European Journal of Endocrinology (2003) **148** 505–513 ISSN 0804-4643

CLINICAL STUDY

Ret/PTC activation does not influence clinical and pathological features of adult papillary thyroid carcinomas

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Abstract

Objective: *RET* proto-oncogene rearrangements (*ret*/PTCs) represent the most common genetic alterations found in papillary thyroid carcinomas (PTCs). Correlation of ret/PTC expression with clinical outcome is controversial. The aim of the present study was to analyze the frequency of *RET* rearrangements in adult PTCs, and to investigate if *ret*/PTCs influence biological behavior and clinical features of the cancers.

Design: *Ret*/PTC rearrangements were looked for in tissue samples of 48 PTCs collected at our institution. Data about clinical and pathological features of the tumors were also reviewed. Three separate association analyses were carried out on the cohort evaluating the effects of, respectively, ret/PTC positivity, preferential RET tyrosine kinase domain (RET-TK) expression, and ret/PTC plus RET-TK positivity, on age, sex, tumor size, staging, number of neoplastic foci, and histological subtype.

Methods: The genetic study was conducted with the RT-PCR–Southern blot technique. Standard Student's *t*-test and Fisher exact test were applied for the association analyses.

Results: The molecular genetic study demonstrated the positivity of ret/PTC1 and ret/PTC3 in 13 of 48 tumors (27.1%), and an exclusive or preferential RET-TK expression in 17 cases (35.4%). None of the three genetico-clinical analyses showed any significant association between ret/PTC expression and the clinical and pathological features of the cancers.

Conclusions: These data indicate that *RET* rearrangements may not play any distinctive role in driving histotype development and cancer progression in these neoplasms. Moreover, they weaken the possibility of using *ret*/PTC as a prognostic marker for papillary thyroid carcinomas.

European Journal of Endocrinology **148** 505–513

Introduction

The *RET* proto-oncogene encodes a membrane tyrosine kinase receptor involved in the transduction of several signals for development, growth, differentiation and migration of neural crest-derived cell lineages (1, 2). *RET* proto-oncogene rearrangements represent the most common genetic alterations found in papillary thyroid carcinomas (PTCs). Moreover, there is evidence to indicate that they are an early genetic event in PTC development (2). These rearrangements derive from the fusion of the *RET* tyrosine kinase domain sequence with the 5′ sequences of heterologous genes, which creates chimeric oncogenes named *ret*/PTCs. They comprise 3 main forms, *ret*/PTC1, 2 and 3, of which *ret*/PTC1 and 3 are definitely more frequent (3–7), and several other rare variants recently described mainly in thyroid cancers from areas contaminated by the Chernobyl nuclear accident, as well as *ret*/PTC4, *ret*/PTC5, *ret*/PTC6, *ret*/PTC7, *ret*/KTN1, *ret*/RFG8, *ret*/PCM-1, and *ret*/ELKS (8).

Ret/PTC1 arises from a paracentric inversion of the long arm of chromosome 10 which creates the fusion oncogene *D10S170(H4)-RET*, owing to the peculiar breakpoints in the regions of *RET* tyrosine kinase domain and of *D10S170 (probe H4)* (9). *Ret*/PTC2 is formed by a recombination between chromosomes 10 and 17 in which gene portions coding for the RET tyrosine kinase domain (RET-TK) and for the promoter of RIα regulatory subunit of cAMP-dependent protein

kinase A form a chimeric gene (10). Finally, *ret*/PTC3, like *ret*/PTC1, derives from a paracentric inversion of the long arm of chromosome 10 which juxtaposes the *RET* tyrosine kinase domain to the 5′ portion of the gene *ELE1* (6). All these fusion proteins are characterized by a cytoplasmic constitutive activation of the tyrosine kinase function (10), which plays a transforming activity as demonstrated in *in vitro* experiments (11) and in transgenic mice models (12–14).

The prevalence of these rearrangements in sporadic PTC from adult subjects varies widely in different studies (2.5–44%) (15–17), due to genetic and/or environmental factors and to methodological differences applied in their search. In pediatric PTCs the prevalence is definitely higher (48–65%) (16–18). But the highest prevalence was reported with pediatric thyroid carcinomas associated with radiation exposure from the Chernobyl nuclear accident (67–87%) (17–19).

One of the most intriguing problems with these rearrangements is their clinical significance. Indeed, correlation between *ret*/PTC rearrangements and biological behavior of the tumors has been controversial. Some report the association of *ret*/PTC with aggressive disease (19–22), while others associate *ret*/PTC with small tumor size and better prognosis (23–25).

