Prognostic Significance of Somatic *RET* Oncogene Mutations in Sporadic Medullary Thyroid Cancer: A 10-Year Follow-Up Study

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Background: Medullary thyroid carcinoma (MTC) is a well-differentiated thyroid tumor that maintains the typical features of C cells. An advanced stage and the presence of lymph node metastases at diagnosis have been demonstrated to be the most important bad prognostic factors. Somatic *RET* mutations have been found in 40–50% of MTCs. Although a relationship between somatic mutations and bad prognosis has been described, data are controversial and have been performed in small series with short-term follow ups. The aim of this study was to verify the prognostic value of somatic *RET* mutations in a large series of MTCs with a long follow up.

Methods: We studied 100 sporadic MTC patients with a 10.2 yr mean follow-up. *RET* gene exons 10–11 and 13–16 were analyzed. The correlation between the presence/absence of a somatic *RET* mutation, clinical/pathological features, and outcome of MTC patients was evaluated.

Results: A somatic *RET* mutation was found in 43 of 100 (43%) sporadic MTCs. The most frequent mutation (34 of 43, 79%) was M918T. *RET* mutation occurrence was more frequent in larger tumors ($P = 0.03$), and in MTC with node and distant metastases ($P < 0.0001$ and $P = 0.02$, respectively), thus, a significant correlation was found with a more advanced stage at diagnosis ($P = 0.004$). A worse outcome was also significantly correlated with the presence of a somatic *RET* mutation ($P = 0.002$). Among all prognostic factors found to be correlated with a worse outcome, at multivariate analysis only the advanced stage at diagnosis and the presence of a *RET* mutation showed an independent correlation ($P < 0.0001$ and $P = 0.01$, respectively). Finally, the survival curves of MTC patients showed a significantly lower percentage of surviving patients in the group with *RET* mutations ($P = 0.006$).

Conclusions: We demonstrated that the presence of a somatic *RET* mutation correlates with a worse outcome of MTC patients, not only for the highest probability to have persistence of the disease, but also for a lower survival rate in a long-term follow up. More interestingly, the presence of a somatic *RET* mutation correlates with the presence of lymph node metastases at diagnosis, which is a known bad prognostic factor for the definitive cure of MTC patients. **(*J Clin Endocrinol Metab* 93: 682–687, 2008)**

Medullary thyroid carcinoma (MTC) is a well-differentiated thyroid tumor that maintains the biochemical and pathological features of the parafollicular or calcitonin-producing C cells from which it derives (1, 2). Its origin makes it a separate entity from the other differentiated thyroid carcinomas. The biological behavior of MTC is much less favorable when

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Abbreviations: CT, Computerized tomography; FNAB, fine-needle aspiration biopsy; MEN 2B, multiple endocrine neoplasia type 2B; MTC, medullary thyroid carcinoma; Pg, prostaglandin.

compared with those of the other well-differentiated thyroid carcinomas, even though it is not as unfavorable as that of anaplastic carcinoma (3). A 10-yr survival of about 50% of MTC patients has been reported in several series. Both the cure and survival of these patients are positively affected by an early diagnosis (4). An advanced stage at diagnosis and, in particular, the presence of lymph node metastases (stage III), have been the most important bad prognostic factors (5, 6). On the basis of this evidence, it is conceivable that only an early diagnosis and complete surgical treatment should provide the only curative approach for MTC patients (4, 7).

A decade ago, we and others (8–11) demonstrated that somatic *RET* gene mutations, which are present in about 40–50% of MTC, could represent a bad prognostic factor for the outcome of MTC patients. These studies were performed in small MTC series with short-term follow-ups. Furthermore, this observation has not been confirmed in several other series (12–15). The aim of the present study was to reevaluate the role of somatic *RET* mutations as a bad prognostic factor in a large series of MTC with a median follow-up of 10 yr. In particular, we analyzed the relationship of somatic *RET* mutations with all the other epidemiological and pathological features of the tumors.

