

RET Proto-Oncogene Mutational Analysis in Patients With Medullary Thyroid Carcinoma

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Abstract

Background: The aim of this study was to identify germline mutation of the RET (rearranged during transfection) gene in patients with Medullary Thyroid Carcinoma (MTC) and their first-degree relatives to find pre-symptomatic carriers for possible prophylactic thyroidectomy.

Methods and results: We examined all six hot spot exons (exons 10, 11, 13, and 14-16) of the RET gene by PCR and bidirectional sanger sequencing in 45 Iranian patients with MTC (either sporadic or familial form) from 7 unrelated kindreds and 38 apparently sporadic cases. First-degree relatives of RET positive cases were also genotyped for index mutation. Moreover, pre-symptomatic carriers were referred to the endocrinologist for further clinical management and prophylactic thyroidectomy if needed. Overall, the genetic status of all of the participants was determined by RET mutation screening, including 61 affected individuals, 22 pre-symptomatic carriers and 29 genetically healthy subjects. In 37.5% (17 of 45) of the MTC referral index patients, seven distinct RET germline mutations were found, including p.C634R (35.3%), p.M918T (17.6%), p.C634Y (11.8%), p.C634F (5.9%), p.C611Y (5.9%), p.C618R (5.9%), p.C630R (5.9%), p.L790F (5.9%) and an uncertain variant p.V648I (5.9%).

Conclusion: RET mutation detection is a promising/golden screening test and provides an accurate pre-symptomatic diagnostic test for at-risk carriers (the siblings and offspring of the patients) to consider prophylactic thyroidectomy. Thus, mutation screening of the RET hot spot exons could be suggested for any MTC patient to find pre-symptomatic RET carriers in their relatives.

Introduction

Calcitonin-secreting parafollicular C cells of the thyroid gland are the origin of 5–10% of thyroid cancers, so called as Medullary Thyroid Carcinoma (MTC). MTC has a worse prognosis than the most common form of thyroid cancer, papillary thyroid carcinoma (PTC), that accounts for 60–80% of thyroid carcinomas and originates from follicular cells [1]. MTC has 2 forms of sporadic (isolated) and hereditary that compromise about 75% and 25% of the cases, respectively. Hereditary forms are transmitted as autosomal dominant pattern of inheritance and are seen either as isolated familial MTC (FMTC) with a prevalence of 10%, or as a syndromic form of cancer, also known as multiple endocrine neoplasia type 2 (MEN2A, MEN2B, which have a prevalence of 85%, 5%, respectively) [2, 3]. In familial medullary thyroid carcinoma, the only lesion present is MTC, but MEN2A is characterized by MTC and pheochromocytoma with or without parathyroid hyperplasia or adenoma [1–3].

PTC and MTC have clear clinical and pathological differences but RET proto-oncogene contributes somehow to the carcinogenesis of both types. Activation of the RET gene by rearrangement (inversion or translocation) appears to be only seen in patients suffering from PTC [5] or thyroid adenoma [6], and germline missense mutations of the RET gene have been shown to be the cause of the hereditary form of MTC (FMTC and MEN2) [7]. Interestingly, somatic mutations in RET have been found in 23–70% of sporadic MTCs and 10–15% of sporadic pheochromocytoma patients, as well [8, 9]. Gain of function mutations in exon 10, 11, 13, 14, and 15 of the RET gene have mostly been reported in FMTC and MEN2A (e.g. codons 609, 611, 618, 620, and 634) but about 95% of the patients with MEN2B have a mutation in exon 16 (codon 918). Therefore, to obtain a reliable screening genetic test for MTC patients, exons 10, 11, 13, 14, 15 and 16 should be considered as the hot spots for RET gene mutations.

