RESEARCH

RET mutated C-cells proliferate more rapidly than non-mutated neoplastic cells

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Abstract

A statistically significant higher prevalence of the *RET* p.Met918Thr somatic mutation, identified by direct sequencing, was previously reported in MTC > 2 cm than in smaller tumors. Aim of this study was to correlate the full *RET* and *RAS* mutation profile, identified by a Next Generation Sequencing approach, with the growth rate, proliferation and tumor size of MTC. Data of 149 sporadic MTC patients were correlated with *RET* mutations and Ki67 positivity. Eighty-one cases had a somatic *RET* mutation, 40 had a *RAS* mutation and 28 were negative. A statistically significant higher prevalence of *RET* mutations was found in MTC > 2 cm. A higher prevalence of *RET* more aggressive mutations, higher allelic frequencies and, higher percentage of Ki67 positive cells were found in larger tumors which had also a worse outcome. Our study highlights the predominant role of *RET* somatic mutations in MTC tumorigenesis. We demonstrate that *RET* mutation prevalence and allelic frequency (AF) are significantly higher in larger tumors. Based on these results, we can conclude that *RET* mutated C-cells's growth and proliferation are more rapid than those of non-mutated cells and give origin to bigger and more aggressive MTC.

Key Words

- medullary thyroid cancer
- RET
- RAS
- Ki67
- allelic frequency
- cells' growth

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Introduction

Medullary Thyroid Carcinoma (MTC) accounts for about 5–7% of all thyroid cancer and can occur in a hereditary (25%) or a sporadic form (75%) (1).

According to the Next Generation Studies published in the last years (2, 3, 4, 5, 6), somatic *RET* mutations are the most frequent alterations found in sporadic MTC. Although a large spectrum of *RET* mutations are described, the commonest alteration is the p.Met918Thr mutation in exon 16 of the *RET* gene (1). In addition to somatic *RET* mutations, sporadic MTC shows the presence of somatic *RAS* mutations that have been mostly reported in *RET* negative tumours and are almost always mutually exclusive with *RET* mutations (7, 8). *RET* somatic mutations have been reported to be a factor of a bad prognosis and are significantly associated with a more aggressive biological behavior and a reduced survival (9, 10, 11). Although less investigated, *RAS* mutations seem to predict a better outcome when compared to somatic *RET* mutations (7, 9).

We previously demonstrated that the presence of a somatic *RET* p.Met918Thr mutation correlated with larger tumor size while it was significantly lower in tumors smaller than 2 cm (12). We hypothesized that p.Met918Thr mutation might not be an early event or that it could be present from the beginning but only in a subpopulation of cells not detectable with the conventional sequencing analysis because of its low sensitivity. This latter hypothesis has been supported by the evidence of *RET* mutation heterogeneity in about 20% of MTC (13, 14). However, due to the low sensitivity of the Sanger method and to the fact that only *RET* exon 11 and 16 have been investigated, the problem of false

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negative cases should be considered as a bias in the former studies.

In the present study, we correlated the prevalence of any type of *RET* and *RAS* somatic mutations, as obtained with a Next Generation Sequencing (NGS) approach, with the MTC tumor size. Moreover, the correlation of their allelic frequency (AF) and the size of the tumor was evaluated to better understand their driver role in the tumoral transformation of the original C cells. Finally, the correlation with Ki67 positivity, that is an index of cell proliferation, with the size and the *RET* mutations was also analyzed.

Patients and methods

Our study group was represented by 149 MTC patients belonging to a larger series of 201 MTC patients submitted to total thyroidectomy and central neck dissection at our hospital whose tumoral tissues were analyzed by NGS for many gene alterations as previously reported (15). To the purpose of this study we included only the 149 cases analyzed on the primary tumor. All patients had no history of familial disease, were negative for the presence of other endocrine neoplasia and no germline *RET* mutations were found.

Clinical, biochemical and pathological data, with particular regard to tumor size, were collected from a computerized database. Cases were classified according to the size of the tumor as follows: group 1, ≤1 cm; group 2, >1 and ≤2 cm; group 3, >2 and ≤3 cm; and group 4, >3 cm.

Data about the presence or absence of *RET* and *RAS* somatic mutations were retrieved from the row data of the previous study (15), including the allelic frequency of the driver mutations.

