REVIEW

RET/PTC rearrangement in benign and malignant thyroid diseases: a clinical standpoint

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Abstract

Cytological examination of fine needle aspiration biopsy is the primary means for distinguishing benign from malignant nodules. However, as inconclusive cytology is very frequent, the introduction of molecular markers in the preoperative diagnosis of thyroid nodules has been proposed in recent years. In this article, we review the clinical implications of preoperative detection of rearrangements of the *RET* gene (*RET*/papillary thyroid carcinoma (*PTC*)) in thyroid nodules. The prevalence of *RET*/PTC in PTC depends on the histological subtypes, geographical factors, radiation exposure, and detection method. Initially, *RET*/PTC was considered an exclusive PTC hallmark and later it was also found sporadically in benign thyroid lesions. More recently, the very sensitive detection methods, interphase fluorescence *in situ* hybridization (FISH) and Southern blot on RT-PCR amplicons, demonstrated that the oligoclonal occurrence of *RET* rearrangement in benign thyroid lesions is not a rare event and suggested that it could be associated with a faster enlargement in benign nodules. For this reason, *RET*/PTC cannot be considered as an absolute marker of PTC, and its diagnostic application must be limited to assays able to distinguish between clonal and oligoclonal expression. Detection of *RET*/PTC by quantitative assays will be useful for diagnostic purposes in cytology specimens when a precise cutoff will be fixed in a clinical setting. Until that time, less sensitive *RET*/PTC detection methods and FISH analysis remain the most appropriate means to refine inconclusive cytology. Future studies with a long follow-up will further clarify the clinical significance of low level of *RET* rearrangements in benign nodules.

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Introduction

Palpable nodules of the thyroid gland are a common clinical occurrence. The majority of such lesions are benign in nature. The differential diagnosis between benign and malignant nodules is based on cytological evaluation of thyroid cells obtained by fine needle aspiration cytology (FNAC), stained by May-Grunwald–Giemsa or Papanicolaou methods (1, 2). However, a relevant number of thyroid nodules exhibit borderline morphological signs of papillary thyroid carcinoma (PTC) representing a difficult diagnostic challenge. Many markers of malignancy have been investigated in the attempt to improve FNAC accuracy (3–6). Among the molecular markers of PTC, oncogenes with a pathogenetic role in the mechanism of thyroid cell transformation are most promising. Rearrangements of the *RET* gene in PTC (*RET*/PTC) was one of the first human oncogene discovered and was the first oncogene proposed as a marker for this tumor (7). The carcinogenetic potential of *RET*/PTC and its expression in different tumors have been extensively studied by different methods in tissues and in cytology

specimens. Early studies concluded that *RET*/PTC was responsible for the development of thyroid cancer and was totally restricted to PTC (8–10). This claimed specificity as a PTC marker raised the possibility for its clinical application in those situations where the conventional cytology yields inconclusive results (11–16). However, the initial enthusiasm was tempered by the finding of *RET*/PTC in benign lesions including Hashimoto's thyroiditis (HT) and adenomas (17–20). The finding that *RET*/PTC expression is not an absolute PTC marker raises concerns on its clinical utility in inconclusive FNAC. Moreover, the finding that *RET* rearrangements can occur only in a fraction of the cells in some PTC raises the alternative hypothesis that *RET*/PTC may also be a common secondary event in the process of thyroid carcinogenesis (21). The subclonal occurrence of *RET* rearrangement in PTC can influence the sensitivity of some methods and might explain why the reported prevalence of *RET*/PTC in papillary carcinomas varies in different studies from 0 to 87% (22, 23). Very recent studies demonstrated that *RET* rearrangement in benign thyroid nodules is not

an uncommon occurrence and suggested that its presence could be associated with a faster nodular enlargement (24–26).

In this article, we review the clinical implications of *RET/PTC* detection in preoperative diagnosis of thyroid nodules and the recent advances in the clinical significance of this oncogene in benign nodules.