Identification of the germline mutation of the RET gene in a patient with MTC provides an accurate pre-symptomatic diagnostic test for the siblings and offspring of the patients. Here we report the results of the mutational analysis of the RET proto-oncogene in 45 Iranian patients with MTC (either sporadic or familial form) from 7 unrelated kindreds and 38 apparently sporadic cases. We examined all six exons (10, 11, 13, 14, 15 and 16) by PCR and direct Sanger sequencing. First-degree relatives of RET positive cases were also genotyped and underwent prophylactic thyroidectomy if they had

the causative mutation. According to the results, 37.5% (17 of 45) of the MTC referral index patients were found to have nine distinct RET germline mutations, including p.C634R (35.3%), p.M918T (17.6%), p.C634Y (11.8%), p.C634F (5.9%), p.C611Y (5.9%), p.C618R (5.9%), p.C630R (5.9%), p.L790F (5.9%) and an uncertain variant p.V648I (5.9%). The distribution of the detected RET mutations was as following; 64.7% in exon 11, 17.6% in exon 16, 11.8% in exon 10, 5.9% in exon 13, and no mutation in exons 14 and 15 was found. Overall, the genetic status of all participants (61 affected individuals, 22 pre-symptomatic carriers and 29 genetically healthy) was determined by RET mutation screening, regarding all six exons of RET (10, 11, 13, 14, 15 and 16). Moreover, 22 genetically pre-symptomatic carriers were referred to the endocrinologist for further clinical management.

Subjects And Methods

Patients and Specimen Collection

Seven unrelated families and thirty-eight apparently sporadic cases with medullary thyroid carcinoma referred to Endocrinology and Metabolism Research Institute of Tehran University of Medical Sciences, participated in this study. First, 45 index cases whose MTC was diagnosed by an endocrinologist and confirmed histopathologically and underwent thyroidectomy based on the ATA guideline were genetically consulted. Then, each index case was evaluated for mutations in six hot spot exons of the RET gene and segregation analysis was done for their at-risk relatives if a causative mutation was found [3]. Overall, 112 participants were examined for RET mutations. All procedures were in accordance with the ethical standards of Tehran University of Medical Sciences and the Helsinki Declaration of 1975 revised in 1983. Informed consent was obtained from all participants or their parents.

Molecular Analysis

Genomic DNA was extracted from EDTA peripheral venous blood using the standard salting out/proteinase K method. Primers were designed to amplify all six exons; 10, 11, 13, 14, 15 and 16, and their exon-intron boundaries using the NCBI Primer-design tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and gene runner software (version 6.0.28). Fifty nanograms of the extracted DNA was used as a PCR template. PCR conditions are available upon request. The PCR products were run on a 2% agarose gel and subsequently, all six exons were bi-directionally sequenced using the ABI3130 automated sequencer and analyzed with the Chromas version 2 (<http://chromas.software.informer.com/2.0/>). The sequence data were compared with the RET sequence data (Ref Seq NM_020975) from the NCBI BLAST human database (<http://www.ncbi.nlm.nih.gov/BLAST/>) and Ensembl human database (GRCh37).

Results

Clinical and molecular findings

Of 45 referred MTC patients, 7 cases had FMTC or MEN syndrome, with more than one affected members. Thirty-eight patients seemed to be apparently sporadic MTC. All six RET exons (10, 11, 13, 14, 15 and 16 exons) were sequenced in 45 index patients. Eight RET known distinct mutations, one unclassified variant, and one novel variant were detected (Table 1).

Table 1
RET mutations and number of affected subjects, carriers and healthy individuals.

ID	Family	Type	Mutation	Polymorphism	Variant	Number of affected	Number of carriers	Number of healthy	ATA risk
Familial Cases									
1	IR-F4	FMTc	L790F	L769L & S836S		4	5	11	A (MOD)
2	IR-F15	MTC	C634R			2			C (H)
3	IR-F18	FMTc	C634R	G691S & S904S		8			C (H)
4	IR-F25	MEN2A	C630R	G691S, L769L & S904S		3	6	4	B (MOD)
5	IR-F31	MEN2A	C634R			4	2		C (H)
6	IR-F33	MEN2A	C618R	G691S		2	3	2	B (MOD)
7	IR-F36	MEN2A	C634R			2	2		C (H)
Sporadic Cases									
8	IR-F11	Metastatic MTC	M918T			1			D (HST)
9	IR-F14	MTC	C611Y	G691S & L769L		1	1	2	B (MOD)
10	IR-F17	MEN2A	C634R			1	1		C (H)
11	IR-F19	MTC			V648I (uncertain)	1			
12	IR-F22	MTC	C634F	G691s, L769L, S836S & S904S + A > T IVS16		1	1	1	C (H)
13	IR-F23	MEN2B (metastatic MTC + ambiguous genitalia)	M918T			1		4	D (HST)
14	IR-F29	MEN2A	C634R			1	1		C (H)
15	IR-F37	MTC	M918T			1			D (HST)
16	IR-F38	MTC	C634Y		H658R (novel)	1			C (H)
18	IR-F39	MTC	C634Y	G691S		1			C (H)