Ki67 proliferative index was evaluated by immunohistochemistry. Five micrometer sections were cut from formalin-fixed paraffin-embedded (FFPE) blocks for 130 out of 149 cases. Ki67 immunostaining was performed automatically by the Ventana Benchmark immunostaining system (Ventana Medical Systems, Inc., Tucson, AZ) using a rabbit monoclonal primary antibody (immunoglobulin (Ig)G) directed against the C-terminal portion of Ki67 (CONFIRM anti-Ki67, clone 30-9; Ventana). Neoplastic cells were considered positive when the nuclei showed an immunoreactivity variable from weak to strong; neoplastic cells without nuclear immunoreactivity were considered as negative. Ki67 score was independently evaluated by two pathologists (L T and F B) who were blinded to the clinicopathologic data. Immunostaining was evaluated using a standard Leica DM4000 microscope with the 'hot spots' method: the field under high power magnification (original magnification, ×40) with the highest apparent Ki67 index was selected. The Ki67 score was defined as the percentage of positive cells among a minimum of 100 neoplastic cells. The results were scored according to the number of Ki67 positive cells: <3% as low, 3–20% as intermediate and >20% as high (16).

An informed consent form for *RET* genetic screening and other clinical procedures was signed by all patients. The present study was approved by the Institutional Review Board and by the 'Comitato Etico Regionale per la Sperimentazione Clinica della Regione Toscana' Prot n 6714, 05/02/2019.

Statistics

The statistical analysis was performed with the Chi-square test and with One-way ANOVA according to the studied variables and using the GraphPad Prism version 7.00 software.

Results

We distinguished 149 MTC cases according to the size of the primary tumor. As shown in Table 1, in this subgroup of primary tissues, we found 81 (54%) cases with a somatic *RET* mutation, 40 (27%) cases with *RAS* mutations and 28 (19%) cases that were negative. Among the *RAS* positive cases, 27 were positive for *HRAS* and 13 for *KRAS* respectively. Data on the presence of the somatic mutations were retrieved by a larger study performed in our center and already published (15).

As shown in Fig. 1 panel A, *RET* somatic mutations were the most prevalent in each group. However, a statistically significant different mutation profile

Table 1 Prevalence of *RET* and *RAS* mutations in the different size group.

Group	Number of patients	*RET* positive, *n* (%)	*RAS* positive, *n* (%)	Negative
A (X ≤ 1 cm)	36	13 (36)	11 (30)	12 (34)
B (1 < X ≤ 2 cm)	55	28 (51)	17 (31)	10 (18)
C (2 < X ≤3 cm)	29	20 (69)	8 (27)	1 (4)
D (3 < X ≤4 cm)	29	20 (69)	4 (14)	5 (17)
Total	149	81 (55)	40 (27)	28 (18)

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($P=0.02$) was observed in the four groups with the highest prevalence (20/29, 69%) of the *RET* mutations found in MTC cases with the largest size (group 3 and 4) and the lowest prevalence in group 2 (28/51, 11.8%) and 1 (13/36, 36%). At variance, *RAS* positive cases and *RET/RAS* negative cases were found to be more frequent in smaller tumors (1 and 2). The mutation profile was also compared between tumors smaller and larger than 2 cm. As shown in Fig. 1 panel B, the prevalence of *RET* positive cases was significantly higher in tumors larger than 2 cm while the prevalence of *RAS* positive and *RET/RAS* negative cases was lower in larger tumors.

We then focused on the different types of *RET* mutations according to the American thyroid association risk classification (17) as highest, high and moderate. As shown in Fig. 2 panel A, a gradual increase of the highest risk *RET* mutation (i.e. p.Met918Thr) has been observed with the increase of tumor size and a simultaneous gradual decrease was observed for high and moderate *RET* mutations ($P=0.0027$). When considering the distribution of the different *RET* mutations in tumors either smaller (group 1 and 2) or larger (group 3 and 4) than 2 cm, we observed that in the group 1+2 *RET* mutations were uniformly distributed (highest 29.3%, high 36.6% and moderate 34%) while in the group 3+4, a significant greater prevalence of the highest *RET* mutations (29/40, 72.5%) was found with respect to the high (7/40, 17.5%) and the moderate (4/40, 10%) *RET* mutations.