***RET* rearrangements: *RET* and the other genes involved**

The *RET* (rearranged during transfection) proto-oncogene is located at chromosome 10q11.2 and encodes a cell membrane receptor tyrosine kinase. It was discovered in 1985 as a gene rearranged in the procedure of an NIH3T3 transfection assay (27). The *RET* proto-oncogene is a main component of the signaling pathway, activated by the glial cell line-derived neurotrophic factor family ligands, and is implicated in the development of kidney and enteric nervous system (28, 29). The *RET* protein comprises an extracellular domain with ligand-binding function composed of four cadherin-like sites, followed by a cysteine-rich loop, a single transmembrane region, and an intracellular domain containing the juxtamembrane domain and the tyrosine kinase domain (30). In the thyroid gland, *RET* protein is strongly expressed in parafollicular C-cells consistently with its role in the development of neural crest-derived structures (29). Although *RET* mRNA has been documented in follicular cell-derived thyroid tumors, to date, *RET* mRNA or protein has never been clearly detected in thyroid follicular cells (31). In some PTCs, the tyrosine kinase domain of *RET* is fused with an heterologous gene that provides the promoter and the 5'-coding region (32). The product of this rearrangement is a chimeric oncogene named *RET*/papillary thyroid carcinoma (*RET/PTC*). Since the original report, at least 11 types of *RET/PTC* variants have been isolated (32). Most of these rearrangements are between *RET* on chromosome 10 and genes located on different chromosomes. By contrast, *RET/PTC1* and *RET/PTC3* are intrachromosomal paracentric inversions because the genes involved *H4* and *RFG* (also designated *ELE1/ARA70/NCOA4*) are also all located on chromosome 10 (32, 33). *RET/PTC1* and *RET/PTC3* account for the vast majority of the variants, while the others are very rare and have little clinical significance. The chimeric *RET/PTC* protein generated by the genetic recombinations lacks the transmembrane domain and the extracellular regulatory region of *RET* but retains the coiled-coil domains in the *RET* partner coding sequences, necessary for the dimerization process. This determines the cytoplasmic location of the chimeric protein, its ligand-independent dimerization, and its autophosphorylation at residues 1015 and 1062 responsible for the oncogenic activity of the *RET/PTC* chimera (34).

Methods of detection for *RET/PTC* rearrangements

Detection of the *RET/PTC* protein in cytology or tissue samples by immunohistochemistry (IHC) would be desirable, so that the pathologist could search for *RET/PTC* in surgical and preoperative cytological samples and at the same time observe the morphology of the sample. In different studies, commercially available or personally produced antibodies for the carboxy-terminal part of *RET* or for the phosphorylated tyrosine 1062 of *RET* were used to detect the presence of the tyrosine kinase domain. This method is based on the assumption that wild-type *RET* is not expressed in PTC or if expressed it is not activated and has the advantage of detecting all *RET* rearrangements (34–36). However, *RET* immunoreactivity in the cytoplasm of PTC is often weak and largely variable, also within the same tumor, making the interpretation of the results challenging. Moreover, very recently, wild-type *RET* expression was demonstrated by IHC and RT-PCR in PTCs in Kuwait (37).

Conventional PCR from genomic DNA is not suitable to detect *RET/PTC* because the rearrangement of *RET* occurs immediately upstream of its tyrosine kinase-encoding domain, whereas the fused gene is truncated at different points in different tumors. Thus, hundreds or thousands base-long intronic sequences separate the exons of the fused genes.

