Break–apart interphase fluorescence *in situ* hybridization assay in papillary thyroid carcinoma: on the road to optimizing the cut-off level for *RET/PTC* rearrangements

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Abstract

Objective: Chromosomal rearrangements of the *RET* proto-oncogene is one of the most common molecular events in papillary thyroid carcinoma (PTC). However, their pathogenic role and clinical significance are still debated. This study aimed to investigate the prevalence of RET/PTC rearrangement in a cohort of *BRAF* WT PTCs by fluorescence *in situ* hybridization (FISH) and to search a reliable cut-off level in order to distinguish clonal or non-clonal RET changes.

Design: Forty *BRAF* WT PTCs were analyzed by FISH for RET rearrangements. As controls, six *BRAF*V600E mutated PTCs, 13 follicular adenomas (FA), and ten normal thyroid parenchyma were also analyzed.

Methods: We performed FISH analysis on formalin-fixed, paraffin-embedded tissue using a commercially available RET break–apart probe. A cut-off level equivalent to 10.2% of aberrant cells was accepted as significant. To validate FISH results, we analyzed the study cohort by qRT-PCR.

Results: Split RET signals above the cut-off level were observed in 25% (10/40) of PTCs, harboring a percentage of positive cells ranging from 12 to 50%, and in one spontaneous FA (1/13, 7.7%). Overall, the data obtained by FISH matched well with qRT-PCR results. Challenging findings were observed in five cases showing a frequency of rearrangement very close to the cut-off.

Conclusions: FISH approach represents a powerful tool to estimate the ratio between broken and non-broken RET tumor cells. Establishing a precise FISH cut-off may be useful in the interpretation of the presence of RET rearrangement, primarily when this strategy is used for cytological evaluation or for targeted therapy.

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Introduction

Papillary thyroid carcinoma (PTC) is the most prevalent form of thyroid cancers, accounting for 80% of all cases. It is characterized by genetic alterations leading to the activation of the MAPK signaling pathway. Together with *BRAF* point mutations, *RET* gene rearrangements represent the two most common molecular events in PTC (1, 2, 3).

The rearranged during transfection (*RET*) proto-oncogene maps to the long arm of chromosome 10 at band q11.2 and encodes for a transmembrane tyrosine-kinase receptor involved in the control of cell differentiation, cell proliferation, and cell survival (4, 5). Oncogenic activation of the *RET* gene via chromosomal

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rearrangement is generally related to radiation exposure and young age (40–70%), but may be found in non-radiated thyroid tumors and in adults (20–40%) (6, 7).

Moreover, a recent study has revealed that 18% of poorly differentiated thyroid carcinomas (PDTC) and 9% of radioactive iodine (RAI) refractory-FDG-PET-positive PDTC harbored *RET/PTC* rearrangements (8).

These rearrangements (balanced inversions or translocations) derive from the fusion of the 3′ portion of the *RET* gene to the 5′ portion of several heterologous genes and create fusion proteins with transforming activity, as demonstrated in *in vitro* experiments and in transgenic mice models (9, 10, 11, 12).

To date, at least 13 different forms of *RET* rearrangement have been documented (13), with *RET/PTC1* (consisting of the fusion of *RET* with the *H4* gene) and *RET/PTC3* (consisting of the fusion of *RET* with the *RFG/ELE1* gene) being the most common (2, 14).

A wide range of prevalence of *RET/PTC* rearrangements in human PTC has been reported, ranging from 3% in Saudi Arabia, 29–35% in Italy, 40% in Canada, to 85% in Australia (15, 16, 17, 18), which can be attributed to ethnical and geographic variability as well as to different sensitivities of detection methods, tumor heterogeneity, age, and radiation exposure (6, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31). Indeed, non-clonal *RET/PTC* rearrangements have been found not only in PTC but also in 10–45% of follicular thyroid adenomas, oncocytic thyroid tumors, and Hashimoto's thyroiditis (30, 32, 33, 34, 35, 36, 37, 38, 39).

The specificity of this rearrangement, as a marker of PTC, has been challenged, and its clinical significance is still under debate. Thus, finding a reliable and biologically relevant strategy for *RET/PTC* detection may have important clinical and diagnostic implications as the detection of *RET/PTC* has been offered as a diagnostic tool for PTC in the surgical and preoperative cytological material (40, 41, 42, 43, 44, 45). Moreover, the emergence of drugs that selectively inhibit RET kinase activity highlights the need of a better understanding of *RET/PTC* distribution within the tumor volume and of standardization of the detection methods for this rearrangement (46, 47, 48, 49). Interphase fluorescence *in situ* hybridization (FISH) represents the gold standard method for detecting gene rearrangements at the single-cell level and is the most sensitive mean for identifying and quantifying intratumoral genetic heterogeneity (50, 51, 52).

