Mutational and gross deletion study of the MEN1 gene and correlation with clinical features in Spanish patients

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Multiple endocrine neoplasia type 1 (MEN1, OMIM 131100) is an autosomal dominant disorder characterised by the combination of endocrine tumours, such as parathyroid tumours, enteropancreatic tumours, anterior pituitary tumours, adrenal gland, and neuroendocrine carcinoid tumours, as well as non-endocrine expression, such as lipoma, facial angiofibroma, collagenoma, and ependymoma. Primary hyperparathyroidism (HPT) is the first manifestation of MEN1 in approximately 90% of patients, although this percentage differed between studies. Gastrinoma is the most frequent enteropancreatic tumour, accounting for approximately 40% of enteropancreatic tumours. It has thus been suggested that MEN1 syndrome should be excluded in patients with gastrinoma. Prolactinomas account for 20% of MEN1 related pituitary lesions, while other reported pituitary tumours are relatively uncommon manifestations of MEN1.

The MEN1 gene is located on chromosome 11q13 and was positionally cloned in 1997. It contains 10 exons and encodes menin, a 610 amino acid protein. Menin is known to be a nuclear protein that represses JunD activated transcription and interacts with other proteins, such as Smad3, nm23, and NF-kB, all of which are involved in the regulation of cell proliferation and development.

Inactivating germline mutations found in MEN1 families/patients indicates that the MEN1 gene is a tumour suppressor gene. More than 200 germline and somatic mutations have been identified to date but no hot spots or genotype-phenotype correlations have been observed. Consequently, carriers in a family with MEN1 should be checked periodically for typical and less frequent expressions of the MEN1 syndrome. Taking into account both the absence of hot spots for typical and less frequent expressions of the MEN1 gene and the lack of genotype-phenotype correlations, it is necessary to establish clinical criteria in order to increase the detection rate of MEN1 germline mutations. It would therefore be more cost effective to perform MEN1 gene mutation analysis of selected patients, rather than of all patients with apparently MEN1 related tumours. This procedure would also avoid unnecessary anxiety for mutation negative patients.

In this study, 28 MEN1 patients with a family history and 27 sporadic MEN1 suspected patients were screened for MEN1 gene germline mutations such as missense, nonsense, or small deletions or insertions, as well as for gross gene deletions. Subsequently, we measured the association between the presence and absence of germline mutation and the age of diagnosis and clinical characteristics.

Subjects and Methods

Patients and families

Informed consent was obtained from all subjects before their participation in this study. Fifty-six unrelated probands with MEN1 were studied (31 females and 24 males), 28 of whom had a family history of the disease. A total of 152 family members, including not only familial but also sporadic MEN1 cases, were included in the study: 49 were affected, and 103 were unaffected at the time of the study. Tables 1 and 2 show the clinical features of 28 familial MEN1 cases and 27 sporadic MEN1 cases.

Classification of patients and clinical criteria

The diagnosis of sporadic MEN1 patients was based on the presence of at least two of the three main MEN1 related endocrine tumours (HPT, enteropancreatic endocrine tumours, and pituitary tumour) without a clear family history of the disease, obtained from the proband and from available relatives. In these cases, the presence of MEN1 related lesion(s) was excluded by biochemical testing.
Familial MEN1 was considered when a MEN1 patient had at least one first degree relative with one of these three tumours.\textsuperscript{2, 3}

A family apparently affected with familial hyperparathyroidism (F66), and another two cases (F27 and 83) with familial neoplasia antecedents compatible with the disease and the existence of members of the family who had died at an early age reported by the index cases were also included.

The diagnosis of primary hyperparathyroidism was established by the simultaneous presence of raised ionised or albumin adjusted serum calcium concentrations and intact parathyroid hormone levels at least twice. Histological study after surgery defined adenoma or hyperplasia. Pituitary adenomas were diagnosed by the presence of a mass lesion on high resolution CT scanning or MR imaging of the sella turcica. Baseline hormonal concentrations and dynamic test results adjusted serum calcium concentrations and intact parathyroid hormone levels were used to diagnose primary hyperparathyroidism. All probands had parathyroid lesions at the time of the study; (n) number of tumours in the family. NFT, non-functioning tumour; PRL, prolactinoma; ACTH, ACTH secreting tumour; GH, GH secreting tumour; mixed, prolactin and ACTH secreting tumour; I, insulinoma; G, gastrinoma.

Table 1 Clinical features of familial MEN1 cases and detected germline mutations

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*Age at diagnosis and sex of probands (F, female; M, male). †Number of subjects with HPT in the family (independently of whether they were hyperplasia or adenomas). All probands had parathyroid lesions at the time of the study; (n) number of tumours in the family. NFT, non-functioning tumour; PRL, prolactinoma; ACTH, ACTH secreting tumour; GH, GH secreting tumour; mixed, prolactin and ACTH secreting tumour; I, insulinoma; G, gastrinoma.

