




Article

Higher *RET* Gene Expression Levels Do Not Represent an Alternative *RET* Activation Mechanism in Medullary Thyroid Carcinoma

Chiara Mulè¹, Raffaele Ciampi¹, Teresa Ramone¹, Alessandro Prete¹, Antonio Matrone¹ , Virginia Cappagli¹ , Liborio Torregrossa², Fulvio Basolo², Rossella Elisei^{1,*}  and Cristina Romei¹

¹ Endocrine Unit, Department of Clinical and Experimental Medicine, University of Pisa, 56124 Pisa, Italy; chiara.mulè@gmail.com (C.M.); raffaele.ciampi@unipi.it (R.C.); teresa.ramone@med.unipi.it (T.R.); alessandro.prete22@gmail.com (A.P.); anto.matrone@yahoo.com (A.M.); virgicap@hotmail.com (V.C.); cristina.romei@unipi.it (C.R.)

² Department of Surgical, Medical, Molecular Pathology, University of Pisa, 56124 Pisa, Italy; libo.torregrossa@gmail.com (L.T.); fulvio.basolo@unipi.it (F.B.)

* Correspondence: rossella.elisei@med.unipi.it; Tel.: +39-050995120

Abstract: This study was designed to investigate whether *RET* (rearranged during transfection) mRNA over-expression could be considered an alternative driver event for the development of medullary thyroid carcinoma (MTC), and if different *RET* isoforms could play a role in MTC tumorigenesis. Eighty-three MTC patients, whose mutational profile was previously identified by next-generation sequencing (NGS) IONS5, were included in this study. Expression analysis was performed by the quantitative reverse transcription-polymerase chain reaction technique. *RET* expression levels were found to be significantly higher in cases with *RET* somatic mutations than in cases that were negative for *RET* somatic mutations ($p = 0.003$) as well as in cases with a somatic mutation, either in *RET* or *RAS* than in cases negative for both these mutations ($p = 0.01$). All cases were positive for the *RET51* isoform expression while only 72/83 (86.7%) were positive for *RET9* isoform expression. A statistically significant higher expression of the *RET51* isoform was found in cases positive for *RET* somatic mutation than in cases either positive for *RAS* mutation ($p = 0.0006$) or negative for both mutations ($p = 0.001$). According to our data, *RET* gene over-expression does not play a role in MTC tumorigenesis, neither as an entire gene or as an isoform. At variance, the *RET* gene, and in particular the *RET51* isoform, is expressed higher in *RET* mutated cases. On the basis of these results we can hypothesize that the overexpression of *RET*, and in particular of *RET51*, could potentiate the transforming activity of mutated *RET*, making these cases more aggressive.

Keywords: *RET*; *RAS*; medullary thyroid carcinoma; mRNA expression



Citation: Mulè, C.; Ciampi, R.; Ramone, T.; Prete, A.; Matrone, A.; Cappagli, V.; Torregrossa, L.; Basolo, F.; Elisei, R.; Romei, C. Higher *RET* Gene Expression Levels Do Not Represent an Alternative *RET* Activation Mechanism in Medullary Thyroid Carcinoma. *Biomolecules* **2021**, *11*, 1542. <https://doi.org/10.3390/biom11101542>

Academic Editor: Vladimír N. Uversky

Received: 2 September 2021
Accepted: 14 October 2021
Published: 19 October 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

The rearranged during transfection (*RET*) proto-oncogene is localized on chromosome 10q11.2 and was first identified in 1985 based on its ability to transform NIH3T3 cells [1]. The *RET* proto-oncogene encodes for a transmembrane tyrosine kinase receptor involved in the control of cell differentiation and proliferation [2]. As well as other growth factor receptors, the *RET* gene may be involved in the development of human cancers through different activating mechanisms [3]. Activating gain of function mutations are specifically related to medullary thyroid carcinoma (MTC) [4], while *RET* gene rearrangements have been reported in papillary thyroid carcinoma (PTC) [4], in lung cancer [5], and chronic myelomonocytic leukemia [6]. Finally, overexpression of the *RET* gene has been demonstrated in the most aggressive estrogen receptor-positive breast cancer [7] and in the more advanced forms of pancreatic [8] and prostate cancer [9].

The *RET* proto-oncogene is subjected to alternative splicing that gives origin to three functional isoforms: *RET51*, *RET9*, and *RET43* [10,11]. Studies on animal models

showed that *RET9* is expressed in several human tissues while *RET51* is only expressed in some of them [12]. Moreover, when compared, *RET9* expression has been found to be higher than *RET51* expression [13]. Conversely, *RET51* isoform expression has been reported to be higher in MTC than in PTC [14], in more aggressive forms of pancreatic cancer [15], and in pheochromocytoma [16], suggesting a specific role of this isoform in determining the aggressiveness of a tumor. As a matter of fact, the two isoforms are characterized by different biochemical and biological properties, and, consequently, they play distinct roles in tumorigenesis and/or development [17].

