Original Article

# Study of the *RET* gene and his implication in thyroid cancer: Morocco case family

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## Abstract

**BACKGROUND:** Multiple endocrine neoplasia type 2A (MEN 2A) is an autosomal dominant inherited cancer syndrome that affects multiple tissues derived from the neural crest. Inheritance of MTC is related to the presence of specific mutations in the *RET* proto-oncogene. Almost all mutations in MEN 2A involve one of the cysteines in the extracellular domain of the *RET* receptor. **AIMS:** The objective of the present study was the biochemical and molecular characterization of the first Moroccan clinically established MEN 2A patient and at-risk family members. **SETTINGS AND DESIGN:** This is a study on a family presented with MTC referred to our institute in 2004. **MATERIALS AND METHODS:** Peripheral blood leukocyte DNA samples were isolated and amplified by polymerase chain reaction followed by restriction enzyme analysis and DNA sequencing. **RESULTS:** We identified a heterozygous germ line missense mutation at codon 634 of exon 11 in the *RET* gene that causes a cysteine to arginine amino acid substitution in the DNA of the proband; this mutation was not found in the DNA of the parents or relatives. **CONCLUSIONS:** The detection of mutated MEN 2A gene carriers enables us to differentiate high-risk members from those who bear the wild-type gene. Occasionally, application of *RET* proto-oncogene testing may lead to the detection of unexpected *de novo* mutation that could be transmitted to children.

**Key words:** Calcitonin, *de novo*, diagnosis, medullary thyroid carcinoma, *RET* proto-oncogene

Multiple endocrine neoplasia type 2A (MEN 2A) is an autosomal dominant inherited cancer syndrome that affects multiple tissues derived from the neural crest.[1,2] The syndrome is characterized by the association of medullary thyroid carcinoma (MTC), pheochromocytoma in 50% of cases and / or primary hyperparathyroidism in 20% of cases.[3,4] The specific germ line point mutations in the *RET* gene have been found to be associated with the inheritance of the MEN 2A phenotype. The human *RET* proto-oncogene, located on chromosome 10q11.2, consists of 21 exons and encodes a transmembrane receptor tyrosine kinase that plays a role in the normal development, differentiation and neoplastic growth of neural crest lineages.[1,4] *RET* protein has an extracellular domain including regions with homology to the cadherin family and a large cysteine-rich region, transmembrane domain and intracellular domain functions in the phosphorylation of tyrosine residues involved in the interaction with downstream targets and activation of signaling pathways.[4,5] Under normal conditions, *RET* receptor is activated by a multicomponent complex involving one of its ligands (glial cell line-derived neurtrophic factor, neurturin, artemin and persephin) and one of their cell surface bound coreceptors (respectively, GFRα-1, GFRα-2, GFRα-3 and GFRα-4).[5] Almost all mutations in MEN 2A involve one of the cysteines in the extracellular domain of RET receptor encoded by exon 11 (codon

634) or exon 10 (codons 609, 611, 618 and 620).[3,4] The detection of germ line mutations in the *RET* gene has important diagnostic and therapeutic impacts: First, genetic screening of patients at risk allows to identify disease gene carriers with very high specificity and sensitivity. Second, total thyroidectomy can be performed based on mutation carrier status in a prophylactic attempt, ideally in a premalignant stage of disease.[6-8] In this paper, we describe a MEN 2A kindred with *de novo* C634R *RET* proto-oncogene germ line mutation in exon 11. Although this phenomenon is not novel, the finding is the first of its kind in Morocco and since *de novo* mutation of MEN 2A has not often been described in the literature, this case could be added to the database.