Molecular analysis of the MEN1 gene

Standard methods\textsuperscript{16} were used to extract genomic DNA from peripheral blood of the patients and their relatives. Mutation screening was performed by CSGE analysis, and variants were confirmed by sequencing analysis. Cases without variant shifts were sequenced to confirm the absence of mutations. The electrophoretic and amplification conditions used for the CSGE analysis have been previously described.\textsuperscript{15} Mutations were named according to standard nomenclature.\textsuperscript{16}

Nucleotide sequencing of PCR products.

The PCR products were purified using columns (EZNA cycle pure kit, OMEGA Biotech) and bi-directionally sequenced with a Big Dye terminator cycle sequencing Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions.

Haplotype analysis

To perform the haplotype analysis in familial cases without germline mutation in the MEN1 gene, we used four previously published extragenic polymorphic DNA markers: D11S1883, PYGM (CA) (GA), D11S913, and D11S1889.

PCR amplification was performed in 20 µl of a mixture containing 100 ng of peripheral blood DNA. The PCR conditions and the primer sequences were obtained from the Genome Data Base (http://www.gdb.org). The PCR product was mixed...
with loading buffer (95% formamide, 0.05% xylene cyanol blue, 0.05% bromphenol blue, 10 mmol/l NaOH), denatured at 95°C for five minutes, and loaded on denaturing 8% acrylamide/7 mol/l urea gels. The gels were run at a fixed power of 75 W for 4.5 hours. After electrophoresis, the gel was silver stained and dried for documentation.

**Southern blot analysis**
A total of 7 µg of genomic DNA from each proband were digested overnight with 30 U of selected, infrequently cutting restriction enzymes (EcoRI, BamHI, SacI) (MBI Fermentas, Germany). Samples were electrophoresed through 0.8% agarose gels and transferred to Zeta-Probe GT Genomic Tested Blotting membranes (Bio-Rad, Hercules, CA, USA) using standard protocols. After transfer, membranes were rinsed in 2 × SSC, dried, and UV cross linked. For hybridisation, filters were incubated at 60°C in 10 ml hybridisation solution (0.5 mol/l SSC, dried, and UV cross linked. For hybridisation, filters were incubated at 60°C in 10 ml hybridisation solution (0.5 mol/l SSC, 7% SDS) for four hours before addition of probe, then incubated at 60°C in 10 ml of the same hybridisation solution. Two low (2 × SSC/0.1% SDS) stringency washes were performed at 60°C, and filters were exposed to Kodak autoradiographic film at −80°C for 48 hours. A probe was generated by restriction digestion with XhoI from a cDNA containing the entire coding region of the gene (clone IMAGE 3138471). Probes were radiolabelled with [α-32P]dCTP using a Megaprime DNA labelling systems kit (Amersham Pharmacia Biotech, UK).

**Statistical analysis**
The association between presence or absence of mutations and other dichotomous variables was determined using Fisher’s exact test. Average ages of diagnosis of patients with and without mutation were compared using the t test.

**RESULTS**

**Germline MEN1 mutations in familial MEN1**
We used sequence analysis of the coding region of the gene to establish that 89.3% of patients with a family history (25/28) had germline mutations scattered throughout the whole coding region. Each family showed a different alteration and 10 out of 25 mutations had not been previously described (40%) (table 1). Some of these results have been partly described by Cebrián et al.22 Three of them generated a change of amino acid: H139P (F38), E45K (F52), and A49F (F64). In order to rule out that the missense mutations not previously described (H139P and A49F) were benign polymorphisms, we performed the study in 200 independent alleles of the control population by means of CSGE and these variants were not present, suggesting the pathogenic character of the change. Moreover, in two of them (E45K and A49F), we were able to perform a segregation analysis to confirm that these variants segregated with the disease.

We identified 77 mutant gene carriers, of which 30 were asymptomatic, and 64 were non-carriers among first degree relatives of the patients with MEN1 mutation (table 1). Moreover, we identified five previously described polymorphisms: 2265-16 C→G (6%), S145S (AGC→AGT, 2%), R171Q (CCG→CAG, 7%), 6025-3 C→G (2%), and D418D (GAC→GAT, 4%) whose frequencies were similar to those reported in the white population.21

In the three families in which CSGE detected no variants, the absence of mutations was confirmed by sequence analysis, and it was possible to perform a haplotype analysis in two of them. Both displayed segregation between the disease and the 11q13 region. In the third family (F66), the haplotype analysis could not be performed because an insufficient number of relatives were available.

We carried out a Southern blot analysis in these three cases to determine if one copy of the MEN1 gene was being inactivated by a method that could not be detected by genomic DNA sequence analysis. Using three restriction enzymes (EcoRI, BamHI, and SacI), we found different rearrangements or gross deletions in two out of three MEN1 families (F28 and F66) (fig 1).