Patients and Methods

Patients

We studied 100 patients, 43 males and 57 females, affected by sporadic MTC with a mean age at diagnosis of 49.6 yr (range 20–83, median 49) and mean follow-up 10.2 ± 5.5 yr (range 3–32, median 10). All patients were submitted to total thyroidectomy and central neck dissection as the minimal standard procedure at the same institution. The lymphadenectomy of the lateral compartment(s) was performed during the first surgical treatment if node metastases were diagnosed before surgery.

All patients had been classified as having the sporadic form of MTC. This classification was based on the apparent absence of *RET* germline mutations, absence of familial history of the disease, and negative clinical and laboratory data for the presence of other endocrine neoplasia.

Clinical data were recorded in a computerized database and analyzed according to the presence or absence of a *RET* mutation.

Informed consent for *RET* genetic screening and other clinical procedures was signed by all investigated subjects.

Genomic DNA extraction and PCR amplification

After 1990, all primary MTC tissues were collected at surgery, immediately frozen in liquid nitrogen, and kept at −80 C. Paraffin-embedded primary MTC tumoral tissues were used for patients operated on before 1990. Blood from all patients was collected in EDTA. Genomic DNA was purified from peripheral blood lymphocytes using the QIAMP DNA mini kit (QIAGEN, Hilden, Germany) and from tumoral tissues using an in-house method based on overnight proteinase K digestion at 55 C, followed by phenol/chloroform extraction and ethanol precipitation. DNA was kept in Tris-EDTA at −20 C. An aliquot of DNA was also stored at −20 C with the intent of searching for new *RET* mutations whenever described.

The *RET* gene exons 10, 11, and 13–16 were analyzed in all cases using PCR and sequencing conditions, as previously reported (16). Whenever a *RET* point mutation altered (creating or deleting) the recognition site of a restriction enzyme, we performed the restriction analysis of the mutated exon to confirm the presence of the *RET* mutation.

Follow-up management

All MTC patients underwent a pentagastrin (Pg) stimulation test for Calcitonin (CT) and neck ultrasound 6 and 12 months after total thyroidectomy. Basal and stimulated CT negative patients were considered disease free with a very low risk of recurrence (17) and were submitted to another Pg stimulation test 3–5 yr later. Patients with detectable levels of basal and/or Pg-stimulated CT were evaluated with imaging techniques [computerized tomography scan, magnetic resonance imaging, and OctreoScan (Pickler, Cleveland, OH)] to reveal the localization of the metastatic disease. Patients with detectable levels of basal and/or Pg-stimulated CT and negative imaging results were defined as affected by a biochemically persistent disease (4).

Histology

MTC histological diagnosis was addressed by typical histological (*i.e.* tumoral cells arranged in trabecular, insular, or sheet-like growth patterns) and immunohistochemical (cells positive for calcitonin and chromogranin) findings.

Statistical analysis

The statistical analysis was performed with the χ^2, *t*, and Mann-Whitney *U* tests according to the studied variables. Survival curves were analyzed using the Kaplan-Meier method, and the statistical significance was assessed by the log-rank test. The multiple logistic regression test was used to determine the independent effect of a somatic *RET* mutation and the other clinical and pathological features on the outcome of MTC patients. A *P* value less than 0.05 was considered significant.

Results

Genetic analysis

Genetic analysis revealed a somatic *RET* mutation in 43 out of 100 (43%) sporadic MTCs (Fig. 1A). The majority of *RET* mutations (34 of 43, 79%) were located at codon 918 of exon 16. The major mutation substituted a methionine (ATG) for a threonine (ACG). The other nine mutations were found in exons 10, 11, and 15. In particular, seven of 43 (16.2%) were at codon 634 of exon 11 (four C634R, two C634W, and one C634Y), one of 43 (2.3%) was a deletion of 48 bp in exon 10, and one of 43 (2.3%) was a 2-bp missense mutation at codon 883 in exon 15 that substituted an alanine (GCT) for a phenylalanine (TTT). No mutations were identified in exons 13 or 14 (Fig. 1B).

