Endocrine-Related Cancer (1998) **5** 37-44

Genetic testing for multiple endocrine neoplasias

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Introduction

Multiple endocrine neoplasias type 1 (MEN 1) and type 2 (MEN 2) represent complex inherited (autosomal dominant traits) syndromes characterized by occurrence of distinct proliferative disorders of endocrine tissues, varying from hyperplasia to adenoma and carcinoma.

MEN 1 syndrome is characterized by parathyroid gland, anterior pituitary and endocrine pancreas tumors. Other endocrine and non-endocrine tumors, such as carcinoids, lipomas, pinealomas, adrenocortical and thyroid follicular tumors, have also been described in MEN 1 patients, occurring at higher frequencies than in the general population (Brandi *et al.* 1987). Recently, also a spinal ependymoma has been found in a patient with MEN 1 syndrome (Kato *et al.* 1996).

Three main clinical MEN 2 syndrome entities are recognized. The first is MEN 2A, characterized by medullary thyroid carcinoma (MTC), primary hyperparathyroidism (PHPT) and pheochromocytoma (PHEO). The second is MEN 2B, which exhibits MTC, usually developing earlier than the MEN 2A-associated disease, PHEO, multiple neuromas of gastroenteric mucosa, myelinated corneal nerves (Gorlin *et al.* 1968) and a typical marphanoid habitus. The third is familial medullary thyroid carcinoma (FMTC) only, seen in families with at least four members with MTC and no objective evidence of PHEO and parathyroid disease on screening of affected and at-risk members, as stated by the International *RET* Mutation Consortium (Mulligan *et al.* 1995).

MEN 1

As mentioned above, MEN 1 syndrome is usually inherited as an autosomal dominant trait, although sporadic cases have been described. Its penetrance can reach up to 100% with increasing age. Combined linkage and allelic loss tumor analysis originally localized the gene defect to chromosome 11q region 12-13 (Larsson *et al.* 1988, Friedman *et al*. 1989, Thakker *et al*. 1989, Bystrom *et al*. 1990). Moreover, the evidence that copies of alleles were lost in tumoral DNA and that the loss derived from the unaffected parent (Larsson *et al.* 1988) seemed to be in agreement with Knudson's 'two hits' hypothesis (Knudson 1971). Description of recombining events at 11q13 loci in MEN 1 patients narrowed the minimal *MEN1* candidate area to an interval consisting of approximately 2 Mb, placing the *MEN1* gene around *PYGM*, flanked by D11S1883 and D11S449 (Courseaux *et al.* 1996). Moreover, DNA markers such as D11S4907 and D11S4908 made it possible to localize the *MEN1* gene between D11S1883 and D11S4907 loci at 11q13 (Debelenko *et al.* 1997). Very recently the *MEN1* gene sequence has been identified by positional cloning and mutational analysis in germ-lines from 14 of 15 MEN 1 kindreds. To date 12 different germ-line mutations have been reported (Chandrasekharappa *et al.* 1997).

A circulating basic fibroblast growth factor (bFGF)-like substance in plasma from MEN 1 patients: a biomarker for diagnosis?

In the past years a bFGF-like substance circulating in the plasma of MEN 1 affected patients has been described (Brandi *et al.* 1986). This bFGF-like factor was shown to be a potent mitogen for parathyroid endothelial cells, and this made possible an evaluation of the role of endothelium in parathyroid tissue tumorigenesis. Ultrastructural histomorphometry studies demonstrated that the proliferation of parathyroid epithelial cells in MEN 1-affected subjects is accompanied by a parallel increase in an associated endothelial component that had not been described in the context of secondary parathyroid hyperplasia (D'Adda *et al.* 1992). This provides strong evidence of an *in vivo* role for the MEN 1-associated bFGF-like circulating factor, either in promoting the first step of polyclonal hyperplasia of parathyroid tissue in MEN 1 syndrome or influencing a late stage of progression of MEN 1-associated parathyroid tumors.