In this study, we investigated retrospectively the prevalence of *RET* rearrangements (both as ret/PTC1 and 3 expression and as the preferential expression of RET-TK, marker of all potential *RET* rearrangements) in a sporadic thyroid carcinoma cohort collected at our center between 1995 and 1999, and correlated the molecular genetic results with the clinical and pathological features of the tumors, trying to understand which role, if any, *ret*/PTC plays in papillary thyroid carcinoma progression.

Materials and methods

Patient population

Forty-eight cases of sporadic papillary thyroid carcinoma from Italian patients aged 22–80 years were studied. Twenty-five were consecutive cases treated surgically between July 1997 and June 1999 at the Institutes of Thoracic Surgery and Oncologic Surgery of the University of Perugia, for which fresh frozen tumor tissues were available. The remaining 23 cases were selected from a consecutive series of 25 PTCs collected between October 1995 and July 1997, which were stored in the files of the Institute of Pathology of our institution. In these cases the material available was paraffin-embedded tissue and they were selected on the basis of the availability of RNA of sufficient integrity to allow genetic analysis.

In 45 cases the only tissue sample analyzed was from the primary tumor, in one case it was exclusively from a metastatic lymph node. Moreover, in two cases we analyzed samples both from the primary tumor and from corresponding lymph node metastases.

The medical records relative to the hospitalization for the thyroidectomy of each patient were reviewed to obtain data about possible radiation exposure, age at diagnosis, and sex.

Histology

Histological slides from the thyroid tumors stained by hematoxylin and eosin were reviewed by at least two pathologists (R F and R R) to confirm the diagnoses, define the pathological T and N stage (pT and pN), and subclassify the variants of papillary carcinomas. In detail, we divided the tumors into two main categories, 'high grade' (including the diffuse sclerosing variant, the tall cell variant and poorly or undifferentiated cancers) and 'low grade', as described previously (26). Moreover, in the low grade group we distinguished two main subtypes, solid/follicular and classic papillary.

Genetic analysis

RNA was obtained from fresh frozen tissues as described by Chomczynski and Sacchi (27). In the 23 paraffin-embedded samples RNA was extracted from two unstained 20-μm thick sections containing at least 70% of tumor tissue, as reported previously (28). In order to exclude the possibility of sample cross contamination during the RNA extraction process, and of RNA or cDNA sample contamination by PCR products present in the laboratory environment, for every five tissue samples we prepared a control sample of the extraction reagents which was submitted to reverse transcription and to all the PCR reactions as an additional negative control. Reverse transcription of either 1 μg total RNA or, in the case of RNA extracted from paraffin-embedded samples, half of the recovered nucleic acids, was conducted at 37 °C with an avian myeloblastoosis virus reverse transcriptase (Finnzymes OY, Espoo, Finland), using a random examers mixture as a primer (New England BioLabs, Beverly, MA, USA), in a total volume of 20 μl. The obtained cDNA was first tested to ensure that the integrity of the RNA was sufficient for analysis. For this purpose a 661-bp sequence of the β-actin cDNA was amplified using primers spanning an exon-exon junction of the gene (29) (Table 1). Only those samples demonstrating clearly detectable levels of the transcript on an ethidium bromide-stained agarose gel were further analyzed (25/25 of fresh frozen tissue samples and 23/25 of paraffin-embedded tissue samples).

All the adequate cDNAs were submitted in the first instance to two PCR reactions using sets of primers designed to amplify the regions bracketing chimeric sequences of the *ret*/PTC1 and *ret*/PTC3 rearrangements (18) (Table 1). Secondly, we tested the samples with primers for the boundaries of exon 7/8

Table 1 Primers used for PCR.

	Size (bp)	**Primer sequences** (5′-3′, a-sense, b-antisense)	**Probes for hybridization** (5′-3′)
β-actin	661	a-TGACGGGGTCACCCACACTGTGCCCATCTA b-CTAGAAGCATTGCGGTGGACGATGGAGGG	
Ret/PTC1	165	a-GCTGGAGACCTACAAACTGA b-GTTGCCTTGACCACTTTTC	GGCACTGCAGGAGGAGAACCGCGA
Ret/PTC3	242	a-AAGCAAACCTGCCAGTGG b-CTTTCAGCATCTTCACGG	GGTCGGTGCTGGGTATGTAAGGA
RET-TK	155	a-GGAGCCAGGGTCGGATTCCAGTTA b-CCGCTCAGGAGGAATCCCAGGATA	ACGCAAAGTGATGTATGGTCT
RET-EC	184	a-GGCGGCCCAAGTGTGCCGAACTT b-CCCAGGCCGCCACACTCCTCACA	GGTCGGTGCTGGGTATGTAAGGA
Calcitonin	302	a-GGACTATGTGCAGATGAAGG b-TAGGAAGGATGCAAGAAGGG	

(extracellular domain, RET-EC) and exons 16/17 (RET-TK) of the *RET* gene (20) (Table 1). The exclusive or preferential expression of the TK domain was considered indicative of TK activation typical of *RET* rearrangements (16, 18).

