RET/NTRK1 Rearrangements in Thyroid Gland Tumors of the Papillary Carcinoma Family: Correlation with Clinicopathological Features

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ABSTRACT

The papillary carcinoma family (PCF) of thyroid tumors includes a wide variety of neoplastic entities regarded as well-differentiated, poorly differentiated, and undifferentiated papillary thyroid carcinomas. Recent studies have established the presence of alternative oncogenic rearrangements of the RET and NTRK1 genes in a consistent fraction (≤50%) of papillary thyroid tumors. RET oncogenic rearrangements are also very frequent (~60%) in Chernobyl radiation-associated papillary thyroid neoplasias, which show an increased aggressiveness in terms of pathological stage at disease onset.

These observations prompted us to study the relationship between the presence or absence of RET and NTRK1 oncogenes and the clinicopathological features (age, sex, histopathology, and pTNMC2 staging) of 76 consecutive, non-radiation-related tumors of the PCF.

As previously reported, statistical univariate analysis revealed a correlation between the combination of RET and NTRK1 (RET/NTRK1) positivity and young age of patients at diagnosis. In addition, a significant association was found between RET/NTRK1 positivity and locally advanced stage of disease at presentation (pT4: P < 0.015). The multivariate analysis confirmed that RET/NTRK1 activation parallels an unfavorable disease presentation, which may correlate with a less favorable disease outcome. Furthermore, within the PCF, the frequency of RET/NTRK1 positivity was not influenced by the different neoplastic subtypes or the tumor versus degree of differentiation.

INTRODUCTION

Classic categorizations indicate that PTC3 accounts for about 80% of thyroid gland tumors. However, according to currently updated criteria (1-7), the papillary histotype appears to encompass several variants, ranging from well-differentiated papillary carcinoma to poorly differentiated papillary carcinoma and including undifferentiated thyroid carcinomas bearing at least focal evidence of papillary differentiation. Taken together, these different entities may be tentatively defined as the PCF.

Recent reports have demonstrated the alternative involvement of the RET and NTRK1 tyrosine kinase receptors in the development of a fraction of PTCs. Somatic rearrangements of these two genes produce several forms of oncogenes, designated RET/PTC1, RET/PTC2, RET/PTC3, TRK, TRK-T1, TRK-T2, and TRK-T3 (8). In all forms, RET or NTRK1 tyrosine kinase domains are fused to the NH2 terminus of different gene products. Significantly, all of these oncogenes are restricted to papillary thyroid tumors (9). Moreover, two recent papers have shown that transgenic mice with thyroid-targeted expression of the RET/PTC1 oncogene develop thyroid carcinomas with considerable similarities to human PTC (10, 11). Differences in the frequency of RET and NTRK1 activation have been reported in PTCs collected from various geographical areas. Our findings demonstrate oncogenic expression of RET and NTRK1 in about 50% of papillary thyroid tumors collected at the Istituto Nazionale Tumori (Milan, Italy). Lower percentages have been described, ranging from 2.5% in Saudi Arabia to 8% in Japan and 29% in Italy (12, 13). The diversity of these figures may suggest not only bias due to various methodologies but also the involvement of different genetic and/or environmental factors.

This observation is strengthened by the increased incidence of PTC that is reported in children living in contaminated areas around Chernobyl. About 60% of them present a RET oncogenic activation (14, 15). However, this high frequency of RET positivity in irradiated children does not rule out the possibility that age per se could play a role in the development of RET-positive tumors (16). In fact, we have recently reported that, in papillary thyroid neoplasias, the frequency of RET and NTRK1 activation is significantly higher when patients are under the age of 30 (P < 0.022). In addition, children are known to be much more sensitive to the tumorigenic effect of external irradiation (17), and the young thyroid displays a high degree of replication and, therefore, an augmented capability to both fix and propagate mutational damage.

The increased aggressiveness of Chernobyl thyroid tumors (supported by a more advanced stage at onset in terms of...
pathological stage; Ref. 18) prompted us to study sporadic (i.e., non-radiation-associated) tumors of the PCF. Therefore, regardless of age, we investigated the correlations between RET and NTRK1 activation and the clinicopathological features of PCF members.

