CD44 and OTP are strong prognostic markers for pulmonary carcinoids

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Pulmonary carcinoids are well-differentiated neuroendocrine tumors, which however show a risk for late recurrence and/or distant metastasis. Histological 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 three genes in relation to patient survival, i.e., CD44, OTP, RET. Our data reveals 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.
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 histological classification in typical and atypical (AC) carcinoids is difficult and its reliability to predict disease outcome varies, we evaluated three genes as potential prognostic markers, i.e., OTP, CD44 and RET.

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

Results: Low mRNA expression levels of CD44 (p=1.8e-5) 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 ACs, absence of CD44 protein was significantly associated with low 20-year survival (p=0.00014 and p=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=5.2e-6). 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.
Introduction

Pulmonary carcinoids comprise a group of well-differentiated neuroendocrine tumors (NETs) 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 WHO classification, lung carcinoids are subclassified as typical carcinoids (TCs) or atypical carcinoids (ACs) (2). ACs are more often characterized by malignant behavior and have a lower 5-year survival rate as compared with TCs (61-88% versus 92-100%, respectively) (3). Metastases will develop in 4-64% of carcinoid patients (TCs: 4-14%, ACs: 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 for pulmonary carcinoids is surgical resection, whereas the use of chemotherapy or radiation therapy for patients with metastatic disease has limited curative potential (1). In the inherited multiple endocrine neoplasia type 1 syndrome approximately 5% of patients develop bronchial carcinoids (5). The MEN1 gene is mutated in approximately 18% of sporadic lung carcinoids (6).

Histological classification of lung carcinoids is difficult and its reliability to predict disease outcome is variable (compare refs (7), (8) and (9)). Furthermore, although most patients remain cancer-free within five 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, high Ki-67 and Bcl-2 expression, deletions of chromosome 11q22.3-q25, and in TCs low membranous expression of the standard splice variant of CD44 (7, 10-14). In order to analyze novel and more reliable prognostic markers for pulmonary carcinoids, we have selected three genes from a gene expression profiling screen of pulmonary carcinoids, comparing cases with a favorable and a poor disease outcome. These genes include the CD44 and orthopedia homeobox (OTP) genes, which were downregulated in tumors from patients with a poor disease outcome, and the RET proto-oncogene that showed upregulated expression in these patients. We analyzed the value of these genes as prognostic indicators using quantitative real-time PCR (qRT-PCR) in a series of 72 pulmonary NETs, and assessed a potential link between mRNA and protein expression using immunohistochemistry. Protein expression was then validated by immunohistochemistry on a series of 352 cases, including 60 out of 72 tumors analyzed by qRT-PCR.

Materials & Methods

Supplementary materials and methods

A detailed description of the used materials and methods in this study can be found in the Supplementary Data.

Collection of tumor material, clinical data and cell lines

We collected frozen material of 56 carcinoid tumors and 16 high-grade neuroendocrine (NE) 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 TCs, 64 ACs, 1 not further subclassified carcinoid, 24 large cell NE carcinomas, 35 SCLCs and 1 not further subclassified high-grade NE carcinoma. Clinicopathological 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 orthopedia homeobox (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 favourable prognosis in TC tumours (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 RT-PCR

RNA isolation from frozen tissue was performed using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). After conversion of RNA into cDNA using the iScript cDNA Synthesis Kit (BioRad, Hercules, CA, USA), qRT-PCR was performed to assess mRNA expression of CD44, OTP and RET, as well as of four housekeeping genes (ACTB, CYP4, GUSB and HPRT) using primers listed in Supplementary Table S1A.

RET mutation and methylation analysis
RET mutation analysis was performed 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 performed using the following primary antibodies: 1) mouse anti-CD44 (standard variant) monoclonal antibody, clone DF1485 (DAKO, Glostrup, Denmark); rabbit anti-OTP polyclonal antibody (HPA039365, Atlas Antibodies, Stockholm, Sweden); rabbit anti-RET polyclonal antibody (HPA008356, Atlas Antibodies). Antibodies were detected by Bright Vision Poly-HRP-anti Mouse/Rabbit/Rat IgG (Immunologic, Duiven, The Netherlands) followed by peroxidase-DAB visualisation. All stained tumor sections were independently scored by two of the authors (DS and RJvS) which were blinded for patient outcome; in case of disagreement, a consensus was reached after analysis by a third observer (EJS). Scoring results were categorized into five 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’s exact test, the Student’s 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 \textit{OTP} and \textit{CD44} and upregulation of \textit{RET} mRNA transcripts.