When comparing the percentage of node positive cases according to the different *RET* mutations, we found 76.5% of *RET* 918 positive patients and 43% of *RET* 634 positive patients having nodes metastases. At variance, only 27% of *RET* negative cases had node metastases.

Clinical evaluation

The mean age at diagnosis and mean follow-up of the 43 MTC patients (20 males, 23 females) carrying somatic *RET* mutations were 53.1 yr (median 55, range 21–83) and 9.1 yr (median 9, range 3–21), respectively. Node and distant metastases were present in 30 of 43 (70%) and 13 of 43 (30%) cases, respectively. According to the Tumor-Node-Metastasis classification (18), five patients had stage I disease (T1N0M0), eight had stage II (T2–4N0M0), 17 had stage III (T1–4N1M0), and 13 had stage IV (T1–4N1M1). At the time of the present study, seven patients were free of disease, 28 had persistent disease, and

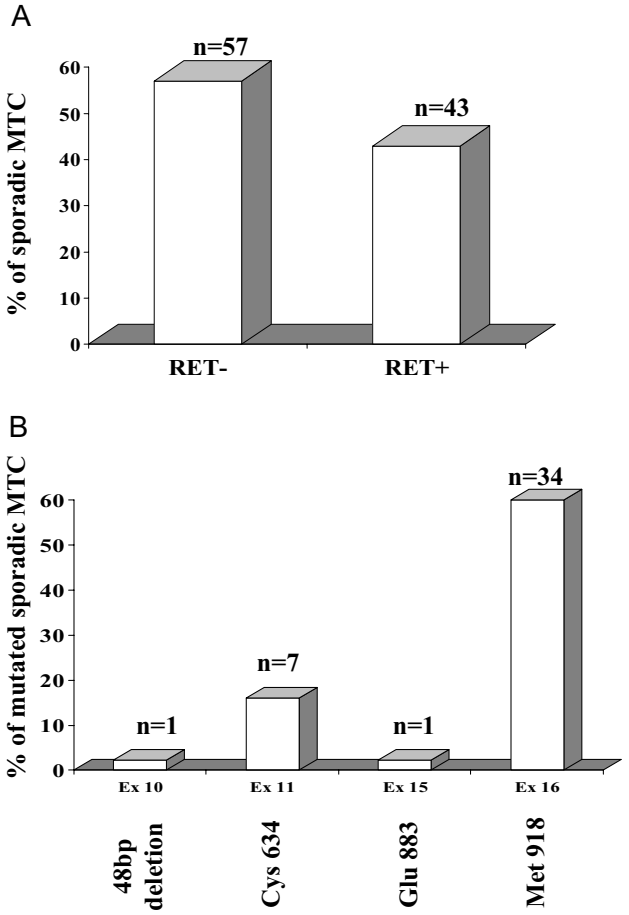

FIG. 1. Prevalence of somatic *RET* mutations found in our series of 100 sporadic MTC cases (A) according to different *RET* gene codons and exons (B). *RET*−, *RET* negative; *RET*+, *RET* positive.

eight were dead from MTC. Among the 28 patients with persistence of the disease, 13 showed a biochemical persistence of the disease, with high levels of basal and/or Pg-stimulated CT, but no evidence of metastases, whereas 15 patients were affected by metastatic disease.

The mean age at diagnosis and mean follow-up of the 57 MTC patients (23 males and 34 females) without somatic *RET* mutation were 47.1 yr (median 48, range 20–71) and 10.7 yr (median 10, range 3–32), respectively. Node and distant metastases were present in 15 of 57 (26.3%) and seven of 57 (12.2%) cases, respectively. According to the Tumor-Node-Metastasis classification (18), 14 patients had stage I disease (T1N0M0), 24 had stage II (T2–4N0M0), 10 had stage III (T1–4N1M0), and nine had stage IV (T1–4N1M1). At the time of the present study, 32 patients were free of disease, 22 had persistent disease, and three were dead from MTC. Among the 22 patients with persistence of the disease, 13 showed a biochemical persistence of the disease, whereas nine patients were affected by metastatic disease.

