs, CD44 and OTP gene expression levels are directly correlated with their respective protein expression levels. Furthermore, low transcriptional as well as protein expression levels are strongly associated with a poor long-term survival rate of pulmonary carcinoid patients. After independent validation, these markers may potentially be implemented in addition to the current histologic classification of pulmonary carcinoid tumors to improve prediction of prognosis.
Our results may also have clinical implications, for example by increasing the frequency of follow-up when these markers are negative, to detect metastatic disease as early as possible.

Disclosure of Potential Conflicts of Interest

W. Van Crieckinge is employed by Pfizer and has a stock interest in Zenyth. F.-C.S. Ramakers and E.-J.M. Speel have honoraria from Speakers Bureau of Pfizer and Lilly and is a consultant/advisory board member of Pfizer. No potential conflicts of interest were disclosed by the other authors.

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