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Identification of somatic mutations of the *MEN1* gene in sporadic endocrine tumours

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Summary Endocrine tumours of the pancreas, anterior pituitary or parathyroids arise either sporadically in the general population, or as a part of inherited syndromes such as multiple endocrine neoplasia type 1 (MEN 1). The mechanisms responsible for the development of sporadic endocrine lesions are not well understood, although loss of heterozygosity (LOH) of the *MEN1* locus on chromosome 11q13 and somatic mutation of the *MEN1* gene have been frequently associated with the development of MEN 1-type sporadic endocrine lesions. To further investigate the role of the *MEN1* gene in sporadic endocrine tumorigenesis, we analysed DNA from 14 primary parathyroid lesions, 8 anterior pituitary tumours and 3 pancreatic tumours for the presence of somatic *MEN1* gene mutations and LOH of seven microsatellite markers flanking the *MEN1* locus. In addition, we similarly analysed 8 secondary parathyroid lesions which arose in patients with chronic renal failure. None of the patients studied had a family history of MEN 1. Three primary parathyroid lesions and one pancreatic tumour (glucagonoma) were found to have lost one allele at the *MEN1* locus. Somatic mutations were identified by SSCP and sequence analysis in one of these parathyroid lesions (P320L) and in the glucagonoma (E179V). These results support previous findings that inactivation of the *MEN1* tumour suppressor gene contributes to the development of sporadic MEN 1-type endocrine lesions but is not associated with the development of parathyroid hyperplasia seen in some renal failure patients. © 2000 Cancer Research Campaign

Keywords: MEN 1; LOH; mutation analysis; endocrine tumour; sporadic

Endocrine tumours predominantly arise sporadically in the general population, but sometimes occur as part of inherited endocrine neoplasia syndromes. Multiple endocrine neoplasia type 1 (MEN 1) is an autosomal dominant disorder characterized primarily by hyperplasia and/or neoplasia of the parathyroid glands, anterior pituitary and endocrine pancreas. The *MEN1* gene is a tumour suppressor gene located at chromosome band 11q13 (Larsson et al, 1988) and was recently identified by positional cloning (Chandrasekharappa et al, 1997).

As well as its involvement in familial MEN 1 and related syndromes, the *MEN1* locus has also been implicated in the development of MEN 1-type sporadic endocrine tumours. Loss of heterozygosity (LOH) of markers flanking the *MEN1* gene has been reported in sporadic primary parathyroid lesions (Tahara et al, 1996; Farnebo et al, 1997a), pancreatic tumours (Teh et al, 1990; Debelenko et al, 1997a), and anterior pituitary tumours (Bates et al, 1997; Dong et al, 1997), as well as in sporadic cases of tumours less commonly associated with MEN 1, such as lung, thymic and gastric carcinoids (Jakobovitz et al, 1996; Debelenko et al, 1997b), lipomas (Dong et al, 1997) and cutaneous tumours (Pack et al, 1997). Mutations in the *MEN1* gene have been detected in all of these tumour types (Heppner et al, 1997; Debelenko et al, 1997c; Zhuang et al, 1997a; Zhuang et al, 1997b; Böni et al, 1998; Vortmeyer et al, 1998).

This study aims to continue investigations into the role of the *MEN1* gene in sporadic endocrine tumorigenesis, by screening for LOH of 11q13 markers and mutations in the *MEN1* gene in a series of sporadic tumours of the pancreas, pituitary and parathyroids. In addition, we aim to investigate the role of the *MEN1* locus in the development of parathyroid lesions that have arisen secondarily due to hypercalcaemia following renal transplantation.