MTC arises from thyroid parafollicular C cells. Its overall incidence is about 0.2–0.8/100,000 people [18] and accounts for about 5% of all thyroid carcinomas. MTC can be inherited (25%) as part of the multiple endocrine neoplasia type 2, or sporadic (75%). The pathogenesis of this tumor is related to activating *RET* mutations that are germline in hereditary cases (approximately 98% of cases) and somatic in sporadic cases (approximately 45% of cases) [19,20]. The genetic landscape of sporadic MTC has been deeply studied, and somatic mutations in the *RET* gene are the major events in its tumorigenesis accounting for up to 80% of cases [21–23]. *H-RAS* (Harvey rat sarcoma virus) and *K-RAS* (Kirsten rat sarcoma virus) mutations are indeed present in about 10–20% of cases and are almost invariably mutually exclusive with *RET* mutations [21,24]. Only other rare genetic alterations have been reported, thus 20–40% of MTC cases are still orphans of a driver mutation [21–23].

The primary objective of the present study was to investigate whether *RET* gene over-expression, a mechanism of *RET* activation different from activating mutation, could be considered an alternative driver event for the development of MTC. As a secondary objective, we also evaluated the expression levels of the two *RET* isoforms (*RET9* and *RET51*) according to the mutational profile.

2. Materials and Methods

2.1. Patients

Eighty-three MTC patients were included in this study. Tissues were collected at surgery, immediately frozen in liquid nitrogen, and stored at -80°C . All samples were previously analyzed by next-generation sequencing (NGS) IONS5, as previously described [21], and the mutational profile of our 83 cases was used to define the groups to be analyzed in the present study. In particular, we distinguished the 3 groups: cases with a somatic *RET* mutation (*RET+*), cases with a somatic *RAS* mutation (*RAS+*), and cases that were negative for *RET* and *RAS* mutations (*RET-* and *RAS-*).

All patients gave their consent to the study that was also approved by the Internal Reviewing Board.

2.2. Expression Analysis

RNA was extracted from fresh tissues using the TRIzol reagent lysis buffer (Invitrogen, Carlsbad, CA, USA) according to the protocol suggested by the manufacturer. Total RNA was quantified using the Qubit RNA HS Assay. cDNA was obtained by reverse transcription using the SuperScript IV VILO and 100 ng of total RNA in a final volume of 20 μL . The amplification of a house-keeping gene (Glucose-6-Phosphate-dehydrogenase, G6PD) was used to verify the quality of cDNA.

To analyze the *RET* gene and *RET* gene isoform expression levels, we used the quantitative reverse transcription-polymerase chain reaction (qRT-PCR) technique with SsoAdvanced SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). All reactions were performed with the BioRad CFX96 instrument.

Primers for the quantitative amplification of the *RET* tyrosine kinase domain were designed using the Primer3 software: (forward 5' \rightarrow 3' AACATCCTGGTAGCTGAGGG and reverse 5' \rightarrow 3' CAGCAGGACACCAAAGACC) and were respectively located on exons 15 and 17. The efficiency and reproducibility of the primers were tested by a standard curve using a serial dilution of TT cDNA. The efficiency of the *RET* TK assay was $E = 97.3\%$,

R^2 was 0.996, and the slope was -3.38 . *RET9* and *RET51* isoforms were amplified using primers and conditions previously reported [14]. qRT-PCR reactions were performed in duplicate. Ct values of the replicates were similar (difference ≤ 0.5).

The *G6PD* housekeeping gene was used to normalize the *RET* gene expression level and its isoforms. It is not easy to find a reference tissue for MTC because normal thyroid tissue is not a counterpart of MTC, so $2^{-\Delta\Delta Ct}$ is not applicable. This is the reason for which we decided to use the ΔCt analysis, a well recognized method for the analysis of the relative expression of genes. Gene expression level was calculated with the ΔCt method ($\Delta Ct = Ct_{RET} - Ct_{G6PD}$), where Ct is the threshold cycle for qRT-PCR. The lowest is the ΔCt value, and the highest is the mRNA expression level.

2.3. Statistical Analysis

Statistical analyses were performed with the Statview 5.0 Program using the Chi-squared test, 1-way ANOVA, and unpaired Student's *t*-test. Differences were considered statistically significant when the *p*-value was less than 0.05.

3. Results

3.1. *RET* Gene Expression

Eighty-three samples previously analyzed by NGS for their mutation profile [21] were analyzed by qRT-PCR for *RET* gene expression. According to those data, cases with a *RET* somatic mutation (*RET+*, $n = 39$), cases with a *RAS* somatic mutation (*RAS+*, $n = 20$) and cases with no *RET* or *RAS* mutation (*RET-* or *RAS-*, $n = 24$). *RET* gene expression was detected in all cases regardless of the presence or absence of the mutations, although at different levels. As shown in Figure 1, a statistically significant higher expression of the *RET* gene was found in *RET+* cases than in *RET-* and *RAS-* cases ($p = 0.005$). Although not statistically significant, *RET* gene expression was found to be higher in *RET+* cases with respect to *RAS+* cases. Similarly, *RAS+* cases had a higher level of *RET* gene expression with respect to *RET-* and *RAS-* cases.

