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Identification of *MEN1* gene mutations in families with MEN 1 and related disorders

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Summary Following identification of the *MEN1* gene, we analysed patients from 12 MEN 1 families, 8 sporadic cases of MEN 1, and 13 patients with MEN 1-like symptoms (e.g. cases of familial isolated hyperparathyroidism (FIHPT), familial acromegaly, or atypical MEN 1 cases) for the presence of germline *MEN1* mutations. The entire coding region of the *MEN1* gene was sequenced, and mutations were detected in 11 MEN 1 families; one sporadic MEN 1 patient, one case of FIHPT and one MEN 1-like case. Constitutional DNA samples from individuals without *MEN1* mutations were digested with several restriction enzymes, Southern blotted and probed with *MEN1* cDNA to analyse for the presence of larger deletions of the *MEN1* gene unable to be detected by PCR. One MEN 1 patient was found to carry such a deletion. This patient was heterozygous for the D418D polymorphism, however sequence analysis of RT-PCR products showed that only the variant allele was transcribed, thus confirming the result obtained by Southern analysis, which indicated loss of a region containing the initiation codon of one allele. © 2000 Cancer Research Campaign

Keywords: MEN 1; mutation analysis; FIHPT

Multiple endocrine neoplasia type 1 (MEN 1) is an autosomal dominant disorder characterized by an inherited predisposition to neoplasia and hyperplasia of the parathyroid glands, anterior pituitary and endocrine pancreas. Also observed are thymic and lung carcinoids, adrenocortical tumours, lipomas, thyroid tumours (Komminoth et al, 1998) and skin lesions such as angiofibromas and collagenomas (Darling et al, 1997). MEN 1 has an estimated prevalence of 0.02–0.2/1000, depending on race and geographic location (Teh et al, 1995). The disease shows almost complete penetrance by age 50 (Komminoth et al, 1998).

The gene responsible for MEN 1 is located on chromosome 11q13, and was shown by linkage analysis and tumour loss of heterozygosity studies to conform to Knudson's two-hit model of tumorigenesis (Larsson et al, 1998). The *MEN1* tumour suppressor gene was recently identified by positional cloning (Chandrasekharappa et al, 1997). The gene covers approximately 8 kilobases (kb) of genomic DNA, is comprised of ten exons and encodes a 2.8 kb ubiquitously expressed mRNA, producing a putative 610 amino acid protein showing no homology to any others in the current databases.

Numerous studies aimed at identifying predisposing *MEN1* gene mutations in MEN 1 families and patients with related disorders have been reported in the literature (for example Agarwal et al, 1997; Bassett et al, 1998; Giraud et al, 1998; Teh et al 1998a). No apparent genotype–phenotype correlations have been identified. Mutations are evenly distributed throughout the coding region of the gene, without obvious hotspots suggestive of functional domains.

In this study, we aimed to characterize the *MEN1* gene for mutations in 12 Australian MEN 1 kindreds and in 21 cases displaying MEN 1-like symptoms, to add to the extensive list of mutations previously reported, and assist with pre-symptomatic testing and genetic counselling of members of these patients' families.

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SUBJECTS, MATERIALS AND METHODS

Patients

Patients were primarily obtained from major public teaching hospitals in Brisbane, as well as from endocrinologists throughout Australia who referred them specifically for mutation testing of the *MEN1* gene. A patient was considered to have familial MEN 1 if they had at least one affected first-degree relative, and provided one or more family members presented with at least two of the three major endocrine lesions (parathyroid, pancreas, anterior pituitary). Sporadic MEN 1 was diagnosed if a patient had at least two of the three major MEN 1 lesions, with no known family history of any endocrine manifestation. Patients were classified as MEN 1-like if they did not fit the above definition of MEN 1, but one of the major MEN 1 lesions was present in at least one family member. Diagnosis of familial isolated hyperparathyroidism (FIHPT) was based on the presence of primary hyperparathyroidism in at least one first-degree relative, in the absence of any other endocrine manifestations. Similar criteria were used for diagnosis of familial acromegaly. A detailed clinical and family history was taken from each available family member, and a peripheral blood sample was drawn for DNA analysis, for which informed consent was given.