For each PCR, 2 μl of the reverse transcribed mixture were amplified with 50 pmol of each primer, 200 μmol/l deoxynucleotide triphosphates, 10 mmol/l Tris–HCl (pH 8.8 at 25 °C), 1.5 mmol/l $MgCl_2$, 150 mmol/l KCl, 0.1% Triton X-100, and 2 U Dynazyme II DNA polymerase (Finnzymes Oy, Espoo, Finland) in a final volume of 50 μl. For β-actin, after 5 min hot-start at 99 °C, 35 cycles of denaturation (95 °C for 1 min), annealing (60 °C for 1 min), and extension (72 °C for 1 min and 30 s) were conducted on a Hybaid PCR Sprint thermal cycler (Hybaid, Ashford, Kent, UK). For *ret*/PTCs, after 5 min hot-start at 99 °C, four cycles of 'touch-down' amplification were performed (progressively lowering the annealing temperature from 61 °C to 57 °C), followed by 40 cycles of regular amplification (95 °C for 1 min, 57 °C for 1 min, and 72 °C for 1 min). Finally, for RET-TK and RET-EC, 5 min of hot-start at 99 °C were followed by 14 cycles of 'touch-down' amplification (64 °C–57 °C) and 27 cycles of regular amplification (95 °C for 1 min, 57 °C for 1 min, and 72 °C for 1 min). Positive controls for amplifications included cDNA samples from two PTCs, one positive for *ret*/PTC1 and one for *ret*/PTC3 (the latter kindly provided by Dr R Elisei, University of Pisa), and from a medullary thyroid carcinoma with constitutive expression of wild-type RET (for amplification of RET-TK and RET-EC).

Ten microliters of the PCR products of ret/PTC1, ret/PTC3, RET-TK and RET-EC were electrophoresed in a 1.8% agarose gel and blotted to a nylon membrane (Schleicher and Schuell, Legnano, Italy). Each filter was then hybridized overnight at 50 °C with the indicated probe end-labeled with ^{32}P (18) (Table 1), washed, and analyzed using a phosphoimager system (Instant Imager, Camberra-Packard, Meriden, CT, USA).

In cases #2, #9, #11, #12, #17, and #19, which tested positive for both RET-TK and RET-EC at comparable levels, we performed an additional PCR reaction using primers for calcitonin (CT) (Table 1) to test for CT expression as a marker of C-cell contamination of the tumor samples. The reaction was conducted applying the same program used for β-actin (annealing temperature 60 °C). As a positive control the cDNA from a medullary thyroid carcinoma was used, and the specificity of the bands obtained was verified by sequence analysis.

Analysis of ret/PTC PCR sensitivities

In order to compare the sensitivities of the PCR reactions used to detect ret/PTC1, ret/PTC3 and RET-TK, in the first instance we performed the reactions using as templates serial dilutions (from 1 pg to 10^{-6} pg) of plasmids containing the full-length cDNAs of ret/PTC1 and ret/PTC3. Moreover, in order to quantify the minimal number of *ret*/PTC-positive tumor cells necessary to obtain a ret/PTC amplicon, we performed PCR reactions for ret/PTC1, followed by Southern blotting, on the cDNA obtained from cell mixtures of serially diluted TPC-1 cells (expressing ret/PTC1) with NIH-3T3 cells (TPC-1/NIH-3T3: $10^7/0$, $10^6/9\times10^6$, $10^5/9.9\times10^6$, $10^4/9.99\times10^6$, $10^3/9.999\times10^6$, $10^2/9.9999\times10^6$, $10/9.99999\times10^6$, $0/10^7$).

TPC-1 and NIH-3T3 cells were grown in Dulbecco's modified eagle medium (GIBCO, Paisley, Scotland, UK) with the addition of 5% fetal bovine serum (GIBCO) and penicillin/streptomycin (GIBCO). RNA extraction, cDNA synthesis, PCR and Southern blot were performed as described above (see Genetic analysis).