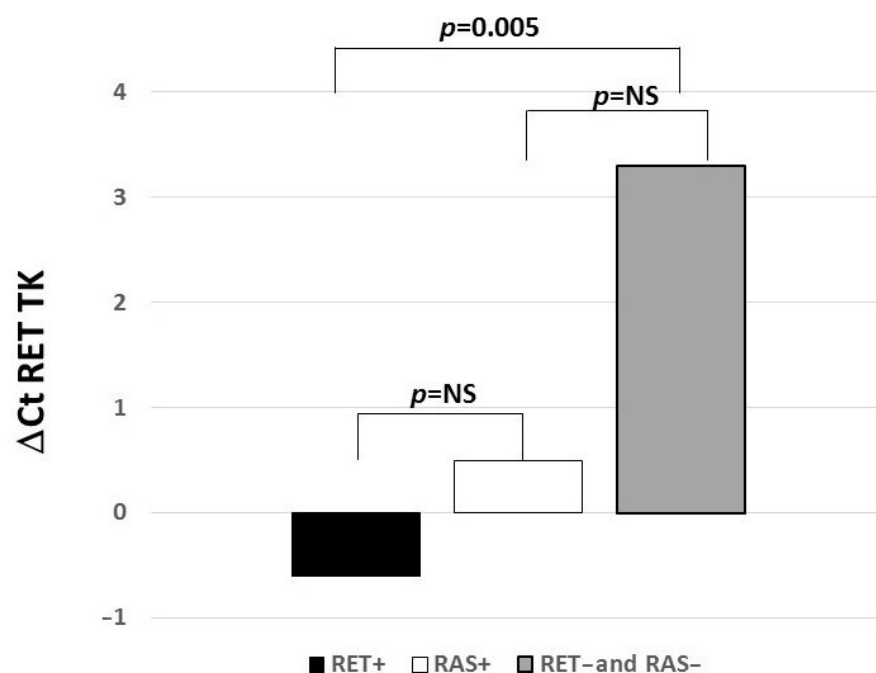


Figure 1. *RET* gene expression levels according to the somatic mutation profile. A statistically significant higher expression level was observed in *RET+*. Gene expression levels are reported as ΔCt ($\Delta Ct = Ct_{RET} - Ct_{G6PD}$) where Ct is the threshold cycle for qRT-PCR. The lowest is the ΔCt value, and the highest is the mRNA expression level.

RET gene expression levels were found to be significantly higher in *RET*⁺ cases with respect to all other cases (i.e., *RET*[−] and *RAS*⁺) ($p = 0.003$) (Figure 2A). Similarly, cases positive for *RET* or *RAS* (*RET*⁺ or *RAS*⁺) showed a higher level of *RET* gene expression with respect to cases negative for both mutations (*RET*[−] and *RAS*[−]) ($p = 0.01$) (Figure 2B).

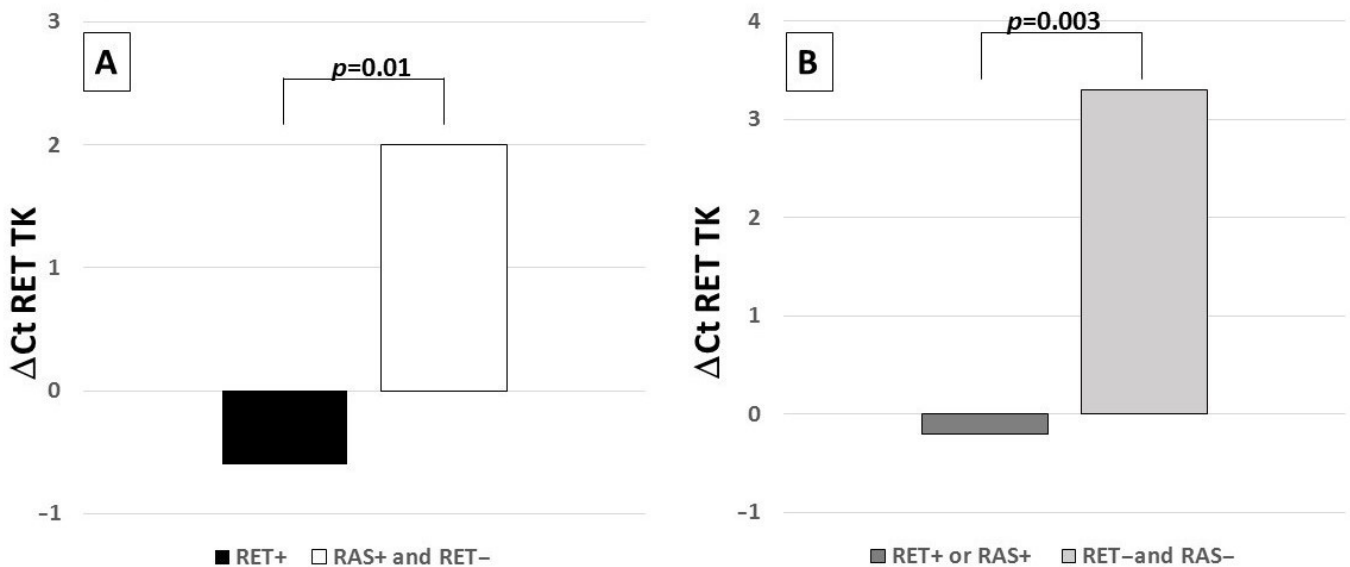


Figure 2. (A): *RET* gene expression levels according to the somatic mutation profile. A statistically significant higher *RET* gene expression level was observed in *RET*⁺ cases with respect to *RET*[−] and *RAS*⁺ cases. (B): gene expression levels according to the somatic mutation profile. A statistically significant higher *RET* gene expression level was observed in *RET*⁺ or *RAS*⁺ cases with respect to cases negative for both gene alterations (*RET*[−] and *RAS*[−]).