Table 1 PCR primers used for mutation analysis

Exon/Gene segment	Primer sequence (5′–3′)	Annealing temp. (°C)	Annealing position[a]
MEN1 cDNA			
1F	CTAGAGATCCCAGAAGCCAC	56	20–39
R	CACTACCCAGGCATGATCC		665–647
2F	ACAGGCACCAAATTGGACAG	58	552–571
R	CACATTGCGGTTGCGACAG		1112–1094
3F	CTGGCTGCTCTATGACCTG	58	902–920
R	CTAGGGACTGCACAAGAAAG		1443–1424
4F	CGACGGCATCTGCAAATGGG	60	1361–1380
R	GGGTTTGGGTAGAGGTGAGG		2059–2040
MEN1 gene			
2F	GTGAGCAGAGGCTGAAGAGG	64	2130–2149
R	ATAACACCTGCCGAACCTCAC		2844–2824
3F	AGGTTGGGTCACAGGCTTG	58	4181–4199
R	CTATGTGGGTGGTGATGGG		4617–4599

[a] Sequence taken from GenBank accession number U93237 (gDNA) and U93236 (cDNA)

DNA and RNA isolation

DNA was isolated from whole blood or lymphoblastoid cell lines (LCLs) using the salt extraction method of Miller et al (1986). Total RNA was isolated from LCL pellets using an RNeasy Mini kit (QIAGEN) as per the manufacturer's instructions.

PCR

Each of the nine coding exons of the *MEN1* gene were amplified using the polymerase chain reaction (PCR). The primers for exons 4–10 were obtained from the European Consortium for MEN 1 and are reported in Lemmens et al (1997). Primers used for amplifying exons 2 and 3 are listed in Table 1. Reactions were performed in 50 µl volumes containing 10 pmol each primer, 5% DMSO, 0.75 U Amplitaq Gold (Perkin Elmer) and approximately 50 ng of template DNA. For PCR of exon 2, 1M betaine was substituted for DMSO. Reactions were cycled on either a Perkin Elmer Cetus or Hybaid Omnigene thermal cycler under the following conditions: 95°C for 12 minutes, followed by 35 cycles of 95°C for 1 minute, 62°C for 1 minute (this annealing temperature differed for some primers; see Table 1), and 72°C for 90 seconds. PCR products were purified by agarose gel electrophoresis and DNA was isolated from the agarose using a QIAquick gel extraction kit (QIAGEN) as per the manufacturer's instructions.

RT-PCR

Five µg of total RNA were used to synthesize the first strand of cDNA using an oligo$(dT)_{12–18}$ primer (Boehringer Mannheim) and a SuperScript II reverse transcriptase kit (Gibco BRL) as per the manufacturer's instructions. Reverse transcriptase PCR (RT-PCR) was performed using the cDNA primers listed in Table 1. Cycling conditions were the same as those described for genomic PCR, using the annealing temperatures listed in Table 1. PCR products were purified as described for genomic PCR.

DNA sequencing

Standard protocols were used for cycle sequencing of PCR products using Big Dye dye terminator reaction premix (ABI prism). Primers used were the same as for PCR. Cycling reactions were performed on a Selby TS-MP96 thermal cycler. Sequences were determined using an ABI377 automated sequencer, and sequence traces were manually analysed for the presence of heterozygous peaks. Base changes were confirmed by sequencing of an independent PCR product.