The aim of this study was to test a new commercially available *RET* break–apart probe on formalin-fixed, paraffin-embedded (FFPE) samples, to investigate the prevalence of *RET/PTC* in a cohort of *BRAF* WT PTCs, to search for a reliable cut-off level in an attempt to distinguish the clonal or non-clonal event of the *RET* rearrangements, and to explore whether *RET/PTC* may be a relevant pathogenic factor.

Materials and methods

Samples collection

Forty cases of *BRAF* WT PTC (31 sporadic; two familial, one familial adenomatous polyposis-associated PTC and six with history of exposure to external beam radiotherapy) were analyzed during the study.

The cases were selected from a consecutive series of 250 PTCs collected from 2003 to 2013 from the files of the Pathology Unit, University of Verona. Previously, all samples had been tested for *BRAF*V600E mutation status (Fig. 1). The histology of all tumor samples was confirmed independently by two pathologists (C C and M B) and classified according to the World Health Organization guidelines (53). As a control group, six *BRAF*V600E mutated PTCs and 13 follicular adenomas (FA) (12 sporadic and one with a history of exposure to external beam radiotherapy) were also tested for *RET* rearrangements (Fig. 1). *BRAF* WT tumor tissue samples were obtained from 37 patients; in three patients with multifocal disease we examined two neoplastic foci (Table 1, cases 2a and 2b, 17a and 17b, 20a and 20b). Moreover, one case of *BRAF*V600E mutated PTCs (Table 1, case 18b) belonged

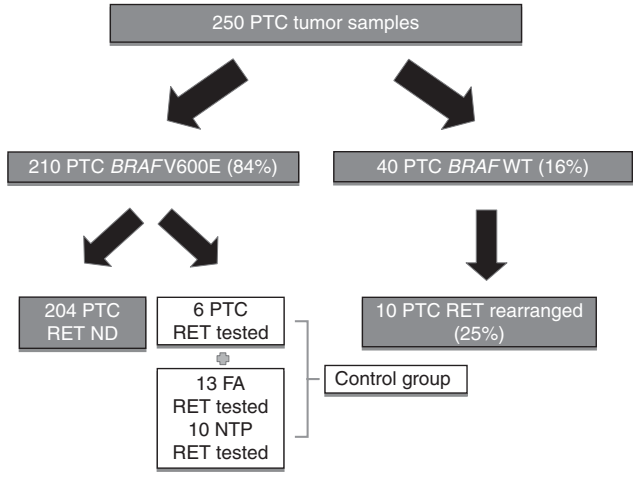

Figure 1
Schematic representation of the study design. ND, not determined.

Table 1 Clinico-pathological and molecular findings in PTC.

