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ORIGINAL ARTICLE

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Differential expression of *RET* isoforms in normal thyroid tissues, papillary and medullary thyroid carcinomas

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Abstract

Pourposes We investigated the expression of *RET9* and *RET51* isoforms in medullary (MTC), papillary (PTC) thyroid carcinoma, normal thyroid tissues, and pheochromocytoma (PHEO) to verify if these isoforms are present also in follicular thyroid cell-derived tissues, and if there is a differential expression of *RET9* and *RET51* in MTC.

Methods Nineteen patients with MTC, 18 patients with PTC, 18 samples of contralateral normal thyroid tissues, and 5 cases of PHEO were included in this study. *RET* isoform expression was studied by real-time RT-PCR.

Results All MTCs and PHEOs were positive for *RET9* and *RET51*. Fourteen/eighteen (77.7%) PTC cases were positive for *RET9* and/or *RET51*, and four were positive for only one of the genes. In normal thyroid tissues, 3/18 (16.7%) cases were negative for both isoforms, 4/18 (22.2%) were positive for both, and 11/18 (61.1%) were positive for only one. *RET* isoforms were expressed at different levels in MTC, PHEO, PTC, and normal thyroid tissues: *RET9* expression was higher in PHEO than in MTC, PTC, and normal thyroid tissues. *RET9* expression was also higher in MTC than in PTC and normal thyroid tissues. No difference was observed between PTC and normal thyroid tissues. A similar pattern of expression was observed for *RET51*. In addition, *RET51* was significantly more expressed than *RET9* in MTC, while *RET9* was the predominant isoform in PHEO.

Conclusions Our study documented the expression of the *RET9* and *RET51* isoforms in normal thyroid and PTC tissues. *RET9* and *RET51* isoforms were also present in MTC and PHEO. *RET51* expression was higher than *RET9* expression in MTC, while there was no difference in the expression of these two isoforms in PTC and normal thyroid tissues. *RET9* was more highly expressed than *RET51* in PHEOs.

Keywords *RET* · Medullary thyroid carcinoma · Papillary thyroid carcinoma · Normal thyroid · Isoforms

Introduction

The *RET* gene encodes a transmembrane tyrosine kinase receptor whose ligands belong to the glial cell-line-derived neurotrophic factor (GDNF) family ligands (GFLs) [1, 2]. These ligands include GDNF, neurturin (NRTN), artemin (ARTN), and persepin (PSPN). Activation of the *ret* receptor promotes cell growth, proliferation, and survival [3]. Northern blot experiments have demonstrated that the *RET* oncogene is expressed in the adrenal medulla, sporadic and familial pheochromocytomas (PHEO), medullary thyroid carcinoma (MTC) and, more generally, in tissues derived from the neural crest [4]. To a lesser extent, the expression of the *RET* oncogene has also been demonstrated in papillary thyroid carcinoma (PTC), as well as follicular thyroid adenoma, follicular carcinoma, and poorly differentiated thyroid tumors [5–7].

The *RET* gene is alternatively spliced at the 3′ end to produce two major isoforms: *RET9* and *RET51*. As shown in Fig. 1, the *RET9* isoform is shorter than the *RET51* isoform, as it is lacking exons 20 and 21 and has a shorter C-terminal tail (i.e., 9 amino acids versus 51 amino acids) [8, 9]. A third isoform, *RET43*, has also been reported,

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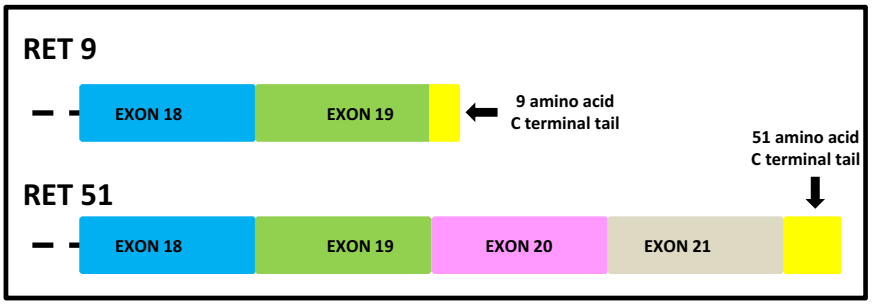