The present investigation, carried out on 76 sporadic consecutive neoplasias of the PCF that were surgically removed from patients, ages 4 to 80 years (mean, 37.5 years), shows that RET/PTC and NTRK1 expression are comparable oncogenic events, representing common features within the papillary carcinoma spectrum. Furthermore, our findings demonstrate a correlation between patients with RET/NTRK1 thyroid carcinomas and both locally advanced stage (pT4) and young age at onset of disease.

PATIENTS AND METHODS

Tumor Specimens and Molecular Analyses. Ninety-two consecutive cases who were diagnosed with PTC and related histotypes, both collected at and referred to the Istituto Nazionale Tumori (Milan, Italy), were studied for RET and NTRK1 oncogenic rearrangements. Unfortunately, for 16 of the 92 patients, it was not possible to gather any information concerning pTNMC2 stage and histological variant. Therefore, only 76 cases were considered informative. All the specimens analyzed in the study were reviewed by two pathologists (S. P. and P. C.) to confirm the original diagnosis and to subclassify the tumors according to updated categorization criteria (1–7). Histological and clinical characteristics are summarized in Table 1.

Detection of oncogenic versions of RET and NTRK1 was performed using different experimental procedures, including Southern blot, extra-long PCR, reverse transcriptase-PCR, and transfection assay (16). RET and NTRK1 rearrangements were first detected using specific probes in Southern blot. Following this assay, we determined RET and NTRK1 oncogenic versions by extra-long PCR of genomic DNA and reverse transcriptase-PCR of RNA. All samples were also analyzed by transfection assay onto NIH3T3 cells. RET- and NTRK1-positive samples in Southern blot were always found to be positive with the other three techniques. On the other hand, negative cases were confirmed not to express the two oncogenes with at least three of the assays indicated above.

Statistical Methods. To study the relationship between RET and NTRK1 activation and the clinicohistopathological features considered, both univariate and multivariate analyses were carried out. In the first case, the degree of association between gene activation and each of the other features was assessed by means of Cramer’s V statistic and the associated P. A Cramer’s V equal to zero denotes the lack of association, whereas a unity value means perfect association. The conventional 5% significance level was adopted for testing purposes.

To assess the joint pattern of association between gene activation and other characteristics of either patient or disease, a multivariate analysis was carried out by means of multiple correspondence analysis (19). This technique is useful for exploratory purposes because it allows a graphical representation of the association between a set of categorical variables to be obtained. To make such a representation more easily interpretable, pT stage, pN stage, MC2 stage, and histology were used as “active” variables (variables determining factorial axes), in relation to their homogeneous meaning as tumor severity indicators. RET/NTRK1 activation, age, and sex were, instead, used as “passive” variables, that is, simply represented on the first factorial plane determined by the active variables.

Further explanations that may help to interpret our findings are supplied below, where the results of the multivariate analysis are described.

RESULTS

Among the 76 tumors analyzed, 35 (46%) were positive for RET/PTC or NTRK1 oncogenic rearrangements. Specifically, RET/PTC oncogenes were detected in 34.2% (26 of 76), and NTRK1 oncogenes were detected in 11.8% (9 of 76) of the evaluated patients. Table 2 shows the relationship between the presence/absence of RET/NTRK1 oncogenic activation and the clinicopathological findings, in terms of pTNMC2, in addition to sex and age.