Statistical analysis

Results are presented as means±S.D. or as a percentage with 95% confidence intervals (CI) as appropriate, calculated using the Poisson distribution. Continuous variables were compared by two-tailed unpaired Student's *t*-test. The Fisher exact test was used to determine two-tailed *P* values in 2×2 contingency tables. *P* values <0.05 were considered significant.

Results

Clinical and pathological characteristics

Forty-eight thyroid carcinomas from 32 women and 16 men were examined (Table 2). The average age at surgery was 45.6 years (range 22–80 years), while the average tumor size was 2 cm (range 0.5–4 cm). Nine patients presented with a microcarcinoma (diameter $\leq$ 1 cm) and 20 had multifocal disease. No patient had an apparent history of neck irradiation. At the time of diagnosis, 20 cases presented with a pathological T4 stage and 17 with a pathological N1 stage. Morphological diagnosis revealed six high grade papillary carcinomas (including one tall cell variant, one diffuse sclerosing variant, two poorly differentiated cancers and two cancers with anaplastic dedifferentiation) and 42 low grade cancers. The low grade tumors included 25 solid/follicular cancers, 16 classic papillary cancers, and one Hürthle cell PTC. All the patients underwent total thyroidectomy and lymph node dissection of the enlarged lymph nodes singled out at surgery.

Table 2 Summary of clinical data. Results are means (range) or number (percentage).

Clinico-pathological features	Number
Sex (F:M)	32:16
Mean age (years)	45.6 (22–80)
Mean tumor size (cm)	2 (0.5–4)
Diameter $\leq$1 cm	9 (19.5%)
pT4	20 (42.5%)
pN1	17 (35.4%)
Multifocality	20 (42.5%)
High grade	6 (12.5%)
Low grade	
Solid/follicular	25 (52%)
Classic papillary	16 (33.3%)
Others	1 (2.1%)

Molecular genetic analysis

RT-PCR followed by Southern blotting demonstrated the presence of ret/PTC1 and ret/PTC3 in 13 of 48 tumors (27.1%, 95% CI 14.4–46.2). In detail, five cancers tested positive for ret/PTC1, five for ret/PTC3, and three simultaneously for ret/PTC1 and ret/PTC3 (Fig. 1).

The search of RET-TK and RET-EC expression showed 17 cases with an exclusive or preferential RET-TK expression (35.4%, 95% CI 20.6–56.7), six cases with both RET-TK and RET-EC expression, and 25 cases negative for both RET-TK and RET-EC expression (Fig. 1). Ten (76.9%) of the ret/PTC-positive tumors presented, as expected, an exclusive or preferential RET-TK expression. However, three of them did not (Fig. 1). In detail, cases #5 and #16 did not show any RET-TK and RET-EC amplification, while case #19 tested positive for both RET-TK and RET-EC at comparable levels. The remaining seven RET-TK-positive–ret/PTC1 and 3-negative tumors might represent cases characterized by the expression of minor ret/PTCs. The contemporary RET-TK and RET-EC amplification could be related to C-cell contamination of the PTC tissues only in sample #2, which also showed expression of calcitonin (Fig. 2). On the other hand, samples #9, #11, #12, #17, and #19 did not show any amplification for calcitonin (Fig. 2). Moreover, in sample #9 the same RET-TK and RET-EC pattern was observed in a corresponding lymph node metastasis, where C-cell growth is highly unlikely (Fig. 1).

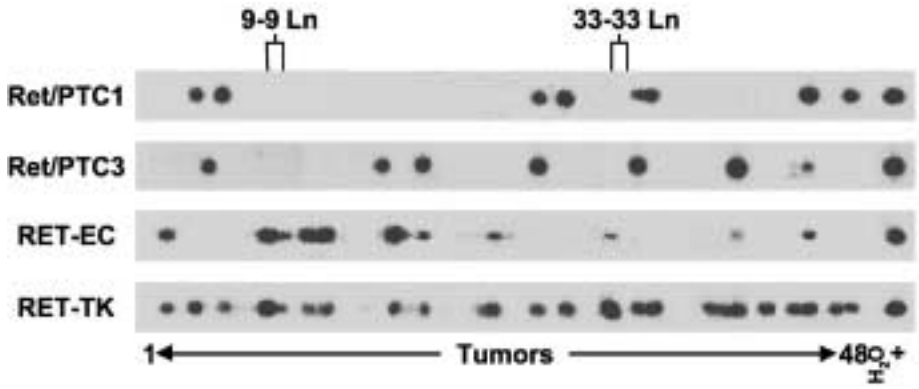

Figure 1 Results of the molecular genetic analysis. RT-PCR followed by Southern blotting demonstrated the expression of ret/PTC1 and ret/PTC3 in 13 out of 48 tumors: five cancers tested positive for ret/PTC1, five for ret/PTC3, and three simultaneously for ret/PTC1 and ret/PTC3. Sixteen cases showed an exclusive or preferential RET-TK expression, six cases both RET-TK and RET-EC expression, and 26 cases neither RET-TK nor RET-EC expression. For tumors #9 and #33, two tissue samples were available, one from the primary lesion and one from a lymph node metastasis (Ln) (see text). H_2O, PCR negative control; +, PCR positive control.