Fig. 1 Schematic representation of the *RET9* and *RET51* isoforms: *RET9* is shorter than *RET51*, as it is missing exons 20 and 21 and has a shorter C terminal tail (i.e., 9 amino acids versus 51 amino acids)

although no evidence of its translational protein product has been provided so far [10]. The *RET9* and *RET51* isoforms are the most highly expressed in human tissues and are usually coexpressed, but *RET9* is commonly present at greater levels than *RET51* [8, 11]. The *RET9* and *RET51* isoforms have been demonstrated to generate differential patterns of gene expression, induce different degrees of cell differentiation and transformation, and play unique roles in human development [12]. Moreover, the *RET9* and *RET51* isoforms are characterized by different biochemical and biological properties, and, consequently, they play distinct roles in tumorigenesis and/or development. In particular, by using shRNA-mediated knockdowns models, a prominent role for the *RET51* isoform in promoting cell survival and the mesenchymal phenotype was demonstrated [13]. So far, scant data exist concerning the expression of these two isoforms in MTC and PTC, and it is unknown which of the two *RET* isoforms is predominantly expressed and contributes more to the development of these tumors.

The aim of this study was to determine the relative expression of the *RET9* and *RET51* isoforms in MTC, PTC, normal thyroid, and PHEO. Moreover, we compared *RET9* and *RET51* isoform levels of expression among samples according to their histotype.

Materials and methods

Samples

Nineteen MTC tissues, 18 PTC tissue, and 18 samples of normal thyroid tissues contralateral to a benign thyroid disease, to avoid any possible malignant cell contamination, were included in this study. Fifteen MTC cases were sporadic, and four were hereditary since the patients were carryng a germline *RET* mutation. Five cases of PHEO belonging to MEN 2A families were also included in this study. Adipose tissue from an obese patient subjected to gastric resection was used as a negative control as derived from the UniGene database (http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Hs.350321), and the TT cell line was used as a positive control. All specimens used in the study were fresh frozen tissues collected at surgery and immediately frozen in liquid nitrogen. Tumoral tissues have been sampled by an experienced pathologist. The histological diagnosis of the tumor confirmed in all cases the malignant nature of collected tissues and the minimal/negligible contamination of normal tissue.

All patients provided written informed consent for the collection and use of their thyroid tissues for the purpose of this study. This study was approved by the Institutional Review Board.

RNA extraction and reverse transcription

The total RNA was extracted from tissues using TRIzol reagent lysis buffer (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. cDNA was prepared using SuperScript™ IV VILO™ and 500 ng of RNA, and genomic DNA was digested using the ezDNAse enzyme a 37° for 2 min.

Quantitative analysis of *RET* isoforms

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to analyze the expression levels of *RET* isoforms with the SsoAdvancedTMUniversal SYBR®Green Supermix (Bio-Rad), and was performed with the BIO-RAD CFX96 instrument. Primers were specifically designed to distinguish the two isoforms. For *RET9*, the forward primer was located on exon 18 (5′->3′: AAGACCTGGAGAAGATGATG), and the reverse primer was on exon 19 (5′->3′: AGTAAATGCATGGGAAATTCT). This amplicon specifically contained the fragment that characterizes this isoform. For *RET51*, the forward primer was located on exon 19 (5′->3′: CTGGTGGACTGTAATAATGC), and the reverse primer was on exon 20 (5′->3′: TTGGATATCTTGGAAACCCA). The most specific and reproducible amplification results were obtained with a melting temperature of 62 °C for 15 min of annealing. Real-time RT-PCR experiments have been performed in triplicates. Only triplicates in which the difference in the Ct value was lower than 0.5 have been accepted; whenever this difference was higher, the experiment has been repeated.

