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Genetic test in multiple endocrine neoplasia type 1 syndrome: An evolving story

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Abstract

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant inherited tumour syndrome expressing various endocrine and non-endocrine lesions and tumours. Since the identification of the causative gene, the oncosuppressor gene *MEN1*, in 1997, genetic testing has revealed an important approach for the early and differential diagnosis of the disease. The finding of a *MEN1* mutation in a patient has important clinical implications for relatives since it allows very early disease diagnosis and identification of carriers, even before biochemical and/or clinical manifestation, permitting their inclusion in a specific program of surveillance and subsequent praecox therapy. Currently, genetic testing for MEN1 consists principally of the sequencing of coding regions and intron-exon junctions of the *MEN1* gene. However, the recent acquisition of novel high throughput technologies will allow the design of innovative, accurate, complete and rapid genetic diagnosis. These new tools are able to increase the strength of the analysis and almost completely eliminate the possibility of false negative results. This review aims to give an overview on genetic testing of MEN1 syndrome, reporting the positive aspects of performing the analysis and the future perspectives for improving the performance of the test, as well as its application in clinical practice.

Key words: Multiple endocrine neoplasia type 1; Genetic test; Clinical practice; Next-generation sequencing

Core tip: Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant inherited tumour syndrome. Mutation analysis of the causative gene, the oncosuppressor *MEN1* gene, is today the best approach for the early and differential diagnosis of the disease. The manuscript gives an overview on current genetic testing of MEN1 syndrome, reporting the positive aspects and clinical utility of performing the analysis. Moreover, the paper aims to report the future perspectives for improving the performance of the test and its application in clinical practice.

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INTRODUCTION

Multiple endocrine neoplasia type 1 (MEN1, OMIM 131100) is a rare (approximately one in 30000) autosomal dominant inherited tumour syndrome with high penetrance (nearly 100% by the age of 50), principally affecting parathyroid glands, entero-pancreatic neuroendocrine tissues and anterior pituitary[1]. A variable combination of about 20 different endocrine and non-endocrine lesions and tumours can randomly occur in families and sporadic cases of the syndrome; despite the heritability of the disease, clinical features may differ in members of the same family and even between homozygote twins[2]. A MEN1 case is clinically defined in a single patient showing at least two of the three main tumours or in a familial case presenting with one of the main syndrome-related tumour and one first-degree relative affected by the syndrome.

MEN1 syndrome occurs as the result of tumour growth and associated metastases which cause the overproduction of hormones. MEN1 patients have been shown to have a reduced life expectancy; approximately 30% to 40% of deaths are associated with malignant neuroendocrine pancreatic tumours (mainly gastrinomas) and thymic or bronchial carcinoid tumour (patients affected by these malignancies have a three-fold higher risk of death)[3]. The improved management of MEN1-associated primary hyperparathyroidism and hypergastrinemia, as well as prophylactic ablation of thymus, have contributed to reduce the mortality of the syndrome. Paradoxically, the longer life expectancy could result in an increased risk for MEN1-associated malignancies. Indeed, current treatments, which are usually the same used for the sporadic tumour counterparts in non-MEN1 patients, are often not as successful in MEN1 patients because of the presence of multiple tumours in different organs, tumours that are generally more aggressive, larger and presenting with recurrences and metastases. An early diagnosis of the disease associated to pre-symptomatic tumour detection and specific therapy, starting as soon as possible, are, at the moment, the best and most effective way to grant MEN1 patients a better prognosis and a reduction of morbidity and mortality. The medical diagnosis of MEN 1 is commonly made by reconstruction of family history, physical examination of signs and symptoms of excess hormones, as well as specific biochemical screenings and imaging tests. These clinical and instrumental approaches usually allow a MEN1 diagnosis only during the fourth or fifth decades of life of the patient, delaying the performance of targeted surgical procedures and/or the initiation of available therapies. Biochemical detection of serum and/or urinary variations in hormones or other molecules deregulated by the presence of one or more MEN1-associated tumour can anticipate the disease diagnosis and tumour detection by about 5-8 years with respect to clinical onset and manifestation[4]. The early recognition of affected subjects and of at-risk individuals within a family can be further anticipated by DNA-testing. Indeed, since the identification of the causative gene, the oncosuppressor gene *MEN1* at the 11q13 region, the genetic test, through *MEN1* gene mutational analysis, has been revealed to be a fundamental approach for early and differential diagnosis of MEN1 syndrome. The advantages of genetic testing are: it requires only a single blood sample, it can be performed at any age and, in theory, it does not need to be repeated during the patient's lifetime. This paper provides a general overview on the current genetic approaches to MEN1 diagnosis, reporting the positive aspects and clinical utility of performing this analysis and explaining the future perspectives for improving the performance of the test and its application in clinical practice.