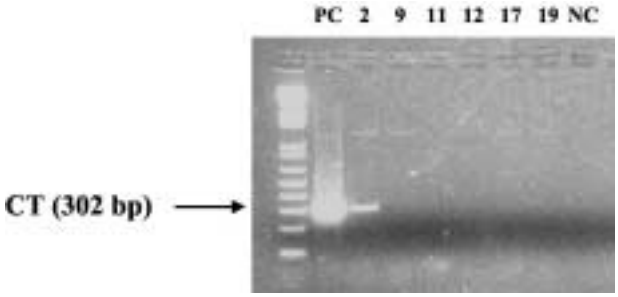

Figure 2 Analysis of calcitonin expression in samples positive for RET-TK and RET-EC at comparable levels. RT-PCR for calcitonin (CT) showed amplification only in sample #2, while samples #9, #11, #12, #17, and #19 were negative for the C-cell marker. PC, positive control, represented by the cDNA derived from a medullary thyroid carcinoma; NC, negative control.

Genetico-clinical analysis

In the first instance three separate genetico-clinical analyses were carried out on the cohort depending on the positivity of ret/PTC1 and 3, or on the preferential expression of RET-TK, or on the positivity of ret/PTC1 and 3 and/or RET-TK. None of these three analyses showed any significant association between ret/PTC expression (evaluated either as ret/PTC1 and ret/PTC3 positivity, or as preferential RET-TK expression) and the different clinical and pathological parameters considered (age, sex, tumor size, pT, pN, number of tumor foci, histological subtype as well as high grade cancers, solid-follicular variants of PTC, classic papillary variants of PTC) (Table 3, panels A and B and C).

Moreover, we looked for the presence of an association between distinct ret/PTC rearrangements (ret/PTC1, ret/PTC3, simultaneous ret/PTC1 and 3 expression, potential new alternative ret/PTCs (RET-TK-positive–ret/PTC-negative cases)) and the tumor features. This analysis also did not show any peculiar genetico-clinical association, with the exception of the finding of 3 multifocal tumors out of 3 in the simultaneously ret/PTC1 and 3-positive PTCs (Table 3, panel D).

Analysis of ret/PTC PCR sensitivities

The analysis of ret/PTC and RET-TK PCR efficiencies showed at least a 1000 times higher sensitivity of ret/PTC1 PCR and a 10 to 100 times higher sensitivity of ret/PTC3 PCR, compared with the PCR for RET-TK (Fig. 3A and B). Moreover, for ret/PTC1 we calculated that the minimum number of positive tumor cells necessary to detect the rearrangement was equivalent to one positive cell in 10^6 negative cells (Fig. 3C).

Discussion

The activation of several oncogenes and the loss of function of some tumor suppressor genes have been shown in thyroid tumors of follicular origin. However, the importance of each of these genes in the carcinogenic process has not been completely elucidated. *Ret*/PTC are very interesting oncogenes both for their relatively high expression frequency in PTCs (16, 17) and for their well known property of being precocious inductors of thyroid carcinogenesis (2, 11–14, 23). Unfortunately, little is known about their role in papillary thyroid cancer progression, and in any case about their role as potential new molecular prognostic markers of the disease.

The aim of our work was to study the prevalence of *ret*/PTC in a cohort of adult PTCs collected in our region and to search for correlations between the molecular genetic results and several clinical and pathological features of the cancers.

In our series, *ret*/PTC1 and 3 rearrangements were found in 13 of 48 (27.1%) adult PTCs, a prevalence slightly lower than that reported in other Italian studies of comparable size (17, 21, 30). This observation should be related to the genetic diversity of the studied population or to a difference in thyroid carcinoma etiological factors present in our region, where most of the patients are from, and not to a lack of sensitivity of the applied technique, as demonstrated by the finding that for RET/PTC1 the RT-PCR–Southern blot method allowed the detection of up to 1 positive cell diluted in 10^6 negative cells and that the PCR for ret/PTC3 allowed the detection of minimal amounts, as low as 10^{-4} pg, of a positive control plasmid.