In the first instance, an early expression of the mitogenic activity would be expected. Lack of correlation between age and the activity of the circulating bFGF-like MEN 1 mitogen was shown in a large MEN 1 kindred

Endocrine-Related Cancer (1998) **5** 37-44 *Printed in Great Britain*
1351-0088/98/005-037 $08.00/0

(Marx *et al.* 1988). However, higher plasma mitogenic activity was present in 'gene carriers' than in unaffected relatives. These findings (i.e. the lack of age dependence and the high circulating levels in *MEN1* gene carriers) suggest the presence of high parathyroid mitogenic activity in plasma very early in life, before endocrine gland hyperfunction occurs (Marx *et al.* 1988). Mitogenic circulating activity was tested in asymptomatic young gene carriers from different kindreds and compared with that of age-matched non-gene carriers. This activity was higher in gene carriers than in the normal counterparts (Benvenuti *et al.* 1997) providing evidence for a precocious role of the MEN 1-associated bFGF-like mitogen in the pathogenesis of parathyroid tumorigenesis. The measurement of MEN 1 growth factor is not used either for diagnostic or follow-up purposes, due to the intrinsic variability of the developed bioassay with areas of overlap between normal subject and MEN 1 patient values (Brandi *et al.* 1986).

Genetic analysis of MEN 1 kindreds before *MEN1* positional cloning

Before positional cloning, 13 marker complexes (17 DNA probes) linked to the *MEN1* gene and spanning a 14% meiotic recombination with the *MEN1* locus in the middle were powerful tools for genetic diagnosis by restriction fragment length polymorphism analysis and were used to attempt to identify the gene carriers in affected kindreds (Larsson *et al.* 1992). Moreover, in the last 8 years an increasing number of very highly informative

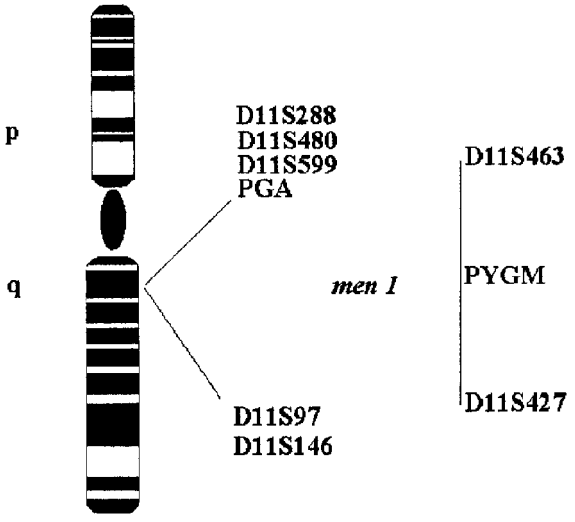

Figure 1 Chromosome 11 ideogram. The shown 11q12-13 region is encompassed from D11S288 (centromeric side) to D11S146 (telomeric side) loci and it spans 14 cM. Loci D11S463, PYGM and D11S427 exhibit recombination 0 with the *MEN1* gene.

chromosome 11q DNA mini-microsatellite sequences have been developed (Weber & May 1989, Waye *et al.* 1997, Tokino *et al.* 1991, Fujimori *et al.* 1992, Iwasaki *et al.* 1992, Litt *et al.* 1993, Qin *et al.* 1993), making it possible to perform genetic diagnosis by PCR-based techniques also, allowing more effective haplotypes for genetic diagnosis to be obtained.

The predictive accuracy of the test for gene carriers can, in fact, reach up to 99.5% when it is possible to exclude meiotic crossing over between the analyzed DNA markers and the disease locus. Four of these DNA markers, D11S288, D11S480, D11S559 and *PGA*, map on the centromeric side of the *MEN1* locus, and two, D11S97 and D11S146, on the telomeric side, while the remaining three, D11S463, PYGM and D11S427, are closely linked to the *MEN1* locus (Fig. 1). In order to identify the mutant chromosome carrying the disease locus, the genetic analysis has been performed only in families with at least two living affected members. In fact, based on the genotypes determined for different marker systems linked to the *MEN1* locus in two affected members in each kindred, we can outline the most likely haplotype carrying the *MEN1* allele in each affected individual (Fig. 2). Following these criteria, and after the haplotype for each family member has been determined, it is possible to identify who inherited the mutant chromosome. The predictive accuracy of the genetic diagnosis depends on linkage distances in percentage crossing over between the *MEN1* locus and the informative DNA markers. The calculated predictive accuracy can exceed 99.5% if three marker systems can be used, two flanking and one showing recombination 0 with the *MEN1* locus, it thus being possible to exclude incorrect results due to meiotic crossings over.

DNA testing for MEN 1 syndrome is a very powerful predictive test that can be applied at any age, providing that the family structure allows linkage analysis. Eight new polymorphisms in 11q13, close to the *MEN1* locus have been discovered and they can be used in genetic testing (Smith *et al.* 1995). Moreover, a set of four microsatellite polymorphic DNA markers (D11S913, D11S1337, D11S987, D11S971) appears to considerably improve the diagnostic value of genotyping patients at risk for developing MEN 1 (Kytölä *et al.* 1995).

