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Somatic mutations in the *RET* proto-oncogene in sporadic medullary thyroid carcinomas

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Abstract

The frequency and prognostic relevance of *RET* proto-oncogene somatic mutations in sporadic medullary thyroid carcinoma (MTC) remain controversial. In order to study somatic mutations in the *RET* proto-oncogene in sporadic MTCs found in the Czech population and to correlate these mutations with clinical and pathological characteristics, we investigated 48 truly sporadic MTCs by sequencing classical risk exons 10, 11, 13, 14, 15 and 16. From the 48 tumors studied, 23 (48%) had somatic mutation in the *RET* proto-oncogene in exons 10, 11, 15 or 16. The classical somatic mutation Met918Thr in exon 16 was only found in 13 tumors (27%). In five cases, multiple somatic mutations and deletions were detected. A statistically significant correlation between the presence of somatic mutation with more advanced pathological TNM stages was observed. Other clinical and pathological characteristics did not show any statistical significant association with the presence or absence of somatic mutation.

Keywords: *RET* proto-oncogene; Medullary thyroid carcinoma; Somatic mutation; Genetics; Prognosis

1. Introduction

Medullary thyroid carcinoma (MTC) is a malignant tumor of the neural crest-derived parafollicular C cells. It occurs in both sporadic (75%) and familial (25%) forms. The familial forms occur either as a familial MTC (FMTC) or as a part of multiple endocrine neoplasia type 2 (MEN 2) syndromes. Mutations of the *RET* proto-oncogene are involved in the pathogenesis of not only MEN 2 syndromes and FMTC, but also of sporadic MTC. Apart from germline mutations in hereditary syndromes, various somatic mutations located in the tyrosine kinase domain and cysteine-rich domain of the *RET* proto-oncogene are also found in truly sporadic MTC. The somatic mutation Met918Thr in exon 16 in the intracellular tyrosine kinase domain as well as minor somatic mutations at codons 768 in exon 13 and 883 in exon 15 have been diagnosed in approximately one-third of sporadic MTCs (Hofstra et al., 1994; Eng et al., 1995a,b; Marsh et al., 1996). Only a few mutations at codons 630 and 634 and deletions have been reported in the extracellular cysteine-rich domain (Romei et al., 1996; Donis-Keller et al., 1993; Hofstra et al., 1996; Ceccherini et al., 1997; Alemi et al., 1997).

The use of a detected somatic mutation as a marker of prognosis has been questioned, its value has been debated with inconclusive results in different studies. Some studies have shown a significant difference in the clinical outcome of sporadic MTC based on the presence of a codon 918 mutation (Zedenius et al., 1995; Jhiang et al., 1996; Romei et al., 1996; Wohllk et al., 1996), whereas others reported no significant difference.

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In this report, we present results from the detection of somatic mutations of the *RET* proto-oncogene with genotype–phenotype correlation analysis in a cohort of 48 patients with sporadic MTCs.

2. Materials and methods

2.1. Patients

Tumor tissues were formalin-fixed and paraffin-embedded (46 tissues) or fresh frozen (6 tissues). The paraffin blocks with tumor tissue were retrieved from the authors' archives (R.K., A.R. and J.D.). All patients had undergone surgery (total thyroidectomy) between 1987 and 2004.

A total of 52 apparently sporadic MTC cases were analyzed for *RET* gene mutations. These patients had no family history of hereditary MTC, pheochromocytoma, parathyroid disease, skeletal abnormalities, mucosal neuromas or Hirschsprung's disease. Table 1 describes the truly sporadic MTC patients' data.

On the basis of our genetic testing, in 4 cases of the 52 (7.7%) apparently clinically sporadic MTC, germline mutations were found: in exon 10 Cys609Tyr, in exon 13 Glu768Asp, in exon 14 Val804Met and a double germline mutation in exons 10 Cys620Phe and 13 Tyr791Phe. These patients were reclassified as FMTC and excluded from our statistical analysis and descriptions.

In addition, four MEN 2 cases were included as positive controls for methodological reasons (MTC tissues obtained from two MEN 2A and two MEN 2B patients).

2.2. Clinical and pathological data

Additional clinical and pathological data was collected including sex, age at diagnosis, serum calcitonin (CT) levels after operation and at the last control (RIA kit, DFL-1200, USA; normal values were considered under 40 pg/ml and increased above 40 pg/ml), pathological TNM classification at the time of operation, presence of local and distant metastases during follow-up period, clinical outcome, length of follow-up period, disease-free interval, tumor size (as the maximum diameter of the tumor), tumor differentiation (defined as well/poorly differentiated according to Shan et al., 1998), vascular invasion and the presence of amyloid and the presence of necrosis.