Conversely, cDNA can be analyzed by RT-PCR using primers annealing to the *RET*-exon 12 and the exons 1 and 6 of *H4* or *ELE1* respectively. Then, the PCR products are electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining. This method to detect *RET/PTC1* and *RET/PTC3* recombinations is easy to perform and accurate. However, the low and heterogeneous expression of *RET* recombinants limits the application of this method and produces a number of false negative results, as demonstrated by comparative analysis of *RET/PTC* detection by RT-PCR and other methods in the same cytology samples (22, 24). Detection of *RET* rearrangements become much more robust when the RT-PCR products are visualized by Southern blot using oligonucleotide probes specific for *RET/PTC1* or *RET/PTC3* (22). Detection of RT-PCR products by Southern blot compared with ethidium bromide resulted in a 2.5- to 5-fold increased sensitivity (24, 35). Moreover, the step of hybridization with specific probes ensures a higher specificity compared with RT-PCR alone. Another method of detection is quantitative real-time RT-PCR. Amplification is achieved by a high number of cycles of amplification followed by post-PCR fluorescence melting curve analysis. Detection of *RET/PTC1* and *RET/PTC3* rearrangement by amplification using real-time RT-PCR did not demonstrate to be more sensitive than RT-PCR (22). However, in this method also, the hybridization step with specific probes increases the specificity and reduces the risk of false positive results.

Dual-color interphase fluorescence *in situ* hybridization (FISH) is based on the microscopic observation of nuclei with overlapping red and green FISH signals (wild-type *RET*), or split FISH signals (rearranged *RET*) if one DNA probe maps the *RET* locus, while the other probe maps a sequence distal to *RET*. Alternatively, the probes can map the *RET* locus or the recombinant genes, and in this case, an overlapping signal indicates a rearranged *RET* gene. This is a time-consuming and laborious method that requires the observation of a large number of nuclei by an experienced operator. However, this method provides important information on the percentage of cells harboring the *RET* rearrangement and on its clonal/non-clonal occurrence (21, 22). More recently, automated interphase FISH was successfully applied to detect *RET/PTC* in PTC primary cultures, a method that, suitably modified for clinical practice, could allow the analysis of a larger number of cells per sample and overcome a possible bias of the operator (38).

RET/PTC in thyroid cancer

RET rearrangements are an exclusive occurrence of thyroid gland. A possible explanation for this restriction is that contiguity between *RET* and the recombinant genes occurs in nuclei of thyroid cells but not in other cell types (39). After its initial discovery in 1985, a large survey conducted in the following decades evidenced the frequent occurrence of *RET/PTC* in PTC and its sporadic oligoclonal occurrence in benign thyroid lesions. The estimated prevalence of *RET* rearrangement in PTC is highly variable among different studies (Table 1). A crucial factor responsible for this wide variability is the detection method. Zhu *et al.* (22) searched for *RET/PTC1* and *RETPTC3*, the two main rearrangements of *RET*, in a cohort of 65 tumor samples from PTC patients using different techniques. They found a broad variability in the prevalence of *RET/PTC* rearrangement as a result of the different sensitivity of the detection methods. *RET* rearrangements were detected in 12 tumors by real-time LightCycler RT-PCR, in 14 by RT-PCR, and in 26 by the more sensitive Southern blot on RT-PCR products. These striking differences reflect the ability of the methods to detect *RET/PTC* when present in a small proportion of neoplastic cells within the tumor. Another important factor responsible for the variable prevalence of *RET* rearrangements among different studies of PTC is their tumor subtype composition. PTC is a heterogeneous tumor including very frequent variants such as classic PTC (cPTC) and follicular PTC (fvPTC), and some uncommon PTC variants such as tall-cell PTC (tcPTC), diffuse-sclerosing, columnar-cell, Hurthle-cell, cribriform, and solid variants (40). The reported prevalence of *RET* rearrangements in cPTC was generally higher than that in fvPTC (Table 2). *RET/PTC*, mostly the *RET/PTC1*, has been detected in a relevant percentage of

Table 1 Prevalence of *RET* rearrangements in sporadic PTC in different studies. Data are presented as sample numbers and (%). RT-PCR was visualized by ethidium bromide.