Expression levels are reported as ΔCt ($\Delta Ct = Ct_{RET} - Ct_{G6PD}$) where *Ct* is the threshold cycle for qRT-PCR. The lowest is the ΔCt value, and the highest is the gene expression level.

3.2. *RET51* and *RET9* Expression

As shown in Table 1, all cases (83/83, 100%) showed a *RET51* isoform expression while only 72/83 (86.7%) showed a *RET9* isoform expression. In the whole series, *RET51* expression levels were significantly higher ($p < 0.0001$) than *RET9* expression levels.

Table 1. *RET9* and *RET51* isoforms expression in our series of 83 MTC cases.

<i>RET</i> Gene Expression	<i>RET9</i> n(%)	<i>RET51</i> n(%)	<i>p</i>
Pos expression	72 (86.7)	83 (100)	Not applicable
Neg expression	11	0	
ΔCt *	4.9	1.8	<0.0001

* Expression levels are reported as ΔCt ($\Delta Ct = Ct_{RET} - Ct_{G6PD}$) where *Ct* is the threshold cycle for qRT-PCR. The lowest is the ΔCt value, and the highest is the mRNA expression level.

As shown in Figure 3, a statistically significant higher expression of the *RET51* isoform was found in *RET*⁺ cases than in *RAS*⁺ cases ($p = 0.0006$) and *RET*[−] and *RAS*[−] cases ($p = 0.001$). No difference in the *RET51* isoform expression levels was found when comparing *RAS*⁺ cases and *RET*[−] and *RAS*[−] cases.

As shown in Figure 4A, a statistically significant higher expression of the *RET51* isoform was found in *RET*⁺ cases than in *RET*[−] and *RAS*⁺ cases ($p = 0.0001$). *RET51* expression levels were found to be significantly higher in *RET*⁺ and *RAS*⁺ positive cases with respect to *RET*[−] and *RAS*[−] cases ($p = 0.006$) (Figure 4B). The *RET9* isoform expression levels were not different in *RET*⁺, *RAS*⁺, and *RET*[−] and *RAS*[−] MTC cases.

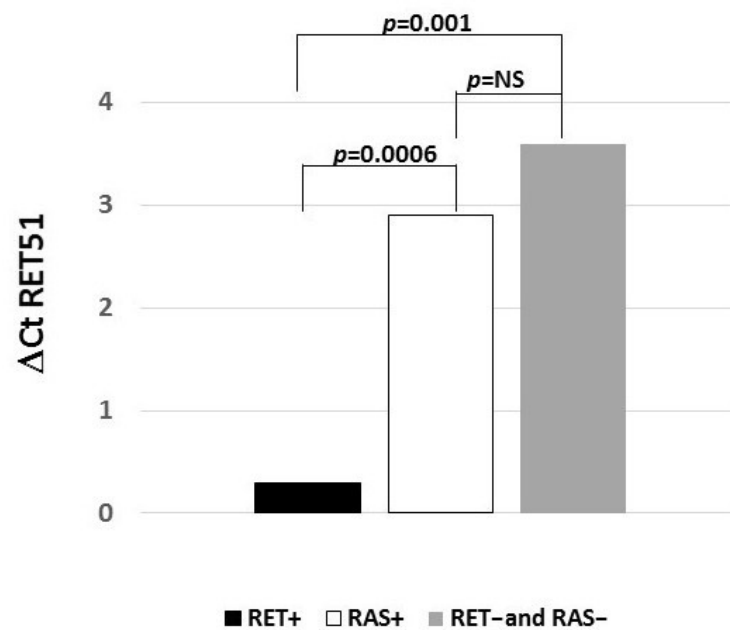


Figure 3. *RET51* isoform expression levels according to the somatic mutation profile. A statistically significant higher expression level was observed in *RET*⁺ cases than in *RAS*⁺ cases and *RET*[−] and *RAS*[−] cases. Gene expression levels are reported as ΔCt ($\Delta Ct = Ct_{RET} - Ct_{G6PD}$) where *Ct* is the threshold cycle for qRT-PCR. The lowest is the ΔCt value, and the highest is the mRNA expression level.