RET mutation and MTC clinical features

We correlated the presence of somatic *RET* mutations with the clinical features and outcome of MTC patients. As shown in Table 1, somatic *RET* mutations were significantly more frequent in larger tumors ($P = 0.03$), in cases with node ($P < 0.0001$) and distant ($P = 0.02$) metastases. No correlation was found between the *RET* mutation and number of lymph node metastases ($P = 0.8$ and $P = 0.5$ by unpaired *t* and Mann-Whitney *U* tests, respectively; data not shown). Somatic *RET* mutations were also correlated with a more advanced stage at diagnosis ($P = 0.004$).

TABLE 1. Somatic *RET* mutations and clinical-pathological features of sporadic MTC

	RET−	*RET*+	*P* value
Age at diagnosis (yr), mean (range)	47.1 (20–71)	53.1 (21–83)	0.1
Sex (F/M)	34/23	23/20	0.5
Mean follow up (yr)	10.7 ± 6.1 (3–32)	9.1 ± 4.5 (3–21)	0.1
T categories (n)			*0.03*
T1 (25)	17	8	
T2 (42)	28	14	
T3 (14)	5	9	
T4 (19)	7	12	
Node metastases (n)			*<0.0001*
N1 (45)	15	30	
N0 (55)	42	13	
Distant metastases (n)			*0.02*
M1 (20)	7	13	
M0 (80)	50	30	
Stage (n)			*0.004*
I (19)	14	5	
II (32)	24	8	
III (27)	10	17	
IV (22)	9	13	
Outcome (n)			*0.0002*
Disease free (39)	32	7	
Persistent disease (50)	22	28	
Dead (11)	3	8	

−, Negative; +, positive; F, female; M, male.

P values in *italics* are statistically significant.

TABLE 2. Correlation of somatic *RET* mutations and other clinical-pathological features of sporadic MTC and final outcome

	Free of disease	Persistent disease	*P* value
Age at diagnosis (yr), mean (range)	51.0 (24–80)	52.7 (20–83)	0.5
Sex (F/M)	25/14	32/29	0.25
Mean follow up (yr)	11 ± 6.1 (3–32)	9.4 ± 5.0 (3–29)	0.13
T categories (n)			*0.0002*
T1 (25)	15	10	
T2 (42)	21	21	
T3 (14)	3	11	
T4 (19)	0	19	
Node metastases (n)			*<0.0001*
N1 (45)	2	43	
N0 (55)	37	18	
Distant metastases (n)			*<0.0001*
M1 (20)	0	20	
M0 (80)	39	41	
Stage (n)			*<0.0001*
I (19)	15	4	
II (32)	22	10	
III (27)	2	25	
IV (22)	0	22	
RET (n)			*<0.0001*
RET + (43)	7	36	
RET − (57)	32	25	

−, Negative; +, positive; F, female; M, male.

P values in *italics* are statistically significant.

Sex, age at diagnosis, and months of follow-up were not different between patients with and without somatic *RET* mutations.

RET mutation as a prognostic factor for the outcome of MTC patients

When comparing the presence of *RET* mutations and the outcome of MTC patients, we found a positive correlation between the presence of the somatic *RET* mutation and the persistence of the disease (disease free *vs*. persistence of disease or dead patients; $P = 0.0002$). No difference was found in the distribution of the somatic *RET* mutations when comparing MTC patients with only biochemical or metastatic disease (data not shown).

In addition to the presence of a *RET* mutation, tumor size ($P = 0.0002$), node metastases ($P < 0.0001$), distant metastases ($P < 0.0001$), and a more advanced stage at diagnosis ($P < 0.0001$) were significantly correlated with a worse outcome. At variance, no correlation of sex or age was revealed (Table 2).