A low degree of association, as measured by Cramer’s V, was observed between RET/NTRK1 positivity and sex (Cramer’s V = 0.066) and histology (Cramer’s V = 0.034). A moderate degree of association was observed in the remaining cases, in which Cramer’s V varied from a minimum of 0.220 (for M stage) to a maximum of 0.348 (for age). In particular, as reported in Table 2, the prevalence of RET/NTRK1 positivity was high in pT4 (65%) versus pT1–T3 (36%) unified cases, in node-positive (53%) and metastatic patients (80%) versus those free from nodal (33%) and distant metastases (46%), and in subjects aged 40 years or less (52%) versus older subjects (17%).

\[
\begin{array}{|c|c|c|}
\hline
\text{Characteristic} & \text{No. of cases} & \% \\
\hline
\text{Sex} & & \\
\text{Male} & 22 & \\
\text{Female} & 54 & \\
\hline
\text{Age at diagnosis (years)} & & \\
\text{Range} & 4–80 & \\
\text{Mean} & 37.5 & \\
\text{Median} & 37 & \\
\hline
\text{Tumor differentiation} & & \\
\text{Differentiated} & 64 & 84.2 \\
\text{Poorly differentiated} & 11 & 14.5 \\
\text{Undifferentiated} & 1 & 1.3 \\
\hline
\text{pT stage} & & \\
\text{pT1} & 13 & 17.1 \\
\text{pT2} & 33 & 43.4 \\
\text{pT3} & 4 & 5.3 \\
\text{pT4} & 26 & 34.2 \\
\hline
\text{pN stage} & & \\
\text{pN0} & 55 & 72.4 \\
\text{pN1} & 6 & 7.9 \\
\text{pNX} & & \\
\hline
\text{MC2 stage} & & \\
\text{M0C2} & 66 & 86.8 \\
\text{M1C2} & 5 & 6.6 \\
\text{MCX} & 5 & 6.6 \\
\hline
\end{array}
\]

\* Comprehensive of microcarcinoma, NOS, and follicular variants.  
\* Comprehensive of tall cell, columnar cell, sclerosing, trabecular, and Sakamoto variants.  
\* Spindle cell (squamous) carcinoma.
Clinical Cancer Research 225

Table 2  Clinicopathological characteristics of 76 tumors belonging to the PCF by RET/NTRK1 oncogenic expression

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Absence</th>
<th>Presence</th>
<th>Cramer’s V</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>28</td>
<td>51.9</td>
<td>26</td>
<td>49.1</td>
</tr>
<tr>
<td>Male</td>
<td>13</td>
<td>59.1</td>
<td>9</td>
<td>40.9</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;20</td>
<td>4</td>
<td>40.0</td>
<td>6</td>
<td>60.0</td>
</tr>
<tr>
<td>20-30</td>
<td>5</td>
<td>33.0</td>
<td>11</td>
<td>68.7</td>
</tr>
<tr>
<td>30-40</td>
<td>14</td>
<td>63.6</td>
<td>8</td>
<td>36.4</td>
</tr>
<tr>
<td>40-50</td>
<td>8</td>
<td>50.0</td>
<td>8</td>
<td>50.0</td>
</tr>
<tr>
<td>&gt;50</td>
<td>10</td>
<td>83.3</td>
<td>2</td>
<td>16.7</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-differentiated</td>
<td>35</td>
<td>54.7</td>
<td>29</td>
<td>45.3</td>
</tr>
<tr>
<td>Poorly + undifferentiated</td>
<td>6</td>
<td>50.0</td>
<td>6</td>
<td>50.0</td>
</tr>
<tr>
<td>pT stage</td>
<td></td>
<td></td>
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<tr>
<td>pT1–pT3</td>
<td>32</td>
<td>64.0</td>
<td>18</td>
<td>36.0</td>
</tr>
<tr>
<td>pT4</td>
<td>9</td>
<td>34.6</td>
<td>17</td>
<td>65.4</td>
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<tr>
<td>pN stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pN0</td>
<td>10</td>
<td>66.7</td>
<td>5</td>
<td>33.3</td>
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<tr>
<td>pN1</td>
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<td>pN2</td>
<td>5</td>
<td>83.3</td>
<td>1</td>
<td>16.7</td>
</tr>
<tr>
<td>MC2 stage</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>MOC2</td>
<td>36</td>
<td>54.5</td>
<td>30</td>
<td>45.5</td>
</tr>
<tr>
<td>M1C2</td>
<td>1</td>
<td>20.0</td>
<td>4</td>
<td>80.0</td>
</tr>
<tr>
<td>M1X</td>
<td>4</td>
<td>80.0</td>
<td>1</td>
<td>20.0</td>
</tr>
</tbody>
</table>

Statistical testing yielded a significant P for pT stage (P = 0.015).