Genetic diagnosis by mutational analysis of the *MEN1* gene

The *MEN1* gene consists of 10 exonic sequences and encodes for a 2.8 kb transcript. Unfortunately, the encoded protein function is still unknown. Mutational analysis for *MEN1* gene has been performed in affected members from 15 typical MEN 1 families. A total of 12 mutations, consisting of 5 frameshift mutations, 3 nonsense mutations, 2 in-frame deletions, and 2 missense

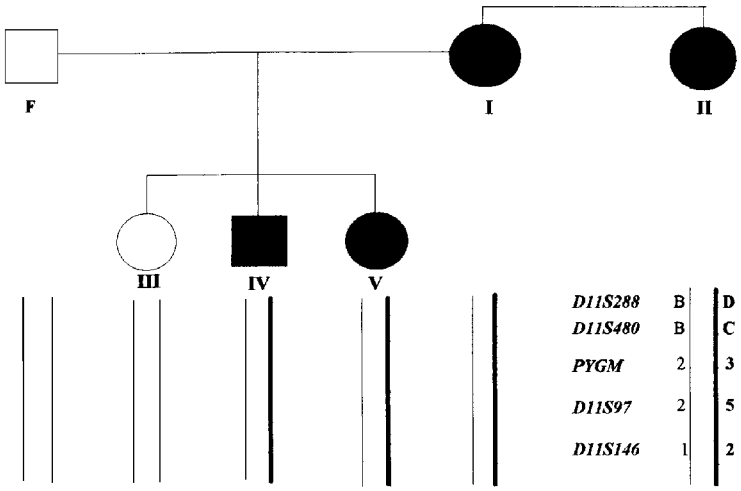

Figure 2 Example of a MEN 1 kindred's pedigree. Full symbols represent affected patients, while open symbols indicate healthy subjects (circle=female; square=male). Under each member, indicated by letters, i.e. F, or roman numbers, i.e. I-V, the resulting haplotype is depicted; examples of analyzed loci on chromosome 11q13 are reported under patient II. The bold lines represent the affected chromosome 11 segregating with the disease.

alterations, have been identified to date. Two mutations were seen twice in non-related families. For 10 different mutations it was confirmed that the observed alteration was inherited in accordance with the MEN 1 phenotype, while none of these mutations were observed in an analysis of normal DNA samples. Primers from intronic sequences have been designed to amplify 9 of 10 exons of the *MEN1* gene from genomic DNA. Sequencing of PCR-amplified product allows direct demonstration of the presence of germ-line alterations (Chandrasekharappa *et al.* 1997).

Actually, such findings represent an important achievement in the comprehension of the molecular basis of the MEN 1 syndrome. Obviously, they will acquire more importance in the study of a possible genotype-phenotype correlation in such syndromes characterized by a largely heterogeneous pathological expressiveness.

Soon, being able to identify the *MEN1* gene will make it unnecessary to have at least two affected members of a specific family. In fact, the mutational analysis can be performed in a single subject, even if they exhibit the sporadic form of the syndrome. However, the identified *MEN1* mutations have still to be assessed for their value in a genetic diagnosis. Until this has been done, linkage analysis, using the large number of DNA markers available from chromosome 11q13, will still have an important role in screening individuals at risk.

MEN 2

Before the possibility of performing genetic tests, first degree relatives of affected members in MEN 2 kindreds were clinically screened every year for MTC, PHEO and PHPT from the age of 6 years to the age of 35, by performing measurements of pentagastrin-stimulated levels of calcitonin, 24 h urinary catecholamines excretion and serum values of both calcium and parathyroid hormone. Because in some clinical centres MTCs have been diagnosed before the age of 6, this was used in advising prophylactic total thyroidectomy before this age in first degree relatives of affected patients (Wells *et al.* 1994). Actually, since identification of *ret* proto-oncogene as the susceptibility gene, both prophylactic thyroid-ectomy and clinical screening have to be limited only to mutant gene carriers.