GENETIC TEST IN MEN1: GENETIC BASIS FOR CLINICAL MANAGEMENT OF PATIENTS

MEN1 syndrome is caused by inactivating mutations of the *MEN1* gene, encoding for the nuclear protein menin. MEN1 exhibits an autosomal dominant pattern of inheritance, and each affected patient has a 50% probability of transmitting the genetic defect and disease predisposition to offspring, independent of sex. Also, all the first-degree relatives of a mutation carrier have a 50% risk of inheriting the mutation and developing the disease. The proposed model for tumorigenesis in MEN1 was according to Knudson's "two hit" hypothesis for tumour suppressor genes[5]. A mutated allele of the *MEN1* gene is inherited from the affected parent or developed at embryonic level (first hit); the second wild type allele is lost at somatic cell level (second hit). Tumours develop after inactivation of both *MEN1* copies at chromosome 11q13, with acquisition of a homozygous recessive state at the tissue level. The absence of wild type menin is responsible for tumour development, but the exact molecular mechanisms of MEN1 tumorigenesis must still be completely clarified and are strongly suspected to be tissue-specific[6]. Moreover, the great clinical variability even between members of the same MEN1 family and/or between patients bearing the same *MEN1* mutation may lead to suspicion regarding the influence of other genetic (*i.e.*, the contemporaneous presence of genetic variations and polymorphisms in genes other than *MEN1*) and non genetic tumour-predisposing cofactors[7]. The presence of a *MEN1* mutation is associated with the development of the

syndrome with a nearly complete penetrance by the age of 50[8]. To date, no cases of mutation carriers who have not developed at least one of the MEN1-associated tumours during their lifetime have been described. Recently, a novel mutation in exon 2 of the *MEN1* gene (Leu143His) has been associated with a limited penetrance and a specific phenotype; family members bearing the mutation presented a mild or even absent primary hyperparathyroidism and multiple well-differentiated functionally active neuroendocrine tumours of the pancreas[9].

In general, the identification of a *MEN1* mutation represents a positive genetic diagnosis of MEN1. Mutation analysis of the *MEN1* gene allows an early identification of subjects who will develop MEN1 syndrome and it is strongly recommended for: (1) index cases meeting the clinical criteria for MEN1 syndrome (sporadic case affected by at least two of MEN1-related main tumours or individual with one of the MEN1-related main tumour and with a first-degree relative affected by at least one of MEN1-related main tumours); (2) index cases not fully meeting clinical criteria for MEN1 but with two or more MEN1-related lesions and/or recurring tumours; and (3) asymptomatic first-degree relatives of a patient with an identified *MEN1* mutation.

Before the discovery and positional cloning of the *MEN1* gene in 1997, the genetic ascertainment of the syndrome consisted of haplotype analysis, using microsatellite markers at the 11q13 locus, in MEN1 affected kindred[10]. The analysis consists of a polymerase chain reaction (PCR)-based haplotype analysis of a series of microsatellite markers flanking, both at centromeric and telomeric sides, the 11q12-13 region containing the *MEN1* gene. Some of these markers exhibit recombination 0 with the *MEN1* gene (*i.e.*, PYGM, D11S463, D11S427) granting the test for carriers to reach up to 99.5% of accuracy and excluding incorrect results due to meiotic crossing over. The analysis requires the availability of a MEN1 family with at least two living clinically affected members, spanning at least two generations, and it allows for the identification, in affected individuals, of the family-specific 11q13 risk haplotype. This screening presents the limitation that it cannot be applied to a single index case. With the reconstruction of haplotypes of all the members of the family it is possible to assess who inherited the predisposing 11q13 haplotype. Since the discovery of the *MEN1* gene this kind of diagnostic approach has been replaced by the PCR-based sequencing analysis of the gene itself. However, the haplotype analysis is still useful and is suggested for the identification of gene carriers in MEN1 families in which a *MEN1* gene mutation has not been identified.