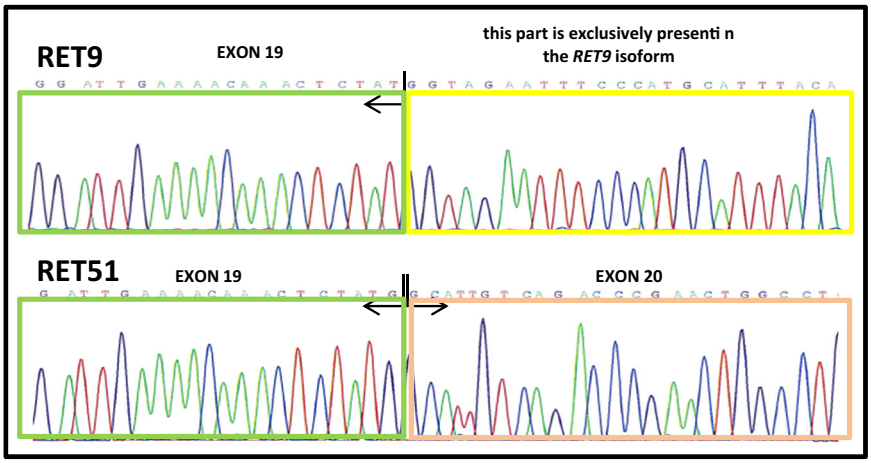

Fig. 2 Electropherograms showing *RET9* and *RET51* sequences obtained by using specifically designed primers to generate two different amplicons. The two isoforms share exon 19 (left); *RET51* has exon 20, while *RET9* has a specific tail

The efficiency, reproducibility, and dynamic range of the SYBER Green I assay were determined by constructing a standard curve using serial dilutions of the TT cDNA. The efficiency of the assay was included in the range of 90–105%, and R^2 was >0.98, with an efficiency $E = -3.2$. The Ct values of the replicates were similar.

The *G6PDH* housekeeping gene was selected to normalize the expression levels of *RET*, because the GTEx-Portal (https://www.gtexportal.org/home/gene/G6PD) shows that it is similarly expressed in different types of endocrine tissues. mRNA gene expression was determined using the ΔCt method [ΔCt = Ct (RET isoform)–Ct (*G6PDH*, ubiquitous referral gene)], in which Ct is the threshold cycle for the quantitative real time RT-PCR. The $2^{-\Delta\Delta Ct}$ (ΔΔCt = ΔCt RET−ΔCt RET adipose tissue) method was applied to analyze the relative changes in gene expression compared with the calibrator that was represented by the adipose tissue. $2^{-\Delta\Delta Ct}$ values ≤1 indicated that *RET* isoforms had a lower expression with respect to the adipose tissue and since this tissue is negative, these cases were considered negative too; $2^{-\Delta\Delta Ct}$ values >1 indicated variable degrees of *RET* isoform expression higher than that observed in the adipose tissue (calibrator) and then considered as positive.

Statistical analysis

Statistical analyses were performed using SPSS (version 21; Armonk, NY: IBM Corp.). Alpha was set at 0.05. The data are presented as the mean ± SD or frequency (percentage). Chi-square test was used to evaluate differences in counts and frequency between isoforms. Paired Student's *t* test was used to test differences in gene expression between the *RET9* and *RET51* isoforms within the same group of patients, while ANOVA was used to evaluate differences in gene expression among groups. Pairwise post hoc comparisons were performed using the Tukey–Kramer method. Variables with skewed distribution were log-transformed before parametric analyses to approximate a normal distribution. Levene's test was used to assess the homogeneity of variances between groups and, in the case of heterogeneous variances, Welch's ANOVA was performed in place of ANOVA.