Southern analysis

Five µg of genomic DNA from each relevant individual were digested overnight separately with 20U of selected infrequent-cutting restriction enzymes (*Eco*RI, *Kpn*I, *Sma*I, *Sac*I, *Sac*II; New England Biolabs). Samples were electrophoresed through 0.8% agarose gels and transferred to Hybond N nylon membranes (Amersham) using standard protocols (Sambrook et al, 1989). Following transfer, membranes were rinsed in 2XSSC, dried, and UV-crosslinked. For hybridization, filters were incubated at 65°C in 100 ml hybridization solution (7% SDS, 0.263 M Na_2HPO_4 pH 7.2, 1 mM EDTA pH 8.0, 1% BSA) for 2–4 hours prior to addition of probe, then hybridized overnight in 10 ml of the same hybridization solution. Two low (2×SSC/0.1% SDS) and two high (0.1×SSC/0.1% SDS) stringency washes were performed at 65°C, and filters were exposed to Fuji autoradiographic film at –70°C for 48 hours. A probe was generated by restriction digestion from a *MEN1* cDNA containing the entire coding region of the gene. Probes were radiolabelled with [α-^{32}P]dCTP using a Rediprime II random primer labelling kit (Amersham) as per the manufacturer's instructions.

RESULTS AND DISCUSSION

Identification of MEN1 gene mutations

A total of 12 patients from MEN 1 families, 8 sporadic MEN 1 cases, one case of familial acromegaly, 5 unrelated patients with FIHPT, and 7 patients demonstrating MEN 1-like symptoms were analysed in this study for the presence of germline mutations of the *MEN1* gene. This was achieved primarily by sequence analysis of exon-specific PCR products from the coding region of the gene. A summary of the clinical details of the patients analysed is presented in Table 2. Germline mutations were identified in 10

Table 2 Clinical presentations of MEN 1 and MEN 1-like patients with *MEN1* gene mutations identified.

MEN 1 Families:

ID	Clinical features	No. affected	Lesions	Exon	*MEN1* mutation	Effect on protein
20002	ZES, ↑PP	5	HPT	10	7773insC	Frameshift
30001		4	Pituitary adenoma, multicentric insulinoma, parathyroid adenoma, HPT		Partial gene deletion†,*	lack of expression
40933		4	Prolactinoma, insulinoma, pituitary adenoma, HPT	2	2536del4	frameshift
41121	Acromegaly, renal calculi	4	HPT		not found	
41131	Renal calculi, ZES	4	Gastrinoma, HPT, pituitary adenoma, lipomata	9	7361del11‡	deletes exon/ intron border
41177	ZES, ↑PP, ↑gastrin, pancreatitis	3	Adrenal tumour, HPT	8	6630T>G	Y353D
41178		4	Gastrinoma, pituitary tumour, HPT	8	6690C>T	P373S
41179	renal calculi	4	Pancreatic tumour, thymic carcinoid, prolactinoma, HPT	9	7312delG≠	frameshift
41180		2	Prolactinoma, HPT	3	4482del4	frameshift
50000	↑gastrin, duodenal ulcer, ZES	4	Lipomata, islet cell tumour, HPT, gastrinoma, pancreatic adenoma	10	7916AGC>T	frameshift
60004	renal calculi, dyspepsia, galactorrhoea	3	Gastrinoma, prolactinoma, malignant thymoma, HPT	9	7254G>C	R415P
96002	renal calculi	4	Lung and thymic carcinoids, prolactinoma, non-functioning pituitary, insulinoma, HPT	9	7278G>A†	W423X

Sporadic MEN 1 patients:

ID	Clinical features	No. affected	Lession	Exon	*MEN1* mutation	Effect on protein
10000	Acromegaly	1	Parathyroid adenoma		not found	
24000	↑prolactin, Ca^{2+}; ↓ phosphate	1	Insulinoma		not found	
40931	Hypercalcaemia, acromegaly	1	Acidophilic pituitary adenoma, HPT, lipomata, bowel polyps		not found	
41076	Acromegaly	1	Prolactinoma, HPT		not found	
41120	Galactorrhoea	1	Prolactinoma		not found	
41181		1	Hyperplastic adrenal, prolactinoma, HPT, insulinoma, glucagonoma	10	7622C>T	$R460^{x}$
50001	Cushing's disease	1	ACTH pituitary adenoma, HPT		not found	
60005		1	Non-functioning pituitary, HPT		not found	