As shown in Fig. 3 panels A and B, the AF of *RET* mutations was significantly higher in larger tumors both when comparing the four different groups (group 1, mean AF 23.42 ± 10.38; group 2, mean AF 29.49 ± 11.16; group 3, mean AF 39.27 ± 9.3, group 4 mean AF 42.48 ± 11.16) and when comparing group 1+2 and group 3+4 (group 1+2, mean AF 27.51± 10.38; group 3+4 mean AF 40.91 ± 12.63). In addition, the *RET* AF was higher in tumors of group 3+4 than in group 1+2 for every type of *RET* mutations (moderate: 46.72% vs 29.72%, high: 39.48% vs 25.52%, highest: 41.52% vs 27.7%, respectively).

In particular, when we focused on the p.Met918Thr, we observed a statistically significant increase in the AF of this mutation with the increase of tumor size (group 1, mean AF 18.16 ± 8.6; group 2, mean AF 28.9 ± 13.8; group 3, mean AF 39.8 ± 13.1, group 4 mean AF 44.3 ± 16.3).

As far as the AF of *RAS* mutations was concerned, although a trend of increase was observed, there was no a statically significant difference neither when comparing the four different groups (group 1, mean AF 29.8 ± 11.9; group 2, mean AF 33.87 ± 12.2; group 3, mean AF 36.13 ± 11.4, group 4 mean AF 46.62 ± 4.6) (Fig. 3 panel C) nor when comparing group 1+2 and group 3+4 (group 1+2, mean AF 32.27 ± 12.08; group 3+4 mean AF 39.63 ± 107) (Fig. 3 panel D).

Ki67 positive expression was high in 15/130 (11.5%), intermediate in 79/129 (60.8%), low in 34/129 (26.2%) and negative in 2/149 (1.5%). A statistically high significant correlation ($P < 0.0001$) was found when the Ki67 low, intermediate and high positivity was analyzed according to the tumor size (Fig. 4, panel A). A positive trend or correlation was also found when the analysis was done with the type of mutation being Ki67 expression higher in *RET* positive cases than in *RAS* positive cases and even lower in *RET/RAS* negative cases (Fig. 4, panel B). Although not statistically significant, we found that, among all mutations, the p.Met918Thr *RET* mutation showed the highest proliferation rate (Table 2). The correlation become significant when the analysis was performed between Ki67 positivity and the AF of *RET* positive cases (Fig. 4, panel C).

Data on the outcome were available in 127/149 cases: 88/127 (69.3%) patients were free of disease while 39/127 (30.7%) patients were either dead or with a persistent disease (i.e. biochemical and/or structural disease). In the group of disease free patients, the prevalence of cases smaller than 2 cm ($n=63$) was significantly higher than those with larger tumors ($n=25$). On the contrary in the group of patients dead or with a persistent disease, the prevalence of cases larger than 2 cm ($n=26$) was

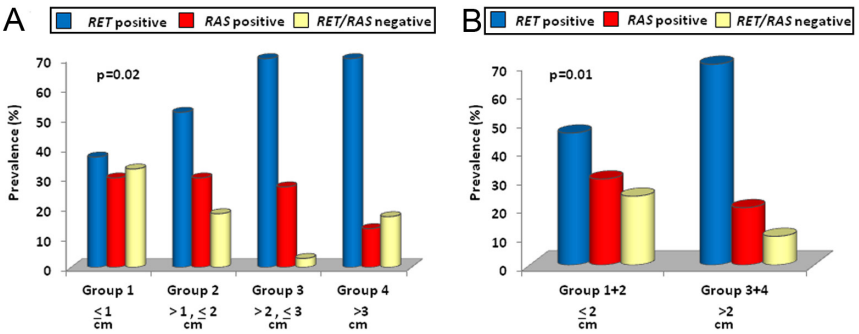

Figure 1
Prevalence of somatic *RET* and *RAS* mutations according to tumor size. A statistically significant difference in the mutations profile was observed both when 4 different groups were considered (group 1, ≤1 cm; group 2, >1 and ≤2 cm; group 3, >2 and ≤3 cm; and group 4, >3 cm ($P=0.02$)) (panel A) and when grouped into 2 bigger groups (1 + 2, ≤2 cm and 3 + 4, >2 cm) ($P=0.01$) (panel B).