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MATERIALS AND METHODS

Patients and tumours

After obtaining informed consent, endocrine lesions from 33 patients undergoing surgery to remove pancreatic (n = 3), anterior pituitary (n = 8) or parathyroid lesions (n = 22) at the Royal Brisbane or Princess Alexandra Hospitals in Brisbane were included in the study. A peripheral blood sample for germline DNA analysis was also acquired from each patient, for which they gave informed consent. Diagnosis of primary hyperparathyroidism was based on the following criteria: elevated serum calcium (>2.6 mmol/l) and/or serum parathyroid hormone (>5.5 μmol/l); and presence of hyperplasia or adenoma following histological analysis of frozen sections of parathyroid tissue. Usually only one of the four parathyroid glands was affected in these patients. Secondary hyperparathyroidism arising in patients with chronic (or end stage) renal failure is associated with hyperplasia of all

four parathyroid glands. A diagnosis of secondary hyperparathyroidism was made in 8 patients, 6 of whom also underwent (successful) renal transplants. In general, any hyperplastic material remaining after diagnostic histopathological studies was analysed. A diagnosis of glucagonoma was made in a patient with elevated fasting plasma glucagon levels (>100 ng/l) and positive staining for glucagon in an islet cell tumour. One case of insulinoma was diagnosed in a hypoglycaemic patient with an insulin-positive islet cell tumour. Pituitary tumours were classified as growth hormone- or prolactin-producing according to clinical evidence of hormone excess, and the remaining tumours were classified as non-functioning. A detailed clinical and family history of each patient was taken to eliminate the possibility of MEN 1 or familial isolated hyperparathyroidism.

Isolation of DNA

Constitutional DNA was isolated from lymphoblastoid cell lines (LCLs) using a method based on that of Miller et al (1986). DNA was isolated from frozen tumour tissue as follows: surrounding stromal tissue was removed from the specimen with a scalpel, then tumorous material was homogenized in lysis buffer (10 mM Tris-HCl, 2 mM EDTA, 400 mM NaCl) using an omni-mixer (DuPont Sorvall), and DNA was isolated using the same protocol as for LCLs.

SSCP analysis

Tumour samples were screened for the presence of somatic mutations using single strand conformation polymorphism (SSCP) analysis (Orita et al, 1989). Exons 3–10 of the *MEN1* gene were amplified by PCR using approximately 50 ng of tumour DNA from each patient in a 10 µl reaction volume as follows: 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTPs (Promega), 10 pmol each primer (listed in Lemmens et al, 1997), 5% DMSO, 0.75 U AmpliTaq Gold DNA polymerase (PE Biosystems) and 1 µCi [α-^{32}P]-dCTP (Amersham). Samples were cycled in an Omnigene (Hybaid) thermal cycler as follows: 95°C for 12 minutes, followed by 35 cycles of 95°C for 1 minute, 62°C (64°C for gene fragment 10.1) for 1 minute and 72°C for 90 seconds. 20 µl of loading dye were added to each PCR product, and 2.5 µl of each sample were electrophoresed under two different gel conditions: (i) 0.6 × MDE gel solution (FMC bioproducts) at 5 W for 16 hours, and (ii) 0.5 × MDE containing 10% glycerol at 7 W for 16 hours. Gels were exposed to X-ray film (Fuji) overnight. Autoradiographs were examined for samples showing shifts in migration patterns compared with known wild type controls.

LOH analysis

Approximately 50 ng of LCL DNA and corresponding tumour DNA from each patient were amplified by PCR using the following microsatellite markers.

D11S4936, D11S4939, D11S4940, D11S449

These markers were amplified as described by Manickam et al (1997), using 0.75 U of AmpliTaq Gold DNA polymerase. Primer sequences for D11S449 were obtained from Debelenko et al (1997d).

PYGM ($CAGA_n$ repeat)

PCR reactions were prepared as described for SSCP analysis and cycled as follows: 95°C for 12 minutes, followed by 25 cycles of 95°C for 1 minute, 62°C for 1 minute and 72°C for 2 minutes. Primer sequences were obtained from Iwasaki et al (1992).

D11S480, D11S913

PCR reactions were prepared as described for SSCP analysis and cycled as follows: 95°C for 12 minutes, followed by 35 cycles of 95°C for 1 minute, 58°C (53°C for D11S913) for 1 minute and 72°C for 90 seconds. Primer information was obtained from Debelenko et al (1997d).

