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CLINICAL STUDY

RET protein expression has no prognostic impact on the long-term outcome of papillary thyroid carcinoma

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Abstract

Background: RET proto-oncogene rearrangements (RET/PTC) are causative events in the pathogenesis of a subset of papillary thyroid cancer (PTC). The prevalence of RET/PTC varies in different countries and according to specific clinical features: it is higher after radiation exposure and it is claimed to be higher in young patients. Conflicting results are reported regarding the prognostic role of RET/PTC activation.

Objective: To investigate the prognostic meaning of RET/PTC rearrangement on the long term outcome of PTC.

Methods: We have studied the expression of the RET encoded protein in 127 papillary thyroid carcinomas by immunohistochemistry using a polyclonal antibody against the tyrosine-kinase domain of the RET protein. These cases have been collected during 1970–1985, and have a mean (±S.D.) period of follow-up of 18.6±3.7 years (range 12–27 years). The results have been compared with the patients' outcome.

Results: The tyrosine-kinase domain of RET was expressed in 82 (64.6%) papillary carcinomas. Among them, RET was highly expressed in 65 (51.2%) cases and moderately expressed in 17 (13.4%). RET expression was absent in 45 (35.4%) cases. No correlation was found between RET expression and other parameters such as sex, age at diagnosis, tumor class and histological variant. Follow-up analysis showed no influence of RET expression on patients' outcome. By multivariate analysis, age (>45 years) and tumor class IV, but not sex and RET expression were adverse prognostic indicators of death.

Conclusion: In conclusion, our analysis indicates that RET expression is frequently found in PTC, and has no influence on tumor outcome.

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Introduction

Papillary thyroid carcinoma (PTC) represents the most common malignant endocrine tumor. In its classical form it is a well differentiated neoplasm that metastasizes mainly to the regional lymph nodes and has favorable prognosis. Besides the classical form, there are several morphological variants that have more aggressive clinical features (1, 2).

RET/PTC oncogene activation has been detected in about 50% of cases of human thyroid papillary carcinomas (3). Three main isoforms (RET/PTC-1, -2 and -3) have been described so far. The RET/PTC-1 oncogene derives from the fusion of the tyrosine-kinase domain of the RET proto-oncogene, which encodes a receptor-type tyrosine-kinase, with the 5′-terminal region of another gene, named 'H4' or 'D10S170'. In RET/PTC-2 and RET/PTC-3 the 5′ portion is represented by the regulatory subunit 'RI' of the cAMP dependent protein kinase A and by a previously unknown gene named 'RFG' respectively. RET/PTC-1 and -3, the most frequently involved, derive from a paracentric inversion of chromosome 10 (4, 5) while RET/PTC-2 originates from a translocation between chromosomes 10 and 17 (6). In addition, several variants of RET/PTC-1 and -3 and other unrelated new rearrangements have been described (7–10).

RET/PTC rearrangements are more frequent in young patients (11) and in patients with a previous history of exposure to ionizing radiation, both external (12) and internal (13–16). The activation of RET proto-oncogene has consistently been found to be restricted to papillary thyroid cancer (17), suggesting that RET oncogene expression is an important and specific event in this particular tumor histotype.

A high rate of RET/PTC activation was reported in papillary microcarcinomas (18, 19) suggesting that it is an early event in thyroid tumorigenesis. Sugg *et al.* (20) found a high rate of RET/PTC rearrangements in 39 microcarcinomas from 21 patients and concluded that these genetic lesions are early events but are not a marker of progression to clinically relevant tumors. In contrast, two other reports (21, 22) have shown that RET/PTC activation is associated with the ability of the tumor to metastasize.

Immunohistochemistry has recently been set up to analyze RET/PTC activation on paraffin-embedded tumoral samples. The rationale for using immunohistochemistry is based on the observation that the RET proto-oncogene is normally expressed in neural crest derived tissues but not in thyroid follicular cells; conversely, its rearranged forms are expressed in thyroid neoplastic cells, as a consequence of a change of the promoter. Therefore, RET expression in follicular neoplastic thyroid cells reflects RET activation. A good correlation between positive immunohistochemistry with anti-RET polyclonal antibodies (the same as used in our study) and RET/PTC rearrangements detected by RT-PCR, has been reported in several studies (3, 15, 20).

