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Original Research

Genetic origin of multifocal sporadic medullary thyroid cancer and C-cell hyperplasia

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Abstract

Objective: Sporadic medullary thyroid cancer (sMTC) mostly presents as a single lesion, but additional tumor foci may be present. The present study aimed to analyze the mutation profile of different tumor foci of multifocal sMTC to verify whether they represent an intra-organ metastatic dissemination or if they are independent tumors. Moreover, the genetics of C-cell hyperplasia (CCH) associated with sMTC was studied to verify whether CCH could be considered preneoplastic or reactive lesions.

Methods: Thirty-eight multifocal sMTCs and 15 sMTCs with associated CCH were included: A total of 106 tumor foci and 25 different CCH areas were studied. The mutational status was analyzed by Next-Generation Sequencing and/or droplet-digital PCR.

Results: Thirty-one/38 (81.6%) sMTCs had a somatic mutation in the main tumor, while 7/38 (18.4%) cases were negative. Thirty/31 (96.8%) mutated sMTCs had a single mutation, while 3 different mutations were detected in 1 case (3.2%). Twenty-eight/31 (90%) mutated sMTCs showed the same mutation profile in the main tumors and in all secondary foci, while 3 cases were discordant. Eleven/15 (73.4%) sMTC with CCH showed a somatic mutation in the main tumor, while 4 (26.6%) were negative. Only 1/11 (9%) mutated cases showed the same mutation in the main tumor and in the CCH.

Conclusions: Our data demonstrate that multiple foci of sMTC share the same driver mutation as the main tumor and support the hypothesis that they are intrathyroidal metastases. Most of the CCH associated with sMTC should not be considered a preneoplastic lesion as they are negative for the mutation of the main sMTC.

Keywords: medullary thyroid cancer, C-cell hyperplasia, multifocality, polyclonality, *RET*

Significance

Our data demonstrate that multiple foci of sporadic MTC are intrathyroidal metastases and not independent tumors and that most of the CCH accompanying sporadic MTC are not preneoplastic lesions.

Introduction

Medullary thyroid cancer (MTC) is a particular type of thyroid cancer since it originates from parafollicular C cells that are not real thyroid cells. Medullary thyroid cancer is rare and accounts for about 3%-5% of all thyroid cancers. Medullary thyroid cancer is sporadic (sMTC) in approximately 75% of cases and hereditary (hMTC) in the remaining 25%.[1]

The pathogenesis of MTC has been extensively studied, and it has been demonstrated that hMTC is almost exclusively caused by germline mutations of the *RET* (REarranged during Transfection) proto-oncogene.[2] *RET* mutations are also present at the somatic level in sMTC in about 40%-80% of cases,[3-7] with this prevalence being much higher in more advanced tumors.[8] In addition to somatic *RET* mutations, *HRAS* and *KRAS* somatic mutations have been described in about 20% of sporadic cases.[3,9,10] Medullary thyroid cancer typically presents as a single lesion, but the occurrence of additional tumor foci, either in the same or in the contralateral lobe, is often observed in familial cases and, although less commonly, also in the sporadic form.[11,12] As reported in a multicentric and international study, the prevalence of multifocality and bilaterality analyzed in more than 300 sMTC cases was 16% and 5.6%, respectively.[13]

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The genetic intra- and inter-tumor heterogeneous distribution of mutations has been reported in about 20% of sMTC when comparing different areas of the same tumor or the main tumor and the corresponding metastatic lesions.[14,15] However, at least to our knowledge, limited data are available on the genetic profile of multifocal MTC, and the question of whether multiple foci of cancer represent different tumors or are the consequence of intra-organ metastatic dissemination of the main tumor remains unanswered.

Medullary thyroid cancer has also been described in association with C-cell hyperplasia (CCH), which has been occasionally reported in sporadic cases and with a higher prevalence in hereditary cases.[16-18] By definition, CCH is characterized by an increased number of normal C cells (more than 50 cells in a low-power field) and can be identified by the presence of intrafollicular C-cell proliferation with varying degrees of dysplasia, typical of invasive solid tumors.[19] C-cell hyperplasia can be classified as reactive or preneoplastic,[17] and it has been hypothesized that preneoplastic CCH could be considered a precursor of MTC.[18,20,21] To our knowledge, the genetic profile of CCH associated with sMTC has been analyzed in very few cases,[16,22] and its clinical and pathological significance remains undefined.