MEN 1-like patients/families

ID	Clinical features	No. affected	Lesions	Exon	*MEN1* mutation	Effect on protein
20005	Hypoglycemia, acromegaly	5	Bronchial carcinoid, caecalsarcoma		not found	
41025		5	Pituitary tumour, HPT, craniopharyngiema		not found	
41082		2	Insulinoma, MTC		not found	
41174	Hypertension, stomach ulcer	3	Thyroid adenoma, HPT	4	4747G>T	R229L
41175	↑PP, peptic ulcer, acromegaly	3	HPT, GH, prolactin-secreting pituitary adenoma		not found	
41176	Renal calculi	1	Sporadic recurrent HPT		not found	
70004		2	Non-secretory pancreatic, HPT		not found	

Familial isolated hyperparathyroidism/acromegaly families:

ID	Clinical features	No. affected	Ages at diagnosis	Exon	MEN1 mutation	Effect on protein
40883	familial acromegaly	3			not found	
41067	FIHPT	3	20–40	2	2543ins18	6 amino acid insertion
41173	FIHPT	2	29		not found	
41182	FIHPT	5			not found	
41183	FIHPT	2	38		not found	
70013	FIHPT	4	18–62		not found	

Abbreviations: HPT = hyperparathyroidism; FIHPT = familial isolated hyperparathyroidism; ZES = Zollinger–Ellison syndrome; PP = pancreatic polypeptide; MTC = medullary thyroid carcinoma. † incorrectly reported as no mutation in Teh et al (1998a). * Further information in Figures 4 and 5. ‡ Incorrectly reported as 7352del11 in Teh et al (1998a). ≠ Incorrectly reported as 4340insA in Teh et al (1998a).

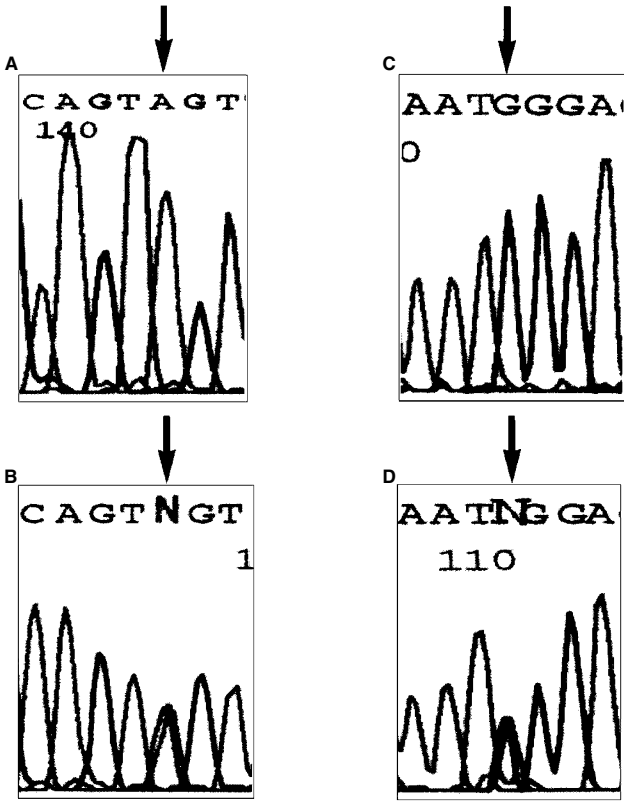

Figure 1 Sequence traces for: (**A**) a portion of wild type exon 8 (complementary strand); (**B**) A–C transversion on reverse strand responsible for missense mutation Y353D; (**C**) wild type exon 9; (**D**) G–A transition resulting in W423^{x}