Samples	Age (years)	Gender	Radiation exposure	Diameter (mm)	Histological variant	Thyroiditis	Stage	Split *RET* signals (%)	*RET/PTC* rearrangement	*BRAF*-V600E status	Follow-up
1	49	F	No	4	Oncocytic	No	I	2	Negative	WT	NED
2a	15	F	No	12	Classical	Yes	I	2	Negative	WT	BPD
2b	15	F	No	5	Classical	Yes	I	0	Negative	WT	BPD
3	26	F	No	16	Follicular	Yes	I	6	Negative	WT	NED
4	44	F	No	15	Follicular	No	I	4	Negative	WT	NED
5	36	F	No	24	Follicular	No	I	4	Negative	WT	NED
6	27	F	No	10	Follicular	Yes	I	6	RET/PTC1	WT	NED
7	19	M	No	40	Follicular	No	I	3	Negative	WT	NED
8	29	F	No	40	Tall Cell	Yes	I	4	Negative	WT	BPD
9	35	F	No	20	Cribriform morular	Yes	I	6	Negative	WT	NED
10	20	F	No	35	Follicular	No	I	2	Negative	WT	NED
11	34	F	No	8	Classical	Yes	I	2	Negative	WT	NED
12	18	F	No	8	Classical	No	I	9	RET/PTC3	WT	NED
13	37	F	No	10	Follicular	Yes	I	4	Negative	WT	NED
14	36	F	No	38	Follicular	No	I	2	Negative	WT	NED
15	24	M	No	45	Follicular	No	I	10	Negative	WT	BPD
16	29	F	No	20	Follicular	Yes	I	10	RET/PTC1	WT	NED
17a	55	F	No	9	Follicular	Yes	II	6	Negative	WT	NED
17b	55	F	No	22	Follicular	Yes	II	8	Negative	WT	NED
18a	43	F	No	10	Cribriform morular	Yes	III	4	Negative	WT	NED
19	65	M	No	45	Follicular	No	III	10	Negative	WT	NED
20a	53	F	No	14	Follicular	Yes	III	8	Negative	WT	BPD
21	69	F	No	48	Solid	No	III	0	Negative	WT	PD
22	57	M	No	55	Solid	No	IVa	4	Negative	WT	BPD
23	75	F	No	60	Classical with minor poorly differentiated component	No	IVc	5	Negative	WT	Dead
24	36	M	No	35	Follicular	No	I	48	RET/PTC1	WT	SpeD
25	11	F	No	45	Solid	No	I	50	RET/PTC3	WT	SpeD
26	37	F	No	11	Solid	Yes	I	13	RET/PTC1	WT	BPD
27	27	F	No	11	Classical	Yes	I	28	RET/PTC1	WT	NED
28	39	F	No	25	Classical	No	I	20	RET/PTC1	WT	SpeD
29	34	F	No	17	Diffuse sclerosing	Yes	I	26	RET/PTC1	WT	NED
30	36	F	No	50	Follicular	Yes	I	50	RET/PTC1	WT	NED
31	11	F	No	20	Diffuse sclerosing	Yes	I	18	RET/PTC1	WT	Lost at follow up
20b	53	F	No	20	Follicular	Yes	III	12	RET/PTC1	WT	BPD
32	26	M	Yes	22	Classical	No	I	30	RET/PTC1	WT	SpeD
33	38	M	Yes	8	Follicular	No	I	0	Negative	WT	NED
34	8	F	Yes	6	Classical	No	I	4	Negative	WT	BPD
35	29	F	Yes	9	Classical	No	I	1	Negative	WT	NED
36	36	M	Yes	3	Classical	No	I	0	Negative	WT	Dead of other disease
37	49	M	Yes	7	Classical	No	I	2	Negative	WT	NED
1	40	F	No	16	Classical	No	I	4	Negative	V600E	NED
2	30	M	No	20	Classical	Yes	I	2	Negative	V600E	NED
3	32	F	No	6	Classical	No	I	8	Negative	V600E	BPD
4	29	F	No	7	Classical	No	I	6	Negative	V600E	NED
5	53	F	No	7	Classical	No	I	6	Negative	V600E	NED
18b	43	F	No	8	Follicular	Yes	III	5	Negative	V600E	NED

to a patient included in the *BRAF*V600E WT group (Table 1, case 18a). Concerning the cases exposed to external irradiation (six PTC and one FA), the patients received radiation therapy for primary cancer (one thymoma, one brainstem glioma, three leukemias, one cerebellar astrocytoma, one rhabdomyosarcoma of the neck), during childhood (four patients) or as adults (three patients). The radiation dose was available only for one

patient and amounted to 18 Gy (Table 1, case 35). Regarding the PTC subset, the tumor latency was as follows: 7, 3, 6, 25, 16, and 45 years respectively (Table 1, cases 32–37); for the FA, the latency was 25 years (Table 2, case 1).

The medical records of each patient (42 with PTC: 37 WT, and five *BRAF*-mutated and 13 with FA) were reviewed to obtain clinical and demographic data. Informed consent was obtained from all patients, as per the recommendations of our Ethics Committee.

Fluorescence *in situ* hybridization

To evaluate *RET/PTC* rearrangements (either inversion 10q11.2 or translocations), FISH was performed using the REPEAT-FREE POSEIDON *RET* (10q11) break–apart probe (Kreatech Diagnostics, Amsterdam, The Netherlands) on FFPE samples.

This commercial probe is designed as a dual-color probe where the two regions across the break-point, the proximal and the distal region to *RET* (10q11), are direct-labeled with Platinum Bright 550 and with Platinum Bright 495 respectively.

The FISH procedure was performed following Kreatech's protocol with modifications designed in our laboratory, in particular regarding the tissue digestion and the hybridization times (54).

In brief, 3 μm thick FFPE tissue sections were mounted on positively charged slides and air dried. Targeted tumor areas were circled with a pen, after review of the corresponding hematoxylin and eosin (H&E) stained slide by a pathologist.

The sections were deparaffinized with two 10-min washes in xylene, hydrated in 100, 85, and 70% ethanol solutions for 10 min each, rinsed in distilled water for 10 min, fixed in methanol:acetic acid 3:1 for 10 min and air-dried. Next, the sections were treated in a 2× SSC solution for 15 min at 37 °C, and then dehydrated in consecutive 70, 85, and 100% ethanol solutions for 1 min each, then dried. The sections were then bathed in 0.1 mM citrate buffer (pH 6) solution at 85 °C for 30 min and again dehydrated in a series of ethanol solutions and dried.

