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

Chiara Mulè 1, Raffaele Ciampi 1, Teresa Ramone 1, Alessandro Prete 1, Antonio Matrone 1✉, Virginia Cappagli 1✉, Liborio Torregrossa 2, Fulvio Basolo 2, Rossella Elisei 1✉, and Cristina Romei 1

1 Endocrine Unit, Department of Clinical and Experimental Medicine, University of Pisa, 56124 Pisa, Italy; chiaramule93@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

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′ -> 3′ AACATCTGTTACGTAGG and reverse 5′ -> 3′ CACGAGGACCCAAAGCC) 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%,
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 (ΔCt = 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).

Expression levels are reported as \(\Delta Ct (\Delta Ct = Ct\ RET - Ct\ G6PD)\) where \(Ct\) is the threshold cycle for qRT-PCR. The lowest is the \(\Delta Ct\) 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.

| RET Gene Expression | RET9 n(%) | RET51 n(%) | \(p\) |
|---------------------|-----------|-------------|-------|
| Pos expression      | 72 (86.7) | 83 (100)    |       |
| Neg expression      | 11        | 0           |       |
| \(\Delta Ct^*\)     | 4.9       | 1.8         | <0.0001 |

*Expression levels are reported as \(\Delta Ct (\Delta Ct = Ct\ RET - Ct\ G6PD)\) where \(Ct\) is the threshold cycle for qRT-PCR. The lowest is the \(\Delta Ct\) 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 ∆Ct (ΔCt = Ct RET – Ct G6PD) where Ct is the threshold cycle for qRT-PCR. The lowest is the ∆Ct 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 ∆Ct (ΔCt = Ct RET – Ct G6PD) where Ct is the threshold cycle for qRT-PCR. The lowest is the ∆Ct 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 Hire 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.

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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.

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