## Materials and Methods

### Case presentation

This is a study on a family from the northwest of Morocco referred to our institute in 2004 and in which one member presented clinical MTC disease. This family has an apparently negative family history for relevant thyroid disorders and hypertension. The study included a total of six individuals from two generations. The index patient [II.3, Figure 1] was an 18-year-old girl. At the time of initial presentation, she presented general symptoms including palpitations, flush and a palpable thyroid nodule. In 2003, she underwent a total thyroidectomy with the lymph node surgery. Histological findings showed the presence of microscopic foci MTC in both lobes. Later, diagnostic imaging investigation comprising metaiodobenzylguanidine (MIBG) scintigraphy revealed intra-adrenal pheochromocytoma. Subsequently, the patient underwent adrenalectomy and histopathology confirmed the diagnosis of pheochromocytoma. The patient had no children and her parents were both alive and did not present any sign related to MEN 2A. A pro forma was completed for the index patient, including patient age, sex, date of diagnosis, therapeutic modalities, clinical status, previous hormonal investigation and histological findings. Classification of the index patient was done based on clinical data according to the international RET mutation consortium definitions.[3] As routinely performed in all MTC patients, the index patient and at-risk family members were submitted to *RET* mutation genetic screening. Prior to this, all individuals included in the study were fully informed and their informed consents were obtained.

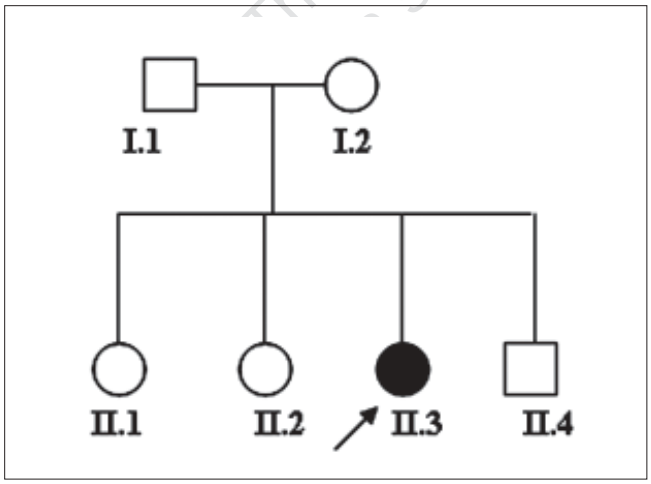


**Figure 1: Pedigree of the present family. Confirmed MTC-affected patient's is colored black. The arrow denotes the index case. Roman numerals represent generation member. Arabic numerals represent individual members.**

### Endocrine testing

For detection of MTC, basal plasma calcitonin concentrations (bCT) were measured by two-site immunoradiometric assay (IRMA) using the ELSA-hCT commercial kit (CIS Bio International, Gif sur Yvette, France). To assess parathyroid function, total calcium was determined using routine diagnostic testing and serum parathyroid hormone level (PTH 1-84) was determined by IRMA method using the ELSA-PTH commercial kit (CIS Bio International, Gif sur Yvette, France). To detect adrenal pheochromocytoma, 24-hour urine specimens were collected for estimation of the extraction of metanephrines. The follow-up of MTC, based on the determination of the bCT level and the carcinoembryonic antigen (CEA) value measured by IRMA method using the ELSA2-CEA commercial kit (CIS Bio International, Gif sur Yvette, France), was done 1 year after surgery to detect persistent or recurrent disease.

### DNA analysis for mutations of the *RET* proto-oncogene

High molecular weight DNA was isolated from peripheral blood leukocyte samples and collected on ethylenediamine tetraacetate according to standard protocols.[9] After measurement of DNA concentration and DNA/protein ratio, the DNA was used for polymerase chain reaction (PCR) amplification. The amplification of the DNA segment containing codon 634 in exon 11 was done as described by Donis-Keller *et al* using the following primers (11F, 5'-CCTCTGGCGGTGCCAAGCCTC-3'; 11R, 5'-CACCGGAAGAGGAGTAGCTG-3').[6] One hundred nanograms of genomic DNA was amplified in a final volume of 50 µl using 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 primer. Genomic DNA was denatured for 5 min at 94°C prior to 35 cycles at 94, annealing at 54°C, elongation at 72°C for 40 s at each temperature followed by 10 min