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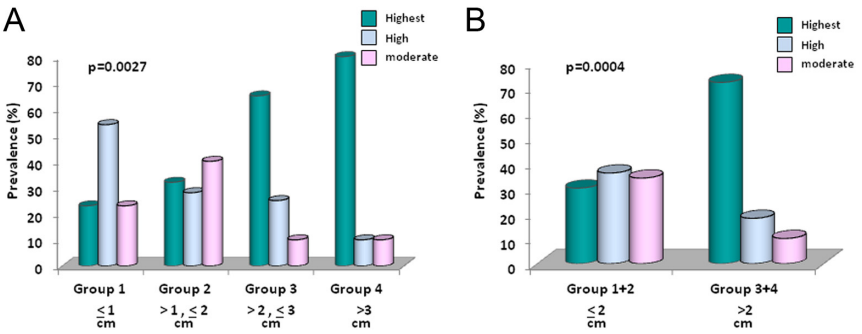

Figure 2
Prevalence of somatic highest, high and moderate *RET* mutations (17) according to tumor size. Four different groups were considered (group 1, ≤1 cm; group 2, >1 and ≤ 2 cm; group 3, >2 and ≤3 cm; and group 4, >3 cm) (panel A); groups A and B vs groups C and D have been considered (panel B). A statistically higher prevalence of the most aggressive *RET* mutations was observed (P= 0.0027 and P = 0.0004, respectively).

significantly higher than those with smaller tumors (n = 13) (P=0.0008).

Discussion

The sporadic form of MTC is mainly characterized by the presence of *RET* (about 40–50% of cases) and *RAS* (about 10–20% of cases) somatic mutations (Catalogue of Somatic Mutation in Cancer https://cancer.sanger.ac.uk/cosmic). The p.Met918Thr mutation in exon 16 is the most common *RET* somatic mutation being present in up to 90% of *RET*-positive cases, while *RAS* gene point mutations in MTC mainly occur in *H*- and *KRAS*, and they are usually mutually exclusive with *RET* mutations. The recent introduction of NGS techniques has largely improved the identification of the molecular alterations involved and causative of many human diseases. Several studies carried out in MTC confirmed the role of *RET* and *RAS* somatic mutations as the main drivers in MTC and only few different genetic alterations have been identified (2, 3, 15). Nevertheless, a rather large portion of cases are negative for the presence of common somatic gene alterations. In the majority of MTC cases, *RET* and *RAS* mutations are mutually exclusive indicating that *RET*-mediated and *RAS* mediated oncogenic transformations occur separately. Since both *RET* and *RAS* alterations lead to an uncontrolled activation of the MAPKinase pathway (18, 19) they are considered driver mutations in MTC.

In the present study, we found that the prevalence of *RET* somatic mutations, any type, was significantly higher in larger than in smaller MTC cases and in particular that the *RET* ATA highest mutation (i.e. p.Met918Thr) was the most represented in the larger tumors. With these results, we confirmed our previous study (12) that was partially affected by the low sensitivity of the method of