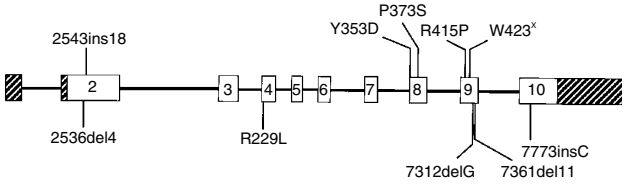

Figure 2 Schematic diagram of the *MEN1* gene showing germline mutations identified. 2543ins18 was identified in a patient with FIHPT, R229L was found in a patient with MEN 1-like symptoms, and the remaining mutations were identified in MEN 1 patients

MEN 1 families, one sporadic MEN 1 patient, one patient with FIHPT and one MEN 1-related case (Table 2). Examples of sequence traces showing the base changes responsible for 2 mutations are shown in Figure 1. A schematic diagram of the *MEN1* gene, showing the approximate locations of the mutations reported here is shown in Figure 2. Only 3 of these mutations (2536del4, 7773insC; R460^{x}) have been reported previously, either in MEN 1 families or in MEN 1-type sporadic endocrine tumors (Agarwal et al, 1997; Bassett et al, 1998).

As has been previously observed, the mutations are distributed throughout the coding region of the gene, although a relatively large proportion of mutations are located in exon 9. This region forms part of a putative site for interaction with JunD (Agarwal et al, 1999), so it is possible that some of the exon 9 mutations reported here may prevent the interaction between menin and JunD.

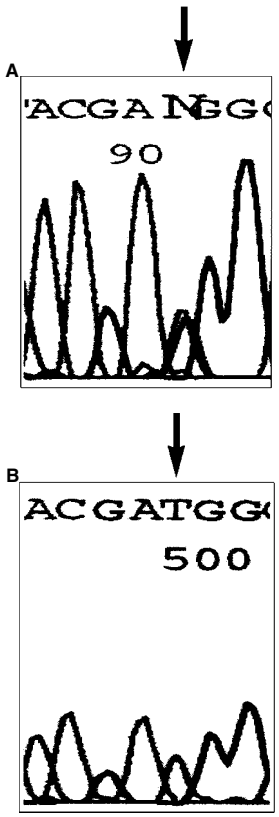

Figure 3 Sequence traces showing loss of expression of one allele in a MEN 1 patient (ID. 30001). (**A**) A portion of exon 9 sequence from genomic DNA showing a heterozygous D418D polymorphism. (**B**) The same sequence by RT-PCR, showing only expression of the polymorphic allele

There have been several other reports previous to this study of germline mutations identified in patients with FIHPT (e.g. Teh et al, 1998b; Shimizu et al, 1997; Ohye et al, 1998; Fujimori et al, 1998; Poncin et al, 1999), and a number of reports of FIHPT families without *MEN1* gene mutations (Agarwal et al, 1997; Giraud et al, 1998; Teh et al, 1998a). Although mutation of the *MEN1* gene in FIHPT is an uncommon event, it should still be considered in genetic screening protocols.

Further analysis of patients with no germline mutations

As a lower number of patients were found to carry germline *MEN1* gene mutations than expected, additional analyses were conducted to determine if one copy of the *MEN1* gene was being inactivated by a method not detectable by sequence analysis of genomic DNA. If a heterozygous sequence polymorphism was identified during germline mutation analysis, RT-PCR was conducted (provided LCLs were available for isolation of RNA) to determine whether loss of mRNA expression of one allele was occurring. This was identified in one familial MEN 1 patient (ID: 30001) out of 11 patients analysed, and the sequence traces demonstrating this are shown in Figure 3.

To investigate whether this loss of expression of one allele of the *MEN1* gene was due to some rearrangement or deletion unable to be detected by PCR, genomic DNA from the patient was digested with a series of infrequent-cutting restriction enzymes