The use of primers only for the chimeric sequences of ret/PTC1 and 3 did not allow us to evaluate the specific expression of minor ret/PTCs, such as ret/PTC2. For this reason we submitted the cDNA samples to another two PCR reactions with primers designed to amplify regions in the RET-EC and RET-TK domains, considering the exclusive or preferential expression of RET-TK to be a general indicator of RET activation through a chromosomal rearrangement. Seventeen cases showed an exclusive or at least a preferential expression of RET-TK (35.4%), six cases tested positive for both RET-TK and RET-EC, and 25 cases did not show any amplification. Ten of the 17 RET-TK-positive tumors were also positive for ret/PTC1, or for ret/PTC3, or for both. The remaining seven RET-TK-positive–ret/PTC1 and 3-negative tumors might represent PTCs characterized by the expression of less frequent ret/PTCs, as well as ret/PTC2 or other more recently identified ret/PTCs (8). The simultaneous positivity for RET-TK and RET-EC could be related to C-cell contamination in only one of the PTC samples. Indeed, in five cases the absence of amplification for calcitonin excluded the presence of C-cells. Moreover, we had one cDNA sample from a lymph node metastasis which showed RET-TK and RET-EC expression superimposable on that observed in the corresponding primary tumor. These data suggest the possibility of wild-type RET proto-oncogene expression in a subgroup of

Table 3 Results of genetico-clinical analyses. (A) Correlation of ret/PTC1 and 3 status with different clinical and pathological parameters of the tumors (ret/PTC+: positive cases; ret/PTC−: negative cases). (B) Effects of the preferential expression of RET-TK on the distribution of tumor features (TK+: tumors with preferential expression of RET-TK; TK−: tumors characterized by the simultaneous positivity or negativity of RET-TK and RET-EC). (C) Correlation of the expression of ret/PTC and/or RET-TK with tumor clinical features (ret/PTC+ & TK+: tumors positive for ret/PTC1 and 3 and/or RET-TK; ret/PTC− & TK−: tumors simultaneously negative for ret/PTC and RET-TK). (D) Correlation of distinct ret/PTC rearrangements with the tumor features (see text). Results are means±S.D. or number with percentage in parentheses.

	A			B			C			D			
Parameter	Ret/PTC+	Ret/PTC−	*P*	TK+	TK−	*P*	ret/PTC+ & TK+	ret/PTC− & TK−	*P*	Ret/PTC1	Ret/PTC3	Ret/PTC1&3	TK+/Ret/PTC−
Sex (F:M)	8:5	24:11	ns	13:4	19:12	ns	13:7	21:9	ns	4:1	1:4	3:0	6:1
Age (years)	49.5±14.6	44.3±16	ns	46.8±14.9	45.6±16.4	ns	48±15.4	44±15.9	ns	49.8±17.4	54.6±15.3	40.3±4.1	44.8±17.8
Tumor size (cm)	2.3±1	1.9±1	ns	1.9±1	2±1	ns	2±1	2±1	ns	2.1±0.9	2.4±1.1	2.6±2	1.4±0.6
Diameter ≤ 1 cm	1 (8.3%)	8 (23.5%)	ns	2 (11.7%)	7 (22.5%)	ns	2 (10.5%)	7 (25%)	ns	1 (20%)	0	0	2 (28.6%)
pT4	6 (46.2%)	14 (41.2%)	ns	8 (47%)	12 (38.7%)	ns	10 (52.6%)	12 (42.8%)	ns	2 (40%)	3 (60%)	1 (33.3%)	3 (42.8%)
pN1	4 (30.8%)	13 (37.1%)	ns	8 (47%)	9 (29%)	ns	9 (47.3%)	8 (28.5%)	ns	0	2 (40%)	2 (66.6%)	4 (57.1%)
Multifocality	6 (46.2%)	14 (41.2%)	ns	8 (47%)	12 (38.7%)	ns	8 (42.1%)	12 (42.8%)	ns	1 (20%)	2 (40%)	3 (100%)	2 (28.6%)
High grade	2 (15.4%)	4 (11.4%)	ns	2 (11.7%)	4 (12.9%)	ns	2 (10%)	4 (14.2%)	ns	0	1 (20%)	1 (33.3%)	0
Solid/follicular	7 (53.8%)	18 (51.4%)	ns	9 (52.9%)	16 (51.6%)	ns	8 (40%)	7 (25%)	ns	3 (60%)	2 (40%)	2 (66.6%)	2 (28.6%)
Classic papillary	4 (30.8%)	11 (31.4%)	ns	5 (29.4%)	10 (32.2%)	ns	10 (50%)	17 (60.7%)	ns	2 (40%)	2 (40%)	0	5 (71.4%)

ns, not significant.

thyroid cancers of follicular origin, as already proposed by other groups (31, 32).