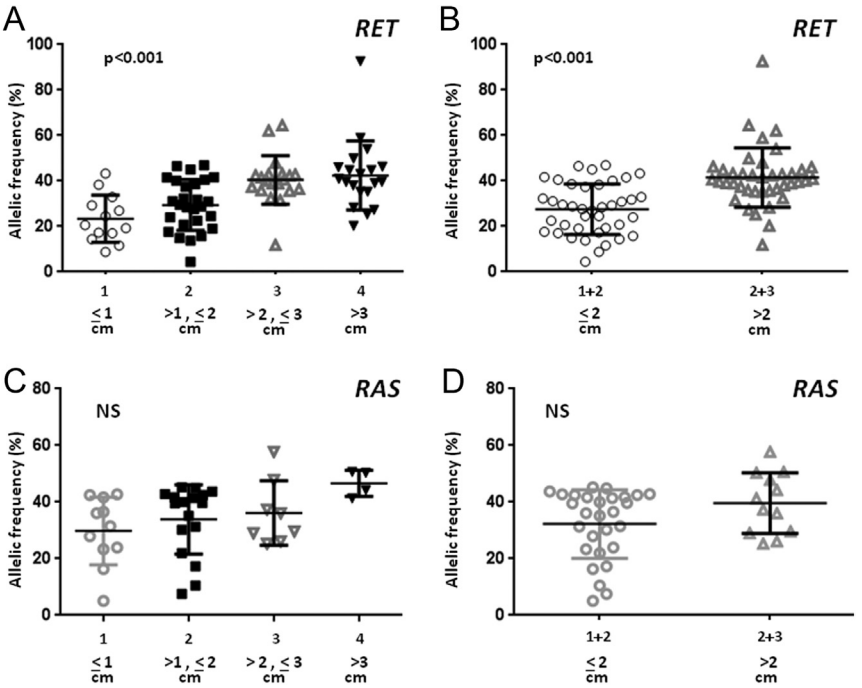

Figure 3
Correlation between the allelic frequency (AF) of the driver mutations and tumor size. *RET* AF is higher in larger tumors. Four different groups were considered (group 1, ≤1 cm; group 2, >1 and ≤2 cm; group 3, >2 and ≤ 3 cm; and group 4, >3 cm) (panel A); groups 1 and 2 vs groups 3 and 4 have been considered. (P < 0.001) (panel B). *RAS* AF was not correlated to tumor size when both the four groups (panel C) and when considered groups 1 and 2 vs groups 3 and 4 (panel D) were considered.

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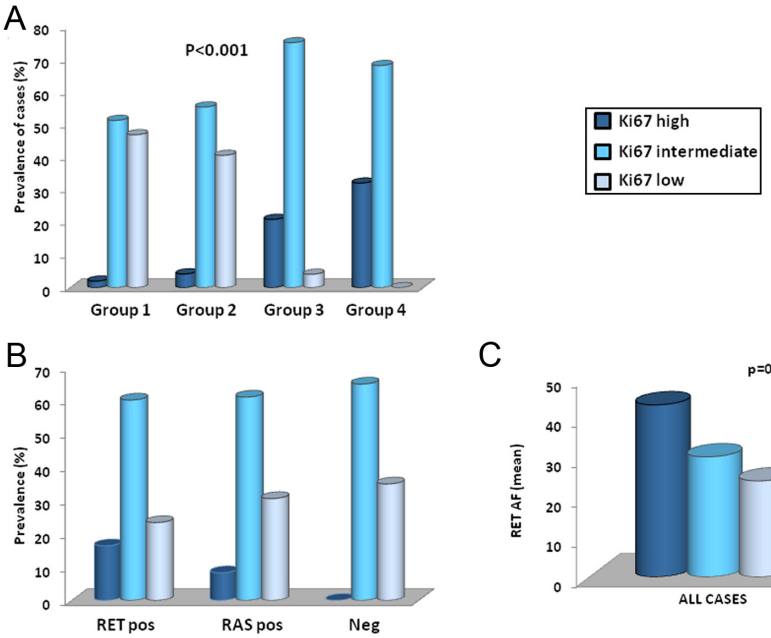

Figure 4
A statistically significant correlation was found when Ki67 low, intermediate and high positivity was correlated to the tumor size (panel A); although not statistically significant, a trend of correlation was observed when Ki67 positivity was analyzed according to the type of mutation (panel B). The correlation of Ki67 positivity was statistically significant when the analysis was performed with the AF of *RET* mutation in *RET* positive cases (panel C).

sequencing used with the risk to have lost some positive cases especially in the smallest tumors. The prevalence of *RET* mutations found with NGS can be considered reliable and we can now confirm that the different prevalence of *RET* in smaller and bigger tumors is true. At variance, a higher proportion of tumors positive for *RAS* somatic mutation or negative for any mutation was observed in smaller tumors. According to these results, we can hypothesize that three types of MTC tumors exist: those with *RAS* or *noRAS/noRET* mutations. At the time of their development they are similar in prevalence but then *RAS* or *noRAS/noRET* cases remain smaller likely because of growing slowly. While those with *RET*, especially p.Met918Thr, mutation becomes bigger likely because of rapidly growing.

A second important observation of the present work is that larger tumors are characterized by a higher AF of *RET* mutations, and in particular of the p.Met918Thr, with a mean AF of 41.54 vs 26.93 in the smaller. This finding indicates that a bigger number of tumoral cells, almost the totality, are mutated in the bigger tumors but not in the smaller tumors. This was not observed for the AF of *RAS* mutations that were present in about 30% of tumoral cells without differences related to the tumor size. These findings support the previous observations that a genetic intra- and inter-tumor heterogeneity and non-clonal origin of some MTC cases exists (13, 14). Considering that *RET* p.Met918Thr mutation has been demonstrated to have the highest transforming ability (20), we can postulate that cells carrying this mutation can duplicate more rapidly and take a growth advantage respect to the non-mutated concomitant cells. The fact that bigger tumors have the *RET* p.Met918Thr mutation more frequently at a higher AF is in line with this hypothesis.