In the pTNM classification (accordingly to the 6th edition of AJCC—American Joint Committee on Cancer, Greene et al., 2002) the T1 category represents tumors 20 mm or less in its greatest dimension and limited to the thyroid, T2 tumors between 21 and 40 mm in its greatest dimension and limited to the thyroid, T3 tumors more than 41 mm in its greatest dimension and limited to the thyroid or with minimal extrathyroid extension and T4 tumors of any size extending beyond the thyroid capsule.

Medullary carcinomas were usually unencapsulated, showing infiltrative growth into surrounding parenchyma. They were composed mostly of polygonal and spindled cells with polymorphous nuclei, bi- or multinucleated cells were present as well.

2.3. Genetic analysis

The mutation analysis was performed with the informed consent of each patient. DNA was extracted in each case from fresh or formalin-fixed paraffin-embedded tumor tissue and corresponding non-neoplastic thyroid tissue or peripheral blood leukocytes by modified phenol–chloroform isolation protocol as described previously (Jindrichova et al., 2003). Each tumor sample included in this study was confirmed to contain a minimum of 70% tumor cells. PCR amplifications of exons 10, 11, 13, 14, 15 and 16 and subsequent double-stranded fluorescent sequencing were performed according to our previously described procedure (Jindrichova et al., 2004).

2.4. Statistical analysis

For statistical evaluation of the data Chi-squared tests, Fisher exact tests and Mann–Whitney tests were used. Values of $p < 0.05$ were considered to be statistically significant.

3. Results

3.1. Frequency of somatic mutations in sporadic MTCs found in the Czech population

Germline mutations (*RET* proto-oncogene mutation detected in tumor tissue as well as in the normal control tissue or blood) were present in 4 out of 52 (7.7%) cases of clinically apparently sporadic MTC; these cases were excluded from further analysis. Forty eight tumors carrying no germline mutation represent truly sporadic MTC. In 23 of them (48%) somatic mutations of the *RET* proto-oncogene were revealed. The detection rate and types of somatic mutations are presented in Table 2. In 13 cases (27%) the classical somatic mutation Met918Thr in exon 16 was found. Mutations in exon 16 were also involved in another 4 cases (8%). Seven tumors carried a somatic mutation in exons 10, 11 or 15. The 6 bp deletion of codons 632–633 in exon 11 was detected in three of these patients. Multiple mutations were found in two MTCs. These two cases were reported by us earlier (Dvorakova et al., 2006). In 25 samples (52%) no mutations in the six screened risk exons of the *RET* proto-oncogene were detected.

3.2. Genotype–phenotype correlation

Table 3 summarizes clinical and pathological characteristics with respect to the presence/absence of somatic mutation. As to sex distribution, there are more females in the group without somatic mutation in comparison to the group with detected somatic mutation (62.1% vs. 37.9%, Fisher exact test, n.s.). The groups did not differ in the age of the patient at diagnosis. Pathological TNM classification was significantly worse in patients with somatic mutation (T1, T2, T3 and T4 in groups with and without mutation; Fisher exact test, $p = 0.022$). If T1, T2 and T3 classes were grouped and compared with the T4 class, the statistical significance was much more obvious (Fisher exact test, $p = 0.003$). Interestingly, all three patients with the deletion in exon 11 belong to the T4 classification. Also, the proportion of patients with increased calcitonin levels after operation and at the last checkup was higher in the group with detected somatic mutation in comparison to those without mutation but the difference did not reach statistical significance (Fisher exact test, n.s.). There is a lower rate of relapse among patients who did not exhibit somatic mutation (40% vs. 60%) and these patients had a better clinical outcome in comparison to the *RET* mutation carriers (Fisher exact test, n.s.). When analyzing tumor size, tumor differentiation, invasion of vessels, presence of amyloid and/or necrosis, no statistically significant parameter was identified, but there is a trend towards worse variants given with somatic mutations (Fisher exact test or Mann–Whitney test, n.s.).

4. Discussion

The role of the *RET* proto-oncogene in the development of sporadic forms of MTC is still not fully understood. The rate of *RET* somatic mutations has been found to vary from 12% to 100% in published literature (Zedenius et al., 1994; Romei et al.,