The gene responsible for MEN 2A syndrome was identified by a combination of physical and genetic mapping, on the long arm of chromosome 10, region q11.2 (Mulligan *et al.* 1993*a*). This region of approximately 480 kb comprises the proto-oncogene *ret*, a gene with 21 exons encoding for a tyrosine kinase receptor (Fig. 3), expressed in MTC, PHEO and, at lower levels, in normal human thyroid tissue and derivatives of neural crest cells (Santoro *et al.* 1990, Nakamura *et al.* 1994). The *ret* oncogene exhibits a restricted pattern of expression in adult human tissues and in a small number of tumors and cell lines (Takahashi & Cooper 1987, Ikeda *et al.* 1990,

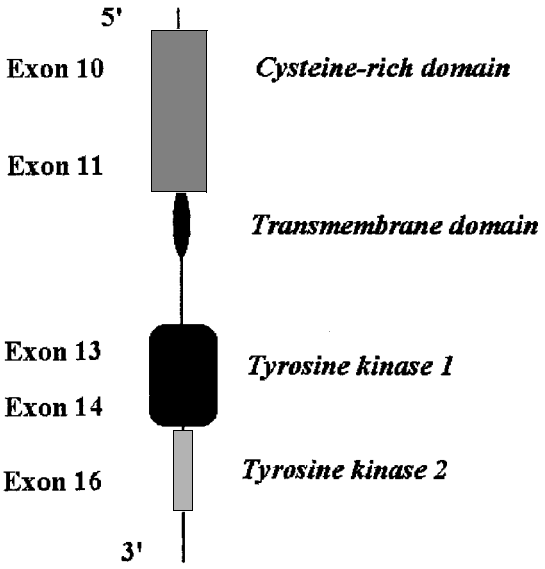

Figure 3 Schematic representation of exons 10, 11, 13, 14 and 16 of *ret* proto-oncogene and corresponding DNA domains (right side).

Santoro *et al*. 1990, Tahira *et al*. 1991, Nakamura *et al*. 1994). The localization of the gene accounting for the MEN 2B syndrome was not distinguishable from that of MEN 2A, thus suggesting that the genetic defect in MEN 2A and MEN 2B syndromes could be due to different allelic mutants localized at the same locus. Allelic losses at the tumor level using contiguous DNA markers to the *Men2* locus (Mulligan *et al.* 1993*b*) were not found in tumors from patients affected by MEN 2A syndrome. In fact, in all but one MEN 2A tumor, heterozygosity for the mutant and wild-type *ret* alleles was retained (Mulligan *et al.* 1993*a*). Subsequently it was possible to reveal missense mutations of the *ret* proto-oncogene in 20 of 23 MEN 2A kindreds, while none was found in 23 normal controls. Moreover 19 of 20 mutations (95%) were in the same conserved residue of cysteine in position 380 of the extracellular domain of the tyrosine kinase protein encoded by the oncogene. Being the same mutation present in two or more affected members of a kindred, but not in healthy subjects of the same family, segregation of the mutant alleles with the disease was evident (Mulligan *et al.* 1993*a*). Interestingly, identical mutations have been described both in MEN 2A and FMTC kindreds (Donis-Keller *et al.* 1993). Probably, different variations somewhere in the *ret* gene or in flanking genes could account for the different phenotypes (MEN 2A versus FMTC) in families with the same mutations (Goodfellow & Wells 1995). In each familial MEN 2A case the *ret* gene mutations in affected patients have been described in exon 10, in one of four cysteine codons (609, 611, 618 and 620) and exon 11, at the cysteine codon 634, at the boundary of the extracellular and transmembrane domains harboring a cysteine rich region (Table 1) (Marsh *et al*. 1994, Mulligan *et al.* 1994, Schuffenecker *et al*. 1994, Xue *et al*. 1994, Zedenius *et al*. 1994, Gagel *et al.* 1995, Komminoth *et al*. 1995, Landsvater *et al*. 1996, Mulligan *et al*. 1995). Two newer mutations in exons 13, codon 768, and 14, codon 804, have been described in families with FMTC (Eng *et al.* 1995*a*, Bolino *et al*. 1995). Mutations of *ret* proto-oncogene have been found in 95% of MEN 2A kindreds and also in 85% of families with familial MTC (Mulligan *et al.* 1995). A single activating mutation on only one allele could be sufficient to determine the neoplastic transformation. Recently, a *ret* ligand has been identified in glial cell line-derived neurotrophic factor (Jing *et al*. 1996, Trupp *et al.* 1996) and interaction of the ligand with the tyrosine kinase receptor causes dimerization of receptor, autophosphorylation on tyrosine residues and cell growth and differentiation.