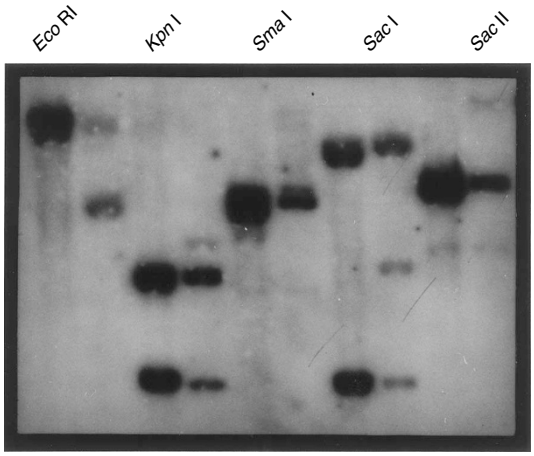

Figure 4 Southern blot analysis of DNA from a MEN 1 patient carrying a large germline deletion. For each enzyme, lane 1 is from a healthy control, and lane 2 is from the MEN 1 patient with a deletion. So that some of the faint bands can be clearly seen, a long exposure time of the autoradiograph has been deliberately used. Band sizes are detailed in Table 3

Table 3 Restriction fragment sizes obtained by Southern analysis in Figure 4 of wild type and MEN1 intragenic deletion DNA.

Restriction enzyme	Fragment sizes (kb)	
	control	mutant
*Eco*RI	12.0	12.0
		6.5
*Kpn*I	4.7	4.7
	3.4	3.4
		5.3
*Sma*I	6.2	6.2
	4.5	4.5
	5.4	1.5
	1.5	12.0
	1.4	15.2
		0.9
*Sac*I	9.2	9.2
	3.4	3.4
	1.1	1.1
		4.8
*Sac*II	6.6	6.6
	5.2	5.2
	1.5	1.5
		15.2

and evaluated by Southern analysis. Digestion with some of these enzymes did produce different-sized bands compared to a control sample (Figure 4). In addition, all patients without a germline mutation were analysed in this fashion using the restriction enzymes *Eco*RI and *Kpn*I, and no aberrant-sized bands were observed (data not shown). Twenty known control samples were also screened with *Kpn*I and *Eco*RI to ensure that these aberrant bands were not just due to population variation (data not shown).

The approximate sizes of the bands in Figure 4 were calculated using a standard curve derived from molecular size markers and are shown in Table 3. From analysis of the aberrant bands, it appears that *Sma*I, *Sac*II and *Kpn*I sites known to be located in intron 1 are lost, as is a *Sac*I site in exon 2. An *Eco*RI site expected to lie about 3.5 kb upstream of the *MEN1* gene would be moved

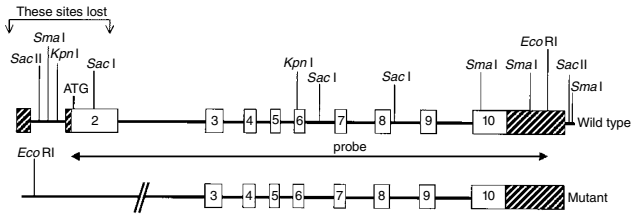

Figure 5 Schematic diagram of the *MEN1* gene and germline deletion in a MEN 1 family. Restriction sites known to be detected by the probe used are marked to demonstrate the rationale behind the predicted deletion

closer as a result of a deletion, thus explaining the smaller bands. From this, it seems that one allele of the *MEN1* gene in this patient contains a large deletion, beginning somewhere upstream of the gene and terminating somewhere before exon 6, thus obliterating the start codon (Figure 5). Analysis with additional restriction enzymes and exon-specific probes may define this deletion further. No unusual clinical features were observed associated with this deletion. Because a similar deletion has been reported previously (Kishi et al, 1998), we suggest such analysis should become a standard procedure in mutation screening protocols.

It remains possible that the *MEN1* gene is inactivated by mechanisms other than those we have described above, such as mutation of intronic or promoter sequences, which may result in reduced transcription or decreased mRNA stability. Mutation of an intron of the *MEN1* gene has been previously reported (Engelbach et al, 1999), resulting in inclusion of 7 bases of intronic sequence into the mRNA and a truncated protein sequence. A number of patients were screened for *MEN1* gene mutations in this study, with no such similar alterations found. However, screening of promoter and intronic sequences, as well as expression analyses should be considered in *MEN1* screening protocols.

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