Twenty µl of loading dye were added to PCR products and 3.5 µl of each sample were electrophoresed through a 6% denaturing polyacrylamide (19:1) gel containing 7 M urea at 1500 V for 3–6 hours, depending on product size. Gels were exposed to autoradiographic film (Fuji) for 1 hour overnight. Heterozygous samples were examined by eye for a reduced intensity of one allele in the tumour DNA lane, compared with the adjacent normal DNA lane. Constitutionally homozygous samples were scored as non-informative.

DNA sequence analysis

Approximately 50 ng of DNA to be analysed were amplified by PCR as described for SSCP, but in a 50 µl reaction volume without radiolabel. Exon 2 of the *MEN1* gene was amplified using the following primers: (forward) 5′-GTGAGCAGAGGCTGAAGAGG-3′ and (reverse) 5′-ATAACACCTGCCGAACCTCAC-3′ with an annealing temperature of 64°C. 1 M betaine was included in this reaction instead of DMSO. The following primers were used for PCR of exon 3: 5′-AGGTTGGGTCACAGGCTTG-3′ (forward) and 5′-CTATGTGGGTGGTGATGGG-3′ (reverse) and annealed at 58°C. PCR products were purified by agarose gel electrophoresis and bands of the correct size were excised. DNA was isolated from the agarose using a QIAquick gel extraction kit (QIAGEN) as per the manufacturer's instructions. 200–300 ng of purified PCR product was sequenced using Big Dye dye terminator reaction premix (PE Biosystems) as per the manufacturer's instructions in a half reaction volume. 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. In addition, sequence traces were aligned to the *MEN1* gene sequence (GenBank U93237) using Mac Vector 4.1.1 (Kodak) software to identify possible homozygous base changes.

RESULTS

A total of 8 sporadic pituitary tumours, 3 pancreatic tumours, 14 primary parathyroid lesions and 8 secondary parathyroid lesions were examined for the presence of allelic deletions of the *MEN1* locus on chromosome 11q13 and mutations of the *MEN1* gene. Clinical details of these tumours are given in Tables 1 and 2.

LOH of microsatellite markers located within an approximately 2.8 megabase (Mb) region flanking the *MEN1* locus, between D11S480 and D11S1337 (Manickam et al, 1997), was detected in 4/33 sporadic endocrine lesions – 1 glucagonoma and 3 primary parathyroid lesions (Tables 1 and 2). Figure 1 shows characteristic LOH patterns detected for each of the 7 markers used.

Table 1 Clinical features and LOH analysis of 22 sporadic parathyroid lesions

Patient ID No.	Age at surgery (years)	1° or 2°	Glands involved	Clinical details	D11S480	PYGM	D11S4940	D11S4939	D11S4936	D11S449	D11S913
40651	82	1°	R sup	adenoma	●	●	–	–	–	●	–
40652	59	1°	R sup	adenoma	○	○	–	–	–	○	○
40654	82	1°	L inf	adenoma	●	●	●	–	●	●	●
40673	44	1°	1 gland	adenoma	–	○	–	–	–	○	○
40703	24	1°	L inf	adenoma	○	○	–	○	○	○	–
40712	24	1°	R sup	adenoma	–	○	○	○	–	–	○
40732	49	1°	L sup, R sup	hyperplasia	○	○	–	○	○	○	○
40734	47	1°	L sup	hyperplasia	○		–	–	–	–	–
40744	36	1°	R inf	adenoma	○	–	○	–	○	○	○
40767	50	1°	R sup	adenoma	○	○	○	–	–	–	–
40769	49	1°	R inf, R sup	adenoma	–	●	●	●	●	●	–
40776	67	1°	R inf	adenoma; diabetes, mild renal impairment	○	○	○	○	○	○	○
40795	82	1°	R inf	adenoma	–	○	○	–	–	–	○
70006		1°			○	○	○	–	○	○	–
40714	44	2°	all 4	renal transplant	–	–	–	○	○	○	○
40715	64	2°	all 4	renal transplant	○	○	–	○	○	○	○
40718	48	2°	all 4	renal transplant	○	○	○	–	–	○	○
40719	36	2°	all 4	renal transplant	○	–	–	○	–	○	–
40723	44	2°	all 4	renal transplant	○	○	–	○	○	○	○
40724	53	2°	all 4	renal transplant	○	○	○	○	○	○	○
40726	62	2°	all 4	diabetes, nephropathy	○	○	○	○	○	○	○
40808		2°	all 4	renal failure	○	○	○	○	–	○	–