The present study aimed to analyze the mutation profile of different tumor foci in multifocal sMTC, as well as of CCH associated with sMTC, to verify whether different tumor foci represent an intra-organ metastatic dissemination of the main tumor or if they are independent tumors, and to determine whether CCH associated with sMTC could be considered a preneoplastic or reactive lesion.

Methods

Study population

In the entire series of multifocal sMTC patients consecutively diagnosed, treated, and followed up at the Endocrine Unit of the University Hospital of Pisa, Italy, between 2005 and 2018, we identified 38 multifocal sMTCs and 15 sMTCs with associated CCH. These cases were selected for genetic analysis. All patients signed informed consent for the use of their clinical, biochemical, and pathological data for research purposes, as well as for the genetic analysis of their tumor tissue. The investigation was approved by a named independent ethical committee and was approved by the "Comitato Etico Regionale per la Sperimentazione Clinica della Regione Toscana" (prot. n. 6714, signed on 05/02/2019), and it was performed according to the Declaration of Helsinki.

Genomic DNA extraction

We analyzed a total of 106 tumor foci, including both main and secondary foci, from 38 sMTCs, and a total of 25 different CCH areas from 15 sMTCs with CCH. DNA from both the tumor foci of the 38 multifocal sMTCs and the CCH areas of the 15 sMTCs was extracted from FFPE samples after microdissection of areas independently identified by 3 pathologists (F.S., B.F., and C.U.), who also estimated the percentage of tumor and CCH cells. DNA extraction was performed using the QIAMP GeneREAD DNA FFPE (#180134, QIAGEN, Hilden, Germany) with an automated QIAcube® Connect system (QIAGEN, Hilden, Germany).

Screening of mutational status

The main tumor was analyzed by Next-Generation Sequencing (NGS) using the Ion Gene Studio S5 System (Ion Torrent; Applied Biosystems, Carlsbad, CA, USA) and a custom panel as previously described.[3] All variants detected by NGS were validated by Sanger Sequencing using a protocol previously reported[14] or by droplet-digital PCR (ddPCR, Bio-Rad QX 200 Droplet Digital™ System, Bio-Rad Laboratories, Hercules, CA, USA) with a Supermix ddPCR for Probes (no dUTP). The mutation of the main tumor was tracked in the secondary tumor foci and CCH areas by ddPCR or Sanger Sequencing (Bio-Rad Laboratories, Hercules, CA, USA RET p.Met918Thr dHsaMDS309834335; RET p.Cys634Arg dHsaMDS251 5168; HRAS p.Gln61Arg dHsaMDV2510576; KRAS p.Gly12 Arg dHsaMDV2510590; RET p.Val804Met dHsaMDS756240 204; RET p.Asp631_633insSer dHsaMDS163586657; HRAS p.Gly12Arg dHsaMDS2510804; HRAS p.Gln61Lys c.182A > T dHsaMDV2510556). A positive and a negative control were included in each run of ddPCR, and we considered the experimental quality to be acceptable only for experiments in which at least 10k droplets were generated. Samples were considered positive when at least 2 droplets had the mutation detected. When the main tumor was found to be not mutated by NGS, the corresponding secondary foci were analyzed by NGS to verify the presence of other alternative driver mutations.

Immunohistochemistry (IHC) for calcitonin

Both secondary tumor foci that did not present the same driver mutation as the main tumor and the CCH areas were analyzed by immunohistochemistry (IHC) for calcitonin to verify their true origin from parafollicular C cells. Immunohistochemistry was performed using the fully automated BenchMark ULTRA system platform (Roche-Ventana Medical Systems, Tucson, AZ, USA), with the antigen-antibody reaction visualized using the Optiview DAB IHC VENTANA Detection Kit Rabbit. We used the polyclonal anti-calcitonin antibody CELL MARQUE (CA, USA).