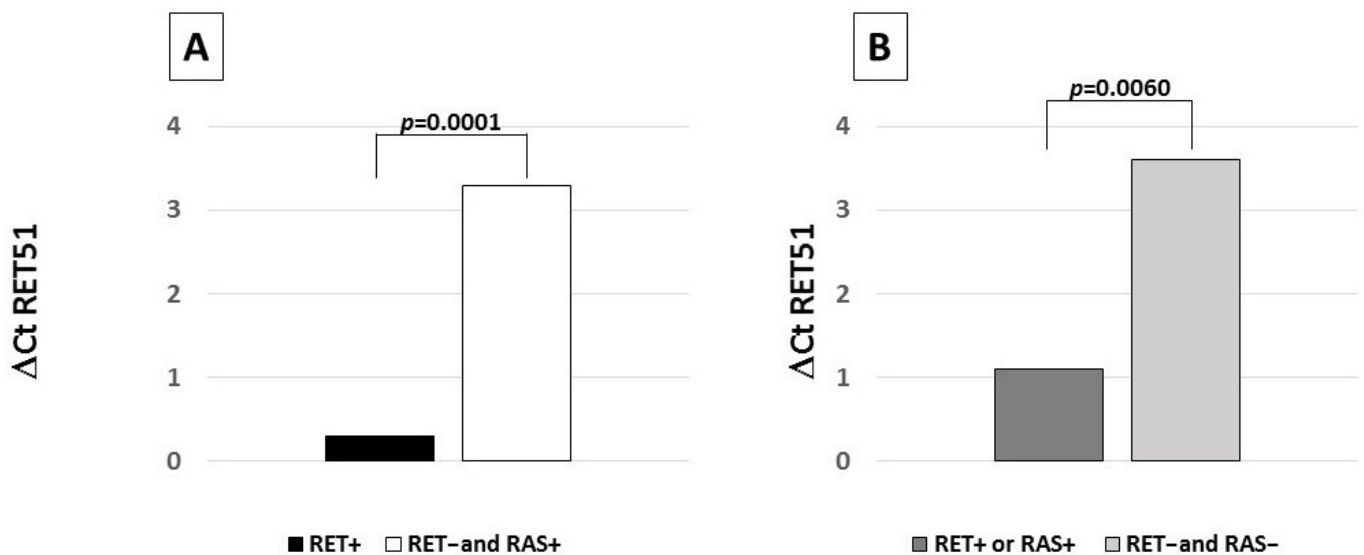


Figure 4. (A): *RET51* isoform expression levels according to the somatic mutation profile. A statistically significant higher *RET51* expression level was observed in *RET*-positive cases with respect to cases negative for a *RET* mutation. (B): *RET51* isoform expression levels according to the somatic mutation profile. A statistically significant higher *RET* gene expression level was observed in positive cases with respect to cases negative for both genes. Expression levels are reported as ΔCt ($\Delta Ct = Ct_{RET} - Ct_{G6PD}$) where *Ct* is the threshold cycle for qRT-PCR. The lowest is the ΔCt value, and the highest are the gene expression levels.

4. Discussion

The *RET* proto-oncogene encodes for a tyrosine kinase receptor involved in the control of cell proliferation and differentiation [4]. Oncogenic mutations that constitutively activate the ret receptor have been reported in human tumors. *RET* mutations involved in the

tumorigenesis processes can be classified as activating gain of function mutations, mainly in sporadic and hereditary MTC [25,26], and as gene rearrangements in PTC [26,27]. In addition to these alterations, increased expression of the wildtype *RET* is involved in the tumorigenesis and progression of some human tumors such as breast [7], pancreas [8], and prostate [9]. Recently, a higher *RET* gene expression has been observed also in MTC with respect to normal subjects [28]. In this study, we evaluated if an alternative mechanism of *RET* activation, such as overexpression, could be involved in MTC tumorigenesis, particularly in those cases that are still orphans of a driver mutation.

Using a qRT-PCR approach we demonstrated that *RET* gene expression levels are higher in MTC cases harboring a *RET* somatic mutation than in cases with a *RAS* somatic mutation or MTC cases without any of these mutations. These findings indicate that the increased rate of *RET* transcription and its higher expression cannot be considered as causative in *RET*– and *RAS*– cases. We recently demonstrated [29] that the prevalence of *RET* somatic mutations is higher in MTC of a larger tumor size, suggesting that the presence of this genetic alteration can induce a higher cell proliferation rate. According to these data, *RET*-mutated MTC cells could have a more active metabolic condition, thus also justifying higher levels of transcription. We could hypothesize that the over expression of *RET* mRNA can potentiate the transforming activity of *RET* mutations, thus concurring to the worse outcome of *RET*-mutated cases with respect to *RET*-negative cases and *RAS*-positive cases. *RET*-mutated tumors (both germline and somatic) show higher transcript levels of many epigenetic regulators than both *RET* wildtype and *RAS*-mutated MTC [30], and we can hypothesize that the higher *RET* gene expression could be due to the same mechanism.