	RT-PCR	SB on RT-PCR	Q-PCR	FISH
Ishizaka <i>et al.</i> (17)		1/11 (9)		
Zou <i>et al.</i> (68)		1/40 (3)		
Nikiforov <i>et al.</i> (45)		11/17 (65)		
Lam <i>et al.</i> (69)				17/40 (43)
Tallini <i>et al.</i> (50)	17/28 (61)			
Sugg <i>et al.</i> (35)		51/84 (61)		
Mayr <i>et al.</i> (70)	8/99 (8)			
Cinti <i>et al.</i> (59)				13/69 (19)
Sheils <i>et al.</i> (71)	12/50 (24)			
Fenton <i>et al.</i> (72)	15/33 (45)			
Chua <i>et al.</i> (73)		37/47 (79)		
Elisei <i>et al.</i> (18)		21/47 (45)		
Puxeddu <i>et al.</i> (74)		13/48 (27)		
Rhoden <i>et al.</i> (75)			18/25 (72)	
Zhu <i>et al.</i> (22)	14/65 (22)	26/65 (40)	12/65 (18)	14/65 (22)
Rhoden <i>et al.</i> (54)			25/34 (74)	24/26 (92)
Frasca <i>et al.</i> (74)	2/50 (4)			
Guerra <i>et al.</i> (24)	8/50 (16)	18/50 (36)		

SB on RT-PCR, Southern blot on RT-PCR; Q-PCR, real-time quantitative RT-PCR

Hurthle-cell variant PTC, in the cribriform variant, which is typically associated with familial adenomatous polyposis, and in hyalinizing trabecular tumor, a borderline thyroid neoplasm with many histological features similar to PTC (41–44).

Ionizing radiations are known to be a relevant cause for genetic alteration and carcinogenesis because they are particularly effective in inducing DNA double-strand breaks. The role of ionizing radiations in thyroid carcinogenesis has been extensively studied in Belarus, Ukraine, and parts of the Russian Federation, which have been affected by the Chernobyl accident. These studies evidenced that the Chernobyl accident resulted in a dramatic increase in the number of thyroid cancers of PTC type with a frequency of rearrangements of the *RET* proto-oncogene higher than that in sporadic PTC (45, 46). The association of *RET* rearrangements with radiation exposure was sustained by the observation that the incidence of PTCs is severely increased in radio-contaminated areas following a linear relationship with dose exposition (47). In the comparative analysis of *RET* rearrangements in sporadic and radiation-induced thyroid tumors performed by Nikiforov *et al.* (45), *RET/PTC1* was shown to be the dominant type within sporadic carcinomas and was strongly related to the classic variant, while *RET/PTC3* was predominant among radiation-induced carcinomas and more frequent in the solid PTC variant. These findings were confirmed by Tallini *et al.* (50) in a study including 201 sporadic PTCs and by Rabes *et al.* (48) and Thomas *et al.* (46) in two large studies of post-Chernobyl PTCs. Interestingly, Elisei *et al.* (18) and Bounacer *et al.* (20) showed that *RET* rearrangements occurred also in benign thyroid nodules with a history of irradiation, further confirming the direct correlation between radiation exposure and this genetic alteration. However, other unknown factors concur to the *RET* rearrangement, as

Table 2 *RET/PTC* expression in PTC subtypes. Data are presented as sample numbers and (%).

References	cPTC	fvPTC	tcPTC
Basolo <i>et al.</i> (77) ^a	11/39 (27.5)	3/12 (25)	14/39 (35.8)
Adeniran <i>et al.</i> (78) ^b	14/53 (26)	1/30 (3)	0/6
Tallini <i>et al.</i> (50) ^a	47/107 (43)	31/8 (26)	
Nikiforov <i>et al.</i> (45) ^a	10/14 (71)	0/1	
Puxeddu <i>et al.</i> (74) ^a	4/15 (27)	7/25 (28)	
Rhoden <i>et al.</i> (75) ^c	3/11 (27)	7/10 (70)	

^aSouthern blot on RT-PCR.^bRT-PCR visualized by ethidium bromide.^cReal-time quantitative RT-PCR.

suggested by the lack of difference in the occurrence of *RET/PTC* in PTC between patients with and without a history of therapeutic irradiation (18). The application of the interphase FISH in post-Chernobyl papillary thyroid tumors led to the interesting observation that irradiated PTCs were composed of a mixture of cells with and without *RET* rearrangements (21). While the oncogenic potential of *RET* rearrangement has been demonstrated using transgenic mouse models, its subclonal occurrence suggests that in some instances it can be a secondary event in PTC development following radiation exposure.