The slides were incubated in 0.75 ml of pepsin (Sigma) solution (4 mg/ml in 0.9% NaCl, pH 1.5) for 15 min at 37 °C, washed again, dehydrated again in graded ethanol solutions (70, 85, and 100%) for 2 min each and dried.

A total of 10 μl *RET* (10q11) break–apart probe was placed on the designated hybridization area and sealed with rubber cement.

A ThermoBrite denaturation-hybridization system (Abbott Molecular) set at 80 °C was used for codenaturation of probe and target DNA for 10 min, before hybridization at 37 °C overnight.

The rubber cement and coverslip were removed and the slides were placed in 0.3% NP-40/2× SSC solution at first for 15 min at room temperature and then at 72 °C for 2 min. The sections were then rinsed in H_2O for 1 min, air-dried, and counterstained with 10 ml of DAPI/Antifade (ProLong Gold Antifade Reagent with DAPI; Life Technologies). The slides were examined using an Olympus BIX-61

Table 2 Clinicopathological and molecular findings in follicular adenoma.

Samples	Age (years)	Gender	Radiation exposure	Diameter (mm)	Histological type	Architectural pattern	Split *RET* signals (%)
1	35	M	Yes	6	Follicular	Normo-macrofollicular	2
2	52	M	No	22	Follicular	Microfollicular and trabecular	4
3	32	F	No	27	Follicular	Microfollocular and trabecular	12
4	54	F	No	25	Follicular	Normo-macrofollicular	3
5	31	F	No	40	Follicular	Microfollicular	4
6	46	F	No	35	Oncocytic	Solid and follicular	2
7	39	F	No	25	Oncocytic	Microfollicular and trabecular	1
8	54	F	No	30	Oncocytic	Normo-macrofollicular	6
9	54	F	No	6	Oncocytic	Microfollicular and trabecular	2
10	60	M	No	40	Oncocytic	Solid-trabecular	1
11	51	M	No	11	Oncocytic	Normo-macrofollicular	4
12	56	M	No	20	Oncocytic	Solid-trabecular	4
13	51	F	No	8	Hyalinizing trabecular	Trabecular	4

NED, not evidence of disease; SPeD, structural persistence disease; BPD, biochemical persistence disease; PD, progression disease.
2a and 2b, 17a and 17b, 18a and 18b, 20a and 20b: each paired sample derived from the same patient.

microscope (Olympus, Hamburg, Germany) with appropriate fluorescence excitation/emission filters. The signals were recorded by a CCD camera (Olympus Digital Camera). For microscopic evaluation, at least 100 intact and nonoverlapping cell nuclei were scored for the presence of a split signal. Only cells with two overlapping signals or one split and one overlapping signal were counted to ensure only complete cell nuclei had been scored. The signal pattern interpretation was as follows: interphase nucleus with two co-localized green/red fusion signals identified normal chromosomes ten, while a separated red and green signals and green/red fusion signals indicated rearranged *RET*.

FISH cut-off level

To establish the cut-off level for *RET/PTC* rearrangements, we performed FISH analysis on ten normal thyroid parenchyma and 100 nuclei were scored for the presence of a split signal. As previously reported, the cut-off value was calculated as mean value +3 S.D. of *RET* rearranged cells (23, 37, 50, 55). The resulting mean value was 3.6% with a S.D. of 2.2%, leading to a positivity threshold of 10.2% ($3.6 \pm 3 \times 2.2$). Therefore, a sample was considered positive if a broken signal was observed in >10.2% of nuclei.

RNA isolation and detection of *RET/PTC* rearrangements from frozen neoplastic thyroid tissue

Total RNA was extracted and reverse transcribed into cDNA. *RET/PTC1* and *RET/PTC3* rearrangements have been investigated by qRT-PCR. In a final volume of 20 μl, we amplified 1 μg cDNA in a mix containing 200 nM final concentration of specific primers and 100 nM of probes.

Primers forward and probes were as follows: *RET/PTC1*, F: 5′-CGCGACCTGCGCAAA-3′; *RET/PTC3*, F: 5′-CCCCAGGACTGGCTTACCC-3′; PTC1 probe, 5′-CAAGCGTAACCATCGAGGATCCAAA-3′; PTC3 probe, 5′-AAAGCAGACCTTGGAGAACAGTCAG-3′.

For both fragments, primer reverse was: *RET/PTC*, R: 5′-CAAGTTCTTCCGAGGGAATTCC-3′. To verify the presence of non-rearranged *RET*, the following primers and probe were used: *RET*, F: 5′-TGCTTCTGCGAGCCC-3′, R: 5′-ATCACCGTGCGGCACAG-3′; *RET* probe 5′-CATCCAGGATCCACTGTGCA-3′. Thermal cycling profile was 3 min at 95 °C followed by 15 s at 95 °C and 1 min at 60 °C for 45 cycles. TPC1 cells with *RET/PTC1* rearrangement and NIH3T3 cells with *RET/PTC3* rearrangement were used to form a standard curve composed by five points (from 1000 to 0.1 ng of cDNA with 1:10 dilution) (56).