Results

Molecular study

Mutational profile of 38 sporadic MTC

Thirty-one out of 38 (81.6%) multifocal sMTCs included in this study had a somatic mutation, while 7 out of 38 (18.4%) cases had no detectable mutations in the main tumor, as analyzed by NGS. Thirty out of 31 (96.8%) mutated multifocal sMTCs had a single mutation, while 1 case had 3 mutations (*HRAS* p.Gln61Arg + *RET* p.Cys634Arg + *KRAS* p.Gly12Arg) in the main tumor (3.2%). As shown in Figure 1, the most common mutation was *RET* p.Met918Thr in exon 16, which was found in 20/31 mutated cases (64.5%). Additionally, we found 5/31 cases (16.1%) with a *RET* mutation in exon 11 (3 cases with an indel: p.Glu632_639delinsHisArg, p.Asp631_633insSer, p.Cys634_Arg635dup, and 2 cases with *RET* p.Cys634Arg), 1 case with p.Ala883Phe in exon 15 (3.2%), and another with p.Val804Met in exon 14 (3.2%). A total of 28/38 (73.7%) cases harbored a *RET* mutation, while only 3/38 (7.9%) carried a single *HRAS* mutation (ie, p.Gln61Arg, p.Gln61Lys, and p.Gly12Arg). The types of mutations and the variant allelic frequencies found in this series, both in the main tumor and their corresponding foci, are reported in Table S1.

Mutational profile of different tumor foci in multifocal sMTC

Overall, 106 tumor foci were analyzed in this study: 38 were represented by the main tumor identified by their larger size,

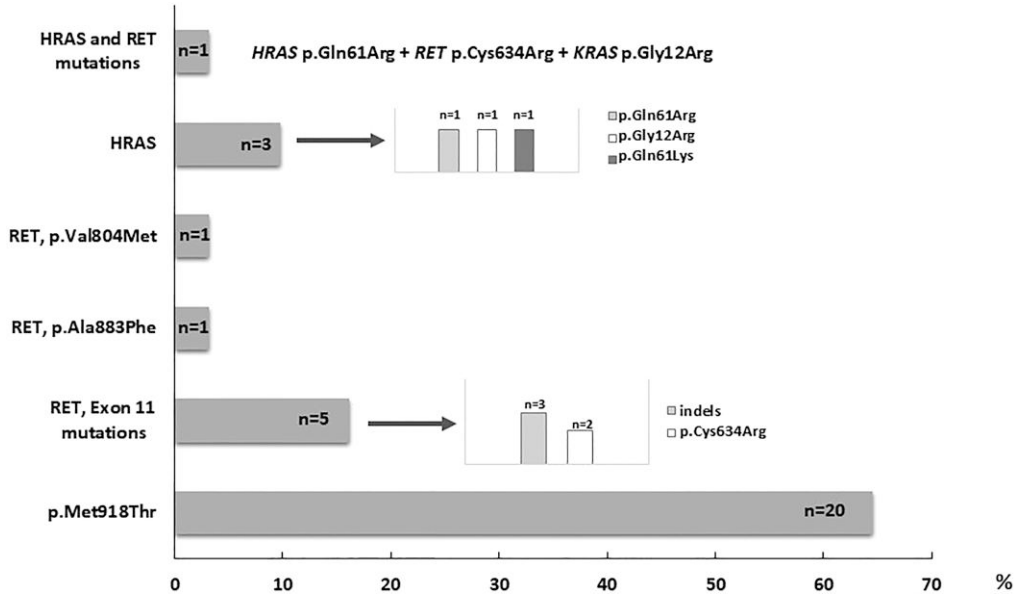

Figure 1. Mutational profile of the 31/38 multifocal sporadic MTC cases identified by NGS. The prevalence of all mutations is reported.