As shown in Table 3, the multivariate analysis, performed by logistic regression test, showed that only the advanced stage at diagnosis and the presence of the somatic *RET* mutation independently correlated with a worse outcome of MTC patients ($P < 0.0001$ and $P = 0.01$, respectively).

Furthermore, when the survival curves of MTC patients with and without somatic *RET* mutations were evaluated, a significantly lower percentage of surviving patients in the group with the somatic *RET* mutation ($P = 0.006$) was demonstrated (Fig. 2).

Discussion

MTC is an aggressive thyroid carcinoma whose prognosis is highly related to an advanced stage at diagnosis (5, 6). Both an early diagnosis and a complete surgical treatment (total thyroidectomy and central neck lymph node compartment dissection, at least) can greatly improve the outcome of these patients (4, 7). The possibility to identify, before surgical treatment, those cases with a greater probability to develop lymph node and/or distant metastases can be of clinical benefit because a more aggressive but, hopefully, resolving therapeutic strategy could be applied. In this study we confirmed a previous observation made by ourselves and others (8–11) more than 10 yr ago that the presence of a somatic *RET* mutation in MTC tumoral tissue represents a bad prognostic factor for the outcome of patients. In the present series, the largest that has been published to our knowledge, we provide evidence that MTC patients with a somatic *RET* mutation not only have a greater probability of unsuccessful treatment but that they have a higher probability of dying of the disease, as demonstrated by their significantly worse 30-yr survival rate with respect to that of patients without somatic *RET* mutations.

Among all somatic mutations, we found that 79% of *RET* mutations were at codon 918 of exon 16 (M918T). It is worth noting that multiple endocrine neoplasia type 2B (MEN 2B) syndrome, which is characterized by the most aggressive form of hereditary MTC, is also mainly associated with the germline M918T mutation (19–21). Among all *RET* mutations, M918T

TABLE 3. Multivariate logistic regression analysis of parameters correlated with a worse outcome of MTC patients

Variable	Odds ratio	95% CI	*P* value
RET mutation	0.2009	0.0589–0.6852	0.01
Stage	0.1574	0.0734–0.3377	<0.0001

CI, Confidence interval.

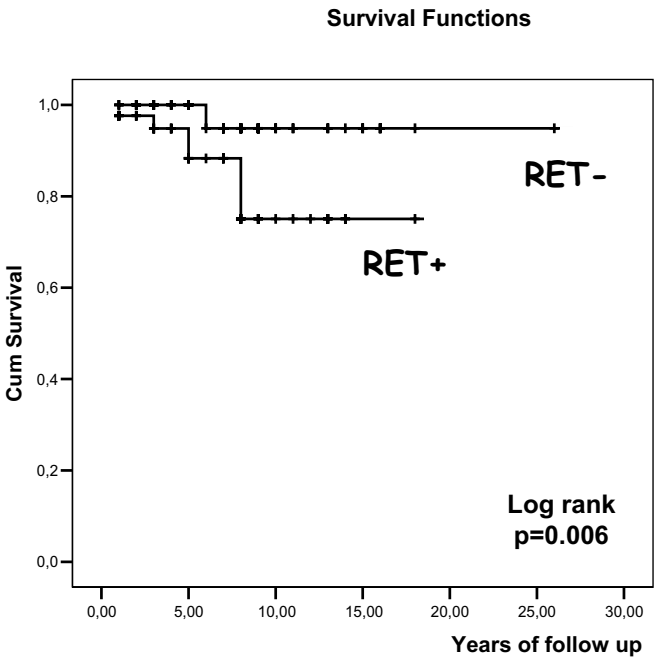

FIG. 2. Survival curves of MTC patients with and without *RET* somatic mutations. Patients with somatic mutations showed a significantly (P = 0.006, by log-rank test) lower survival. Cum, Cumulative; *RET*−, *RET* negative; *RET*+, *RET* positive.