Agarose gel PCR

The generic rearrangement for *RET* (RET/PTCX) was analyzed searching for the expression of tyrosine kinase (TK) and extracellular (EC) domains using the following primers: *EC*, F: 5′-GGCGGCCCAAGTGTGCCGAACTT-3′, R: 5′-CCCAGGCCGCCACACTCCTCACA-3′; *TK*, F: 5′-TGGTTCTTGGAAAAACTCTAG-3′, R: 5′-CTGCAGGCCCCATACAATTT-3′. Only samples showing TK expression and not associated with EC were considered positive for rearrangement. Thermal cycling conditions included an initial step (94 °C for 10 min) followed by 35 cycles at 60 °C and a final extension (72 °C for 10 min). TPC1 cells (rearranged for *RET/PTC1*) were used as a positive control and BCPAP cells (carrying the *BRAF*V600E mutation) were used as a negative control (42).

BRAF status

BRAF sequence was screened for V600E mutation by pyrosequencing. DNA was first amplified using 'RotorGene 6000' (Corbett Research, St. Neots, Cambridgeshire, UK) and then sequenced using PyroMark Q96 ID system. PCR was performed with the following conditions: initial denaturation at 95 °C for 3 min; 40 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; final step 60 °C for 5 min with TaKaRa Ex Taq (Qiagen). PCR amplification and mutational analysis were performed in accordance with the Diatech manual (anti-EGFR MoAb response BRAF status).

Statistical analyses

For statistical analysis, the unpaired Student's *t*-tests, the χ^2, and the Fisher's exact test were used, as appropriate. Statistical significance was defined at $P<0.05$. The *P* values were corrected for multiple testing according to Bonferroni. All analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com).

Results

The clinicopathological and molecular features of the 46 PTC (40 PTC *BRAF* WT and six PTC with *BRAF*V600E mutation) and 13 FA cases are given in Tables 1, 2 and 3. The mean age of the patients with *BRAF* WT PTC and with FA was 35.5 and 46.7 years respectively.

Table 3 Clinicopathological features of *BRAF* WT PTC patients. One case of FAP-associated papillary carcinoma; one case of sporadic cribriform-morular variant of papillary carcinoma; one case of a recurrence nodule in thyroid bed.

Parameter	Number
Mean age (years)	35.5 (8–75)
Mean tumor size (mm)	22.5 (3–60)
Diameter ≤1 cm	13 (32.5%)
Multifocality	12 (33%)
Thyroiditis	16 (44%)
pT1-2	18 (49%)
pT3-4	19 (51%)
pN1	18 (49%)
Stage (AJCC 2009)	
I–II	31 (84%)
III–IV	6 (16%)

In the former group, there were 28 females and nine males, resulting in a female:male ratio of 3.1:1. In the latter group, there were seven females and five males with a sex ratio of 1.4:1.

The mean tumor size of the *BRAF* WT PTC samples was 22.5 mm and 32.5% of them were microcarcinomas. Moreover, multifocality was present in 33% of the samples, and lymph node metastases were found in about 49% of the patients. Following the TNM staging (57), 31 patients were at stage I and II (Table 3).

Assuming that *BRAF*V600E mutation and *RET/PTC* aberration are usually mutually exclusive (58, 59), the overall prevalence of RET rearrangement, including all 250 PTCs, was 4% whereas if we consider only the *BRAF* WT PTC samples, the prevalence was 25% (10/40 cases) (Fig. 1).

In detail, eight out of ten *RET*-positive cases showed a high percentage of split, ranging from 18 to 50%, while two cases harbored 12 and 13% of positive nuclei, respectively (Table 1). In the cases with low percentage of split, the aberrant cells were found scattered in the contest of cells harboring normal chromosome 10, without clustering. *RET* rearrangement was observed in nine sporadic PTCs (two solid, two classical, three follicular, two diffuse sclerosing variants) and in one with a history of exposure to external beam radiotherapy (classical variant; Fig. 2). Considering only the group of PTCs exposed to radiation, the frequency of *RET* rearrangement was 17% (one out of six cases with 30% of rearranged cells). The patient had received whole total body radiotherapy for leukemia 7 years before the diagnosis and removal of thyroid cancer (Table 1, case 32). Overall, the patients are young in age, the majority have lymph node metastases at the diagnosis and have PTC variants at the histology, frequently linked to *RET* genotype (Table 1).