There is great interest in finding genetic markers that predict the behavior of thyroid tumors, as histological and clinical features may fail to do so. The value of ret/PTCs as prognostic factors is still controversial. Some authors report the association of *RET* rearrangements with aggressive disease (19–22), while others associate them with small tumor size and better prognosis (23–25). Moreover, three recent studies could not show any significant difference between ret/PTC-positive and ret/PTC-negative cancers, either in terms of clinical features or in terms of histotype distribution (17, 31, 33). In this study, we investigated the presence of associations between the expression of RET rearrangements and several clinical and pathological features of the tumors known to play a role in the prognostic stratification of thyroid cancers. We were unable to find any significant association either with ret/PTC1 and 3 expression or with RET-TK preferential expression. These data do not exclude the possibility that RET activation may play a role as an independent prognostic factor, which could be evaluated only after the follow-up of a sufficient number of patients for a sufficient time to have a significant number of tumor relapses and cancer-related deaths. However, they seem to indicate, at least, that ret/PTCs may not specifically influence local aggressiveness and histotype development in PTCs occurring spontaneously in an adult population.

Three of our 13 ret/PTC-positive cases did not show any preferential RET-TK expression. These tumors may represent a group of cancers characterized by a lower ret/PTC expression, confined only to some cells, so that the more sensitive search for ret/PTC proved positive, while the search for RET-TK proved negative. A large variability in the level of ret/PTC expression in PTCs has already been described, and recently higher ret/PTC expression was found in tumors of patients with low risk clinical parameters and lymphatic involvement (34, 35). In our hands this was not the case, as a sub-analysis performed moving the ret/PTC-positive–TK-negative cases to the ret/PTC-negative group (data not shown) did not give us any significant association between ret/PTC expression and the clinical and pathological features of the tumors.

Finally, the search for an association with specific *RET* rearrangements (*ret*/PTC1, *ret*/PTC3, *ret*/PTC1 and 3, and potential new alternative *ret*/PTCs) showed only a potential association between multifocality of the tumor and the simultaneous expression of ret/PTC1 and 3 (100% of ret/PTC1 and 3-positive cases were multifocal against a prevalence of 20–40% multifocal lesions in the other groups). Independently of the lack of statistical significance for the low number of cases, this last observation was interesting, because there is still debate about the origin of multifocal tumor lesions. A recent paper