Alternative splicing of the *RET* gene at the 3' end has been described to produce two major isoforms: *RET9* and *RET51* [10,11]. A *RET43* isoform has also been reported, although no evidence of its translational protein product has been provided so far [11]. The two isoforms are characterized by different biochemical and biological properties, and, consequently, they play distinct roles in tumorigenesis and/or development [17]. In particular, *RET51* more effectively enhances cell proliferation and motility as well as maintains a more mesenchymal phenotype than *RET9* [12,13] and is characterized by greater transforming potential [17,18]. In addition, previous studies using *RET* gene overexpression models have shown that *RET51* has a greater transforming potential compared to *RET9* [31–33]. In keeping with these observations, *RET51* isoform expression is higher in MTC than in PTC [14] and in the more aggressive forms of pancreatic cancer [15], suggesting a specific role of this isoform in determining the aggressiveness of a tumor. In the present series, we evaluated the expression of the two *RET* isoforms, and we correlated the expression levels with the mutation profile. Interestingly, we found that all investigated cases were positive for *RET51* expression while *RET9* expression was found in 72/83 MTC cases, thus suggesting a predominant role of the longer isoform in MTC tumorigenesis. This predominant role has been confirmed by the observation that *RET51* expression levels are higher than that of *RET9*.

We previously observed that overall *RET51* isoform is more expressed than *RET9* in MTC [14]. In the present study we demonstrated that *RET51* isoform expression is higher in MTC cases harboring a *RET* somatic mutation with respect to cases with either a *RAS* somatic mutation (i.e., *RAS*+) or any somatic mutation (i.e., *RET*– and *RAS*–). At variance, no different levels of expression were found when analysing *RET9* isoform expression. As reported by Le Hirt et al. [16] it is likely that, in tumors caused by *RET* mutations, the presence of higher amounts of the long isoform can confer a selective growth advantage as demonstrated by the evidence that, in PC12 cells, the *RET51* isoform displays a more prominent potential as compared to the corresponding *RET9* isoforms [31,34]. This is in line with our previous evidence that sporadic MTC cases with a *RET* somatic mutation show a more rapid growth rate with respect to not-mutated cases in which *RET51* isoform is less expressed [29].

5. Conclusions

In conclusion, according to our data, *RET* gene over-expression does not play a role in MTC tumorigenesis, neither as an entire gene or as isoforms. At variance, *RET* gene, and in particular the *RET51* isoform, is expressed higher in *RET*-mutated cases. Taking into consideration that the *RET51* isoform seems to be able to confer a selective growth advantage, our previous results, showing that *RET* mutated cases have a high percentage of the mutated allele and that the corresponding tumors are usually bigger than not-mutated cases, are further supported. Moreover, the overexpression of *RET* could potentiate the transforming activity of mutated *RET*, making these cases more aggressive.

Author Contributions: Conceptualization, C.M. and C.R.; Data curation, A.P., A.M. and V.C.; Methodology, R.C. and T.R.; Resources, L.T. and F.B.; Supervision, R.E. All authors have read and agreed to the published version of the manuscript.

Funding: This study has been supported by grants to R.E. from Associazione Italiana per la Ricerca sul Cancro (AIRC, Investigator grant 2018, project code 21790), Agenzia Italiana del Farmaco (AIFA, project code AIFA-2016-02365049).