All six PTC samples, carrying *BRAF*V600E mutation detected through pyrosequencing, showed a percentage of *RET*-positive cells under the cut-off threshold (range 2–8%) (Table 1). Both molecular aberrations were mutually exclusive.

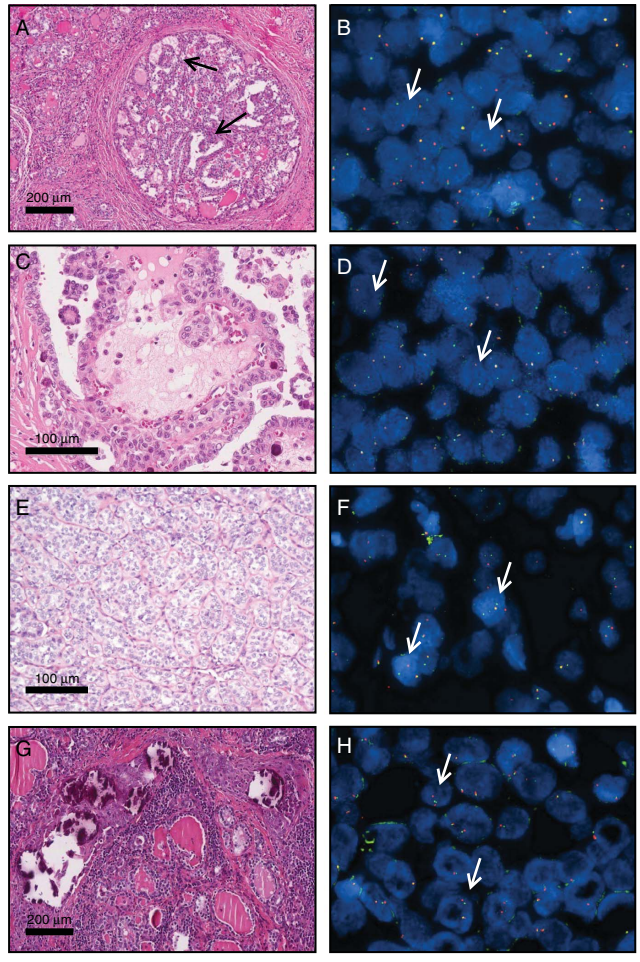

Figure 2
Histology and corresponding FISH images of representative *RET* rearranged PTC samples. (A and B) Classical variant PTC with predominant follicular growth pattern (case 29, Table 1). Black arrows indicate papillary structures. (C and D) Classical variant PTC with a history of exposure to external beam radiotherapy (case 35, Table 1). (E and F) Solid variant PTC (case 27, Table 1). (G and H) Diffuse sclerosing variant PTC (case 33, Table 1). White arrows indicate the rearranged copy of *RET*. A full colour version of this figure is available at http://dx.doi.org/10.1530/EJE-14-0930.

The comparison between FISH and qRT-PCR results are depicted in Table 1.

All ten *RET* positive PTC cases analyzed by FISH matched well with qRT-PCR data. In particular, nine out of ten cases (n. 20b, 24, 26, 27, 28, 29, 30, 31, and 32) showed detectable *RET/PTC1* mRNA, while one case (n. 25) exhibited *RET/PTC3* mRNA.

Controversial data were obtained in five cases showing a frequency of rearrangement very close to the cut-off level. Cases 6, 12, and 16, displaying 6, 9, and 10% of aberrant nuclei, respectively, showed detectable *RET/PTC3* or *RET/PTC1* mRNA, while samples n.15 and n.19 exhibited no detectable *RET/PTC1*, *RET/PTC3*, and tyrosine kinase domain mRNA expression and 10% of split FISH signals.

Moreover, the remaining 25 *BRAF* WT PTC cases and all six *BRAF*V600E cases were negative by both methods.

Finally, we found *RET/PTC* activation in one spontaneous FA (one out of 13 cases, 7.7%), harboring split signals in 12% of the nuclei, above the cut-off threshold (Table 2).

The comparison between *RET* rearranged and non rearranged PTCs is summarized in Table 4. No significant differences were found concerning the clinicopathological features, with the exception of the frequency of extrathyroidal invasion which was significantly higher in tumors with *RET* rearrangement than those harboring *RET* WT ($P=0.027$; Table 4).

Discussion

RET/PTC was the first chimeric gene with oncogenic potential described in a tumor of epithelial origin and represents one of the major genetic alterations found in PTC (1, 60).

For almost two decades, the pathogenic role of this hybrid gene both in sporadic PTC (adult and pediatric) and in PTC developing after ionizing radiation exposure, has been considered a dogma, but the detection of *RET*-positive cells in benign thyroid lesions and the discovery of heterogeneous distribution of this rearrangement within an individual nodule have called into question the belief (22, 24, 25, 37, 61).