and 68 were secondary foci (42 ipsilateral and 26 contralateral). Considering the main tumor and the additional secondary foci, we observed that 5 different tumor foci were present in 1 sMTC (2.6%), 4 foci in 4 sMTCs (10.5%), 3 foci in 18 sMTCs (47.4%), and 2 foci in 15 sMTCs (39.5%) (Figure 2A). Twenty-eight out of 31 mutated multifocal sMTC (90.3%) showed the same driver mutation in all investigated foci, while 3 cases were discordant. As shown in Figure 2A, 2 of these 3 discordant cases (*n* 29 and *n* 30) showed no mutation in the secondary foci. The mean size of these negative foci was 1.2 ± 0.3 cm (median 1.1 cm) and was like the mean size of the foci in which the driver mutations were detected and confirmed (1.7 ± 1.5 cm, median 1.1 cm). To confirm that tumor foci negative for somatic mutations were indeed real MTC foci, our pathologist performed IHC for calcitonin in the tissue slices adjacent to those used for genetic analysis: The positive immunohistochemical staining for calcitonin confirmed that the tumor foci were MTC (Figure 3).

Only 1 case out of 31 (3.1%) (*n* 31 in Figure 2A) had a heterogeneous mutation profile in the main tumor with 3 different somatic mutations (*HRAS* p.Gln61Arg + *RET* p.Cys634Arg + *KRAS* p.Gly12Arg) that were all present in 1 additional secondary ipsilateral focus while another focus in the same lobe presented only the *HRAS* p.Gln61Arg + *RET* p.Cys634Arg mutations and the other secondary focus, located in the contralateral lobe, showed only the *RET* p.Cys634Arg somatic mutation (Figure 2B).

Seven cases were found to be negative in the main tumor (18.4%). The corresponding secondary tumor foci were analyzed by NGS and were found to be negative for all the mutations included in the screening panel, thus confirming the consistency of the genetic profile between the main and secondary foci also in these negative cases. The IHC for calcitonin confirmed that these were true MTC foci.

Molecular analysis of CCH tissues from FFPE

Eleven out of 15 selected sMTCs with CCH (73.3%) were found to be positive for the presence of a somatic mutation in the main tumor, while 4 (26.7%) were negative. Only 1 of the 11 mutated cases was found to harbor the same mutation (*HRAS* p.Gln61Arg) identified in the main tumor, also in the associated CCH. In contrast, the CCH areas associated with the 10 mutated cases were negative for the mutation found in the main tumor. In the single positive case, CCH was in the same lobe as the main tumor (Figure S1). The corresponding normal tissue was not affected by the *HRAS* mutation, demonstrating that this mutation was not present at the germline level. C-cell hyperplasia samples from the 4 negative cases were analyzed by NGS and found to be negative for all the mutations included in the screening panel. Hematoxylin/eosin staining and IHC for calcitonin confirmed that these areas were all true CCH.

Discussion

The high prevalence of multiple tumor foci in hMTC is likely due to the constitutive *RET* activation in every single C cell, thus inducing tumor development at different sites. However, although at a lower prevalence, the presence of multiple tumor foci is also observed in sMTC. For these cases, a clear explanation and the possible molecular causes responsible for multifocality are not yet well understood, and the question of whether different tumor foci should be considered intrathyroidal metastases of the main tumor, or if they are independent tumor foci, remains unanswered.

Our data identified a positive correlation between the presence or absence of the driver mutations in the main tumor and the secondary foci. These findings strongly support the hypothesis that the foci of multifocal sMTC are indeed due to intra-thyroid monoclonal metastatic dissemination, as previously suggested.[23] Similarly, a concordant mutation profile was detected also in other human tumor, such as ovarian[24] and hepatocellular[25] carcinomas, and this finding has been interpreted as the evidence that the multiple cancer foci are the result of an intra-organ metastatic dissemination. This conclusion is of great clinical interest, especially today, when there is a trend toward more conservative medicine, and the possibility of treating some cases of sMTC with lobectomy alone is