In the present study, we have analyzed RET expression by immunohistochemistry in a large group of patients with PTC, followed for at least 12 years. The correlation of RET expression with clinical and histological parameters has been studied to determine the prognostic role of RET proto-oncogene activation.

Patients and methods

Patients

The study group included 127 patients with PTC, treated with total thyroidectomy (plus lymphadenectomy in 52) from 1970 to 1985 and followed at regular intervals up to the present time. After surgery, all patients underwent thyroid residue ablation with ^{131}I therapy (mean ± S.D.: 81 ± 22 mCi; range: 30–140 mCi). The subsequent follow-up strategy was based on periodic measurements of serum thyroglobulin (Tg) whilst on and off L-thyroxine suppressive therapy, and diagnostic ^{131}I whole body scan (WBS). In the case of elevated serum Tg and/or abnormal uptake in the WBS, patients were treated with therapeutic doses of ^{131}I up to normalization of serum Tg and disappearance of abnormal areas of uptake in the WBS. This procedure was necessary in 70 patients (55.1%) with regional or distant metastases. The cumulative dose of radioiodine received by these patients was 387 ± 350 mCi (range 60–1585 mCi). Surgical reintervention on the neck was performed in 15 (11.8%) patients. Two patients were treated by surgery for isolated bone metastasis. Five (3.9%) patients were treated with external radiotherapy for persistent tumor in the neck deprived of ^{131}I uptake, 2 (1.6%) patients received chemotherapy and 2 (1.6%) chemotherapy plus external radiotherapy for diffuse metastatic and local disease.

As reported in Table 1, 88 patients were females (69.3%) and 39 were males (30.7%), with a female to male (F:M) ratio of 2.2:1. Mean (± S.D.) age at diagnosis was 38.3 ± 17 years (range: 8–81 years). According to the tumor stage classification proposed by DeGroot *et al.* (23), 42 patients (33.1%) had class I tumors (tumors limited to the thyroid gland), 51 (40.2%) had class II tumors (lymph node metastases), 12 (9.4%) had class III tumors (tumor extending outside the thyroid gland) and 13 (10.2%) had class IV tumors (distant metastases). In 9 (7.1%) patients it was not possible to ascertain the class of tumor.

At the end of follow-up, 116 (91.3%) patients were alive, 95 (74.8%) were free of disease and 21 (16.5%) had persistent disease. Eleven patients (8.7%) had died from thyroid cancer.

Table 1 Epidemiological and clinical data of 127 patients (88 females, 39 males; F:M, 2.2:1; age at diagnosis 38.3 ± 17 years (range 8–81)) with papillary thyroid cancer.

	n	%
Tumor class		
I Intra-thyroidal tumor	42	33.1
II Lymph node metastases	51	40.2
III Extra-thyroidal	12	9.4
IV Distant metastases	13	10.2
Unknown	9	7.1
Outcome		
Remission	95	74.8
Persistence	21	16.5
Death	11	8.7
Post-surgical treatments		
Residue ablation	127	100
Additional ^{131}I	70	55.1
Neck reintervention	15	11.8
Bone surgery	2	1.6
External radiotherapy	5	3.9
Chemotherapy	2	1.6
Chemotherapy + external radiotherapy	2	1.6
Histological variants		
Classical	63	49.6
Follicular	37	29.1
Columnar	3	2.4
Tall cells	8	6.3
Solid	16	12.6

Mean (±S.D.) follow-up was 18.5±3.8 years (range 12–27 years) in alive patients, and 4.1±3.7 years (range 1–12 years) in dead patients.

Thyroid tissue

Immunohistochemical studies were performed on archival material stored in the local Division of Pathology. Histology (reviewed in all cases) was classical variant in 63 (49.6%), follicular variant in 37 (29.1%), solid variant in 16 (12.6%), tall cell variant in 8 (6.3%) and columnar variant in 3 (2.4%) (Table 1). Normal peritumoral tissues were used as controls.