The expression levels of three genes selected from an expression profiling study, i.e., \textit{CD44}, \textit{OTP} and \textit{RET}, were analyzed by qRT-PCR on frozen material of 56 carcinoid tumors and 16 high-grade lung NE carcinomas (see Table 1A), 4 normal tissues and 9 NE cell lines. In Figure 1A-C, the qRT-PCR results are provided as scatterplots. The gene expression levels of \textit{CD44} and \textit{OTP} in the different tumor subtypes were highly correlated (p=3.15e^{-7}). The mean relative gene expression levels of \textit{CD44} and \textit{OTP}, normalized to the levels of the \textit{ACTB} and \textit{CYPA} housekeeping genes, showed a large variation within the group of carcinoid tumors, but were close to zero in high-grade NE carcinomas (Figure 1A-B and 1D-E). \textit{RET} expression did not differ significantly between these two groups, but was slightly lower in high-grade lung NETs as compared to carcinoids (Figure 1C and 1F). Relative expression values of \textit{CD44} (Figure 1D) and \textit{OTP} (Figure 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), while the values for \textit{RET} (Figure 1F) were increased. Cut-off values for survival analyses were determined using time-dependent area under the ROC curve examination, and chosen to maximise sensitivity and specificity for adverse disease outcome (Figure 1G-I). Downregulation of \textit{CD44} and \textit{OTP}, and upregulation of \textit{RET} had a large
negative impact on 20-year overall survival, both within the complete group of lung NETs

\((CD44: p=1.1\times10^{-7}; OTP: p=6.7\times10^{-5}; RET: p=0.011)\) and within the group of carcinoids (Figure 1J-L). Within the qRT-PCR series, the differential expression levels for all three genes outperformed histological subclassification into TCs and ACs \((p=0.029\), not shown\) as indicators of patient outcome (see p-values in Figure 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, since this gene is mutated in the multiple endocrine neoplasia type 2 syndrome and in other sporadic NE 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 performed mutation and methylation analyses of five carcinoid cases with high \(RET\) gene expression levels and a poor prognosis and five 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 ten tumors \(\text{data not shown}\).

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 performed immunohistochemistry for their encoded proteins on a series of 292 lung carcinoids and 60 high-grade pulmonary NE carcinomas \(\text{see Table 1B}\), including 60 of the 72 cases included in the qRT-PCR analyses \(\text{Supplementary Table S2}\). A preselection was made for extra ACs 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 Figure 2, for normal (lung) tissue in Supplementary Figure S1 and for high-grade lung NE carcinomas in Supplementary Figure S2.

The immunohistochemistry results were correlated with clinical follow-up data, the outcome of which is shown in Figure 3. Also, histopathological subclassification into TCs and ACs was correlated to disease outcome. AC histology was strongly associated with poor disease outcome (p=8.2e-7; Figure 3A).

CD44

CD44 displayed a strong membranous staining pattern for most positive carcinoid cases (Figure 2A), while a small number of the CD44-negative tumors (Figure 2B) showed a weak nuclear staining in dispersed cells. Most cases showed a homogenous staining pattern for CD44, while a small minority of cases had somewhat more heterogeneous immunoreactivity pattern. Normal lung displayed positivity of basal cells in the bronchial epithelium and in seromucous glands (Supplementary Figure 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, while 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 (Figure 3B), as well as within the subgroup of ACs (Figure 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 cut-off point of 2%; our unpublished observations).

The tumor cells of high-grade lung NETs were usually CD44 negative (50 cases, 94.3%), although strong membranous staining in the proximity of necrotic areas was regularly seen.
Also within the total group of NETs, absence of CD44 was significantly associated with a low 20-year overall survival rate ($p=6.2\times10^{-8}$; 171 positive cases and 130 negative cases) and distant metastasis ($p=1.6\times10^{-10}$).