The pathogenetic role of *RET/PTC* in spontaneous thyroid cancer is also supported by the observation of its frequent occurrence in micro-PTC. Fusco *et al.* (49) detected *RET/PTC* in microscopic papillary foci of thyroid nodules with borderline morphological features of malignancy. Tallini *et al.* (50) found a strong correlation between *RET* rearrangements and micro-carcinomas ($P < 0.01$), and accordingly, Sugg *et al.* (35) observed that *RET/PTC* was more prevalent in micro-PTCs than in clinically evident PTCs (77 vs 47%). A different oncogenic potential of *RET/PTC1* and *RET/PTC3* has been proposed. Rabes *et al.* (48) observed that tumors carrying the *RET/PTC3* rearrangement had a shorter latency and a more aggressive clinical behavior. *RET/PTC3* was more frequent than *RET/PTC1* in tcPTC, an aggressive PTC variant, and its expression in transgenic mice generated solid tumor variants of thyroid cancer with more aggressive and metastatic behavior (8, 51).

***RET/PTC* in benign diseases**

While initially *RET/PTC* was considered to be a specific marker for thyroid cancer, further studies reported the presence of this genetic alteration in different benign thyroid diseases. In this regard, the crucial factor has been represented by the introduction of highly sensitive detection methods that led to discover *RET* recombination in a higher percentage of PTCs and in a significant proportion of different benign thyroid lesions. The first paper reporting the presence of *RET* rearrangements in thyroid specimens of patients affected with HT was performed by Wirtschaffter *et al.* (52). The authors

detected *RET/PTC* in 95% of samples by means of a highly sensitive RT-PCR using two rounds of amplification with at least 40 cycles per round. The same prevalence was confirmed in a further study by means of a highly sensitive Taqman PCR (53). Recently, Rhoden *et al.* (54) searched for *RET/PTC* in HT samples by means of different techniques. Analysis by dual-color FISH and by a combination of real-time PCR and laser-capture microdissection showed consistent results detecting *RET/PTC* in 68 and 62% respectively. The detection of *RET* rearrangements within HT samples might be related to the presence of occult PTCs in a relevant percentage of these patients, an hypothesis supported by several studies reporting the frequent occurrence of PTC in patients affected with HT (52, 55). The detection of *RET/PTC* in a broad percentage of both PTCs and HTs is quite interesting as these two conditions show many significant similarities. Indeed, follicular HT cells typically harbor cytological features and molecular markers similar to PTC and genetic alterations such as allelic DNA loss and karyotypical abnormalities (56–58). However, analysis by FISH and careful microscopic examination reported *RET/PTC* in rare follicular cells in the majority of thyroid glands with HT but excluded the possibility that these cells were collected from microscopic foci of PTC (54). Although the expression of *RET/PTC* in rare cells within HT could be an early step of carcinogenesis, no evidence is provided supporting a link with the development of clinically significant PTC, and the biological and clinical significance of this finding remains unclear.