Moreover, the clinical significance of *RET/PTC* rearrangements is still debated. Indeed, some authors have suggested that *RET* rearrangements are associated with local invasion and distant metastases (17, 26, 62, 63, 64, 65) while other authors associated with early-stage small PTCs and better prognosis (30, 66, 67, 68, 69, 70). However, these studies assumed that all types of rearrangement have comparable properties and considered them as a group (19).

Thus, the current challenge in using *RET/PTC* analysis affects the interpretation of dataset results. Finding an accurate, reliable, and clinically pragmatic strategy for *RET/PTC* detection becomes imperative because the detection of *RET/PTC* has been offered as a diagnostic tool for PTC in the surgical and preoperative cytological material (3, 21, 40, 41, 42, 43, 44). FISH is considered as the assay of choice for rearrangement detection on formalin-fixed surgical samples (71) and according to Marotta *et al.* (21), at present, it is the most suitable method for detecting clonal changes. Moreover, the application of interphase FISH on thyroid tumors is appropriate as tumors of endocrine glands are known to have a low growth rate (72).

The aim of this study was to investigate the prevalence of *RET* rearrangement by interphase FISH analysis in a cohort of *BRAF* WT PTC and to search for a reliable cut-off value in order to distinguish the occurrence of clonal or non-clonal *RET* changes and to explore whether *RET/PTC* may be a relevant pathogenic factor.

In our series, we found a total of ten out of 40 (25%) *BRAF* WT PTC samples with broken *RET* above the cut-off level, a prevalence slightly lower than that reported in other Italian studies of comparable size, ranging from 27.5 to 35% (16, 30, 62, 73, 74).

This finding could be explained by the significant decrease in *RET/PTC* over the years and the equivalent increasing rate of *BRAF*V600E and *RAS* mutations in PTC, possibly attributed to the decreased exposure to ionizing radiation in the last decades or to new pollutants (75, 76, 77, 78, 79).

Table 4 Comparison between the clinico-pathological data of rearranged and non-rearranged *RET/PTC* samples.

	Rearranged *RET*	Non-rearranged *RET*	*P* value
Gender (M:F)	1:4	1:2.9	NS
Mean age (years±S.E.M.)	31 (±4.1)	37.1 (±3.2)	NS
Tumor diameter (mm±S.E.M.)	25.6 (±4.3)	17 (±3.1)	NS
Multicentricity	2 (20%)	10 (37%)	NS
pT3	9 (90%)	9 (33.3%)	0.027
Thyroiditis	6 (60%)	10 (37%)	NS
Lymph node involvement	7 (70%)	11 (40.7%)	NS
Stage (AJCC 2009)			NS
I–II	9 (90%)	22 (81.5%)	
III–IV	1 (10%)	5 (18.5%)	
Histological subtype			NS
Follicular	3 (30%)	15 (50%)	
Classical	3 (30%)	8 (26.7%)	
Tall cell	0 (0%)	1 (3.3%)	
Others	4 (40%)	6 (20%)	

Moreover, the prevalence of 4% of *RET*-positive samples in our consecutive series of 250 PTCs is consistent with Jung *et al*. (76) who documented the decreasing in *RET/PTC* rearrangement from 11 to 2%. This prevalence was calculated assuming that *RET* rearrangement and *BRAF*V600E mutation are mutually exclusive as reported in some studies which consider the two genetic alterations as separated events in PTC without overlap (58, 59). Moreover, de Biase *et al*. (80) demonstrated that *BRAF*V600E is present in virtually all/the majority neoplastic cells in many mutated PTCs supporting the idea of that this genetic alteration is a founding event, acquired early during PTC development.

All six *BRAF*V600E samples tested for *RET* rearrangements exhibited split signal under the cut-off level. This finding indirectly confirms the pertinence of the 10.2% threshold for distinguishing non-clonal from subclonal or clonal *RET/PTC* rearrangement given that *BRAF*V600E mutation and *RET/PTC* aberration are usually mutually exclusive (58, 59). Moreover, this cut-off level parallels previous studies, which used a cut-off level of between 5 and 10% to separate cases from false-positives (22, 81, 82, 83).

We detected *RET/PTC* rearrangement in one of the six patients (17%) with a history of exposure to external beam radiotherapy. In the context of thyroid irradiation, this finding is to be considered a low figure, although the values reported in the literature have been decreasing recently (71, 75, 84). The high variation of *RET/PTC* rearrangements reported in different series can be due to differences in the prevalence of this alteration in specific age groups or due to the different time of latency of the tumors (3, 71, 84, 85).