The family IR-F4 was an extended kindred with two affected individuals in the second generation (II₂ and II₃), two affected (III₃ and III₁₀) and three carriers in the third generation (III₅₋₇), and two carriers in the fourth generation (IV₂ and IV₈) (Fig. 1-a). The 44-year-old proband III₃ and other affected individuals of the pedigree had a known heterozygous mutation (c.2370G > T) in exon 13 (p.L790F). According to the ATA risk classification, p.L790F mutation is stratified as

level A, indicating moderate risk of aggressive MTC with ~ 10% incidence of PHEO. Carriers of this mutation are advised to undergo prophylactic thyroidectomy only if it occurs as FMTC or they have a high level of calcitonin. Therefore, these carriers were referred to the endocrinologist for more clinical follow-up.

The proband of the family IR-F15 (II₂) also had a known mutation (c.1900T > C) in exon 11 (p.C634R). Her mother (I₂) died because of MTC and was not available but two other family members did not have any RET mutations in the six tested exons (Fig. 1-b).

The family IR-F18 was another family with three affected daughters (II₂, II₆ and II₇), one affected son (II₄), three affected grandchildren (III₃, III₄ and III₈), and one carrier granddaughter (III₆) (Fig. 1-c). A known heterozygous mutation in exon 11 [c.1900T > C (p.C634R)] was found in all affected individuals (II₂, II₄, II₆, II₇, III₃, III₄ and III₈) and one carrier granddaughter (III₆). Interestingly, individual III₆ had two genetically normal triplets. According to the ATA risk classification, she was advised to undergo prophylactic thyroidectomy before 5 years of age. The family IR-F25 was an extended kindred consisting of a healthy father (II₃) and an affected mother (II₄) with one affected son (III₁), one affected daughter (III₂), two carrier daughters (III₄ and III₆), one phenotypically healthy son (III₃) and one genetically healthy daughter (III₅), three carrier grandsons (IV₁, IV₄ and IV₉), one carrier granddaughter (IV₂), and three genetically healthy grandchildren (IV₃, IV₇ and IV₈) (Fig. 1-d). The 63-year-old proband (II₄) was diagnosed with MEN2A (MTC, pheochromocytoma (PHEO), and hyperparathyroidism) and underwent thyroidectomy, parathyroidectomy and adrenalectomy. Sequencing of six RET exons of the proband and her affected children revealed a heterozygous mutation (c. 1888T > C) in exon 11 (p.C630R) classified as a pathogenic variant. All carrier relatives of c. 1888T > C mutation with elevated calcitonin levels, III₄, III₆, IV₁, IV₄ and IV₉, underwent prophylactic thyroidectomy at the time of diagnosis (age: 36, 24, 13, 12, and 5 years old, respectively). The 19-year-old carrier granddaughter (IV₂), who had a normal calcitonin level, did not undergo prophylactic thyroidectomy. However, according to the ATA risk level, p.C630R RET mutation is stratified as level B with a moderate risk of aggressive MTC (MOD) and the patients are recommended to receive prophylactic thyroidectomy before the age of 5 years old.

The family IR-F31 was another kindred comprising an affected father (I₁), a healthy mother (I₂), two affected daughters (II₂ and II₄), one affected son (II₅), one genetically healthy son (II₇), and two carrier grandsons (III₃ and III₄) (Fig. 1-e). MEN2A was diagnosed in the 45-year-old grandfather as the proband with clinical manifestations of MTC and pheochromocytoma. He underwent thyroidectomy and adrenalectomy. His affected children also had MEN2A, including MTC and pheochromocytoma. We identified a heterozygous known mutation in exon 11 (c.1900T > C, p.C634R) in this family which has been classified as a pathogenic variant with ATA risk level C. Prophylactic thyroidectomy is recommended for this mutation before 5 years of age because of high risk of aggressive MTC (H). To conduct a follow-up study, two carrier grandsons (III₃, III₄) were referred to the endocrinologist and underwent prophylactic thyroidectomy at age 5.

