

RET Proto-oncogene mutation analysis in Medullary Thyroid Carcinoma in Moroccan families: Primarily study

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Résumé

BACKGROUND: Specific missense mutations in the RET proto-oncogene are known to be associated with inherited medullary thyroid carcinoma (MTC) in the context of three clinical syndromes including multiple endocrine neoplasia type 2A (MEN2A), MEN2B and familial MTC (FMTC).

AIM: Our purpose was the identification of RET mutations in Moroccan families with inherited or sporadic MTC.

MATERIALS AND METHODS: In this study, six unrelated Moroccan families with MTC corresponding either to one MEN 2A, one FMTC and four apparently sporadic MTC and their relatives were studied. Median age at diagnosis was 17 years (MEN2A and FMTC) and 32 (sporadic MTC). Total thyroidectomy was performed in all affected patients. Genomic DNA was extracted from peripheral blood leukocytes, and exons 10, 11, 13 to 16 of the RET gene were amplified by polymerase chain reaction (PCR) and examined by DNA sequence and/or restriction enzyme analysis to detect mutations in purified amplicons.

RESULTS AND CONCLUSIONS: A common germline mutation TGC>CGC at codon 634 of exon 11 in the RET gene that resulted in amino acid substitution from cysteine to arginine was present in one unrelated MEN2A individual ; this mutation was *de novo*, it was not found in the DNA of the parents or relatives. The DNA sequence of the PCR products from the second MEN2A family showed a mutation TGC>TTC at codon 634 of exon 11 in the RET gene leading to a cysteine to phenylalanine amino acid change in two individuals. Mutations were not identified in blood DNA of the remaining patients. RET mutations found in Moroccan patients with MTC are similar to those previously reported in several MTC families worldwide. This indicates that RET mutations are highly conserved and that MTC etiology does not depend to environmental factors or ethnical differences.

Mots clés : Medullary thyroid carcinoma, MEN2A, RET Proto-oncogene

Introduction

Medullary thyroid carcinoma (MTC) derives from the parafollicular C cells and may develop in either sporadic (75 %) or hereditary form (25%). The familial form is an autosomal dominant inherited disease characterized by the presence of MTC and other endocrine tumors such as pheochromocytoma and / or parathyroid adenoma in multiple endocrine neoplasia type 2A (MEN2A), pheochromocytoma ganglioneuromatosis, mucosal neuromas and/ or skeletal abnormalities in MEN2B. Familial MTC (FMTC) is characterized by the familial occurrence of MTC without other lesions [1]. In 1993, RET proto-oncogene was shown to be involved in the pathogenesis of the hereditary form of MTC.

Germline mutations of codons 609, 611, 618, 620, and 634 in exons 10 and 11 have been observed in MEN2A and FMTC. Germline mutations of codon 918 (exon 16) have been reported in 95 % of patients with MEN2B [2, 3]. In sporadic form, somatic RET proto-oncogene mutations of tumors tissue have been found in codon 768 (exon 13) or 918 (exon 16) [4].

Traditionally, biochemical screening for elevated basal and stimulated serum calcitonin levels has been used to identify the patients at-risk to develop hereditary MTC. Unfortunately, false test results have occurred with biochemical screening, resulted in some gene carriers being missed [5]. Genetic identification of asymptomatic RET gene carriers now allows the accurate diagnosis of MEN2 at an early stage of the disease. The prophylactic total thyroidectomy can be

performed in individuals with germline RET mutation considering that they are at-risk for developing MTC [6]. In the present report, our purpose was the identification of RET mutations in Moroccan patients affected with inherited or sporadic MTC.

Matériel et Méthodes

Patients and their family members

A total of 25 Moroccan individuals were enrolled in this study. Six unrelated Moroccan families with MTC (5 females and 1 male, aged 18-70 years) corresponding either to one MEN 2A, one FMTC and four cases considered having apparently sporadic MTC as extensive questioning revealed no history of inherited MTC disease. MTC was diagnosed at variable ages in these kindred. Median age at diagnosis was 17 years (MEN2A and FMTC) and 32 (sporadic MTC). One patient died of his disease.