In order to validate FISH results, we performed qRT-PCR assay in our study cohort composed of 40 *BRAF* WT and six *BRAF*V600E PTC cases. The FISH results matched well with qRT-PCR in 41 PTC cases (31 *BRAF* WT and six *BRAF*V600E PTCs), whereas five cases (n. 6, 12, 15, 16, and 19), showing a frequency of rearrangement very close to the cut-off level, were discordant (Table 1).

This discrepancy may reflect the genetic heterogeneity within an individual tumor, the different sensitivity of the detection approaches used in the study and the samples type used for the comparative analysis, e.g. FFPE material for FISH analysis and frozen tissue for qRT-PCR, representing different regions of the same tumor presumably with different distribution of *RET*-positive cells (3, 22). Moreover, the documented highly variable levels of *RET/PTC* expression in PTC, the identification of which is strictly dependent on factors that affect the sensitivity, could contribute to explaining the inconsistencies in detection rates between the DNA-based method (FISH) and RNA-based assay (qRT-PCR). The existence of quantitative variation in the expression levels should be taken into account to investigate the correlation of *RET/PTC* with clinical findings (83, 86).

The clinical significance of *RET/PTC* remains unclear, with conflicting results between the studies. Considering the clinicopathological features evaluated in our series, we found no correlation of *RET/PTC* expression with age, gender, tumor size, histological variant, multifocality, lymphocytic infiltration, and lymph node metastasis, but the frequency of extrathyroidal invasion in tumors with *RET/PTC* expression (9/10, 90%) was significantly higher than those of *RET/PTC* negative (9/27, 33%, $P=0.027$), as already reported in three other studies (87, 88, 89). However, follow-up analysis seems to indicate no influence of *RET* expression on patients' outcome, although the short follow-up period makes it difficult to draw definitive and firm conclusions on the prognosis. According to Tallini *et al*. (68), only one case of PTC with minor poorly differentiated component (case 23) was negative for *RET* rearrangement, confirming the low potential (the apparent inability) of *RET/PTC*-positive PTC to progress to a less differentiated phenotype.

As in the study of Soares *et al*. (69), our series did not include papillary microcarcinoma, carrying *RET* rearrangement. However, a high prevalence of *RET* rearrangement has been detected in papillary microcarcinomas by Viglietto *et al*. (67), leading the authors to conclude that this genetic alteration is an early event in PTC tumorigenesis and occurs in tumors with less propensity to evolve toward clinically more aggressive forms. Also Corvi *et al*. (90) found *RET* activation in 11 microcarcinomas out of 21 (52%) using FISH method. It is likely that these discrepancies could be attributed to the different study populations evaluated.

We found *RET/PTC* activation in one spontaneous FA (one out of 13 cases, 7.7%), harboring split signals in 12% of the nuclei. Although, initially, *RET* rearrangements were considered as a specific marker for PTC, they have been sporadically reported in nodules classified as benign at histology by means of different detection methods (21, 29, 32, 46). The biological significance of *RET/PTC* in benign lesions remains difficult to explain, if we exclude the occurrence of microfoci of PTC within an otherwise benign nodule. Some authors have hypothesized that the *RET*-positive adenomas are composed of a mixture of cells with and without rearrangement (non-clonal event), while others suggested that adenomas

RET/PTC-positive may grow faster than those *RET/PTC*-negative (23, 32, 36, 46).

In conclusion, this study demonstrates that interphase break–apart FISH analysis proves a reliable and sensitive strategy to detect *RET/PTC* activation in thyroid tumors, comparable with RT-PCR or Southern blot analysis with the advantage to allow, on histology sections, the direct correlation between the histopathological features and the distribution of *RET* rearrangements in the tumor/nontumor cells. It also represents a powerful tool to estimate the ratio between broken and non-broken *RET* cells in an individual tumor, with the possibility to separate the clonal (driver mutation) from subclonal event (passenger mutation) and to quantifying intratumoral genetic heterogeneity.

Finally, the identification of a precise laboratory FISH cut-off appears to be a pivotal prerequisite in the interpretation of the presence of *RET* rearrangement, particularly when *RET/PTC* detection is used for cytological evaluation of malignancy or for targeted therapy.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

All authors approved the final version of the manuscript. Study concept and design: C Colato, M Ferdeghini, M Chilosi, F Pacini, and M Brunelli. Acquisition of data: C Colato, C Vicentini, S Cantara, S Pedron, P Brazzarola, I Marchetti, and G D Coscio. Analysis and interpretation of data: C Colato, C Vicentini, S Cantara, F Pacini, M Chilosi, M Ferdeghini, and M Brunelli. Drafting of the manuscript: C Vicentini and C Colato.

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