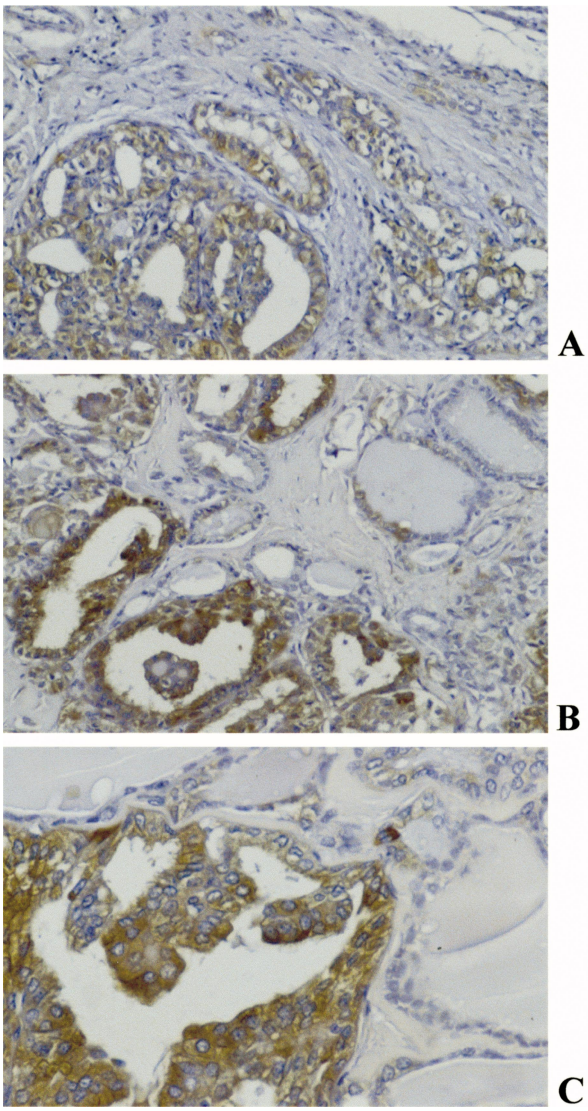

Figure 1 Two representative examples of RET protein expression in papillary thyroid carcinoma (follicular variant), one with weak RET expression (panel A; magnification×100), and one with high RET expression at low (panel B; ×40) and high (panel C; ×400) magnification. Normal thyroid tissue not expressing RET protein is visible in both panels C and B.

Immunohistochemistry

RET expression was evaluated using a polyclonal antibody raised against the tyrosine-kinase (TK) domain of RET (anti-RET^{TK}), as previously characterized (3, 15, 24). After formalin fixation, processing, and paraffin embedding, 3–5-μm-thick sections were prepared. Paraffin sections were dewaxed in xylene and rehydrated through graded alcohol. The sections were then placed in a solution of methanol and 2% hydrogen peroxide for 30 min, washed in distilled H_2O and PBS and incubated overnight at 4 °C in a humidified chamber with primary antibody diluted 1:100 in PBS. After the sections were stained with the avidin-biotin immunoperoxidase complex technique, the immunoreactivity was visualized by incubating the slides in diaminobenzidine. Then the slides were washed, counterstained with hematoxylin, dehydrated with alcohol and xylene and mounted with a permanent mounting medium. Negative controls were performed by omitting the primary antibody. The immunoreactivity was cytoplasmic.

Sections were scored independently by two pathologists (F B and L P) using a method described previously (25): the intensity of staining was scored from 0 to 3 (0=absent; 1=weak; 2=moderate and 3=strong); the proportion of malignant cells positively stained was scored from 0 to 4 (0=no positive cells; 1 =≤ 10% positive cells; 2 = 11–50%; 3 = 51–75% and 4 = 76–100%). The two scores were added to yield the total score (0–7). Immunoreactivity was defined as

Table 2 RET expression according to sex, different histological sub-types and tumor class.