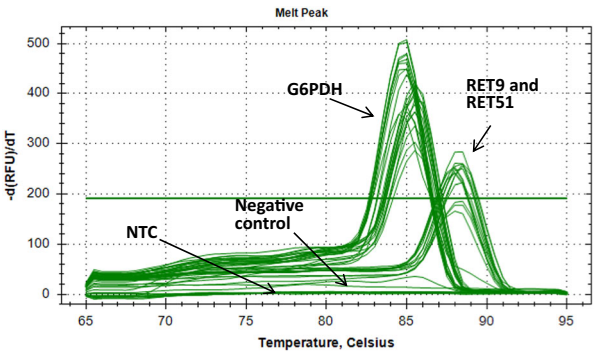

Fig. 3 Melting curves of the SYBER Green I amplification for both *RET* isoforms and G6PDH. A single peak was obtained, indicating very good performance of the qRT-PCR assay

Results

Amplification of the *RET9* and *RET51* isoforms

As shown in Fig. 2, by using specifically designed primers for the amplification of *RET* isoforms, we were able to generate two different amplicons corresponding to the *RET9* and *RET51* isoforms. As shown in Fig. 3, we also obtained single peaks in the melting curves of the SYBER Green I amplification for both *RET* isoforms with the same primers, indicating very good performance of the qRT-PCR assay.

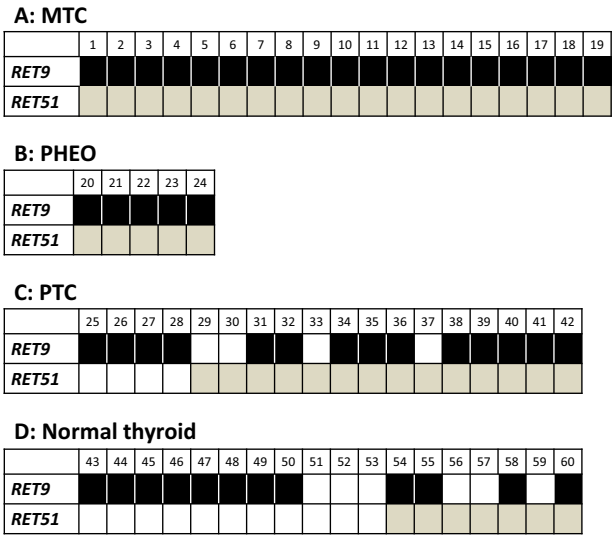

Fig. 4 *RET9* (black squares) and *RET51* (gray squares) positive cases in MTC (**a**), PHEO (**b**), PTC (**c**), and normal thyroid tissues (**d**). MTC and PHEO tissues were positive for both *RET9* and *RET51*. Fourteen/18 PTC cases were positive for *RET9* and/or *RET51*, and four were positive for only one. In normal thyroid tissues, 3/18 (16.7%) cases were negative for the two isoforms, 4/18 (22.2%) were positive for both, and 11/18 (61.1%) were positive for only one. PTC samples and normal thyroid tissue samples did not belong to the same patients

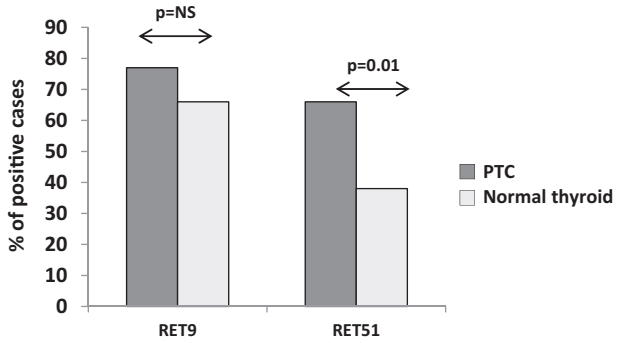

Fig. 5 Comparison between the prevalence of *RET9*- and *RET51*-positive cases in PTC and normal thyroid tissues. No statistically significant difference was observed in the prevalence of *RET9*- and *RET51*-positive cases, while *RET51*-positive cases were higher in PTC than in normal thyroid tissues