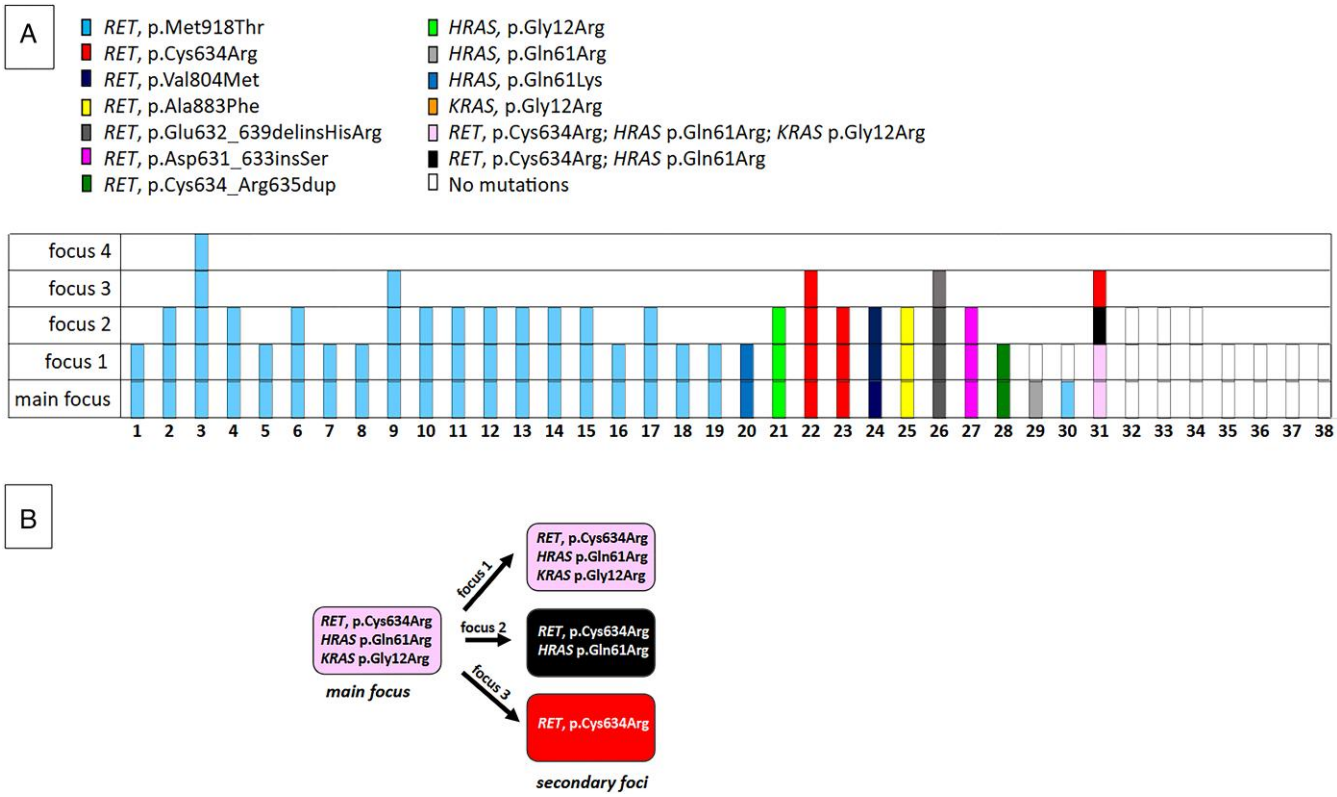

Figure 2. (A) Mutational profile of both the main tumor and secondary foci of the entire series of 38 multifocal MTC cases. Different colors are used to indicate different driver mutations. Cases 29 and 30: The mutation was found in the main tumor but not in the secondary foci; case 31: Three different mutations were found in the main tumor, which were differently present in the secondary foci. (B) Detailed description of the different distribution of the 3 mutations of case 31 in the main tumor and the corresponding foci.