**OTP**

The large majority of cases had a homogenous OTP staining pattern. Three different OTP staining patterns could be discerned (Table 2). A strong nuclear staining, either with or without a cytoplasmic reactivity, was seen in 207 carcinoid cases (71.6%; Figure 2C), while exclusively cytoplasmic staining was seen in 32 cases (11.1%; Figure 2D), and 50 carcinoid cases (17.3%) were negative (Figure 2E). In case of nuclear reactivity, >90% of the tumor cells were generally positive. OTP transcription levels and nuclear protein expression levels (Supplementary Table S2) were strongly correlated ($p=5.9\times10^{-11}$). The subset of cases that lost nuclear staining, but retained cytoplasmic reactivity, as well as the completely negative cases, showed low *OTP* gene expression levels.

In normal lung, cytoplasmic reactivity for OTP was evident in bronchial epithelial cells, macrophages and in seromucous glands (Supplementary Figure S1B).

Absence of nuclear OTP staining was correlated to a poor prognosis, within the complete group of carcinoid tumors (Figure 3D) and within TCs (Figure 3E). The difference in survival between cases with or without nuclear immunoreactivity became particularly prominent after five years following first diagnosis. Absence of nuclear OTP expression was correlated to the occurrence of distant metastasis ($p=0.00014$). Similarly, absence of cytoplasmic OTP staining was associated with adverse disease outcome within the complete group of carcinoids ($p=0.00038$). Also the subdivision of OTP expression according to the three immunostaining patterns was strongly related to patient outcome within the group of pulmonary carcinoids (Figure 3F), the patients with nuclear OTP reactivity having the best outcome, patients with
cytoplasmic staining displaying intermediate survival and negative tumors having the worst
disease outcome.

High-grade NE lung carcinomas most often did not exhibit nuclear OTP staining (Table 2).

Only five cases (8.5%) exhibited nuclear reactivity (Supplementary Figure S2B), eight cases
(13.6%) retained cytoplasmic expression, and 46 cases (78.0%) were negative
(Supplementary Figure S2C). Interestingly, two OTP-positive, limited-stage SCLCs
(Supplementary Figure S2B) were disease-free 5 and 10 years after surgery, respectively.

Also within the combined group of NETs, absence of nuclear (p=3.6e-12; 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-9). Again, the subdivision into
three OTP staining categories (Supplementary Figure 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-35). When 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 one of the
two, negativity for the other was of strong additive value in predicting poor outcome within
the total group of carcinoids (Figure 4A) as well as the total group of pulmonary NETs
(Figure 4B).

RET

In lung carcinoids, RET did not show the typical membranous staining pattern as described
for normal ileum and colon (19) (Supplementary Figure S1C), but was located
cytoplasmically, often showing a punctate staining pattern (Figure 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 Figure 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 performed on tumor subgroups using clinicopathologic parameters (age at diagnosis, histopathological distinction between TCs and ACs, 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 ACs, only stage was significant in multivariate analysis, although CD44 immunostaining showed a trend towards significance (p=0.069). Within the group of TCs, 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 TCs, 64 ACs) we could demonstrate 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 TCs and ACs, the latter being
largely neglected in the literature. Since the standard variant of CD44 was shown to be the
most potent prognostic marker in the study by Granberg _et al._ (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 NE 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 _et al._ (11), the
presence of the standard variant, as well as the CD44 variant 9, was associated with a positive
outcome within 43 TCs. In a study of Sun _et al._ (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 orthopaedia homeobox (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 directly coupled to its mRNA
expression and strongly correlated to CD44 protein expression. *OTP* gene expression levels were close to zero in NE cancer cell lines and in most high-grade NE carcinomas, where the typical nuclear staining pattern was very rare. Since nuclear OTP was also present in two limited stage SCLC cases (both CD44-negative) with good outcome, OTP might also be a prognostic indicator in high-grade NE 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 was severely abrogated in Otp−/− mice (33). Amir-Zilberstein et al. (34) reported that Otp can be induced by stress, leading to transcriptional activation of e.g. corticotropin-releasing hormone. Only recently, Kim et al. (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 NE tissues and NE (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 ACs, whereas the loss of nuclear OTP immunoreactivity has a strong prognostic value within the group of TCs. In addition, they partially compensate each other’s shortcomings from a methodological 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 histopathological distinction between TCs and ACs, 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 TCs. In the group of ACs, CD44 showed a trend towards 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 ACs 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 two small studies demonstrated interobserver variation to be present and more prominent in ACs than in TCs (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 (rearranged during transfection) 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 
et al. (16), who did not study its effect on gene expression levels. We did not identify RET 
methylation in ten 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 multiple 
endocrine neoplasia type 2 (MEN2) and Hirschprung 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 et al. (41) did 
not find mutations in 11 lung carcinoids and 7 SCLCs. Futami et al. (42) identified two 
identical mutations in two out of six 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 histological 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, in order to detect metastatic disease as early as
possible.
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Reference List