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*Age at diagnosis and sex of probands (F, female; M, male). NFT, non-functioning tumour; PRL, prolactinoma; ACTH, ACTH secreting tumour; GH, GH secreting tumour; Mixed tumour, prolactin and ACTH secreting tumour; I, insulinoma; G, gastrinoma; Gl, glucagonoma. †Mutation not previously described. Fa, father; Mo, mother; S, son. ‡Results partially described by Cebrián et al.22

Table 2

Clinical features of sporadic MEN1 cases and detected germline mutations
Germline MEN1 mutations in sporadic MEN1

We analysed 27 sporadic MEN1 cases and identified heterozygous germline mutations located throughout the entire coding region of MEN1 gene in nine cases (33%) (table 2). Some of these results have been partially described by Cebrián et al.19 The parents of five of these cases could be analysed, thereby confirming that the mutations were de novo (table 2). In the 18 cases without germline mutation, we performed a Southern blot analysis but did not detect any rearrangements. 

Aside from the five polymorphisms previously mentioned, we identified another variant, A541T (GCA→ACA, 2%), whose frequency was similar to that described in the white population.20–22

Genotype-phenotype correlations

We defined two groups to compare their clinical features. The first included all familial and sporadic cases with germline mutations or with confirmed segregation with 11q13 (n=37). The second group comprised all cases without germline mutation (n=18). The analysis was not biased given that table 1 shows clinical data and the age of development of the disease from the index patients of the families. Fig 2 shows the mean age at which the first lesion was diagnosed in both groups: 33 years (50th centile =32) in cases with germline mutation and 53.6 years (50th centile=54.5) in sporadic cases without mutation. This difference was statistically significant (p<0.0001). Fig 3 shows the clinical characteristics of both groups. Worthy of note is that nearly 100% of all cases without mutation. This difference was statistically significant (p=0.001).

DISCUSSION

To date, in MEN1 families there is no specific correlation between the kind of tumours and the MEN1 germline mutation. Its clinical presentation, age of onset, and natural history of the disease vary even in members of the same family, probably owing to modifying genes which can influence the penetrance of disease phenotypes.1 Thus, the MEN1 carriers in a family with either typical or atypical expression of MEN1 should be monitored similarly for expression of MEN1 tumours.7 It is essential to establish useful clinical criteria to perform a MEN1 mutation test, not only in MEN1 families but also in sporadic MEN1 cases, in which the identification of MEN1 germline mutation has clear consequences in the follow up. 

In our series of Spanish patients, we tried to identify which tumour combination would most probably show a MEN1 germline mutation in patients without a clear familial history.
of the disease, and to discover if all typical MEN1 families could be explained by alterations in the MEN1 gene.

**Germline MEN1 mutations in MEN1 families and sporadic cases**

A total of 89.3% (25/28) of familial MEN1 cases showed germline mutations in the MEN1 gene. Each case had an individual alteration and all but seven of them generated a truncated protein. The type of mutation was characteristic of the tumour suppressor gene, and the frequency of these mutations was similar to that reported by other authors.17-24

No germline mutation was detected in three families analysed. A haplotype study was conducted in those two families with a sufficient number of relatives and we were able to confirm that the 11q13 region segregated with the disease in both families. As gross deletions have been previously described to be a MEN1 inactivation mechanism,23-27 we investigated the presence of this type of alteration in cases without germline mutation. Rearrangements were detected in two out of three analysed families, implying that this type of analysis should become a standard procedure in mutation screening of the MEN1 gene. Following the strategy described in this study, we were able to define the molecular alteration of the MEN1 gene to explain more than 96% of Spanish families. Regarding the family in which 11q13 segregated but had no germline mutation (F22, table 1), the existence of alterations in other regions of the gene or the presence of one or more additional genes in this region requires further investigation. There is controversy over the existence of genetic heterogeneity in this syndrome, as genetic linkage studies have suggested that the familial MEN1 trait always arises from the same gene,2 while, on the other hand, this idea has already been opposed by other authors.19-24

To date, the 27 sporadic cases constitute the largest series of patients with sporadic MEN1 to be reported. We detected germline mutations in nine of them (33.3%). This incidence of MEN1 gene mutations in sporadic cases was similar to that described by Hai et al25 (40%) but was lower than that reported by other authors,26-28 probably because of the different selection criteria used in these studies.

In five out of nine patients with germline mutations (13.8% of all detected mutations, including gross deletions), we were able to confirm by analysis from parental DNA that these mutations were de novo. In the rest of the patients, it was not possible to obtain the collaboration of their relatives to carry out the analysis. Although none of the relatives had symptoms related to the disease, we cannot establish whether these cases involved incomplete penetrance or were indeed de novo cases. The confirmation of a de novo mutation in near to 14% of our series is of great clinical importance both for the probands and for the genetic counselling of the rest of family, for whom carrier status may be determined and a suitable clinical and biochemical follow up instigated for the early detection of the disease.