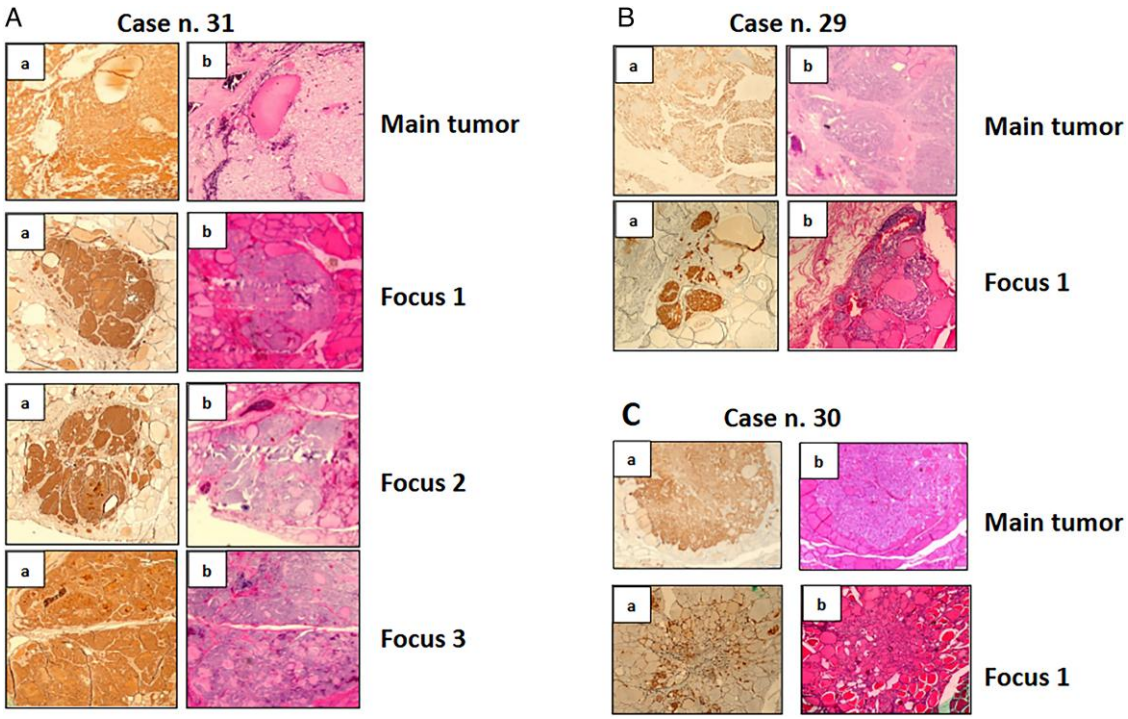

Figure 3. Histology of the 3 discordant cases (20× magnification): In panel A, case 31, the immunohistochemical staining for calcitonin (A) and hematoxylin/eosin staining (B) of the main tumor and the other 3 foci are shown; in panels B, case 29, and C, case 30, the immunohistochemical staining for calcitonin (A) and hematoxylin/eosin staining (B) of the main tumor and the other foci are shown.

being evaluated.[26-28] According to our findings, if at the time of surgery, the main tumor is found to be unique within the entire lobe, it will be very unlikely that the other secondary foci will recur in the other lobe over time.

Three multifocal sMTC cases in our series did not share the same mutation profile. In particular, 1 of the 3 cases showed different simultaneous mutations, both *RET* (1 mutation) and *RAS* (2 different mutations), in the main tumors that were not all present in the 4 secondary foci. Indeed, we previously found that some metastatic lymph nodes presented a mutational profile different from the main tumor, and we had already hypothesized the possibility that multiple cell clones existed within the same tumor.[14] The polyclonality of sMTC has been previously reported and in particular, Eng et al. demonstrated the simultaneous presence of *RET*-mutated and non-mutated cells in the same tumor tissue.[14,15] This could be the same case that we observed in the 2 discordant cases in our series, where the secondary foci did not show the mutation of the main tumor: The secondary foci could have originated from the non-mutated cells mixed to the mutated cells in the main tumor. It could be argued that the small size of the secondary foci and the possible contamination from non-tumoral cells might have impaired the detection of the mutation, making the negative result a "false" negative. However, the median size of the secondary tumor foci was the same in both positive and negative cases, thus excluding that the small size could have been a problem for the mutation detection. Moreover, the high sensitivity of ddPCR, performed with specific primers for the investigated mutation, further guarantees the accuracy of the negative result.

The overall prevalence of somatic *RET* mutations in this series of multifocal sMTC cases was higher than expected when considering the *RET* prevalence in sMTC consecutively collected (73.7% vs 64.5%).[3] It has been previously shown that the prevalence of *RET* somatic mutations increases with either the aggressiveness of the disease[8] and the tumor size greater than 2 cm.[29] Since the multifocality correlates with high-grade tumors and advanced disease[23,30] and since the mean size of the primary tumor analyzed in this study was 1.7 cm, a higher prevalence of *RET* mutations could be expected.