	RET-positive (group A (strong) + group B (weak))		**RET-negative** (group C)	
	n	%	*n*	%
Sex				
Female (*n* = 88)	53	60.2	35	39.8
Male (*n* = 39)	29	74.4	10	25.6
Histotype				
Classical (*n* = 63)	40	63.5	23	36.5
Follicular (*n* = 37)	22	59.4	15	40.6
Columnar (*n* = 3)	2	66.7	1	33.3
Tall Cells (*n* = 8)	6	75.0	2	25.0
Solid (*n* = 16)	12	75.0	4	25.0
Class				
I (*n* = 42)	21	50.0	21	50.0
II (*n* = 51)	37	72.5	14	27.5
III (*n* = 12)	9	75.0	3	25.0
IV (*n* = 13)	10	76.9	3	23.1
Unknown (*n* = 9)	5	55.6	4	44.4
Total (*n* = 127)	82	64.6	45	35.4

P = NS for all values.

Table 3 RET expression according to age at diagnosis.

	RET-positive (group A (strong) + group B (weak))		RET-negative (group C)	
Age	*n*	%	*n*	%
5–29 years ($n = 44$)	29	65.9	15	34.1
30–44 years ($n = 43$)	27	62.8	16	37.2
45–90 years ($n = 40$)	26	65.0	14	35.0

P = NS for all values.

Table 4 RET expression according to outcome.

	RET-positive (group A (strong) + group B (weak))		RET-negative (group c)	
Outcome	*n*	%	*n*	%
Remission ($n = 95$)	60	63.2	35	36.8
Persistence ($n = 21$)	16	76.2	5	23.8
Death ($n = 11$)	6	54.5	5	45.5
Total	82	64.6	45	35.4

P = NS for all values.

Table 5 Risk factors of death from cancer.

Risk factor	*P*-value	Relative risk
Age (years)	0.0729	
31–45 vs 0–30	N.S.	
>45 vs 0–30	0.0028	2.25
RET protein expression		
Group B vs C	N.S.	
Group A vs C	N.S.	
Sex	N.S.	
Tumor class	0.003	
II vs I	0.0549	0.57
III vs I	N.S.	
IV vs I	0.002	2.8

N.S., not significant.

strong when the total score was ≥4 (group A), weak when it was between 1 and 3 (group B) and negative when it was 0 (group C).

Statistical analysis

Survival curves were plotted by the Kaplan-Meier method. Statistical analysis of survival was performed by the Log Rank test and Cox proportional hazard model as specified. Chi square analysis was used for all the other statistics.

Results

RET protein expression by immunohistochemistry

A total of 82 (64.6%) PTC expressed RET protein with different patterns of intensity and numbers of cells. Using the score derived from the two parameters, 65 tumors (51.2%) had strong RET expression (group A), 17 (13.4%) had positive but weak expression (group B), and 45 (35.4%) had no expression (group C). A significant positive correlation ($r = 0.87$; $P < 0.0001$, by simple regression analysis) was found between the degree of cell staining and the proportion of cells expressing the RET protein.

Immunohistochemical detection of RET protein was consistently observed in the cytoplasm of the neoplastic cells (Fig. 1). RET protein expression was absent in the normal peritumoral tissues.

Correlation with clinical data

As shown in Table 2, sex, histological sub-types and tumor class did not influence the expression of RET. Similarly, RET expression was not different in the various age groups: in particular, younger patients were not associated with a higher frequency of RET expression (Table 3). Expression of RET according to the final outcome is reported in Table 4: positive RET