Initially, the majority of studies excluded the occurrence of *RET/PTC* in benign nodules. Nevertheless, *RET* rearrangements have been sporadically reported in nodules classified as benign at histology. In 1991, Ishizaka *et al.* (17) detected for the first time *RET/PTC* in four of 19 (21%) follicular adenomas by means of RT-PCR. A later study performed by Elisei *et al.* (18) by means of Southern blot on RT-PCR found *RET* rearrangements in 13.9% of naturally occurring benign nodules. In 2000, Cinti *et al.* (59) discovered three of 19 follicular adenomas harboring *RET/PTC* by means of FISH demonstrating that only 10–20% of cells carried the mutation. A recent study performed by Guerra *et al.* (24) further confirmed these findings as *RET* rearrangements were detected in 13.3% of nodules showing benign histology by means of Southern blotting after RT-PCR amplification. The latter study clearly demonstrated the relevance of the methodology applied as only one of the four positive cases was detected by application of RT-PCR amplification alone. Two studies performed a comparative analysis of *RET* recombination in benign lesions with and without radiation exposure. Bounacer *et al.* (20) detected *RET* rearrangements only in follicular adenomas (45%) of patients with a history of external irradiation, but not in naturally occurring benign nodules. Elisei *et al.* (18) demonstrated a significantly higher occurrence of the rearrangement

in benign nodules of patients exposed to irradiation than in spontaneous lesions. This evidence further confirms the strike association between radiation exposure and *RET* rearrangements also in benign thyroid lesions.

Pathophysiological aspects of RET/PTC

There are several lines of evidence pointing to *RET/PTC* as a driving event in thyroid carcinogenesis. The rat thyroid cells, PCCl3, change morphology, loose their differentiated functions, and are no longer TSH dependent for growth when stably transformed by *RET/PTC3* (60). However, acute expression of *RET/PTC* is sufficient to block the expression of TSH receptor and generation of cAMP, but it is insufficient to allow cells to grow in the absence of TSH, suggesting that *RET/PTC* is a weak tumor-initiating factor and that secondary genetic or epigenetic changes are required for full transformation into a cancer cell (61). Transgenic mice expressing *RET/PTC1* develop thyroid papillary carcinomas with features very similar to those shown by human PTC. These tumors are characterized by nuclear grooves and ground glass cells, slow growth rate, TSH responsiveness, continued expression of thyroglobulin, and loss of radioiodide-concentrating activity (8, 62). Similarly, *RET/PTC3* transgenic mice develop thyroid hyperplasia and locally invasive solid subtype of PTC with lymph node metastasis (51). However, not all the *RET/PTC* transgenic animals develop thyroid cancer, and it appears only after a long latency period. This evidence suggests that other molecular events are required for the development of thyroid carcinomas. This concept is further supported by the finding that *RET* rearrangements can occur only in a fraction of the cells in some PTC, thus being a secondary event in these tumors, and by the presence of *RET/PTC* in sporadic cells in HT and benign nodules. Radiation exposure, cellular over-proliferation, production of free radicals, and chemokine secretion have been hypothesized to account for the occurrence of intrachromosomal rearrangements in these benign thyroid lesions (63).

The biological significance of *RET/PTC* in benign lesions is a challenging task. A recent study has tried to answer the question whether this oncogene changes the fate of benign nodules. Sapio *et al.* (26) searched for *RET* rearrangements in a cohort of benign thyroid nodules in patients subjected to a careful clinical and ultrasonographic follow-up for 3 years. The researchers did not find evidence of cancer development but demonstrated that nodules harboring *RET/PTC* grow more rapidly than those without the oncogene. This finding is consistent with the hypothesis that even the presence of a small portion of cells harboring *RET/PTC* may have biological and clinical consequences in benign nodules. While the mechanisms underlying the more rapid growth of these nodules remain unclear, it can be

hypothesized that the pro-inflammatory transcriptional program activated by *RET/PTC*, including upregulation of various cytokines, chemokines, and their corresponding receptors, can stimulate thyroid cell proliferation through a autocrine/paracrine mechanism extended to the neighboring cells and involving a larger part of the nodule (64–66). More studies with longer follow-up are needed to clarify the clinical significance of low level of *RET* rearrangements in benign nodules.