C-cell hyperplasia, which is often present in hMTC and less frequently in sMTC, has been classified as physiologic/reactive or preneoplastic and can be focal, diffuse, or nodular.[20] While preneoplastic CCH is usually associated with hMTC, it is still unclear whether CCH associated with sMTC can be considered a preneoplastic or reactive lesion.[18,20] Limited data are available in the literature about the genetics of CCH that would clarify this question. According to the 2 published studies, a total of 12 sMTC cases associated with CCH have been analyzed for the presence of *RET* mutations in the main tumor and in the CCH.[16,22] However, among them, only 3 main tumors were *RET*-mutated, and in all cases CCH was negative for *RET* mutations. In our series, we analyzed 15 sMTCs with CCH, and 11 of them had a *RET* mutation in the main tumor. We confirmed that CCH was negative for the driver mutation found in the main tumor in all but 1 case. Again, one might argue that the CCH areas were very small, and we could have dissected normal thyroid cells other than CCH. To resolve this doubt, our pathologists performed calcitonin IHC on the tissue slice adjacent to the slice used for genetics, demonstrating that we microdissected a real CCH area. This finding strongly suggests that CCH associated with sMTC is not preneoplastic but more likely to be reactive. We had only 1 single case in which the CCH harbored the same *HRAS* Q61R mutation found in the main tumor. A possible explanation for this finding is that this CCH area was indeed a real, and unique in our series, preneoplastic CCH that, if the thyroid gland were not removed, could have evolved into a true tumoral focus.

Our study has some limitations, and particularly the relatively restricted set of investigated genes. For this reason, we cannot exclude that the discordant cases share common mutations in genes not assessed in our targeted NGS panel.[3] For these cases, extensive NGS profiling could be more informative and beneficial. However, the 17 genes studied with our custom panel are those mainly involved in thyroid tumorigenesis and, considering that in several studies,[3-5] only RET and RAS have been found in MTC, it is very unlikely that other genetic alterations could be present.

In conclusion, our data demonstrate that multiple foci of sMTC share the same driver mutation as the main tumor and definitively confirms the hypothesis that they are intrathyroidal metastases and not independent tumors. In some polyclonal cases, the intrathyroidal metastatic foci may show different genetic profiles, in agreement with the concept that some sMTC are polyclonal. Most of the CCH associated with sMTCs are reactive rather than preneoplastic. These findings have a significant clinical impact: In the era of personalized medicine, an accurate understanding of the clonal origins of cancer and the evidence that different tumor foci may or may not have the same genetic alterations could be relevant in defining the appropriate personalized cancer treatment.

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Supplementary material

Supplementary material is available at *European Journal of Endocrinology* online.

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Authors' contributions

Jacqueline Fátima Martins de Almeida (Investigation [equal], Methodology [equal], Validation [equal], Writing—original draft [equal]), Cristina Romei (Conceptualization [equal],

Data curation [equal], Writing—original draft [equal], Writing—review & editing [equal]), Teresa Ramone (Methodology [equal], Validation [equal]), Roberta Casalini (Methodology [equal]), Raffaele Ciampi (Data curation [equal], Formal analysis [equal], Supervision [equal]), Beatrice Fuochi (Methodology [equal]), Francesca Signorini (Validation [equal]), Clara Ugolini (Supervision [equal], Validation [equal], Visualization [equal]), Virginia Cappagli (Conceptualization [equal], Data curation [equal], Investigation [equal]), Laura Sterian Ward (Project administration [equal], Writing—review & editing [equal]), and Rossella Elisei (Conceptualization [equal], Funding acquisition [equal], Investigation [equal], Writing—review & editing [equal])

Conflict of interest: There are no conflicts of interest for any author. R.E. is on the editorial board of EJE. She is not involved in the review or editorial process for this paper, on which they are listed as authors.

Ethics approval

The investigation was approved by a named independent ethical committee and was approved by the "Comitato Etico Regionale per la Sperimentazione Clinica della Regione Toscana" (prot. n. 6714, signed on 05/02/2019).

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