1° = primary hyperparathyroidism; 2° = secondary hyperparathyroidism; L = left; R = right; sup = superior; inf = inferior; ○ = no LOH; ● = LOH; – = not informative; blanks indicate information unavailable

Table 2 Clinical features and LOH analysis of sporadic pancreatic and anterior pituitary lesions

Patient ID No.	Age at surgery (years)	Tumour type	Hormone over-secreted	Symptoms	D11S480	PYGM	D11S4940	D11S4939	D11S4936	D11S449	D11S913
40653	70	pancreas	gluc		●	●	●	–	–	–	●
40711	82	pancreas	ins	hypoglycaemia	○	○	○	○	○	○	–
41042	51	pancreas	ins		○	○	○	○	–	–	–
40716	57	pituitary	NF		○	○	○	○	–	○	○
40717	59	pituitary	NF	hypopituitarism	–	○	–	–	–	○	–
40741	76	pituitary	NF		–	–	–	○	–	○	○
40768	50	pituitary	pro		○	○	○	○	–	○	○
40773	31	pituitary	pro	galactorrhoea, headaches	○	○	–	○	–	○	–
40785	39	pituitary	GH	acromegaly, headaches	○	○	○	–	–	○	–
40788	27	pituitary	NF		○	○	○	○	○	○	○
40819		pituitary	GH		○	–	○	–	○	○	

gluc = glucagon; ins = insulin; GH = growth hormone; pro = prolactin; NF = clinically non-functioning; ○ = no LOH; ● = LOH; – = not informative; blanks indicate information unavailable

Exons 3–10 of the *MEN1* gene were analysed for the presence of somatic mutations in all 33 sporadic tumours using SSCP. Band-shifts (Fig. 2) were detected in the glucagonoma sample and in one of the parathyroid lesions also showing LOH. Sequencing of the relevant exons resulted in the identification of 2 missense mutations, in exons 3 and 7 respectively. Patient 40653–001 was found to have a 4387A>T base change, resulting in a protein change of E179V, and the mutation in patient 40769–001 was 6071C>T (P320L). The P320L mutation has been previously identified in a Japanese MEN 1 patient (Tanaka et al, 1997), but the E179V mutation is novel. The corresponding constitutional DNA from these patients was found to be wild type, thus confirming that the mutations occurred somatically. Sequence traces demonstrating this are shown in Figure 3.

In addition to SSCP analysis of the other two parathyroid adenomas displaying LOH, the entire coding region of the *MEN1* gene was sequenced in these samples. No mutation was detected in DNA from these tumours.

During the process of mutation analysis, a number of sequence polymorphisms were detected. These include the commonly-reported D418D and R171Q variants, as well as an A342A polymorphism (6138G>A), which we report here for the first time. This polymorphism was only identified once out of approximately 100 MEN 1 patient samples and sporadic endocrine tumour sample analysed, and was found in the tumour sample with the P320L mutation. For these reasons, and also because the protein sequence is not altered, this base change was not deemed to be a somatic mutation.

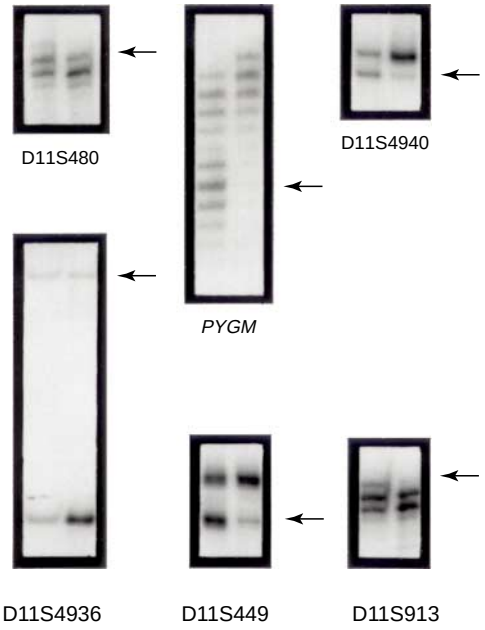

Figure 1 Representative patterns of LOH detected for 6 of the 7 microsatellite markers analysed. For each marker, lane 1 is LCL DNA, and lane 2 is corresponding tumour DNA. The PYGM tumour lane also shows possible microsatellite instability