Expression analysis of the *RET* isoforms

According to the $2^{-\Delta\Delta Ct}$ analysis, positive and negative cases were identified. As shown in Fig. 4 (panels a and b), all MTC and PHEO tissues were positive for both *RET9* and *RET51*. In the PTC group (Fig. 4c), 14/18 (77.7%) cases were positive for *RET9* and/or *RET51*. Specifically, 10/18 (55.6%) were positive for both isoforms, while the others were positive for only one of the isoforms; 4/18 (22.2%) were positive for *RET9*, and 4/18 (22.2%) were positive for *RET51*. In the normal thyroid group (Fig. 4d), 3/18 (16.7%) cases were negative for both isoforms, 8/18 (44.5%) were positive only for *RET9*, 3/18 (16.7%) were positive only for *RET51*, and only 4/18 (22.2%) were positive for both isoforms. When comparing the prevalence of *RET9* and *RET51* positive cases, we observed slightly different distributions of the two isoforms, with a significantly higher prevalence of *RET51* positive cases in PTC than in normal thyroid tissues (Fig. 5).

The two *RET* isoforms were also expressed at different levels in MTC, PHEO, PTC, and normal thyroid tissues ($p < 0.001$). As shown in Fig. 6a, *RET9* isoform expression was, on average, relatively higher in PHEO than in MTC, PTC, and normal thyroid tissues. Level of *RET9* expression was also relatively higher in MTC than in PTC and normal thyroid (all adj. $p < 0.05$). No statistically significant difference was observed between PTC and normal thyroid tissues (adj. $p > 0.05$). A similar pattern of expression levels was observed for *RET51* (Fig. 6b).

The expression levels of the *RET9* and *RET51* isoforms were compared between each of the four groups of tissues. As shown in Fig. 7a, on average, *RET51* was significantly more expressed than *RET9* in MTC (fold difference = 2.8, 95% CI: 1.6 to 5.1, $p = 0.002$), while *RET9* was the predominant isoform in PHEO (fold difference = 4.7, 95% CI: 1.8 to 12.8, $p = 0.01$) (Fig. 7b). *RET51* (mean value of $2^{-\Delta\Delta Ct} = 226.4$) was more expressed than *RET9* (mean value of $2^{-\Delta\Delta Ct} = 146.8$) also in PTC, but this difference was not statistically significant. No differences were observed in the expression levels of the two isoforms in normal thyroid tissues (data not shown).

Correlation between *RET* isoforms expression and somatic mutations in MTC

In our series of 19 MTC cases, 4/19 carried a germline *RET* mutation (1 M918T, 1 C634R, 1 C634S, and 1C618S), 5/19 had a somatic *RET* mutation (three M918T, two C634R), 4/19 had a *HRAS* somatic mutation and 6/19 had no mutations. As shown in Table 1, not mutated MTC samples had a significantly higher expression of *RET51* isoform with respect to *RET9* isoform ($p = 0.04$). No difference was observed in the two RET isoform expression levels in *HRAS*- and *RET*-positive cases.

Discussion

The *RET* oncogene is involved in the pathogenesis of both MTC and PTC, although with different mechanisms of activation [14]. The expression of *RET* is not ubiquitous, but is present in tissues derived from the neural crest [4]. A

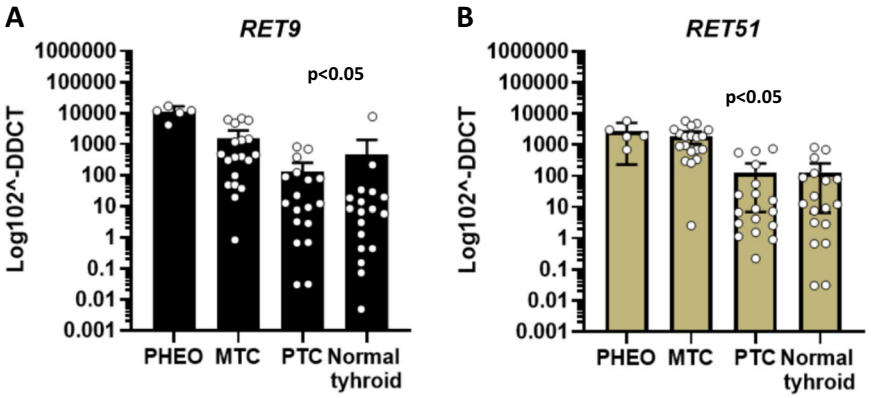

Fig. 6 *RET9* (**a**) and *RET51* (**b**) mRNA expression levels expressed as $\log_{10}2^{\Delta\Delta Ct}$. *RET* isoform expression is higher in PHEO than in MTC, PTC, and normal thyroid tissues, and was relatively higher in MTC than in PTC and normal thyroid tissues. No statistically significant difference was observed between PTC and normal thyroid tissues. Circles indicate a single case