Table 1 - Clinicopathological characteristics of the formalin-fixed paraffin-embedded and frozen tissue series of pulmonary carcinoids and high-grade neuroendocrine carcinomas

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<td>24</td>
</tr>
<tr>
<td>SCLC</td>
<td>8</td>
<td>35</td>
</tr>
<tr>
<td>Not further subclassified</td>
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<td>1</td>
</tr>
<tr>
<td><strong>Lymph node metastasis at diagnosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>10</td>
<td>37</td>
</tr>
<tr>
<td>Absent</td>
<td>36</td>
<td>212</td>
</tr>
<tr>
<td>Unknown</td>
<td>10</td>
<td>43</td>
</tr>
<tr>
<td><strong>Distant metastasis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At diagnosis</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>During follow-up</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>Absent</td>
<td>46</td>
<td>271</td>
</tr>
<tr>
<td>Unknown</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td><strong>Stage at diagnosis</strong></td>
<td></td>
<td></td>
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<tr>
<td>IA</td>
<td>26</td>
<td>158</td>
</tr>
<tr>
<td>IB</td>
<td>10</td>
<td>37</td>
</tr>
<tr>
<td>IIA</td>
<td>6</td>
<td>26</td>
</tr>
<tr>
<td>IIB</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>IIIA</td>
<td>3</td>
<td>19</td>
</tr>
<tr>
<td>IIIB</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>IV</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>Unknown</td>
<td>7</td>
<td>38</td>
</tr>
<tr>
<td><strong>Total number of cases</strong></td>
<td>56</td>
<td>292</td>
</tr>
</tbody>
</table>

Abbreviations used: AC, atypical carcinoid; FFPE, formalin-fixed paraffin-embedded; LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung cancer; TC, typical carcinoid
Table 2 - Immunohistochemistry results of CD44 and OTP protein expression in neuroendocrine lung tumours

<table>
<thead>
<tr>
<th>CD44</th>
<th>n</th>
<th>Membranous staining&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Negative&lt;sup&gt;a&lt;/sup&gt;</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&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Nuclear and cytoplasmic staining&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Exclusive cytoplasmic staining&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Negative&lt;sup&gt;a&lt;/sup&gt;</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 used: AC, atypical carcinoids; LCNEC, large cell neuroendocrine carcinomas; n, number of cases; SCLC, small cell lung cancers; and TC, typical carcinoids

<sup>a</sup>Samples with staining intensity ≤ 1 were regarded negative, while samples with staining intensity > 1 were considered positive; as described in the Supplementary Materials & Methods.
Figure Legends

Figure 1 – Quantitative RT-PCR results for CD44, OTP and RET.

(A-C) Scatterplots showing the relative mRNA expression values of CD44 (A), OTP (B) and RET (C) for typical carcinoids (TC), atypical carcinoids (AC), large cell neuroendocrine carcinomas (LCNEC), small cell lung cancers (SCLC), neuroendocrine cell lines (NECL) and normal tissues (NT), normalized to the geometric mean of the expression levels of the housekeeping genes ACTB and CYP A. The mean relative expression levels for the respective subgroups are indicated. The only NE cell line showing CD44 expression was the insulinoma cell line CM (relative expression level 0.157, A). Levels of OTP expression were virtually absent in all cell lines analyzed. Only the pancreatic endocrine cell line QGP exhibited considerable RET expression (relative expression level 0.083, C).