After the positional cloning of the *MEN1* gene and its identification as the gene for MEN1 syndrome, the genetic diagnosis of MEN1 consists in the mutation analysis of the coding regions (exons 2-10) and intron-exon junctions of this gene. The great majority of laboratories worldwide currently use selective PCR-based amplifications of *MEN1* exons and splicing sites, followed by specific PCR-based incorporation of dideoxynucleotides according to Sanger sequencing strategies. The *MEN1* gene consists of 10 exons encoding for a main mRNA transcript of 2.8 Kb. Less commonly mRNA transcripts have been also described, presenting variations of the 5'-untranslated region but not of the coding region[11]. The entire exon 1 (in some cases referred as two distinct exons, as reported on the NCBI Gene database: Gene ID: 4221) and part of exons 2 and 10 are non coding regions, and, thus, exon 1 is conventionally non-included in the mutation screening. Mutations in the *MEN1* promoter region have not been described[5]. About 70%-90% of MEN1 families and sporadic cases result to be carriers of a *MEN1* mutation; some studies reported that 5%-10% of patients do not have an identifiable mutation in the coding region or in splice sites of the gene[7]. They may have mutations in the promoter, introns or untranslated regions that normally are not analysed in genetic tests, or they may bear a gross gene deletion/insertion that cannot be identified by the classical *MEN1* sequencing analysis. Large gene deletions, insertions or other gross rearrangements can be detected by Southern blot analysis or by other gene dosage procedures. Multiplex ligation-dependent probe amplification (MLPA) is a quantitative highly sensitive and accurate multiplex PCR technique able to detect copy number changes within a gene and, thus, identify the presence of entire exons and/or whole gene loss or other gross intra-genic modifications. MLPA diagnostic screening should be considered in MEN1 index cases with a negative *MEN1* sequencing test, to detect large *MEN1* coding region deletions/duplications. In case of both sequencing and MPLA negative screenings, the application of familial haplotype analysis should be considered[12].

Patients with MEN1-like clinical phenotypes but negative to *MEN1* genetic tests may also represent phenocopies. Phenocopies may mimic MEN1 either by occurrence of a single sporadic endocrine tumour within a MEN1 kindred or of two endocrine tumours/lesions associated with different aetiologies and, thus, they can confound clinical diagnosis of the disease. It is estimated that phenocopies may arise in up to 5% of MEN1 families[13]; their recognition, also by specific genetic tests, is very important in the clinical management of MEN1 patients. In particular, the multiple neoplasia type 4 (MEN4) syndrome, a rare hereditary tumour syndrome presenting clinical manifestations overlapping with those of MEN1 but caused by inactivating mutations of the *CDKN1B* gene, should be strongly suspected, and the *CDKN1B* gene should be screened for mutations. Indeed, to date, nine different inactivating mutations of the *CDNK1B* gene (including missense, nonsense and frameshift variations and mutations in the 5'UTR of the gene) have been identified in 9 patients with a clinical MEN1-like syndrome but without any identified

MEN1 mutation[14]. However, because of the rarity of the MEN4 syndrome, the very small number of *CDKN1B* mutated patients, and the still unclear data about molecular mechanisms underlying MEN4 tumorigenesis, specific surveillance programs and specific clinical and therapeutic guidelines are not yet available for this syndrome, and the clinical management of patients overlaps that of MEN1 syndrome. Recently Longuini *et al*[15] found an association between the *CDKN1B* rs2066827 polymorphic variant and tumour multiplicity in patients bearing a *MEN1* germline mutation. In MEN1 patients over 30 years of age with a *MEN1* truncating mutation, the presence of rs2066827 T allele was strongly higher in subjects with susceptibility to tumours in multiple glands (3 or 4 affected glands *vs* 1 or 2 affected glands). These data need to be confirmed by additional studies and by functional analyses but they seem to suggest that the *CDKN1B* gene may act as a disease modifier in MEN1 syndrome in association to *MEN1* mutation, influencing the severity of disease clinical outcomes. In addition, less than 2% of clinical MEN1 patients without *MEN1* mutation may have mutations or polymorphisms in members of the cyclin-dependent kinase inhibitor (CDKN) family, other than *CDKN1B*, such as *CDKN1A, CDKN2B*, or *CDKN2C* genes encoding, respectively, the $p21^{cip1}$, $p15^{Ink4b}$, $p15^{Ink4c}$ proteins, which all negatively regulate the cell cycle progression and cell growth[7]. Genetic analyses of these genes should be considered in patients with classical clinical manifestations of MEN1 but have resulted negative for *MEN1* and *CDKN1B* mutations.