The higher prevalence of *RET* somatic mutations, and in particular of the p.Met918Thr, in larger tumors is in keeping with the evidence that larger tumors have a worse outcome (21, 22) as shown also in the present series. On this regard, it is useful to recall that *RET* p.Met918Thr is also the germline mutation of the MEN 2B, whose MTC is the most aggressive and rapidly growing tumor among all genotype of MEN 2 (23). These findings are in keeping with the hypothesis that *RET* mutations, particularly p.Met918Thr, identify a subgroup of tumors rapidly growing for the presence of a highly transforming mutation and with a more aggressive behavior with respect to those with *RAS* mutations or without any mutations. The great correlation of Ki67 positivity, that is a well-recognized index of tumor proliferation, with the greater size of the tumor and with a higher percentage of AF of *RET* in *RET* positive cases can be considered the

Table 2 Ki67 expression level in RET positive cases.

Mutation	Number of patients	Ki67		
		Low, *n* (%)	Intermediate, *n* (%)	High, *n* (%)
Highest[a]	38	5 (14.8)	23 (29.5)	10 (66.6)
High	26	8 (23.5)	16 (20.5)	2 (13.4)
Moderate	7	3 (8.8)	4 (5.2)	0
Total	71	16	43	12

[a]36 M918T and 2 A883F; by chi square: $P = 0.089$.

proof of the fact that *RET* positive cells take a growth advantage with respect to the negative cells (i.e. higher percentage of AF of the mutation), divide and reproduce more rapidly (i.e. higher Ki67 positivity) thus determining a rapid increase of the tumor respect to *RET* negative, either *RAS* positive or negative, cases.

Simultaneously, we can consider that, at least cases with *RAS* mutations, are more clonal and less aggressive similarly to *RAS* positive follicular adenomas and carcinomas (24). This behavior has been also reported in colon cancer (25, 26) in which both *KRAS* and *NRAS* mutations are usually present in the majority of neoplastic cells and have been considered a clonal event in the tumoral transformation. It has been proposed (27, 28) that, at variance with *PIK3CA* and *NOTCH1* mutations that instead seem to have a biological behavior similar to that of *RET* mutations (13, 14), *RAS* mutations would likely accumulate at the beginning of tumor formation thus representing the main driver alteration.

In conclusion, this study highlights the predominant role of *RET* somatic mutations in MTC tumorigenesis and demonstrates a high prevalence of these alterations in all size categories. We demonstrate that *RET* mutation prevalence and *RET* mutation AF are significantly higher in larger than in smaller tumors indicating that *RET* alterations are clonal event in cases larger than 2 cm and subclonal event in small MTC cases. At variance, both the prevalence and the AF of *RAS* mutations are similar in MTC size categories. Finally, bigger tumors, that have higher prevalence of *RET* mutations as well as higher level of Ki67 positivity, showed a more aggressive behavior and a worse outcome.

Declaration of interest

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

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Author contribution statement

C R designed the study, performed the in silico analysis and prepared the manuscript. T R and C M performed all DNA preparation and the MLPA experiments. A P, V C and L L contributed to patients selection and collection of clinical data. L T and F B reviewed histological slides and gave the final diagnosis of MTC. R C performed all NGS experiments. R E is the team leader, she contributed to the preparation of the manuscript.

References

1 Romei C, Ciampi R & Elisei R. A comprehensive overview of the role of the RET proto-oncogene in thyroid carcinoma. *Nature Reviews: Endocrinology* 2016 **12** 192–202. (https://doi.org/10.1038/nrendo.2016.11)

2 Simbolo M, Mian C, Barollo S, Fassan M, Mafficini A, Neves D, Scardoni M, Pennelli G, Rugge M, Pelizzo MR, *et al*. High-throughput mutation profiling improves diagnostic stratification of sporadic medullary thyroid carcinomas. *Virchows Archiv* 2014 **465** 73–78. (https://doi.org/10.1007/s00428-014-1589-3)

3 Agrawal N, Jiao Y, Sausen M, Leary R, Bettegowda C, Roberts NJ, Bhan S, Ho AS, Khan Z, Bishop J, *et al*. Exomic sequencing of medullary thyroid cancer reveals dominant and mutually exclusive oncogenic mutations in RET and RAS. *Journal of Clinical Endocrinology and Metabolism* 2013 **98** E364–E369. (https://doi.org/10.1210/jc.2012-2703)