Results from the multivariate analysis are displayed in Fig. 1. In Fig. 1, distinct modalities of the active variables were represented on the first factorial plane by open circles. The distance between two points is intended to be a measure of their similarity. For instance, M1C2 and poorly differentiated and undifferentiated histologies (Undiff.) are relatively close. This means that this type of histology is relatively prominent in metastatic tumors. Unfavorable disease characteristics, such as pT4 stage, presence of nodal (pN), or distant metastases (M1C2), or poorly differentiated and undifferentiated histologies, were placed in the left quadrants. Equally, within the passive variables (filled circles), the parameters displayed in the left quadrants were associated with less favorable characteristics. Importantly, RET/NTRK1 oncogenic activation was represented to the left (Fig. 1A), thus suggesting the existence of an association between this feature and the presence of unfavorable disease characteristics. However, the moderate displacement from the origin of RET/NTRK1 modalities was consistent with a low degree of association, as shown by univariate analyses. Due to the modest number of RET/PTC2- and NTRK1-positive cases, their statistical representation was not suitable to distinguish any biological feature specifically conferred by these two rearrangements. Conversely, both RET/PTC1 and RET/PTC3 rearrangements occurred with higher frequencies (13 and 11 of 26 positive cases, respectively) and could therefore be graphically represented. Interestingly, in this case, RET/PTC3 oncogenes were placed more to the left than were RET/PTC1 alterations, thus suggesting that the former molecular event might be associated with a more aggressive tumor (data not shown). Taken as two homogeneous groups, RET/PTC and NTRK1 oncogenic rearrangements were topologically strictly related (Fig. 1B). Therefore, these types of oncogenic pathways do not imply a different pattern of association with pTNMC2 or histological characteristics.

Finally, as shown in Fig. 1, C and D, young age (≤20 years) and male sex also tend to imply less favorable disease characteristics.

In our analysis, RET/PTC and NTRK1 rearrangements were not associated with the degree of tumor differentiation. In fact, although the number of undifferentiated carcinomas was not sufficient to draw any final conclusion, the combined frequency of RET/PTC and NTRK1 expression in well-differentiated carcinomas (47%) did not differ significantly from that of poorly differentiated and undifferentiated papillary carcinomas (50%) grouped together (Table 3). In addition, all the histological variants (with the exception of tall cell carcinomas) presented similar frequencies of RET/PTC and NTRK1 activation and no particular unbalance in the frequencies of the oncogene subtypes.

**DISCUSSION**

We previously reported that PTC exhibits a significant association between RET/NTRK1 oncogenic activation and younger age of patients at diagnosis. Here, we have expanded the analysis to the entire spectrum of the PCF, and we have shown that, although the significance is borderline (P < 0.056), this association is confirmed by the univariate analysis.

A reevaluation of the previously published data collected from post-Chernobyl pediatric PTCs (14, 15) indicated that 12 of the 18 cases for which pathological staging was available were pT4 and that 9 (75%) of them showed RET/PTC positivity.
Given that post-Chernobyl neoplasias are also characterized by a short latency and aggressive features at onset (i.e., thyroid capsule and adjacent soft tissue invasion, high incidence of nodal involvement and intraglandular tumor dissemination; Refs. 17 and 20–22), the assumption that RET/PTC activation might be associated with a more aggressive tumor presentation must be taken into account. Our data do not contradict this hypothesis. In fact, the univariate analysis showed that, in sporadic tumors of the PCF, the combined RET/NTRK1 oncogenic expression is associated with a more invasive phenotype at presentation (pT4; $P < 0.015$). Examination of the cumulative effect of age, sex, histopathology, pTNM staging, and the combined or separated RET/PTC and NTRK1 positivities indicates that these oncogenic events parallel RET/NTRK1, an unfavorable disease presentation, which, in turn, may correlate with a less favorable disease outcome. Unfortunately, due to the lack of follow-up relative to our patients, we can not interpret the results in a prognostic context. Nevertheless, here we outline a definite relationship between different known molecular events and thyroid carcinogenesis. In fact, although PTCs and follicular thyroid carcinomas originate from the same follicular epithelium, they are characterized by a widely different array of clinical, morphological, and molecular features and are therefore regarded as distinct neoplastic entities. In this context, our findings reinforce the hypothesis that alterations of tyrosine kinase genes are causatively related to papillary thyroid tumorigenesis, assimilating RET and NTRK1 oncogenic expression to the same transformation pathway (8). On the contrary, thyroid follicular neoplasias have been frequently associated with the activation of p21 Ras (23–25).