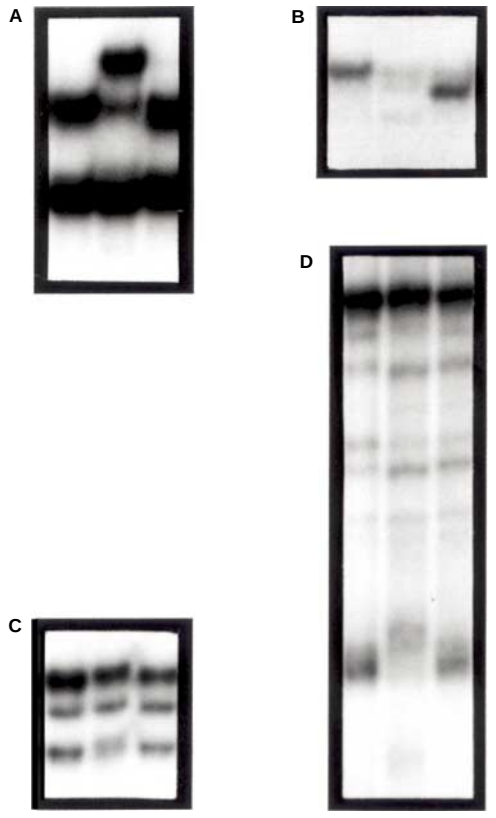

Figure 2 Autoradiographs showing SSCP band-shifts for 2 somatic mutations. (**A**) Migration pattern for exon 3.1 in 0.6 X MDE. Lane 1 is wild type, lane 2 is the E179V missense mutation, and lane 3 contains a sample carrying a R171Q polymorphism. (**B**) Migration pattern for exon 3.1 in a gel containing 10% glycerol, loaded as per. **A**. (**C**) Exon 7 PCR products in 0.6 X MDE. Lanes 1 and 3 are wild type, and lane 2 contains the P320L missense mutation. (**D**) The same samples as in **C**, in a gel containing 10% glycerol.