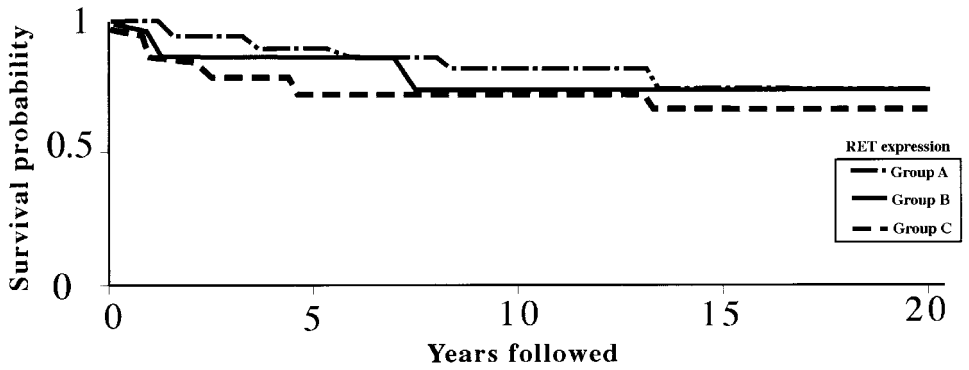

Figure 2 Survival probability (cancer related deaths only), by Kaplan-Meier method, in relation to RET protein expression. The probability of survival is similar for patients of groups A, B or C ($P = 0.79$, by Long Rank test).

expression (group A + group B) was not associated with a specific outcome. This was true even when tumors with weak RET expression (group B) were excluded from the statistical comparison or were grouped together with tumors with no RET expression (group B + group C).

The cumulative survival rate at 20 years was 91.3%. As shown in Fig. 2, the survival probability did not differ according to the pattern of RET expression ($P = 0.799$).

By multivariate analysis (Cox proportional hazard model; Table 5), significant independent predictors of death were: age >45 years (relative risk: 2.25) and tumor class IV (relative risk: 2.8). Sex and RET expression had no influence on survival.

Discussion

The relationship between RET/PTC and clinical–histological features has been studied by several authors with conflicting results. In some studies, RET/PTC rearrangements have been found more frequently in microcarcinomas and in the classical variant of PTC (17–19). No rearrangements were found in poorly differentiated or in undifferentiated tumors by Tallini *et al.* (3). The same authors found no correlation between RET/PTC activation and tumor size, clinical stage of the disease at presentation and presence of metastases (3). RET/PTC has been associated with tumors of patients of younger age, those with the best prognosis, and in addition has been correlated with markers of low proliferative activity, such as MIB-1 (26, 27). Altogether, these studies lead to the tentative conclusion that RET/PTC is predominant in well differentiated papillary thyroid carcinomas which do not progress to less differentiated phenotype.

On the other hand, other authors have found a significant association between RET activation and papillary tumors exhibiting a more advanced and aggressive phenotype at presentation, including the association with distant metastases (21, 22, 28). These authors speculated on the possibility that RET expression might be associated with an adverse final outcome.

Apart from these data, the role of RET activation on tumor outcome has never been addressed in a large series of patients with long term follow-up. Our study has been designed to investigate this aspect, using immunohistochemistry for RET protein on archival material from a retrospective series of papillary thyroid cancer patients followed for a mean time of 18.5 ± 3.8 years.

The anti-RET antibody used in this study reacts against the tyrosine-kinase domain of RET which is present in any form of the rearranged RET protein, as well as in the normal RET protein. Since RET proto-oncogene is not expressed in normal follicular cells (3, 29), we can assume that the detection of the TK domain with our antibody represents evidence that a RET rearrangement has taken place. This statement is validated by previous studies, showing that our antibody correlates well with RET oncogene activation detected by RT-PCR (3, 15, 20), and by the constant absence of RET expression in the normal peritumoral tissues of our patients.

In our study, no difference was found in the survival of RET-positive or RET-negative cases. The significant adverse prognostic variables found in this study were those usually reported in several large series of PTCs: older age and distant metastases at diagnosis. We can speculate that, while RET is an important initiating event, the activation of other oncogenes or the inactivation of tumor suppressor genes are responsible for the progression to more aggressive and treatment-resistant tumors. The behavior of a human RET-positive papillary thyroid cancer resembles that experimentally found in transgenic mice expressing RET/PTC-1, which develop slowly progressive thyroid tumors not causing the premature death of the animals (30). The higher prevalence of RET rearrangement found in young patients by previous studies (11, 19) was not confirmed in our series.

In conclusion, our study shows that RET-positive papillary thyroid carcinomas are not associated with a prognosis different from RET-negative cases. RET expression gives no prognostic information in the clinical management of papillary thyroid carcinoma.

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