Genetic analysis

Before genetic testing, all patients and their at-risk family members had given their informed consent in accordance with institutional ethics guidelines and national regulations. Genomic DNA was isolated from peripheral blood leucocytes using standard salting-out technique [7]. Polymerase chain reaction amplification (PCR) of exons 10, 11, 13 to 16 of the RET gene was performed according to previously described methods [8]. Briefly, 100 ng of genomic DNA was amplified in a final volume of 50 µl reaction containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 µM dNTPs, 1.25 units of Taq polymerase and 0.5 µM of each primers (Tab.1). Genomic DNA was denatured for 5 min at 94°C prior to 35 cycles at 94, 54 and 72 °C for 40 sec at each temperature followed by 10 min 72°C polishing step. The amplified DNA was analysed on a 2 % agarose gel. An aliquot of the PCR product was digested with appropriate restriction enzyme. The presence of the mutation was confirmed by direct sequencing of the purified PCR product using the Sanger method in an automated sequencer, according to the manufacturer's instructions (ABI PRISM 3130 Genetic Analyzer, Applied Biosystems Inc., Foster City, CA, USA)

Résultats et discussion

We examined seven unrelated families suffering from MTC and found germline mutations in 3 of them.

MEN2A family. Figure 1 shows the pedigree of the kindred. The proband (II.3) a 18- year- old female was referred for screening MEN2A. She had high basal calcitonin levels. She was subjected to total thyroidectomy in 2003. The pathological findings was bilateral MTC. 24-hour urine of epinephrine and norepinephrine were abnormal indicating pheochromocytoma. Screening for

hyperparathyroidism was negative at time of diagnosis. By DNA sequencing analysis a heterozygous substitution of TGC for CGC was identified at the codon 634, which detremines a cysteine to arginine change (Fig 1.). The patient had no children and there is no family history of inherited MTC. We consider this case to be an exemple of *de novo* RET mutation.

FMTC family. The genealogy of the family is shown in figure 2. A total of five individuals from two generations were screened for RET mutations. Two first-degree relatives, patient II-2 a 23-year- old girl and patient II-3 a 24- year-old girl presented increased basal calcitonin levels.

Treatment consisted of total thyroidectomy was performed and examination of the thyroid histology showed microscopic MTC in both sisters. There has been no evidence of pheochromocytoma and parathyroid hyperplasia to date in both patients. Exon 11 PCR product sequencing analysis of individuals II-2 and II-3 showed a germline heterozygous missense TGC to TTC substitution in codon 634. This transversion leads to replacement of a cysteine with phenylalanine (Fig 2.). They had most likely inherited the mutation from her father who died early of his disease.

SMTC cases. No germline mutations in exon exon 10, 11, 13, 14, 15 or 16 in the RET in peripheral blood DNA of samples were detected; thus discarding inherited disease.

In all our available surviving at-risk families members no molecular abnormalities were detected , they were shown to bear the wild type gene and they could therefore be excluded from further clinical screening.

The RET proto-oncogene is expressed in cells of neuronal and neuroepithelial origin and encodes a receptor tyrosine kinase. Mutations of RET proto-oncogene have been well characterized and several groups have studied the disease phenotype-genotype [8, 9]. Our laboratory established a MEN2 molecular genetic diagnosis program in Morocco. The program will cover a large number of MTC patients and their relatives in the near futur.