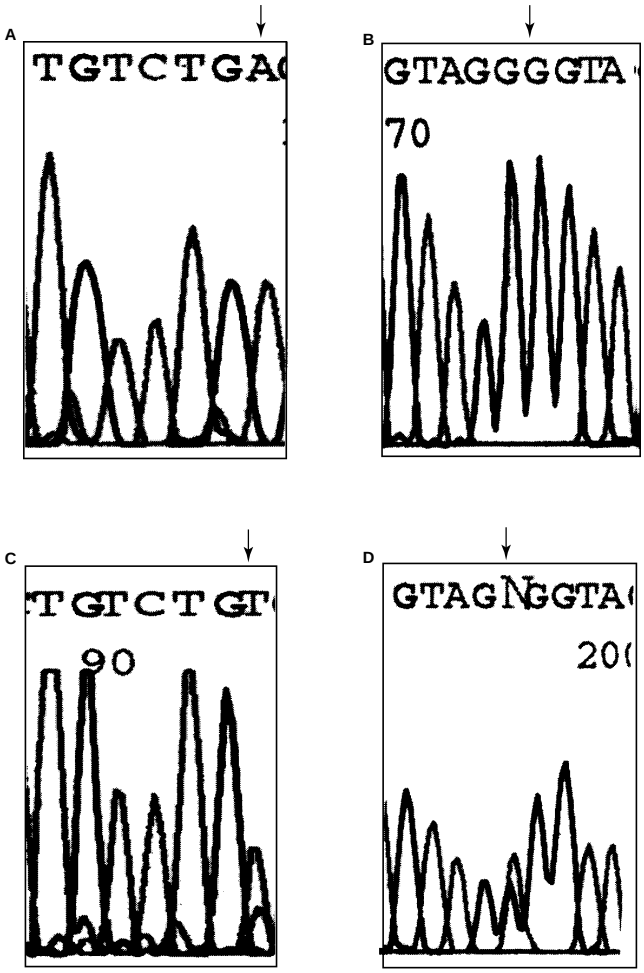

Figure 3 Sequence traces showing somatic *MEN1* gene mutations identified. (**A**) A portion of exon 3 sequence in LCL and (**B**) same sequence in the corresponding glucagonoma showing A–T transversion responsible for E179V. (**C**) Portion of exon 7 complementary strand from LCL and (**D**) in corresponding parathyroid adenoma showing G–A (reverse strand) transition resulting in a P320L substitution.

DISCUSSION

The role of the *MEN1* gene in sporadic endocrine tumorigenesis is well established. To further analyse the extent to which this gene is involved in the development of such tumours, we analysed 14 primary and 8 secondary parathyroid lesions, 3 pancreatic tumours and 8 pituitary tumours for the presence of allelic deletions and somatic mutations.

Of the 14 primary parathyroid lesions analysed, 3 (21%) showed LOH at the *MEN1* locus, and 1 (7%) of these carried a somatic mutation. Previous studies have found the rate of allelic deletion of the *MEN1* locus in this type of tumour to be similar, at approximately 30% (Carling et al, 1998; Farnebo et al, 1998). Moreover, *MEN1* gene mutations have previously been identified in 9–21% of parathyroid lesions studied (Heppner et al, 1997; Shan et al, 1998), comparable with our results. Parathyroid tumorigenesis can occur by other mechanisms such as cyclin D1 overexpression (Rosenberg et al, 1991), however this possibility was not assessed in this study. Of the 8 secondary parathyroid lesions examined, no LOH or *MEN1* gene mutations were detected. This

result supports those of previous studies (Farnebo et al, 1997a,b), suggesting that hyperparathyroidism arising as a consequence of renal failure occurs following some other genetic trigger.

Of the three pancreatic adenomas analysed, a glucagonoma was found to have both LOH and a somatic *MEN1* mutation. The low number of such lesions precludes statistical comparisons between this and other studies. No LOH or mutations were detected in any of the pituitary tumour samples. Once again, a relatively small number of samples was analysed, but previous studies reflect a similar finding, wherein 10% or fewer pituitary tumours show involvement of the *MEN1* locus (Zhuang et al, 1997b; Prezant et al, 1998). Numerous other loci have been implicated in pituitary tumorigenesis, including the *gsp* oncogene (Landis et al, 1989), and the *TP53* (Bates et al, 1997) and *CDKN2A* (Woloschak et al, 1996) tumour suppressor genes.

In the four tumours with LOH, allelic loss was detected across the entire region studied. This confirms the findings of others, in which deletions of the wild type allele in sporadic endocrine tumours are generally large and cover many kilobases (Heppner et al, 1997). Since some of the tumours analysed were uninformative at the microsatellite markers closest to the *MEN1* gene (e.g. PYGM and D11S4940), it is formally possible that some small allelic deletions might have gone undetected. However, because large deletions are usually observed, this is unlikely. It is possible that the two tumours carrying LOH at the *MEN1* locus without detectable *MEN1* gene mutations have the second *MEN1* allele inactivated by some other mechanism such as transcriptional silencing by promoter methylation, although we were not able to test this in this study, as RNA was not available from these tumours.

In summary, we have found LOH at the *MEN1* locus in 3 sporadic primary parathyroid lesions and in 1 sporadic glucagonoma. Somatic mutations in the *MEN1* gene were identified in one of these parathyroid lesions and in the glucagonoma. This supports previous findings that the *MEN1* gene is involved in the development of a subset of sporadic MEN 1-type endocrine lesions. Also confirmed are previous results suggesting that the development of secondary hyperparathyroidism occurs by some other genetic mechanism, rather than mutation of the *MEN1* gene.

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