The family IR-F33 had a known RET mutation (c.1852T > C) in exon 10 (p.C618R) (Fig. 1-f). The proband (II₅) was first referred for RET genetic testing because of a high calcitonin level (above 1500) and histopathological results showed MTC. Initially, a heterozygous known mutation (c.1852T > C) was seen in exon 10 (p.C618R) in the proband (II₅) at age 27 and subsequently, this variant was found in his affected father I₁ and three carrier siblings (II₁, II₂ and II₃). According to the ATA risk classification, p.C618R mutation is stratified as level B (MOD) and carriers are recommended to undergo prophylactic thyroidectomy before 5 years of age. Follow-up visits showed that the calcitonin level was above the normal range (300 pg/ml) in II₅ even after prophylactic thyroidectomy.

Thirty-eight index patients who seemed to be apparently sporadic MTC were tested for RET mutation at six mentioned exons. The results showed that 10 out of 38 patients had germline RET mutations. In addition, in four families, we found

four carriers of a causative mutation in presymptomatic children who had affected parents, including family IR-F14 with c.1832G > A mutation in exon 10 (p.C611Y), families IR-F17 and IR-F29 with c.1900T > C mutation in exon 11 (p.C634R) and family IR-F22 with c.1901G > T mutation in exon 11 (p.C634F).

In the family IR-F17, a 32-year-old affected mother (II₈) with a p.C634R mutation had a -year-old daughter with elevated calcitonin who was found to have the same mutation as observed in her mother. The proband in the family IR-F29 (III₁₆) had an asymptomatic 10-year-old boy who carried the same mutation (p.C634R). Moreover, a 15-year-old carrier of p.C611Y in the family IR-F14 with an ATA risk level B (MOD) and a 12-year-old carrier of p.C634F in the family IR-F22 were found. All of the carriers in these four families were referred to the endocrinologist for further clinical follow-up and prophylactic thyroidectomy. Furthermore, three index patients in families IR-11, IR-F23 and IR-F37 had a known RET germline mutation (c.2753T > C) in exon 16 (p.M918T) which is a MEN2B specific mutation with ATA risk level D (highest risk of aggressive MTC-HST). However, no available persons in their families had this mutation. The apparent sporadic patient IR-F39 had a known RET mutation (c.1901G > A) in exon 11 (p.C634Y). Likewise, another apparently sporadic patient (IR-F38) had a RET germline mutation (p.C634Y) and a novel uncertain variant (c.1973A > G) causing p.H658R, both in exon 11. RET novel variant p.H658R was predicted to be of uncertain significance by InterVar, benign by polyphen2, disease causing by mutation taster and tolerated with SIFT score 0.5 by SIFT web server (Table 2). RET sequencing results of another sporadic patient (IR-F19-II₇) showed an uncertain variant, c.1942G > A, in exon 11 (p.V648I). This variant was predicted to be likely benign by InterVar, benign by polyphen2, disease causing by mutation taster and tolerated with SIFT score 0.43 by SIFT web server (Table 2).

Table 2
Variants according to *in silico* analysis

	Variant	InterVar	Polyphen2	Mutation Taster	SIFT	MEN2 database	Report
1	p.H658R	Uncertain significance	Benign with 0.006 score: Sensitivity 0.97 Specificity 0.75	Disease causing	Tolerated (SIFT score 0.5)		Novel
2	p.V648I	Likely benign	Benign with 0.006 score: Sensitivity 0.97 Specificity 0.75	Disease causing (NMD, amino acid sequence change, protein features might be affected, splice site changes)	Tolerated (SIFT score 0.43)	Uncertain	[24] (2002)

Discussion

Medullary Thyroid Cancer (MTC) is responsible for about 10% of thyroid cancers but its prognosis is worse than the most common thyroid cancer, papillary thyroid cancer (PTC). However, 25% of MTCs are hereditary, known as MEN2 syndromes, including MEN2A, MEN2B, and FMTC. Germline missense mutations in hot spot exons of the RET gene (exons 10, 11, 13–16) cause 90% of all MEN2 syndromes. Biochemical tests used for screening at-risk cases of MEN2 have false positive/negative results. By contrast, RET mutation detection is a promising/golden screening test. Accurate identification of genotype-phenotype correlation in MEN2 syndromes is essential for pre-symptomatic detection of

carriers and clinical management of the affected subjects. In addition, RET genetic testing can contribute to distinguishing between three types of MEN2 syndromes that have some manifestation similarities.