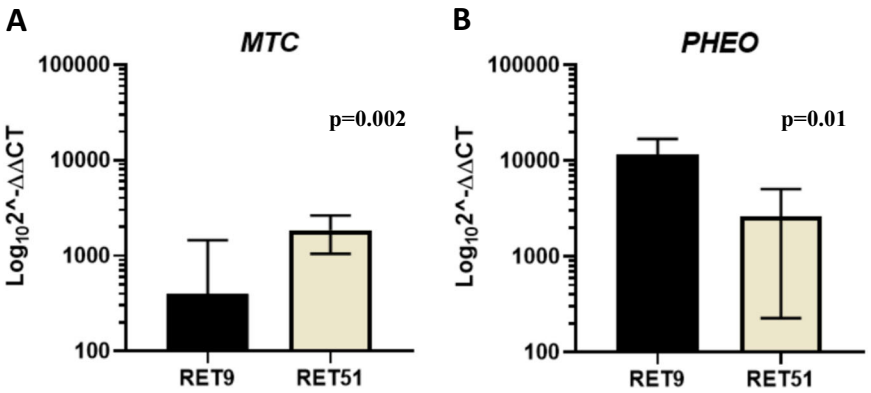

Fig. 7 Comparison of *RET9* and *RET51* isoform expression levels in MTC (**a**) and PHEO (**b**). *RET51* was significantly more highly expressed than *RET9* in MTC, while *RET9* was the predominant isoform in PHEO

Table 1 Correlation between RET9 and RET51 expression and somatic mutation profile

	Sex	Type of tissue	Somatic mutation	Germline mutation	$2^{-\Delta\Delta Ct}$ RET 9	$2^{-\Delta\Delta Ct}$ RET 51	*P*
974	M	Primary	HRAS G13R		1323	689	
144	F	Primary	HRAS Q61R		5873	5792	NS
1075	F	Primary	HRAS Q61R		6793	2256	
703	M	Primary	HRAS Q61R		474	1734	
			Mean value		**3615.75**	**2617.75**	
1049	F	Primary	NM		19	604	
584	F	Primary	NM		458	1871	**0.04**
879	F	Primary	NM		37	257	
1031	M	Primary	NM		48	704	
1050	F	Primary	NM		380	3125	
1292	M	Primary	NM		398	948	
			Mean value		**223.33**	**1251.5**	
1212	M	Primary	RET M918T		4804	3019	
509	M	Primary	RET M918T		95	296	
991A6	F	Primary	RET C634R		300	897	
2052	M	Primary	RET C634R		48	335	NS
2125	M	Primary	RET M918T		0,84	2.5	
2252	M	Primary		RET C634S	300	4067	
1615	M	Primary		RET C634R	1184	1897	
2149	F	Primary		RET M918T	1448	1807	
2113	M	Primary		RET C618S	6165	4770	
			Mean value		**1593.88,** $p = \text{NS}$	**1898.94,** $p = \text{NS}$	

slight expression of *RET* has also been demonstrated in follicular thyroid cells [13, 15]. Two major isoforms, *RET9* and *RET51*, are reportedly expressed in MTC, PHEO, and PTC, but little data concerning their level of expression have been reported so far [11, 13, 16].