We have studied six Moroccan families with MEN2A, FMTC and SMTC in order to provide additional information for the disease genotype-phenotype characteristics. The identification of the mutation in the RET gene in groups of inherited MTC confirms the clinical diagnosis and identifies family members with MEN2A or FMTC syndrome. Germline mutations were found in 28,57 % of these probands of all MTC families. This result is in agreement with the data stating that up to 25 % of MTC cases are inherited [10]. In agreement with data from other contries all detected mutations were found exclusively in exon 11 at codon 634 but there were

different amino acid substitutions: replacements of cysteine with arginine or phenylalanine [10, 11]. This means that RET gene mutations are highly conserved despite ethnical variations or environmental factors specific to our study population. In MEN2A family the replacement Cys 634 Arg was present; whereas in FMTC family the Cys 634 Phe mutation was detected which usually described as a cause of MEN2A. It could be explained by the misclassification of MEN2A with low penetrance of pheochromocytoma or there could be an influence of RET polymorphisms or other modifier genes that protect the FMTC from the development of pheochromocytoma [12].

Strong correlations have been found between MEN2A disease phenotypes and specific RET mutations [10, 11]. The very small number of our samples does not allow us to draw widespread conclusions on phenotype/genotype correlations. But, according to the literature, the presence of mutations at codon 634 is associated with a high risk of pheochromocytoma [10, 11]. In this study, despite similar age at diagnosis, individuals with hereditary thyroid carcinoma harboring codon 634 mutations, exhibit a highly variable disease presentation depending on the type of nucleotide amino acid substitution. Thus, individual with Cys 634 Arg phenotype had only pheochromocytoma; in contrast, individuals with Cys 634 Phe phenotype did not present any sign of pheochromocytoma and parathyroid abnormality. There is a potential explanation for this difference in the clinical manifestation of the disease is that our cases were still young patients which may have not allowed the biochemical abnormalities of MEN2A to become manifest. In fact, there are a few reports indicating that disease penetrance, age at onset, and clinical manifestation of the disease can be quite variable within carriers of the same RET mutation [13].

Genetic screening is very useful in sporadic MTC families. In our materials, the sequence analysis of blood DNA of the four sporadic MTC probands was negative for the mutation of the RET gene in the examined codons. Mutations in other domains of RET gene or molecular alterations in other genes might be involved in genesis of sporadic MTC [14, 15]. Unfortunately, we did not be able to perform analysis of the tumor DNA of the affected patients because tumor specimens were not available in all cases.

The identification of the RET proto-oncogene mutations responsible for MEN2 syndrome provides the opportunity to find mutation carriers in families at risk and simplifies the management of kindreds with this disease. Considering the very small number of our observations we have demonstrated that biological, clinical, histological and genetic characteristics of MTC in Moroccan patients with inherited or sporadic MTC are similar to those already described in several MTC families worldwide.

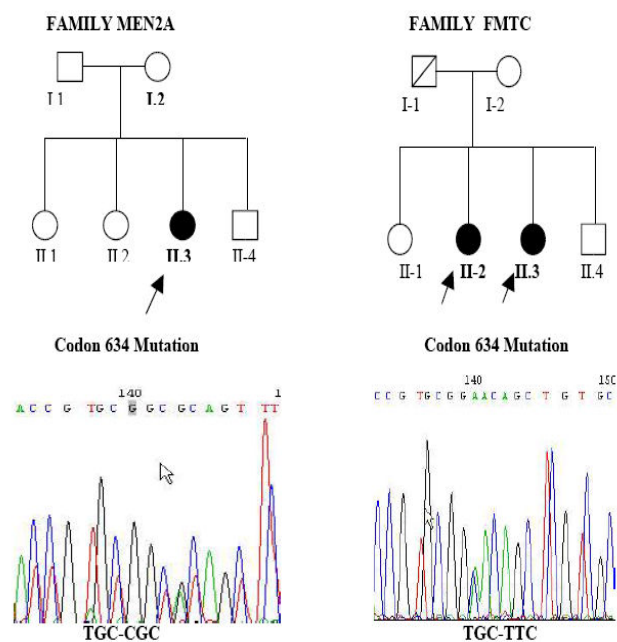


Fig. 1 Description of mutations within exon 11 of the RET proto-oncogene in two families with inherited medullary thyroid carcinoma. Partial pedigrees of the two families are shown. In each case the proband is indicated by an arrow. Genotypically positive individuals have solid symbols, negative individuals have open symbols. Deceased individuals were untested.

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