According to the results, 37.5% (17 out of 45) of the MTC index patients had nine distinct RET germline mutations, including; p.C634R (35.3%-in 6 patients), p.M918T (17.6%- in 3 patients), p.C634Y (11.8% - in 2 patients), p.C634F (5.9% - in 1 patient), p.C611Y (5.9%-in 1 patient), p.C618R (5.9%-in 1 patient), p.C630R (5.9%-in 1 patient), and p.L790F (5.9%-in 1 patient). The distribution of the detected mutations in hot spot exons of RET gene was as follows; 64.7% (11/17) in exon 11, 17.6% (3/17) in exon 16, 11.8% (2/17) in exon 10, 5.9% (1/17) in exon 13, and no mutations in exons 14 and 15 were found. We were able to determine the clinical condition of all 112 participants by RET screening test, including 61 affected individuals, 22 pre-symptomatic carriers and 29 genetically healthy subjects. Pre-symptomatic carriers were referred to the endocrinologist for further clinical evaluation and possible prophylactic thyroidectomy.

In this study 7 Iranian kindreds with hereditary MTC had four distinct RET germline mutations (4 families had p.C634R mutation, and three other families had p.C630R, p.L790F and p.C618R mutations). Three families (IR-F18, IR-F31, and IR-F36) with p.C634R mutation had FMTC or MEN2A phenotypes (MTC, PHEO and PHPT). Based on the ATA guideline, p.C634R mutation is seen in MEN2A syndromes. Our findings support the role of p.C634R mutation in MEN2A manifestation. The ATA guideline also indicates that p.C634R mutation is seen in FMTC. Substitution mutations at codon 634 in exon 11, especially cytosine to arginine, are predominately seen in Iranian MEN families [15–19]. It has been stated that mutations at codon 634 are the most frequent mutation in Caucasians [20]. This mutation comprised 53% of all detected mutations in this study. The present study showed that mutations in exon 11, especially at codon 634, are commonly observed in the Iranian population, which is consistent with other studies [15–19].

Interestingly, follow-up studies in IR-F25 family showed that a 19-year-old carrier, IV-2, did not manifest any MEN2A phenotypes unlike other carriers. The carrier IV-2 might have some other modifier genes which give her an opportunity to be a healthy carrier with a normal calcitonin level until 19 years of age. In addition, a review of the literature showed that p.L790F mutation is reported for the second time in Iranian families with FMTC [21]. Similarly, it is a second report of p.C618R mutation in Iranian MTC families (18). Nevertheless, p.C618R mutation is the most common mutation in MEN2A in Saudi Arabian families (22).

The present study showed five distinct RET mutations in 10 out of 38 (26.3%) apparently sporadic Iranian MTC cases, including two p.C634R, two p.C634Y, one p.C634F, three p.M918T, and one p.C611Y mutations. Additionally, one RET uncertain variant (p.V648I) was detected in one apparently sporadic MTC Iranian patient for the first time. Moreover, it is a first report of an apparently sporadic MTC Iranian patient who had a p.C634R mutation and a novel H658R uncertain variant. Further molecular studies are needed to identify the clinical effect of these two variants.

On the other hand, in this study, p.M918T mutation was another common mutation in sporadic Iranian MTC cases, which is inconsistent with findings in European populations (16,21). To the best of our knowledge, p.M918T mutation has been reported in two cases and has not been detected in Iranian Azari and other populations [15–19]. Different genetic backgrounds could be the reason of the mentioned conflicting results which requires more investigations. On the other hand, it seems that some modifier polymorphisms that were found in some carriers of RET mutation in our study might contribute to MTC manifestations [23]. Additional genetic evidence is required to clarify the additive effect of modifier polymorphisms

In conclusion, evaluation of RET mutations in hot spot exons resulted in genetic diagnosis of 37.5% of the Iranian MTC patients. The genotype of all participants in this study was precisely determined and 22 pre-symptomatic carriers were referred for further clinical management and prophylactic thyroidectomy. Thus, RET mutation screening could serve as an essential detection method for any MTC patient to find pre-symptomatic RET carriers.

Declarations

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Competing interests

The authors declare that they have no competing interests.

Informed consent

Informed consent was obtained from all participants or their parents.