72°C polishing step. A negative control without DNA was applied in each reaction. The amplified DNA was analyzed on a 2% agarose gel. The presence of mutation was detected by digesting an aliquot of the PCR product with the restriction enzymes HhaI, RsaI and Hae III at 37°C for 3 h. Then, the product was examined on a 2.5% agarose gel and the bands visualized by ethidium bromide staining. The only informative digestion was that with HhaI. A new site for this enzyme is, in fact, generated by the Cys 634 Arg mutation; as a consequence, the amplified band of 235 bp is digested in two lower bands of 174 and 61 bp. The 235-bp band, corresponding to the nonmutated allele, was still detectable, indicating that the mutation was present in a heterozygous state [Figure 2]. The presence of the mutation was confirmed by direct sequencing using the same primers as for PCR amplification. Thus, PCR product of exon 11 was purified using exonuclease I and shrimp alkaline phosphatase (Amersham Life Science, Cleveland, OH) to remove the excess of primers and deoxyribonucleotides. The purified products were subjected to 25 cycles (96°C for 20 s, 50°C for 10 s and 60°C for 4 min) with sense or antisense primer using fluorescence-based dideoxyterminator cycle sequencing (ABI PRISM big dye terminator cycle sequencing ready reaction kit with AmpliTaq Polymerase, FS, PE applied bio system, Warrington, United Kingdom). The products were eluted through a Centri-Sep spin column and subjected to gel electrophoresis. Data collection and analysis were performed on an automate 3130 DNA sequencer (ABI PRISM 3130 genetic analyzer, applied bio system Inc, Foster City, CA, USA). A heterozygous mutation appeared: A transition occurred at position 634, replacing a T with a C resulting in the substitution of a cysteine with an arginine [Figure 3]. The presence of mutations in *RET* in the hot spot exons has been reported in a number of publications. Thus, to ensure that no other relevant mutations have been overlooked, the exons 10, 13, 14, 15 and 16 were amplified and sequenced from genomic DNA using primers and conditions described by Brendt *et al.*[5] No mutations were detectable in these exons.

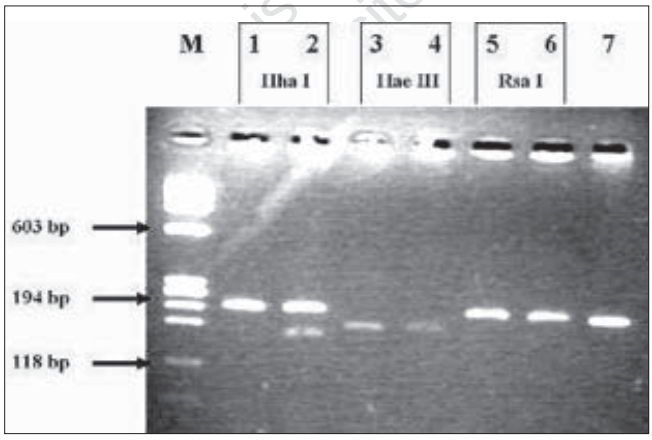


**Figure 2: *RET* exon 11 was amplified by PCR using oligonucleotides primers for exon 11. The size of the PCR product was 235 base pairs (bp) in normal allele. The aliquots of the products were digested with the restriction enzymes Hha I, Hae III and Rsa I as indicated (lane 2, 4 and 6). Germ line DNA from unrelated MEN 2A was used as control (lane 1, 3 and 5). Affected individual showed additional 174 bp and 61 bp fragments (lane 2). Lane M illustrates the migration of the molecular size marker ΦX 174 RF DNA/Hae III fragments. Lane 7: Normal individual.**

## Results

We found by endocrine screening test an elevated serum bCT. The evaluation for suspected pheochromocytoma demonstrated increased urinary metanephrines [Table 1]. These laboratory results confirmed the clinical diagnosis of MEN 2A with a clinical picture characterized by MTC and pheochromocytoma. One year after surgery, the serum CEA level was within the normal range, whereas serum bCT level remained elevated; the value was 119 pg/ml. The normal serum calcium and serum intact parathyroid hormone values indicated that the parathyroid glands were unaffected.