In this study, we demonstrated that *RET9* and *RET51* are expressed both in normal follicular cells and in PTC, as well as in MTC and PHEO. However, while these isoforms were present in all MTC and PHEO tissues examined, the two isoforms can be expressed either simultaneously or not in both PTC and normal tissues. In particular, cases completely negative for the expression of both isoforms were present only in normal thyroid tissues. For a more accurate analysis, mRNA expression studies should be completed with the analysis at tissue level of the protein expression (i.e., immunohistochemistry and/or western blot). Unfortunately, we had the big limit of the amount of fresh tissue that was not enough for performing protein expression experiments. However, the protein analysis was recently performed by Lian et al. [13] who demonstrated that the two isoforms were expressed, although at different levels, in all MTC, PTC, and benign thyroid lesions. When comparing our results with those of Lian et al., we found some controversial data likely due to the different aims of the studies since they concentrated their attention on the levels of *RET* isoforms expression in different hystotypes of thyroid cancer and in benign thyroid tissues. At variance with us, they found that all cases were positive for *RET* isoforms expression. Scanty data are available on wild-type *RET* expression, and doubts about the possibility that positive cases could be due to C cell contamination are present [15]. However, our normal thyroid tissues were not all positive and, in the majority of cases, were positive either for *RET9* or *RET51*, making C cell contamination unlikely.

According to the number of positive *RET9* and/or *RET51* cases, we found that *RET51* was significantly more frequently expressed in PTC than in normal thyroid tissues. This finding is in agreement with the distinct roles of the two isoforms since *RET51* more effectively enhances cell proliferation and motility, as well as maintains a more mesenchymal phenotype than *RET9* [12, 13]. *RET51* is also characterized by greater transforming potential [17, 18].

Our study also demonstrated that the two *RET* isoforms were expressed at different levels in MTC, PHEO, PTC, and normal thyroid tissues. Higher expression levels for both isoforms were found in PHEO tissues, which were higher than those in MTC and PTC. The lowest levels were observed in normal thyroid tissues. This observation is in agreement with the findings of Lian et al. [13], who obtained their results by immunohistochemistry, while we measured mRNA expression. Although mRNA expression is not necessarily correlated with protein expression, the combination of our results with those of Lian et al. [13] strongly support the evidence that both isoforms of wild-type *RET* are expressed in normal thyroid tissues and, to a much higher level, in tumor tissues, particularly those derived from C cells. In agreement with the evidence of a greater transforming ability for *RET51* than *RET9* [17, 18], when we compared the expression levels of the two isoforms within the same tumor type, we found that *RET51* was significantly more highly expressed than *RET9* in MTC, but not in PTC or normal thyroid tissues. In general, MTC is more aggressive than PTC, and greater levels of *RET51* expression can be hypothesized as at least one of the concurrent causes for this aggressiveness. Moreover, *RET51* expression was found to be higher than *RET9* expression in not mutated tumors, suggesting the hypothesis that a higher expression of the more aggressive *RET* isoform could represent a driver event in MTC tumorigenesis. This hypothesis requires to be confirmed in a larger series of MTC cases.

This study demonstrated that all our cases of PHEO were positive for the expression of both *RET* isoforms. This finding is in line with data from Le Hir et al. [11], who analyzed a large number of PHEOs that were either sporadic or familial. These authors concluded that the familial forms of PHEO exhibited higher expression of *RET51* than the sporadic forms. We cannot make this comparison because our cases were all familial in nature with MEN 2 A. Our data demonstrated that in this small series of PHEOs, the *RET9* isoform was significantly expressed at higher levels than the *RET51* isoform. By carefully examining the data from Le Hir et al. [11], we observed that even in those cases, both sporadic and familial, *RET9* expression levels were higher than *RET51* expression levels. The majority of PHEO cases have a good clinical course and no malignant features [19, 20]. In line with this, our PHEOs were not histologically malignant, and the treated patients were all cured.

In conclusion, our study documents the mRNA expression of *RET* isoforms in a subgroup of normal thyroid tissues, in all PTCs (although some were positive for only one of the two isoforms), and in all cases of MTC and PHEOs. *RET51* was more frequently expressed in PTC than in normal thyroid tissues. *RET51* expression was also higher than *RET9* expression in MTC and particularly in *RET/RAS* wt cases, suggesting a possible pathogenic role of this isoform through its increased expression. Finally, *RET9* was more highly expressed than *RET51* in familial PHEOs.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest that could affect the impartiality of the reported research.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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