Ethics approval

All procedures were in accordance with the ethical standards of Tehran University of Medical Sciences and the Helsinki Declaration of 1975 revised in 1983

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Figures

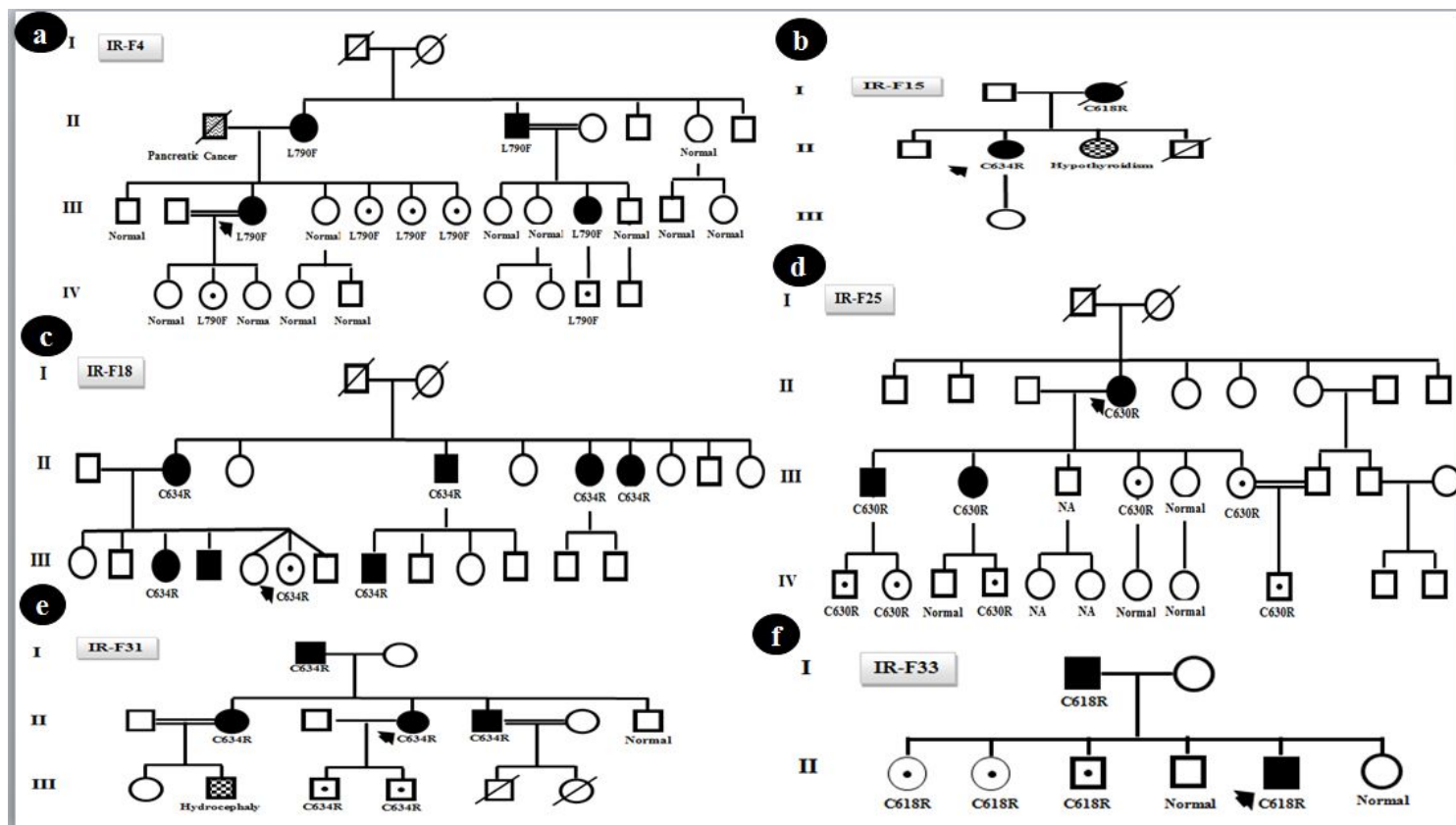


Figure 1

Pedigrees of families with MEN2. Blackened symbols: affected individuals; dotted symbols: asymptomatic carriers; dashed symbols: individuals with symptoms other than MEN2; dashed symbols: deceased.