Lemos and Thakker[7] in 2008 created a database of published *MEN1* mutations by searching the NCBI PubMed scientific publication database for article in English. No uploaded mutation database has been published or released after that. They reported a total of 1133 different germline and 203 somatic mutations. Twenty-four polymorphisms (12 in the coding region, of which 10 synonymous and 2 non-synonymous, 9 in the introns and 3 in the untranslated exonic regions) have also been described. *MEN1* mutations are scattered along the entire 1830 bp coding region without showing any hot spot, making genetic analysis labour-intensive. Mutations are different in their types: 20% missense mutations, 23% nonsense mutations, 41% frameshift insertions or deletions, 6% in-frame insertions or deletions, 9% splice site mutations and 1% large deletions. About 80% of all identified *MEN1* mutations are responsible for a truncated protein unable to reach the nucleus of the cells and exerting its role of oncosuppressor[1].

Unfortunately, no genotype-phenotype correlation has been reported, and the identification of a specific *MEN1* mutation and/or a specific mutated region of menin does not allow the prediction of clinical outcomes of the disease, strongly reducing the importance of genetic information for the individual clinical management of both affected and asymptomatic mutation carriers. However, the individuation of any *MEN1* mutation in an individual gives indications for a lifelong routine of clinical surveillance for MEN1-associated tumours and lesions, indicates some surgery procedures and enables the specific mutation analysis for first-degree relatives in order to individuate mutation carriers. In the presence of a *MEN1* mutation lifelong specific clinical surveillance is suggested, as specifically reported in Table 1. These guidelines are reported according to the most recent "clinical practice guidelines for Multiple Endocrine Neoplasia type 1" drafted by the leading worldwide specialists in this field[16]. Moreover, a positive test can also determine the type of surgical intervention for MEN1-associated primary hyperparathyroidism, leading to the decision for a total parathyroidectomy to prevent future recurrences of parathyroid adenomas, instead of the selective ablation of only the adenomatous gland/glands in subjects with a negative *MEN1* genetic test. In *MEN1* mutation carriers also a prophylactic thymectomy is also strongly suggested, at the same time as the parathyroid surgery, to prevent the occurrence of thymic carcinoids and related malignancies and to remove possible intra-thymic ectopic/supra-numerary parathyroid glands. A positive test may also direct prenatal decisions, such as the performance of *in vitro* fertilization with pre-implantation genetic selection of non-mutated embryos or *MEN1* prenatal genetic test in a naturally occurring foetus. Conversely, a negative test in a MEN1 family member is an indication for no further biochemical and instrumental MEN1-related screenings and for no prenatal genetic testing.

FUTURE PERSPECTIVES IN *MEN1* GENETIC TEST

As mentioned above, the great majority of laboratories worldwide performing the genetic test for MEN1 syndrome, uses PCR-amplification of exons and splicing sites, followed by the PCR-based Sanger sequencing method. Only some of these laboratories also perform MPLA or linkage analyses in case of a negative sequencing result. These approaches fail to detect possible mutations in non coding and regulatory regions as well as identify phenocopies. The identification of possible phenocopies for the correct differential analysis of tumour endocrine syndromes would require a sequential traditional Sanger sequencing of all the associated genes, an extremely time-consuming approach. Next generation sequencing (NGS) is a novel sequencing technology that could allow these limitations to be bypassed, increasing the strength, efficacy, completeness and speed of genetic analysis. Application of NGS to the genetic diagnosis of the disease permits, at the same time, the sequencing of the coding regions, the introns, the untranslated and the regulatory regions, allowing the large intra-genic deletions/duplications to be recognised and the identification of novel

Table 1 Suggested guidelines for biochemical and instrumental surveillance screenings in individuals bearing any *MEN1* gene mutation

Tumour/lesion	Recommended age of begin screenings (yr)	Biochemical screenings	Frequency	Instrumental screenings	Frequency
Parathyroid adenoma	8	Fasting total serum calcium concentration (corrected for albumin) and/or ionized-serum calcium concentration Fasting serum concentration of full-length PTH	Yearly	None	NA
Gastrinoma	20	Fasting serum gastrin concentration	Yearly	None	NA
Insulinoma	5	Fasting serum glucose and insulin concentration	Yearly	None	NA
Other pancreatic neuroendocrine tumours	Under 10	Serum concentration of chromogranin-A, glucagone, pancreatic polypeptide, vasoactive intestinal peptide	Yearly	Abdominal CT, MRI or EUS	Yearly
Anterior pituitary adenoma	5	Serum concentration of prolactin and insulin-like growth factor-1	Yearly	Head MRI	Every 3-5 yr (depending on results of biochemical screenings)
Adrenal gland tumour	Under 10	None unless symptoms or signs of functioning tumour and/or tumour > 1 cm are recognised by imaging	NA	Abdominal CT or MRI	Yearly with pancreatic imaging
Thymic and bronchial carcinoid	15	None	NA	Thoracic CT or MRI	Every 1-2 yr