4 Wei S, LiVolsi VA, Montone KT, Morrissette JJD & Baloch ZW. Detection of molecular alterations in medullary thyroid carcinoma using next-generation sequencing: an institutional experience. *Endocrine Pathology* 2016 **27** 359–362. (https://doi.org/10.1007/s12022-016-9446-3)

5 Ji JH, Oh YL, Hong M, Yun JW, Lee HW, Kim D, Ji Y, Kim DH, Park WY, Shin HT, *et al*. Identification of driving ALK fusion genes and genomic landscape of medullary thyroid cancer. *PLoS Genetics* 2015 **11** e1005467. (https://doi.org/10.1371/journal.pgen.1005467)

6 Heilmann AM, Subbiah V, Wang K, Sun JX, Elvin JA, Chmielecki J, Sherman SI, Murthy R, Busaidy NL, Subbiah I, *et al*. Comprehensive genomic profiling of clinically advanced medullary thyroid carcinoma. *Oncology* 2016 **90** 339–346. (https://doi.org/10.1159/000445978)

7 Ciampi R, Mian C, Fugazzola L, Cosci B, Romei C, Barollo S, Cirello V, Bottici V, Marconcini G, Rosa PM, *et al*. Evidence of a low prevalence of ras mutations in a large medullary thyroid cancer series. *Thyroid* 2013 **23** 50–57. (https://doi.org/10.1089/thy.2012.0207)

8 Fussey JM, Vaidya B, Kim D, Clark J, Ellard S & Smith JA. The role of molecular genetics in the clinical management of sporadic medullary thyroid carcinoma: a systematic review. *Clinical Endocrinology* 2019 **91** 697–707. (https://doi.org/10.1111/cen.14060)

9 Vuong HG, Odate T, Ngo HTT, Pham TQ, Tran TTK, Mochizuki K, Nakazawa T, Katoh R & Kondo T. Clinical significance of RET and RAS mutations in sporadic medullary thyroid carcinoma: a meta-analysis. *Endocrine-Related Cancer* 2018 **25** 633–641. (https://doi.org/10.1530/ERC-18-0056)

10 Mian C, Pennelli G, Barollo S, Cavedon E, Nacamulli D, Vianello F, Negro I, Pozza G, Boschin IM, Pelizzo MR, *et al*. Combined RET and Ki-67 assessment in sporadic medullary thyroid carcinoma: a useful tool for patient risk stratification. *European Journal of Endocrinology* 2011 **164** 971–976. (https://doi.org/10.1530/EJE-11-0079)

11 Elisei R, Cosci B, Romei C, Bottici V, Renzini G, Molinaro E, Agate L, Vivaldi A, Faviana P, Basolo F, *et al*. Prognostic significance of somatic RET oncogene mutations in sporadic medullary thyroid cancer: a 10-year follow-up study. *Journal of Clinical Endocrinology and Metabolism* 2008 **93** 682–687. (https://doi.org/10.1210/jc.2007-1714)

12 Romei C, Ugolini C, Cosci B, Torregrossa L, Vivaldi A, Ciampi R, Tacito A, Basolo F, Materazzi G, Miccoli P, *et al*. Low prevalence of the somatic M918T RET mutation in micro-medullary thyroid cancer. *Thyroid* 2012 **22** 476–481. (https://doi.org/10.1089/thy.2011.0358)

13 Romei C, Ciampi R, Casella F, Tacito A, Torregrossa L, Ugolini C, Basolo F, Materazzi G, Vitti P & Elisei R. RET mutation heterogeneity in primary advanced medullary thyroid cancers and their metastases. *Oncotarget* 2018 **9** 9875–9884. (https://doi.org/10.18632/oncotarget.23986)