Molecular and biochemical studies of *ret* mutation

Recent studies provide evidence of a ligand-independent dimerization of the receptor protein encoded by the *ret* gene in MEN 2A mutations. Therefore, the mutant receptor is permanently activated in its intrinsic tyrosine kinase enzymatic activity. In this model the MEN 2A mutation causes the loss of one cysteine residue in the extracellular domain of the *ret* receptor so that the unpaired wild-type cysteine residue is now available to form an abnormal intermolecular disulfide bond between two close mutant receptors (Asai *et al*. 1995, Santoro *et al.* 1995). Therefore, this dominant activating change represents the first evidence of an inherited transmission of a dominant transforming gene in human malignant neoplasias. The effects of mutations of exons 13 (Table 1) and 14 are not clear to date. It is thought that the mutation of exon 13 may determine a kinase activity alteration by changing the substrate specificity or the ATP-binding capacity of the receptor (Eng *et al.* 1995*b*), while exon 14 mutation, valine with leucine substitution, could activate the receptor by altering the interaction with normal cellular substrates or modifying the amount of substrate susceptible to phosphorylation.

Exon 16, codon 918 (Table 1), is found to be mutated in patients affected by MEN 2B characterized by a stronger aggressiveness of MTC and by the presence of typical mucosal neuromas and the marphanoid habitus (Hofstra *et al.* 1994). The mutation consists of substitution of a threonine residue for a methionine. The position of the amino acid residue at 918 is within the catalytic core region of the tyrosine kinase domain of the protein encoded by the *ret* gene. The MEN 2B mutation causes the

Table 1 Description of main *ret* proto-oncogene mutations described in patients affected by MEN 2A, 2B and FMTC syndromes.*

Exons	Mutation	Restriction enzymes	Size of wild-type fragments	Size of mutated fragments	Amino acid mutation
Exon 10					
Codon 611	TGC→TGG	*NlaIV*	78	65+13	Cys→Trp
Codon 618	TGC→CGC	*CfoI*	187	131+56	Cys→Arg
	TGC→TAC	*RsaI*	187	129+55	Cys→Tyr
	TGC→TTC	*MboII*	158	124+34	Cys→Phe
	TGC→AGC	*MboII*	158	139+19	Cys→Ser
Codon 620	TGC→CGC	*BstUI*	187	138+49	Cys→Arg
	TGC→TTC	*TaqI*	187	138+49	Cys→Phe
Exon 11					
Codon 634	TGC→CGC	*CfoI*	416	357+59	Cys→Arg
	TGC→TGG	*CfoI+Fnu4HI*	416	357+59	Cys→Trp
	TGC→AGC	*DdeI*	194	134+60	Cys→Ser
	TGC→GGC	*HaeIII*	416	357+59	Cys→Gly
	TGC→TAC	*RsaI*	170	113+57	Cys→Tyr
	TGC→TNC	*Fnu4HI*	62+14	76	Cys→Phe →Ser
Exon 13					
Codon 768	GAG→GAC	*AluI*	130+98	228+130 + 98	Glu→Asp
Exon 16					
Codon 918	ATG→ACG	*FokI*	79+38	137	Met→Thr

*Mutations at codon 609 of exon 10 and at codon 804 of exon 14 have also been described (see the text).

activation of the receptor kinase activity to a lesser extent than does the MEN 2A mutation, altering, moreover, autocatalytic and substrate specificity (Santoro *et al.* 1995). Moreover, the receptor with this mutation is also able to bind to and phosphorylate substrates that are more typical for non-receptor tyrosine kinases, such as c-src and c-abl (Carlson *et al.* 1994, Borrello *et al*. 1995, Santoro *et al*. 1995). A recent report shows that the MEN 2B mutant tyrosine kinase domain exhibits both biological and biochemical activity higher than the wild-type domain. The MEN 2B mutant receptor can reach a full activation only after homodimerization. Moreover, the altered substrate specificity may play an extremely important role for the proliferation rate of mutant cells and for ontogenesis of affected tissues (Borrello *et al*. 1995).