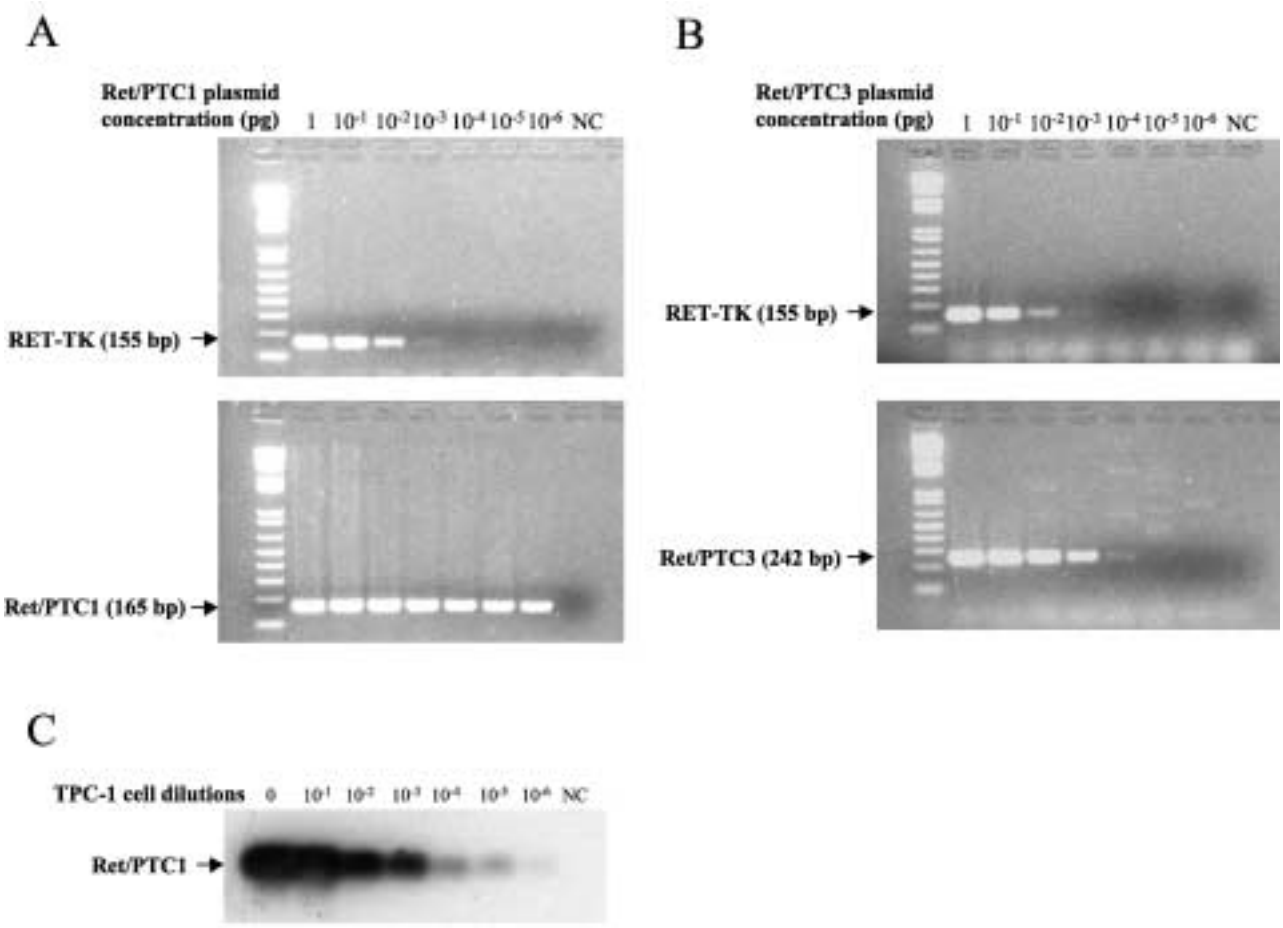

Figure 3 Analysis of ret/PTC and RET-TK PCR sensitivities. (A) Comparison of the sensitivities of the PCRs for RET-TK and ret/PTC1, using as templates serial dilutions of a plasmid containing the full-length cDNA of the oncogene, showed that the PCR for ret/PTC1 was at least 1000 times more sensitive than the PCR for RET-TK. NC, negative control. (B) Comparison of the sensitivities of the PCRs for RET-TK and ret/PTC3, using as templates serial dilutions of a plasmid containing the full-length cDNA of the oncogene, showed that the PCR for ret/PTC3 was 10 to 100 times more sensitive than the PCR for RET-TK. NC, negative control. (C) Quantification of ret/PTC1 PCR absolute sensitivity showed that the minimum number of ret/PTC1-expressing TPC-1 cells necessary to detect the rearrangement was equivalent to one positive cell in 10^6 negative cells (NIH-3T3 cells). Zero represents the result obtained with non diluted TPC-1 cells, while the values 10^{-1}–10^{-6} refer to the dilution of TPC-1 cells in NIH-3T3 cells. NC, negative control.

pointed out that in multifocal disease individual tumors, characterized by diversity in ret/PTC profiles, arise independently (36). Our data, showing the highest prevalence of multifocal thyroid carcinomas in the group of tumors expressing simultaneously ret/PTC1 and 3, seem to confirm the possibility of a multiclonality of multifocal disease.

In summary, we were unable to find any significant association between the expression of *RET* rearrangements, evaluated either as ret/PTC1 and 3 or preferential RET-TK expression, and several clinical pathological features of PTCs. Moreover, we were unable to find any potential association between the tumor features considered and the expression of specific RET rearrangements. Our data may indicate that *RET* rearrangements, although essential for the development of a subgroup of papillary thyroid carcinomas (11–14), do not play any significant distinctive role in driving the histotype development and the progression, in terms of local invasive capacity, of these neoplasms. We hypothesize that other genetic events may be needed in addition to ret/PTC activation for the expression of a complete malignant phenotype and for cancer progression. Alternatively, we propose that ret/PTCs may have the same non specific weight in PTC development and progression as other alternative genetic events involved in follicular cell carcinogenesis. Both these hypotheses weaken the possibility of using *ret*/PTC as a valuable molecular genetic prognostic marker for papillary thyroid

carcinomas occurring spontaneously in the adult population.

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Received 29 October 2002
Accepted 5 February 2003