14 Eng C, Mulligan LM, Healey CS, Houghton C, Frilling A, Raue F, Thomas GA & Ponder BA. Heterogeneous mutation of the RET proto-oncogene in subpopulations of medullary thyroid carcinoma. *Cancer Research* 1996 **56** 2167–2170.
15 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. (https://doi.org/10.1016/j.isci.2019.09.030)
16 Fuchs TL, Nassour AJ, Glover A, Sywak MS, Sidhu SB, Delbridge LW, Clifton-Bligh RJ, Gild ML, Tsang V, Robinson BG, *et al.* A proposed grading scheme for medullary thyroid carcinoma based on proliferative activity (Ki-67 and mitotic count) and coagulative necrosis. *American Journal of Surgical Pathology* 2020 **44** 1419–1428. (https://doi.org/10.1097/PAS.0000000000001505)
17 Wells SA Jr, Asa SL, Dralle H, Elisei R, Evans DB, Gagel RF, Lee N, Machens A, Moley JF, Pacini F, *et al.* Revised American Thyroid Association guidelines for the management of medullary thyroid carcinoma. *Thyroid* 2015 **25** 567–610. (https://doi.org/10.1089/thy.2014.0335)
18 Mulligan LM. GDNF and the RET receptor in cancer: new insights and therapeutic potential. *Frontiers in Physiology* 2018 **9** 1873. (https://doi.org/10.3389/fphys.2018.01873)
19 Stalnecker CA & Der CJ. RAS, wanted dead or alive: Advances in targeting RAS mutant cancers. *Science Signaling* 2020 **13** eaay6013. (https://doi.org/10.1126/scisignal.aay6013)
20 Cosci B, Vivaldi A, Romei C, Gemignani F, Landi S, Ciampi R, Tacito A, Molinaro E, Agate L, Bottici V, *et al.* In silico and in vitro analysis of rare germline allelic variants of RET oncogene associated with medullary thyroid cancer. *Endocrine-Related Cancer* 2011 **18** 603–612. (https://doi.org/10.1530/ERC-11-0117)
21 Gharib H, McConahey WM, Tiegs RD, Bergstralh EJ, Goellner JR, Grant CS, van Heerden JA, Sizemore GW & Hay ID. Medullary thyroid carcinoma: clinicopathologic features and long-term follow-up of 65 patients treated during 1946 through 1970. *Mayo Clinic Proceedings* 1992 **67** 934–940. (https://doi.org/10.1016/s0025-6196(12)60923-9)
22 Machens A & Dralle H. Biological relevance of medullary thyroid microcarcinoma. *Journal of Clinical Endocrinology and Metabolism* 2012 **97** 1547–1553. (https://doi.org/10.1210/jc.2011-2534)
23 Elisei R, Matrone A, Valerio L, Molinaro E, Agate L, Bottici V, Viola D, Giani C, Cappagli V, Latrofa F, *et al.* Fifty years after the first description, MEN 2B syndrome diagnosis is still late: descriptions of two recent cases. *Journal of Clinical Endocrinology and Metabolism* 2019 **104** 2520–2526. (https://doi.org/10.1210/jc.2018-02102)
24 Paniza ACJ, Mendes TB, Viana MDB, Thomaz DMD, Chiappini PBO, Colozza-Gama GA, Lindsey SC, de Carvalho MB, Alves VAF, Curioni O, *et al.* Revised criteria for diagnosis of NIFTP reveals a better correlation with tumor biological behavior. *Endocrine Connections* 2019 **8** 1529–1538. (https://doi.org/10.1530/EC-19-0459)
25 Normanno N, Rachiglio AM, Lambiase M, Martinelli E, Fenizia F, Esposito C, Roma C, Troiani T, Rizzi D, Tatangelo F, *et al.* Heterogeneity of KRAS, NRAS, BRAF and PIK3CA mutations in metastatic colorectal cancer and potential effects on therapy in the Capri GOIM trial. *Annals of Oncology* 2015 **26** 1710–1714. (https://doi.org/10.1093/annonc/mdv176)
26 Dienstmann R, Elez E, Argiles G, Matos I, Sanz-Garcia E, Ortiz C, Macarulla T, Capdevila J, Alsina M, Sauri T, *et al.* Analysis of mutant allele fractions in driver genes in colorectal cancer – biological and clinical insights. *Molecular Oncology* 2017 **11** 1263–1272. (https://doi.org/10.1002/1878-0261.12099)
27 Joung JG, Oh BY, Hong HK, Al-Khalidi H, Al-Alem F, Lee HO, Bae JS, Kim J, Cha HU, Alotaibi M, *et al.* Tumor heterogeneity predicts metastatic potential in colorectal cancer. *Clinical Cancer Research* 2017 **23** 7209–7216. (https://doi.org/10.1158/1078-0432.CCR-17-0306)
28 Sottoriva A, Kang H, Ma Z, Graham TA, Salomon MP, Zhao J, Marjoram P, Siegmund K, Press MF, Shibata D, *et al.* A big bang model of human colorectal tumor growth. *Nature Genetics* 2015 **47** 209–216. (https://doi.org/10.1038/ng.3214)

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