As for the histology, the frequency of RET/NTRK1 positivity is not influenced by the different subtypes and/or the degree of differentiation of the analyzed tumors. A recent report (26) considering sporadic versus radiation-induced PTCs showed a correlation between the type of RET/PTC oncogenic rearrangement and tumor morphology. In particular, RET/PTC1 was predominantly activated in sporadic tu-
mors of the NOS subtype, whereas RET/PTC3 was frequently expressed in radiation-related tumors that were classified as solid variants of the follicular PTC subtype. In their experience, the authors found the latter tumor variant to be well represented in radiation-induced papillary thyroid tumors but relatively uncommon in sporadic carcinomas. In agreement with these data, in our series of 76 sporadic cases, we observed only 2 specimens of the solid subtype. Interestingly, one presented with a RET/PTC3 rearrangement and the other expressed the TRK oncogene. In our series, the well-differentiated variants of PTC (microcarcinoma, follicular, and NOS), show a percentage of RET/NTRK1 oncogenic expression (from 42 to 50%) that is similar to that of poorly differentiated and undifferentiated carcinomas (50%) considered together. Moreover, we find that RET/PTC1 (13 of 26) and RET/PTC3 (11 of 26) oncogenic events occur with similar frequencies and are casually distributed within the different tumor variants. On the other hand, our data fit well with a previous report (27) showing that “occult” microcarcinomas, considered an early papillary lesion, display RET/PTC oncogenic activation in about 40% (11 of 26) of cases. RET/PTC expression may, therefore, act as an initiating genetic event in the generation of PTC. In fact, different reports have demonstrated that transgenic mice with thyroid-targeted expression of the RET/PTC1 oncogene develop thyroid carcinomas that closely resemble human PTC. However, our results do not support the concept that RET/PTC activation, as an initial genetic alteration, predicts evolution toward a more differentiated phenotype (27). Our findings show that the frequency of RET activation in differentiated PTCs is similar to that observed in less differentiated ones. Therefore, additional unidentified genetics events are required to confer a more malignant behavior to papillary thyroid tumors. In this context, several reports have proven that p53 mutations are associated with the most aggressive variants of thyroid tumors (poorly differentiated and undifferentiated carcinomas) and, thus, that they represent a late genetic event in human thyroid carcinogenesis (28–31).

In summary, this study shows that RET and NTRK1 oncogenic expression seem to activate comparable transformation pathways. In addition, these oncogenic events are not restricted to well-differentiated papillary thyroid tumors but represent a common genetic alteration throughout the spectrum of the PCF (including both poorly differentiated and undifferentiated variants).

Furthermore, our findings show that tumors of the PCF carrying RET/NTRK1 activation are more likely to present with locally advanced disease at onset (pT4), a feature that mostly parallels a more aggressive behavior. This concept is reinforced by the significant association between RET/NTRK1 expression and pT4 stage when only well-differentiated PTCs are considered among the PCF (Cramer’s V = 0.305, P < 0.015; data not shown).

Finally, the association between the presently reported oncogenic events and the younger age of patients seems to confirm a higher sensitivity of the young thyroid gland to the effects of epigenetic factors, as suggested by tumors of children exposed to the Chernobyl nuclear accident (17).

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RET/NTRK1 and Papillary Thyroid Tumors

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