has had the highest penetrance and transforming activity (22, 23). On the basis of these considerations, we derive the conclusion that the worst outcome of somatic *RET* positive MTC patients is related to the presence of this highly transforming mutation. Unfortunately, the other somatic *RET* mutations are rare, and a comparison between patients with the somatic mutation at codon 918 *vs.* those with the other types of somatic mutations is not possible in our series. However, it is worth noting that among the other nine somatic mutations, seven were located at codon 634, which is the most frequently altered codon in MEN 2A, and one was at codon 883, which, although less frequent than M918T, also has been described as being associated with MEN 2B. On the contrary, none of the noncysteine coding codons with *RET* mutations, which have been demonstrated to have less transforming activity (24, 25) and to be more frequently associated with familial MTC (26), was found in this series of sporadic MTC. Another evidence supporting the hypothesis that the type of *RET* mutation is related to the degree of aggressiveness of the MTC is that tumors with mutation at codon 918 showed the highest prevalence of lymph node metastases (76.5%), with respect not only to negative cases (27%), but also those with *RET* mutation at codon 634 (43%).

The presence of metastatic nodes at the first surgical treatment is a well-known negative prognostic factor for the complete cure of the disease (6). In the present series, we demonstrated a statistically significant correlation between the mutation and lymph node metastases at diagnosis. This correlation can justify the negative role of somatic *RET* mutation in the outcome of these patients. In our opinion this finding also has a practical implication because the *RET* mutation can now be determined by analyzing DNA extracted from the tumoral thyroid nodule obtained by fine-needle aspiration biopsy (FNAB) (11). Although it is accepted that the surgical dissection of the lateral node compartment should be included whenever a presurgical diagnosis of node metastases is achieved, it is still under debate whether a modified radical neck dissection with removal of nodes in the ipsilateral or bilateral compartment should be performed on principle (27, 28). This controversy is of great significance because patients with MTC can be cured by the initial surgical treatment, thus, the most appropriate procedure should be chosen at that time. However, radical neck dissection can result in significant morbidity (29) and should be avoided if the tumor is still intrathyroid (stage I), a stage that has had a very low risk of recurrence and death (4–7). The identification of a *RET* mutation in the DNA extracted from FNAB could allow for preoperative stratification of patients at high (*RET* positive) and low (*RET* negative) risk of developing lymph node metastases, and, as a consequence, the surgical strategy could include or omit radical neck dissection, respectively. However, because it has been reported that in some MTCs *RET* somatic mutations are not present in all tumoral cells (30), the possibility to obtain negative *RET* cells with FNAB makes less sensitive the suggested presurgical genetic screening.

In the era of the development of new targeted therapies, the demonstration that *RET* mutations are related to a worse outcome also has a direct therapeutic implication. *RET* positive cases might be treated with tyrosine kinase inhibitors and, in particular, with those having a higher affinity for *RET* (31). Although tumor *RET* gene mutation analysis could help to predict patients who will respond to *RET*-targeted therapies, unfortunately, the genetic test is not commercially available, and, at present, it is performed only in thyroid cancer-dedicated clinical units.

In conclusion, we demonstrated that the presence of a somatic *RET* mutation correlates, independent of other clinical and pathological features, with a worse outcome of MTC patients, not only in terms of the highest probability for disease persistence after surgery, but also for a lower survival rate at the long-term follow-up. This finding validates the role of somatic *RET* mutations as a bad prognostic factor for MTC. More interestingly, the presence of a somatic *RET* mutation correlates with the presence of lymph node metastases at diagnosis, thus suggesting a role of somatic *RET* mutation screening before surgery to have the decision of including bilateral neck node dissection in the standard surgical procedure of total thyroidectomy and central neck dissection. As far as a routine clinical application is concerned, this diagnostic approach should be cautiously suggested and eventually performed only in very specialized centers.

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