By DNA testing, we identified a heterozygous germ line missense mutation at codon 634 of exon 11 in the *RET* gene that causes a cysteine to arginine amino acid substitution in the DNA of the proband and then confirmed the diagnosis established from clinical and

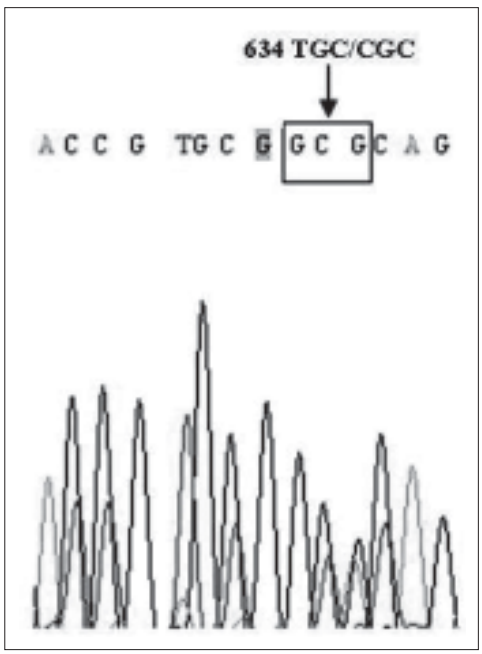


**Figure 3: Partial sequence of exon 11 of the *RET* proto-oncogene showing a T to C transition at the first nucleotide of codon 634 (TGC to CGC) causing a cysteine to arginine amino acid substitution. Exon 11 was sequenced with both sense (not shown) and antisense primers (upper panel)**

**Table 1: Laboratory findings after surgery**

| Parameter | 1 month after surgery | 12 months after surgery | Normal range |
|---|---|---|---|
| Serum calcitonin (pg/ml) | 44.8 | 119.86 | 0-10 |
| Serum *CEA (ng/ml) | ND | 1.47 | 0-10 |
| Serum intact-‡PTH (pg/ml) | ND | 31.25 | 8-62 |
| Serum calcium (mmo1/1) | 2.27 | 2.34 | 2.15-2.67 |
| Urinary metanephrine (µg/ml.24 h) | >1000 | ND | <340 |
| Urinary normetanephrine (µg/ml.24 h) | >3000 | ND | <440 |

*CEA: Carcinoembryonic antigen, ‡PTH: Parathyroid hormone, ND: Denote not done

biochemical parameters. Next, presymptomatic identification of the carriage of mutated *RET* proto-oncogene was attempted in the remaining family members: Three sisters, one brother and both parents. They were shown to bear the wild-type gene [Figure 4] and they could therefore be excluded from further clinical screening. Finally, using a parental test (data not shown), we ruled out the possibility of no paternity; therefore, the finding was a case of *de novo RET* gene mutation associated with MEN 2A that could be transmitted to the children. In addition, results of known *RET* gene polymorphisms analyses were negative in our MTC case (data not shown).

## Discussion

Mutations in the hot spot exons and newly detected risk exons 5 and 8 of the *RET* proto-oncogene have been well characterized and several groups have studied the disease phenotype-genotype, allowing prediction of the clinical manifestations of specific mutations in MEN 2A kindred.[10-12] Many studies of *RET* mutations in inheritable MTC have been published in different countries. Here, we report, for the first time in our country, the identification of a case of MEN 2A associated with *de novo* C634R *RET* sequence alteration. Missense mutations at codon 634 coding for one of five cysteine residues of the extracellular part of the protein are frequent in MEN 2A; $Cys^{634}$ Arg, in particular, is the one most frequently associated with this syndrome.[3] The mutation detected in our case, as well as most other studies, is concentrated in the cysteine residues of the *RET* receptor. This mutation results in ligand-independent receptor dimerization and autophosphorylation, converting the mutated allele to a dominant transforming gene.[7,13-16]