Application of RET/PTC testing in fine needle biopsy of thyroid nodules

Since its discovery, *RET/PTC* was proposed as a hallmark of PTC useful to refine inconclusive cytology assessment of thyroid nodules. Although the amount of RNA obtained from fine needle biopsy is generally low, it does not appear to be a limitation, and in many studies, the survey of *RET/PTC* in cytology samples, even in needle washout, has been successfully performed. To use this oncogene as a diagnostic marker, it is essential to know its prevalence in malignant and benign lesions in a clinical setting, in order to determine the sensitivity and specificity of a *RET/PTC*-based assay. Unfortunately, the broad variability of *RET/PTC* prevalence in PTC reported in different studies is a strong limitation. This is in part due to geographic and environmental factors, but also to the application of different detection methods. Thus, the comparison of different studies is impaired by the lack of a unique and validated technique of detection. In the studies considered in Table 3, the oncogene was detected in cytology samples by means of different methods in 63 of 283 PTCs (22.3%). Southern blot on RT-PCR was twice over more sensitive than RT-PCR alone (28.7 vs 11.5%). In the study

Table 3 Survey of RET/PTC expression in cytology samples with definitive histology diagnosis. Data are presented as sample numbers and (%). RT-PCR was visualized by ethidium bromide.

	Histology	RT-PCR	SB on RT-PCR	Q-PCR
Cheung <i>et al.</i> (14)	PTC		16/33 (48.5)	
	B		0/39	
Domingues <i>et al.</i> (15)	PTC	3/11 (27.2)		
	B	1/11 (9.1)		
Sapio <i>et al.</i> (79)	PTC	1/6 (16.7)		
	B	0/72		
Pizzolanti <i>et al.</i> (13)	PTC	0/33		
	B	0/32		
Nikiforov <i>et al.</i> (16)	PTC			5/40 (12.5)
	B			0/38
Cantara <i>et al.</i> (11)	PTC		11/74 (14.9)	
	B		0/165	
Musholt <i>et al.</i> (80)	PTC	2/22 (9.1)		
	B	0/63		
Guerra <i>et al.</i> (24)	PTC	8/50 (16.0)	18/50 (36.0)	
	B	1/30 (3.3)	4/30 (13.3)	
Overall	PTC	14/122 (11.5)	45/157 (28.7)	5/40 (12.5)
	B	2/208 (1.0)	4/234 (1.71)	0/38

PTC, papillary thyroid carcinoma; B, benign nodules; SB on RT-PCR, RT-PCR visualized by Southern blot; Q-PCR, real-time quantitative PCR.

by Cantara *et al.* (11), searching for *RET/PTC* allowed to correctly diagnose two PTCs of 87 nodules classified as benign at cytology and two PTCs of 41 in the indeterminate category. In two other studies, two PTCs of 52 nodules and nine of 23 were identified in the indeterminate category by *RET/PTC* detection (14, 16). These results suggest that searching for *RET/PTC* can be used as a complementary approach to FNAC, increasing the overall sensitivity of a preoperative cytological diagnosis.

In most of the studies, *BRAF* mutation was also investigated, demonstrating only a small overlap between the two oncogenes so that searching for both markers further improves the sensitivity of molecular diagnosis. In the study by Pizzolanti *et al.*, the sensitivity of a molecular assay using *BRAF*^{V600E} alone was 69% and increased to 78% in combination with *RET/PTC* (16). Similarly, in the studies by Nikiforov *et al.* (16) and Cantara *et al.* (11) searching for both oncogenes increased the sensitivity from 45.0 to 57.5% and from 42.3 to 56.4% respectively (11, 13). Noteworthy in these two studies, is the detection of *RET/PTC* disclosed two false negatives over 99 benign FNACs and identified five PTCs over 146 indeterminate or inadequate FNACs.