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board (Comitato Etico Regionale per la Sperimentazione Clinica della Regione Toscana) protocol code 6714, 05/02/2019.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data available on request due to restrictions, e.g., privacy or ethical.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Takahashi, M.; Ritz, J.; Cooper, G.M. Activation of a novel human transforming gene, *ret*, by DNA rearrangement. *Cell* **1985**, *42*, 581–588. [[CrossRef](#)]
2. De Groot, J.W.B.; Links, T.P.; Plukker, J.T.M.; Lips, C.J.M.; Hofstra, R. *RET* as a Diagnostic and Therapeutic Target in Sporadic and Hereditary Endocrine Tumors. *Endocr. Rev.* **2006**, *27*, 535–560. [[CrossRef](#)] [[PubMed](#)]
3. Mulligan, L.M. GDNF and the *RET* Receptor in Cancer: New Insights and Therapeutic Potential. *Front. Physiol.* **2019**, *9*, 1873. [[CrossRef](#)] [[PubMed](#)]
4. Romei, C.; Ciampi, R.; Elisei, R. A comprehensive overview of the role of the *RET* proto-oncogene in thyroid carcinoma. *Nat. Rev. Endocrinol.* **2016**, *12*, 192–202. [[CrossRef](#)] [[PubMed](#)]
5. Kohno, T.; Ichikawa, H.; Totoki, Y.; Yasuda, K.; Hiramoto, M.; Nammo, T.; Sakamoto, H.; Tsuta, K.; Furuta, K.; Shimada, Y.; et al. *KIF5B-RET* fusions in lung adenocarcinoma. *Nat. Med.* **2012**, *18*, 375–377. [[CrossRef](#)] [[PubMed](#)]
6. Ballerini, P.; Struski, S.; Cresson, C.; Prade, N.; Toujani, S.; Deswarte, C.; Dobbelsstein, S.; Petit, A.; Lapillonne, H.; Gautier, E.-F.; et al. *RET* fusion genes are associated with chronic myelomonocytic leukemia and enhance monocytic differentiation. *Leukemia* **2012**, *26*, 2384–2389. [[CrossRef](#)] [[PubMed](#)]
7. Griseri, P.; Garrone, O.; Sardo, A.L.; Monteverde, M.; Rusmini, M.; Tonissi, F.; Merlano, M.; Bruzzi, P.; Nigro, C.L.; Ceccherini, I. Genetic and epigenetic factors affect *RET* gene expression in breast cancer cell lines and influence survival in patients. *Oncotarget* **2016**, *7*, 26465–26479. [[CrossRef](#)] [[PubMed](#)]
8. Zeng, Q.; Cheng, Y.; Zhu, Q.; Yu, Z.; Wu, X.; Huang, K.; Zhou, M.; Han, S.; Zhang, Q. The Relationship between Over-expression of Glial Cell-derived Neurotrophic Factor and Its *RET* Receptor with Progression and Prognosis of Human Pancreatic Cancer. *J. Int. Med. Res.* **2008**, *36*, 656–664. [[CrossRef](#)] [[PubMed](#)]
9. Ban, K.; Feng, S.; Shao, L.; Ittmann, M. *RET* Signaling in Prostate Cancer. *Clin. Cancer Res.* **2017**, *23*, 4885–4896. [[CrossRef](#)] [[PubMed](#)]
10. Tahira, T.; Ishizaka, Y.; Itoh, F.; Sugimura, T.; Nagao, M. Characterization of *ret* proto-oncogene mRNAs encoding two isoforms of the protein product in a human neuroblastoma cell line. *Oncogene* **1990**, *5*, 97–102.
11. Myers, S.M.; Eng, C.; A Ponder, B.; Mulligan, L.M. Characterization of *RET* proto-oncogene 3' splicing variants and polyadenylation sites: A novel C-terminus for *RET*. *Oncogene* **1995**, *11*, 2039–2045.
12. Lee, K.-Y.; Samy, E.T.; Sham, M.-H.; Tam, P.K.-H.; Lui, V.C.-H. 3' Splicing variants of *ret* receptor tyrosine kinase are differentially expressed in mouse embryos and in adult mice. *Biochim. Biophys. Acta BBA Gene Struct. Expr.* **2003**, *1627*, 26–38. [[CrossRef](#)]
13. Ivanchuk, S.M.; Myers, S.M.; Mulligan, L.M. Expression of *RET* 3' splicing variants during human kidney development. *Oncogene* **1998**, *16*, 991–996. [[CrossRef](#)]
14. Ramone, T.; Romei, C.; Ciampi, R.; Tacito, A.; Piaggi, P.; Torregrossa, L.; Ugolini, C.; Elisei, R. Differential expression of *RET* isoforms in normal thyroid tissues, papillary and medullary thyroid carcinomas. *Endocrine* **2019**, *65*, 623–629. [[CrossRef](#)]