(D-F) Differences between the mean values ± SD of gene expression levels between the total group of carcinoids (CD) and carcinomas (CAR) for CD44 (D, 95% CI -0.283/-0.152), OTP (E, 95% CI -0.382/-0.224) and RET (F, not significantly different), and in carcinoids from patients with a favorable (F) and a poor (P) disease outcome for CD44 (D, 95% confidence interval [CI] 0.166/0.349), OTP (E, 95% CI 0.176/0.437) and RET (F, 95% CI -0.233/0.012).

(G-I) Time-dependent Receiver Operating Characteristic (ROC) curves depicting the trade-off between the sensitivity and specificity of CD44 (G), OTP (H) and RET (I) expression in predicting disease outcome.

(J-L) Kaplan-Meier analyses depicting the difference in 20-year overall survival for high (solid lines) or low (dotted lines) mRNA expression levels of CD44 (J, cut-off value 0.109), OTP (K, cut-off value 0.132), and RET (L, cut-off value 0.0256). Cut-off values were determined using the ROC-curves depicted in (G-I). The period is indicated in months. Censored cases are indicated by an asterisk.
Abbreviations used: AUC = Area under the curve.

**Figure 2 – CD44, OTP and RET immunohistochemistry of pulmonary carcinoids.**

Representative immunohistochemical staining patterns for CD44 (A-B), OTP (C-E) and RET (F).

(A) Typical carcinoid (TC) 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 (AC) 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) TC 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) AC of a patient alive 10 years after diagnosis. This case has lost nuclear OTP staining but retains strong cytoplasmic localization. (E) TC of a patient that died 5 years after initial diagnosis, which lost OTP expression completely.

(F) Liver metastasis of an AC 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 Figure S1, and for high-grade lung neuroendocrine tumors in Supplementary Figure S2.

**Figure 3 – Survival analyses of pulmonary carcinoids using histopathology and protein expression of CD44 and OTP as prognostic indicators.**
Kaplan-Meier analyses showing 20-year overall survival rates for (A, B, D, F) the complete group of carcinoid tumors, (C) atypical carcinoids (ACs) or (E) typical 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 TCs and ACs and for which follow-up data was available. The solid line indicates TCs and the dotted line refers to ACs.

(B, C): Correlation between survival and CD44 immunostaining. Dotted lines indicate staining intensities ≤ 1, while solid lines depict cases with staining intensities > 1. Overall survival is depicted for the 264 out of 282 cases with available follow-up from the complete group of carcinoids (B) or for the 59 out of 61 ACs with available follow-up only (C). (D-F): Correlation between survival and OTP immunostaining. Overall survival for nuclear OTP reactivity for the 269 out of 289 cases with available follow-up from the complete group of carcinoids (D) or for the 207 out of 225 TCs with available follow-up only (E). Dotted lines indicate staining intensities ≤ 1, while solid lines depict cases with staining intensities > 1.

(F): Gradient of OTP immunostaining for the 269 out 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.

**Figure 4 – Survival analyses of pulmonary neuroendocrine tumors in relation to a combination of CD44 and OTP protein expression**

Kaplan-Meier analyses showing 20-year overall survival rates for (A) the 261 out of 279 carcinoids with available follow-up and (B) the 298 out of 331 cases of the combined group of carcinoids and high-grade neuroendocrine carcinomas with available follow-up, using the combined CD44 and OTP immunohistochemistry results. The period is indicated in months.
Censored cases are indicated by an asterisk. The solid lines indicate cases with both CD44 and OTP (nuclear and/or cytoplasmic) protein expression. The dashed lines indicate tumors which have lost CD44 or OTP expression, and the dotted lines indicate tumors negative for both proteins.
Figure 1
Figure 2
Figure 3
Figure 4
Clinical Cancer Research

CD44 and OTP are strong prognostic markers for pulmonary carcinoids


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