**Genotype-phenotype correlations**

Recently, some menin domains have been defined as being important for menin function and essential for its interaction with other proteins, such as JunD or nm23.11-15 We have tried to find some associations between mutations located in these domains and more aggressive phenotypes than those produced by mutations located outside these regions. Our study and others24,25 have all pointed the phenotype to be independent of the location of the mutation. As carriers of germline MEN1 mutations without a family history evidently showed the same clinical behaviour as MEN1 patients with antecedents of the disease, we established two different groups (cases with germline mutation or confirmed segregation v cases without germline mutation) to formulate clinical criteria to identify disease gene carriers among patients with apparently sporadic MEN1 in the Spanish population. The mean age of diagnosis in the two groups was significantly different: 33 years in patients with germline mutation and 53.6 years in cases without alteration (p<0.0001) (fig 2). Among sporadic cases only, all patients younger than 40 years of age had a germline mutation except two women, 15 and 39 years old, and a 38 year old man, who will be discussed in greater detail below. These results support those of other authors,13 who identified novel MEN1 families from MEN1 suspected patients younger than 35 years and/or multiple MEN1 related lesions in a single organ or two distinct organs affected.

Regarding the clinical aspects (fig 3), we observed no differences between the development of HPT or gastrinoma and the presence or absence of mutations in the MEN1 gene. An explanation could be the prevalence of HPT in the population older than 40 years, or that pancreatic islet tumour can typically cause symptoms from hormone excess at this age, and then gene carriers probably will develop enteropancreatic tumours later.

Five patients developed carcinoid tumours and all of them had germline mutations. These data support the suggestion of Chanson et al26 that MEN1 gene analysis should be obligatory in patients with thymic or bronchial carcinoid. One striking result was that 37.8% (14/37) of cases with germline mutation developed prolactinoma, whereas only 11.1% (2/18) of patients without mutation had it.

Significantly, one of these cases was a 15 year old girl (C4, table 2) who, owing to her age at diagnosis, was considered a de novo case, with no mutation detected either by sequence analysis or by Southern blotting, pending confirmation or ruling out the existence of mutations in other regions of the MEN1 gene, such as the promoter. This difference between cases with and without mutation in the development of prolactinoma was not great enough to be statistically significant (p=0.106) for the small sample size currently available.

That said, our results suggest that there is a need to study the MEN1 gene in all patients younger than 40 years of age who present the combination of HPT and prolactinoma, because 14/16 cases with prolactinoma had germline mutation, whereas only one case with germline mutation developed GH secreting pituitary tumours, 8/18 (44.4%) patients without mutation had this tumour in combination with HPT. It is worth highlighting the coincidence in clinical manifestations in our patients without mutation and those provided by Hai et al.25 The patients in these studies had developed HPT and GH secreting pituitary tumour. These data support the view that the association of HPT and GH secreting pituitary tumours should be considered a distinct entity from MEN1, a hypothesis supported more firmly because two of these patients were the subjects aged 38 and 39 years mentioned before. This would explain why these patients do not have a mutation in the MEN1 gene. This hypothesis was suggested before the MEN1 gene was cloned.30 When the gene was isolated, GH secreting tumours were found to be more frequent in cases suspected of having sporadic MEN1 but without germline mutation.26,28,30-33 In our series, there was a strong association between the presence of GH secreting tumour and the absence of mutation in the MEN1 gene (p=0.001), indicating that these patients represent a different entity with a distinct aetiology to MEN1 syndrome.

To summarise, we have found germline mutations (including Southern blot analysis) in 96.4% of MEN1 families and 33.3% of sporadic cases. We have not observed any association between the location of mutations and the clinical characteristics of the patients. Our results suggest the following.

- Patients with only two of the three main clinical manifestations of the disease, without a family history and older than 40 years of age, should not be included in the study of MEN1. Hence, they are probably not MEN1 cases, but instead phenocopies generated by mutations in other genes or by random development of HPT and other MEN1 related tumour owing to the high frequency of some of these pathologies in the population older than 40 years of age.
Patients with any type of carcinoid tumour should be studied in order to rule out the possibility of MEN1 syndrome.

- Analysis of the MEN1 gene should be obligatory for patients with HPT and prolactinoma, because 87.5% (14/16) of cases had germline mutation.

- The association of GH secreting tumours and HPT seems to be a different entity to MEN1 disease, and the gene responsible for this condition remains to be localised.

- We recommend the analysis of gross deletions in the MEN1 gene in families without germline mutation as this type of alteration was detected in 2/3 of such cases.

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