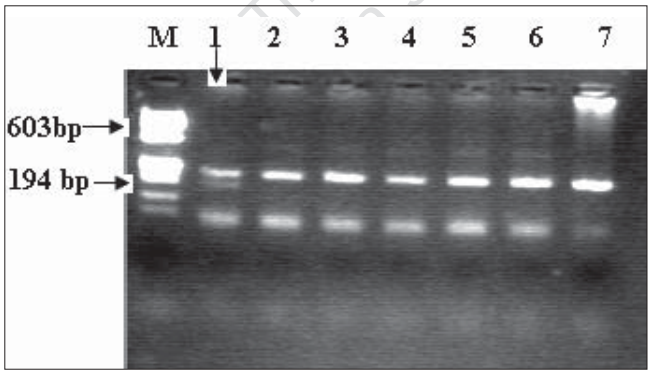


**Figure 4: Germ line DNA from the two sisters, one brother and the two parents (lane 2-6) were tested for the presence of the mutation $Cys^{634}$ Arg by PCR and Hha I restriction enzyme digestion. The *RET* mutation detected in the case index (lane 1) was de novo mutation. Germ line DNA from unrelated MEN 2A was used as control (lane 7). Lane M illustrates the migration of the molecular size marker ΦX 174 RF DNA/Hae III fragments.**

In classical MEN 2A cases, the substitution of cysteine by arginine in the mutations involving codon 634 is significantly predictive for the development of pheochromocytoma and hyperparathyroidism disease.[17,18] However, in the kindred that we have described here, the parathyroid glands seem to be unaffected. An explanation for this clinical presentation is that the patient was young and hyperparathyroidism could develop later in advanced age. This fact is in agreement with some 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.[17,18] It also possible that early thyroidectomy with removal of some parathyroid glands had altered the natural course of the parathyroid disease. Furthermore, the results derived from *RET* gene polymorphisms analyses do not permit us to exclude the possible role of the other variants in *RET* or other related genes in the final presentation of the disease.[19]

Elevated bCT levels in MEN 2A patient are indicative of the presence of MTC; persistent high values after total thyroidectomy predict usually residual lymph node metastases or distant tissues. Calcitonin production by the pheochromocytoma has also been described in a few reports, but actually it is an exceptional rarity case report.[20] In our case, the persistent high calcitonin level during the first postoperative year with normal CEA level is in fact consistent with incompleteness of the first surgical procedure. This clinical finding is quite in line with data emphasizing the need for a systematic locoregional lymphadenectomy in addition to thyroidectomy.[21,22]

In the literature, rare cases of *de novo RET* mutations have been described in which germ line alterations are

found in one affected person and his offspring but not in his parents. Usually, the mutated allele was of paternal origin, suggesting that paternal *RET* proto-oncogene may be sensitive to mutation during spermatogenesis or during cell division after early fertilization.[23] Deeper investigations would be needed to elucidate the underlying mechanisms for this relationship. Unfortunately, we could not establish whether the parental alleles were involved in the transmission of the disease because our laboratory suffers from lack of different intragenic polymorphic markers.

## Conclusion

The present report convincingly highlights the importance of molecular genetic testing methods offered to MEN 2A patients. The detection of mutated MEN 2A gene carriers enables us to differentiate high-risk members from those who bear the wild-type gene. Occasionally, application of *RET* proto-oncogene testing may lead to the detection of unexpected *de novo* mutation that could be transmitted to children. Since *de novo* cases of MEN 2A are not often described in the literature, this case could be added to the database. Future studies from Morocco could be on determination of *RET* proto-oncogene and characteristics of MTC disease in our population.



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