CT: Computed tomography; MRI: Magnetic resonance imaging; EUS: Endoscopic ultrasound; NA: Not applicable.

unexpected causative mutations. This method allows the enlargement of nucleotide sequencing from single-gene to multi-gene-disease-targeted panels and up to the entire genome using platforms and instruments capable of producing hundreds of gigabytes of genetic data in a single run. The technique gives a less-expensive and higher-throughput alternative to DNA sequencing compared to the traditional Sanger method. Moreover, NGS provides a high degree of flexibility for the level of resolution required for any single genetic analysis and a sequence run can be specifically tailored to produce more or less data and/or to screen one or more preselected regions of the genome or a specifically designed set of genes. Two alternative approaches for the detection of gene mutations are available: (1) the sequencing of the entire human genome, which facilitates the discovery of genes and regulatory elements, even unknown, associated with diseases; and (2) NGS targeted multi-gene sequencing, using a platform including a selected panel of genes (usually including all the coding, non coding and regulatory regions of each gene), which allows the identification of disease-causing mutations for the genetic diagnosis of a specific disease or a group of related disorders (*i.e.*, hereditary endocrine tumours). The first approach is suggested for diseases with a suspected genetic origin but for which the causative gene/genes are not yet known. The second approach will be useful for the differential genetic diagnosis of hereditary endocrine tumours by the design of a specific platform including all the genes associated, up to date, to these pathologies.

In 2013 Rattenberry *et al*[16] tested a targeted NGS platform, including 9 causative genes, for the genetic diagnosis of patients with pheochromocytoma and paraganglioma, allowing this NGS-based test to be established, validated and introduced into diagnostic practice. Very recently Welander *et al*[17] enlarged the targeted NGS platform for the mutation analysis of pheochromocytoma and paraganglioma, including 14 different susceptibility genes, proving to be a cost- and time-reducing effective genetic diagnostic method for these tumours. A similar NGS-targeted approach was applied to the detection of mutations in thyroid cancer (ThyroSeq panel including 12 cancer genes with 284 mutational hot spots)[18], and also in this case NGS was revealed to be an optimal genetic test for multiple genes with high accuracy and very good sensitivity.

These promising results suggest possible development and application of an NGS targeted platform for the mutation analysis of multiple endocrine neoplasia syndromes and inherited endocrine tumours in the near future. The classical Sanger monogenic sequencing method should be maintained as a pre-screening test in all the suspected MEN1 probands and also for the research of a specific mutation in relatives of *MEN1* mutated individuals, while targeted NGS analysis should be applied routinely to patients showing clinical manifestations of MEN1 but resulting to be negative from classical Sanger sequencing of the *MEN1* and the *CDKN1B* genes.

CONCLUSION

The possibility of genetic testing has facilitated increased praecox and specific diagnosis of MEN1 syndrome. Even if it is a rare disease, and despite the fact that the presence of a specific *MEN1* mutation does not allow the prediction of clinical phenotype, the

finding of a *MEN1* mutation in a patient has important implications for his/her clinical surveillance and for clinical management of first-degree relatives. The application of genetic diagnosis, and the subsequent early identification of at-risk individuals have contributed, in the last two decades, to the reduction of morbidity and mortality of patients.

A genetic positive test, predicting the future development of MEN1, may ultimately have an important psychological impact on the proband and his/her family, often causing distress, anxiety, depression, *etc*. Clinical practice guidelines for MEN1[16] recommend that MEN1 patients and their families be followed by a multidisciplinary team of specialists, with experience in the diagnosis and treatment of endocrine tumours, including genetic counselling before and after the genetic tests are carried out. The genetic counselling, performed by a professional from the discipline of medicine or genetics, should include the correct and detailed explanation of psychosocial benefits and risks of the genetic test result, as well as its influence in clinical management and quality of life of the patient and his/her relatives. In general, collaboration between genetic counsellors, laboratories and clinicians could help a patient (and his/her family) to understand the clinical meaning of his/her genetic test result, whether positive, negative, or indeterminate, and better plan preventive screenings and therapeutic approaches.

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