By comparison between the haplotypes of healthy and affected individuals it is possible to identify the carrier status also in clinically asymptomatic patients. The involvement of parathyroid glands, together with the presence of PHEO, seems to be correlated with mutations of the most commonly affected codon, codon 634, in MEN 2A syndrome. It could be explained at the molecular level with the full ligand-independent activation of the mutant tyrosine kinase receptor in MEN 2A, in contrast to the MEN 2B mutation as mentioned above. Thus the MEN 2A fully activated receptor could activate the mitogenic pathways in parathyroid cells that normally express the *ret* gene (Pachnis *et al*. 1993), while the partial MEN 2B mutant tyrosine kinase receptor activation may not be sufficiently 'strong' to cause parathyroid hyperplasia. Moreover, the full activation mechanism in MEN 2A syndrome resembles a ligand-receptor activation of the wild-type receptor and could account for the absence of the lesions that are typically associated with the MEN 2B syndrome, where the molecular mechanism is represented by a change in the catalytic substrate specificity of the mutant receptor (Santoro *et al.* 1995, Songyang *et al*. 1995). In normal tissue development *ret* activation depends on the availability of a putative ligand in a defined period, while in MEN 2A mutant tissue *ret* activation is not ligand-dependent and it could occur before the normal differentiation substrate availability so that activation of a mitogenic pathway may result (Borrello *et al.* 1995).

The study of *ret* mutations may become useful also for the evaluation of sporadic MTC, where *de novo* mutations can occur at the constitutional level. Interestingly, in sporadic MTC the MEN 2B mutations are frequent (approximately 25% of tumors) while somatic cysteine codon MEN 2A mutations have never been described (Eng *et al*. 1995*a,b,* Hofstra *et al*. 1994). These data may be explained according to the somatically occurring MEN 2B-like mutation activating altered substrates responsible

for thyroid C cell proliferation (Borrello *et al.* 1995). The finding of sporadic somatic MEN 2A-like mutations could be possible only if they occur within the period in which the natural ligand is not available (i.e. during the thyroid ontogenesis), being otherwise acting as the wild-type receptor.

Inherited *ret* mutations are also associated with Hirschsprung's disease, an enteric disorder characterized by aganglionosis and defects of intestinal peristalsis. *Ret* mutations in this particular disease are shown to be recessive at the cellular level and they are usually inactivating causing a loss of function or inactivation of the receptor (Pasini *et al.* 1995). Genetic counseling in these patients is of great clinical relevance.

Genetic alterations of the *ret* oncogene have been found to be associated also with sporadic thyroid carcinomas (Grieco *et al.* 1990, Ishizaka *et al.* 1990, Lanzi *et al*. 1992, Bongarzone *et al*. 1993, 1994, Jhiang *et al*. 1994, Sozzi *et al*. 1994).

Positive and negative aspects of *ret* mutational analysis

The mutational analysis of *ret* proto-oncogene can be easily performed by PCR techniques (Lips *et al.* 1994, Marsh *et al*. 1994, McMahon *et al*. 1994). Since *ret* mutations have been found in 92% of MEN 2 kindreds, mutational analysis can be used to confirm clinical diagnosis and to identify asymptomatic family members with this syndrome. For first degree relatives of an affected member, or one known to be a gene carrier, DNA testing should be performed, especially for children under 6 years of age (Wells *et al.* 1994) or before prophylactic thyroidectomy. Genetic tests are very accurate, 100% in families known to have mutations, and easily reproducible, and provide identification of asymptomatic affected members.

However, the new DNA testing-based screening strategy shows positive and negative aspects. DNA testing is able to identify not more than 92% of families with a clear clinical diagnosis of MEN 2, even if, in the kindreds in which *ret* proto-oncogene activating mutation has been identified, it is 100% accurate in confirming gene-carrier status in family members with the syndrome (Eng 1996). Before excluding non-gene carriers from further clinical screening, it would be advisable to repeat DNA tests in a different genetic laboratory, analyzing a second blood sample to exclude administrative, sampling or laboratory errors (about 3-5%). Since gene carrier status may be determined prenatally or in very early childhood, the question is raised whether the removal of the thyroid at an early age produces a higher cure rate in affected subjects. The answer to this important question is still unclear. Until now there are two different opinions: one is to perform thyroidectomy as soon as possible at any age, and the other one is to wait to 6 years of age in order to reduce morbidity and to insure a good long-term quality of life. Ten percent of relatives of patients with apparently sporadic MTC represent familial cases on the basis of the pentagastrin test (Ponder *et al.* 1988), although DNA tests identify only about 90% of kindreds with MEN 2A. For these reasons, it seems not totally reliable to use DNA tests alone for the screening of relatives of an apparently sporadic MTC patient.

In the case of families affected by the MEN 2B form, genetic testing should begin at least 2 years earlier due to the precocity of onset of MTC.

Acknowledgements

This work was supported by grants of the Associazione Italiana per la Ricerca sul Cancro (to MLB), from CNR/PF ACRO (INV. 95.00316 PF 39) and by MURST 60% (to MLB).

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