Unlike *BRAF*^{V600E}, *RET/PTC* is also sporadically detected in non-malignant thyroid lesions at low expression level. By means of Southern blot on RT-PCR, *RET* rearrangement was found in 13.3% of cytology aspirates from nodules classified as benign at histology after careful microscopic observation that did not disclose microscopic foci of malignancy (24). In the same study, only one of the four positive cases was detected by application of RT-PCR amplification alone, demonstrating that the sensitivity of the detection method is a crucial factor. The finding of *RET/PTC* in non-malignant nodules renders its application in a diagnostic assay more problematic.

Conclusions

FNAC is the gold standard for the differential diagnosis of thyroid nodules. Unfortunately, inconclusive results present a large gray zone even for skilled pathologists. The discovery of genetic mutations involved in the pathogenesis of thyroid tumors has provided the basis for further improvements of the pre-surgical diagnostic methods. The most extensively studied mutation in this diagnostic context is *BRAF*^{V600E} (67). Because this highly specific PTC marker is expressed only in about half of PTCs, more cancer markers are needed. Its demonstrated pathogenetic role, its prevalence of about 20%, and its low overlapping with *BRAF*^{V600E} have made *RET/PTC* one of the most promising genetic alteration in a multiple marker diagnostic assay. However, the initial enthusiasm has been tempered by the demonstration that this oncogene is also found in non-malignant thyroid nodules. The biological and clinical significance of *RET/PTC* in benign thyroid

lesions is still controversial. FISH analysis demonstrated that in HT, in adenomas, and also in a significant percentage of PTCs, *RET/PTC* is present as oligoclonal occurrence (21, 22, 54, 59). The expression of *RET/PTC* in HT can be explained as an event secondary to chemokine stimulation induced by the large lymphoid infiltration present in this disease. Similarly, chronic stimulation by lymphoid infiltration, radiation exposure, or other factors in benign and malignant nodules could be responsible for the occurrence of *RET* rearrangements in sporadic cells. Although several observations have associated HT to thyroid tumors, including the occurrence of cytological alterations similar to those found in PTC and an increase in the risk for papillary carcinoma, to date there are no evidence supporting the concept that *RET/PTC*-expressing HT is a PTC precursor.

In a very recent study, Sapio *et al.* (26) followed for 3 years 19 nodules diagnosed as benign at FNAC that harbored *RET/PTC1* or *RET/PTC3*. Clinical and ultrasound examination did not provide evidence for a malignant transformation, and the final histopathology of three of these nodules was benign hyperplastic nodules. Of note is that *RET/PTC*-positive nodules displayed a fourfold faster growth with respect to their *RET/PTC*-negative counterparts. Thus, while at the moment a nodule with benign cytology and *RET* rearrangement disclosed by means of Southern blot on RT-PCR must be considered benign in nature, it should be subjected to a careful follow-up for its propensity to a fast enlargement. Searching for *RET/PTC* by highly sensitive methods might be useful in choosing the most appropriate and timely therapeutic option for benign thyroid nodules.

At the same time, highly sensitive methods are not appropriate to distinguish between benign and malignant nodules in inconclusive cytology. Searching for *RET/PTC* by a less sensitive method may have the drawback of leaving some PTCs undiagnosed but has the advantage of reducing false positive findings. Indeed, while sporadic cells harboring a *RET* rearrangement can be present in benign nodules, its clonal occurrence is exclusive to PTC. Hence, the less sensitive RT-PCR is more suitable for diagnostic purposes than Southern blot on RT-PCR. Rhoden *et al.* (54) demonstrated the possibility to distinguish between HT and PTC by *RET/PTC* in quantitative methods. Because *RET/PTC* cannot be considered as an absolute marker of PTC, its application for diagnostic purposes in cytology samples is limited to less sensitive methods or must wait for a precise cutoff in a quantitative assay, fixed in a clinical setting. Until that time, FISH analysis remains the most appropriate means to detect the clonal occurrence of *RET/PTC*, suitable to refine inconclusive cytology. Future studies with a long follow-up of benign nodules will clarify the clinical significance of low level of *RET* rearrangements.

Declaration of interest

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

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