15. Lian, E.Y.; Hyndman, B.D.; Moodley, S.; Maritan, S.M.; Mulligan, L.M. RET isoforms contribute differentially to invasive processes in pancreatic ductal adenocarcinoma. *Oncogene* **2020**, *39*, 6493–6510. [[CrossRef](#)]
16. Le Hir, H.; Charlet, N.; Gimenez-Roqueplo, A.-P.; Mannelli, M.; Plouin, P.-F.; De Franciscis, V.; Thernes, C. Relative Expression of the RET9 and RET51 Isoforms in Human Pheochromocytomas. *Oncology* **2000**, *58*, 311–318. [[CrossRef](#)]
17. Richardson, D.; Rodrigues, D.M.; Hyndman, B.D.; Crupi, M.J.F.; Nicolescu, A.; Mulligan, L.M. Alternative splicing results in RET isoforms with distinct trafficking properties. *Mol. Biol. Cell* **2012**, *23*, 3838–3850. [[CrossRef](#)]
18. Miranda-Filho, A.; Lortet-Tieulent, J.; Bray, F.; Cao, B.; Franceschi, S.; Vaccarella, S.; Maso, L.D. Thyroid cancer incidence trends by histology in 25 countries: A population-based study. *Lancet Diabetes Endocrinol.* **2021**, *9*, 225–234. [[CrossRef](#)]
19. Marsh, D.J.; Learoyd, D.L.; Andrew, S.D.; Krishnan, L.; Pojer, R.; Richardson, A.-L.; Delbridge, L.; Eng, C.; Robinson, B.G. Somatic mutations in the RET proto-oncogene in sporadic medullary thyroid carcinoma. *Clin. Endocrinol.* **1996**, *44*, 249–257. [[CrossRef](#)]
20. Wells, S.A.; Pacini, F.; Robinson, B.G.; Santoro, M. Multiple Endocrine Neoplasia Type 2 and Familial Medullary Thyroid Carcinoma: An Update. *J. Clin. Endocrinol. Metab.* **2013**, *98*, 3149–3164. [[CrossRef](#)]
21. Ciampi, R.; Romei, C.; Ramone, T.; Prete, A.; Tacito, A.; Cappagli, V.; Bottici, V.; Viola, D.; Torregrossa, L.; Ugolini, C.; et al. Genetic Landscape of Somatic Mutations in a Large Cohort of Sporadic Medullary Thyroid Carcinomas Studied by Next-Generation Targeted Sequencing. *iScience* **2019**, *20*, 324–336. [[CrossRef](#)]
22. Agrawal, N.; Jiao, Y.; Sausen, M.; Leary, R.; Bettgowda, C.; Roberts, N.; Bhan, S.; Ho, A.S.; Khan, Z.; Bishop, J.; et al. Exomic Sequencing of Medullary Thyroid Cancer Reveals Dominant and Mutually Exclusive Oncogenic Mutations in RET and RAS. *J. Clin. Endocrinol. Metab.* **2013**, *98*, E364–E369. [[CrossRef](#)]
23. Simbolo, M.; Mian, C.; Barollo, S.; Fassan, M.; Mafficini, A.; Neves, D.; Scardoni, M.; Pennelli, G.; Ruggie, M.; Pelizzo, M.R.; et al. High-throughput mutation profiling improves diagnostic stratification of sporadic medullary thyroid carcinomas. *Virchows Archiv* **2014**, *465*, 73–78. [[CrossRef](#)]
24. Moura, M.M.; Cavaco, B.M.; Leite, V. RAS proto-oncogene in medullary thyroid carcinoma. *Endocr. Relat. Cancer* **2015**, *22*, R235–R252. [[CrossRef](#)]
25. Mulligan, L.M.; Kwok, J.; Healey, C.S.; Elsdon, M.J.; Eng, C.; Gardner, E.; Love, D.; Mole, S.; Moore, J.K.; Papi, L.; et al. Germ-line mutations of the RET proto-oncogene in multiple endocrine neoplasia type 2A. *Nat. Cell Biol.* **1993**, *363*, 458–460. [[CrossRef](#)]
26. Salvatore, D.; Santoro, M.; Schlumberger, M. The importance of the RET gene in thyroid cancer and therapeutic implications. *Nat. Rev. Endocrinol.* **2021**, *17*, 296–306. [[CrossRef](#)]
27. Fusco, A.; Grieco, M.; Santoro, M.; Berlingieri, M.T.; Pilotti, S.; Pierotti, M.A.; Della Porta, G.; Vecchio, G. A new oncogene in human thyroid papillary carcinomas and their lymph-nodal metastases. *Nat. Cell Biol.* **1987**, *328*, 170–172. [[CrossRef](#)]
28. Shakiba, E.; Movahedi, M.; Majd, A.; Hedayati, M. Investigating the expression and promoter methylation of RET gene in patients with medullary thyroid cancer with unmutated RET. *J. Cell. Physiol.* **2019**, *234*, 16304–16311. [[CrossRef](#)]
29. Romei, C.; Ramone, T.; Mulè, C.; Prete, A.; Cappagli, V.; Lorusso, L.; Torregrossa, L.; Basolo, F.; Ciampi, R.; Elisei, R. RET mutated C-cells proliferate more rapidly than non-mutated neoplastic cells. *Endocr. Connect.* **2021**, *10*, 124–130. [[CrossRef](#)]
30. Sponziello, M.; Durante, C.; Boichard, A.; Dima, M.; Puppini, C.; Verrienti, A.; Tamburrano, G.; Di Rocco, G.; Redler, A.; Lacroix, L.; et al. Epigenetic-related gene expression profile in medullary thyroid cancer revealed the overexpression of the histone methyltransferases EZH2 and SMYD3 in aggressive tumours. *Mol. Cell. Endocrinol.* **2014**, *392*, 8–13. [[CrossRef](#)]
31. Rossel, M.; Pasini, A.; Chappius, S.; Geneste, O.; Fournier, L.; Schuffenecker, I.; Takahishi, M.; Van Grunsven, L.A.; Urdiales, J.L.; Rudkin, B.B.; et al. Distinct biological properties of two RET isoforms activated by MEN 2A and MEN 2B mutations. *Oncogene* **1997**, *14*, 265–275. [[CrossRef](#)] [[PubMed](#)]
32. Ishiguro, Y.; Iwashita, T.; Murakami, H.; Asai, N.; Iida, K.-I.; Goto, H.; Hayakawa, T.; Takahashi, M. The Role of Amino Acids Surrounding Tyrosine 1062 in Ret in Specific Binding of the Shc Phosphotyrosine-Binding Domain1. *Endocrinology* **1999**, *140*, 3992–3998. [[CrossRef](#)] [[PubMed](#)]
33. Iwashita, T.; Kato, M.; Murakami, H.; Asai, N.; Ishiguro, Y.; Ito, S.; Iwata, Y.; Kawai, K.; Asai, M.; Kurokawa, K.; et al. Biological and biochemical properties of Ret with kinase domain mutations identified in multiple endocrine neoplasia type 2B and familial medullary thyroid carcinoma. *Oncogene* **1999**, *18*, 3919–3922. [[CrossRef](#)] [[PubMed](#)]
34. Pasini, A.; Geneste, O.; Legrand, P.; Schlumberger, M.; Rossel, M.; Fournier, L.; Rudkin, B.B.; Schuffenecker, I.; Lenoir, G.M.; Billaud, M. Oncogenic activation of RET by two distinct FMTC mutations affecting the tyrosine kinase domain. *Oncogene* **1997**, *15*, 393–402. [[CrossRef](#)]