

Multiple endocrine neoplasia type 1 (*MEN1*) gene mutations in a subset of patients with sporadic and familial primary hyperparathyroidism target the coding sequence but spare the promoter region

W Karges, K Jostardt¹, S Maier, A Flemming, M Weitz, A Wissmann, B Feldmann, H Dralle², P Wagner³ and B O Boehm

Division of Endocrinology, Department of Internal Medicine, University of Ulm, Ulm, Germany

¹Department of Molecular Human Genetics, Department of Pediatrics, Inselspital, University of Berne, Berne, Switzerland

²Department of Surgery, Martin Luther University of Halle-Wittenberg, Halle/Saale, Germany

³Department of Surgery, Rosenheim General Hospital, Rosenheim, Germany

(Requests for offprints should be addressed to W Karges, Division of Endocrinology, Department of Internal Medicine, University of Ulm, Robert Koch Strasse, 89081 Ulm, Germany; Email: wolfram.karges@medizin.uni-ulm.de)

Abstract

Germ line mutations of the multiple endocrine neoplasia type 1 (*MEN1*) tumour suppressor gene cause MEN1, a rare familial tumour syndrome associated with parathyroid hyperplasia, adenoma and hyperparathyroidism (HP). Here we investigated the role of the *MEN1* gene in isolated sporadic and familial HP. Using RT-PCR single-strand conformational polymorphism screening, somatic (but not germ line) mutations of the *MEN1* coding sequence were identified in 6 of 31 (19.3%) adenomas from patients with sporadic primary HP, but none in patients ($n=16$) with secondary HP due to chronic renal failure. *MEN1* mutations were accompanied by a loss of heterozygosity (LOH) for the *MEN1* locus on chromosome 11q13 in the adenomas as detected by microsatellite analysis. No DNA sequence divergence within the 5' region of the *MEN1* gene, containing the putative *MEN1*

promoter, was detectable in HP adenomas. Clinical characteristics were not different in HP patients with or without *MEN1* mutation. Heterozygous *MEN1* gene polymorphisms were identified in 9.6% and 25% of patients with primary and secondary HP respectively. In a large kindred with familial isolated familial HP, *MEN1* germ line mutation 249 del4 and LOH was associated with the HP phenotype and a predisposition to non-endocrine malignancies. We suggest that the bi-allelic somatic loss of *MEN1* wild-type gene expression is involved in the pathogenesis of a clinically yet undefined subset of sporadic primary HP adenomas. *MEN1* genotyping may further help define the familial hyperparathyroidism–*MEN1* disease complex, but it seems dispensable in sporadic primary HP.

Journal of Endocrinology (2000) **166**, 1–9

Introduction

Primary hyperparathyroidism (HP) is the most frequent disease manifestation in patients with multiple endocrine neoplasia type 1 (*MEN1*) syndrome, a rare familial tumour syndrome characterized by the development of multiple parathyroid, pituitary, and gastroenteropancreatic neoplasias. Virtually all patients with *MEN1* syndrome carry germ line mutations of *MEN1* (Agarwal *et al.* 1997), a highly conserved (Karges *et al.* 1999) tumour suppressor gene (Chandrasekharappa *et al.* 1997, The European Consortium on *MEN1* 1997) characterized by a 1833 base pair translated region encoded on nine exons. The gene product of *MEN1*, *menin*, is a 610 amino acid nuclear protein interacting with the AP1 transcription factor JunD

(Agarwal *et al.* 1999). In affected tissues, *MEN1* wild-type gene expression is typically abrogated through allelic loss of heterozygosity (LOH) for chromosome 11q13 harbouring the *MEN1* gene locus (Ludwig *et al.* 1999).

With an estimated incidence of 25–30/10⁶ per year (Zahrani & Levine 1997), primary HP is among the most common endocrine disorders in Western countries, but only a minority of primary HP occurs in association with the *MEN1* syndrome. The vast majority of patients with primary HP do not have a positive family history or concomitant endocrine neoplasia, and are thus considered sporadic cases. The molecular basis of sporadic HP is not well understood. Mutations and LOH of p53 (Cryns *et al.* 1994a, Hakim & Levine 1994) or retinoblastoma (RB) (Cryns *et al.* 1994b) tumour suppressor genes are rarely,

Table 1 Clinical characteristics of patients with sporadic primary HP and secondary HP. Values are means \pm s.d.

	Patients with primary HP		Patients with secondary HP (controls)
	Without <i>MEN1</i> mutation	With <i>MEN1</i> mutation	
No. of patients (male/female)	25 (9/16)	6 (2/4)	16 (5/11)
Age at diagnosis (years)	60 \pm 14	58 \pm 12	54 \pm 11
Serum calcium (mmol/l)*	3.11 \pm 0.32	2.85 \pm 0.16	2.76 \pm 0.42
Serum PTH (pmol/l)*	24.6 \pm 18.4	15.8 \pm 4.73	58.8 \pm 36.4
No. of adenomas resected	1.2 \pm 0.6	1.6 \pm 0.4	3.3 \pm 1.1

There was no significant difference in any of these characteristics when patients with primary HP either with or without *MEN1* mutation were compared (Mann–Whitney U test and χ^2 test).

*Serum levels of calcium and PTH at time of surgery.

if at all, found in sporadic parathyroid adenomas. In these tumours, LOH for chromosome 11q13 has frequently been identified (Larsson *et al.* 1995), compatible with a role of *MEN1* in tumorigenesis. Extending previous observations (Heppner *et al.* 1997, Carling *et al.* 1998, Farnebo *et al.* 1998), we here characterize the *MEN1* tumour suppressor gene and promoter structure in patients with isolated sporadic and familial primary HP.

Materials and Methods

Patients

Between 1996 and 1998, consecutive patients who were referred for parathyroid surgery for sporadic primary HP ($n=31$) and secondary HP associated with chronic renal failure ($n=16$) were studied. The diagnosis of sporadic primary HP was based on elevated serum calcium (≥ 2.75 mmol/l) and parathyroid hormone (PTH; ≥ 6.8 pmol/l) levels, and a negative family history. One patient with HP associated with familial *MEN1* carrying germ line *MEN1* mutation 678 del6 has been described earlier (patient 1 in Ludwig *et al.* 1999). Patients with secondary HP due to chronic renal failure had elevated serum creatinine levels (587.7 ± 297.7 mmol/l, mean \pm s.d.) and hyperphosphataemia (2.15 ± 0.89 mmol/l, mean \pm s.d.). Additional patient characteristics are shown in Table 1.

Familial isolated primary hyperparathyroidism (fiHP) was studied in a Caucasian family from southern Germany. The index patient (male, age 33 years, patient 3 in Fig. 4A) had typical HP with elevated levels of calcium (3.3 mmol/l) and PTH (10.5 pmol/l), which returned to normal after surgical resection of two orthotopic parathyroid adenomas in 1998. He had undergone chemotherapy for acute myelogenous leukaemia (FAB M2, t[8;21], CD19⁺) in 1997, and has been in complete remission since then. His identical twin brother (patient 4 in Fig. 4A) was diagnosed with HP in 1999 (calcium,

2.8 mmol/l; PTH, 66 pmol/l). In their father (age 60 years, patient 2 in Fig. 4A), a parathyroid adenoma had been resected in 1990, with no recurrence of HP since then. Hepatic metastases of an undifferentiated bronchogenic adenocarcinoma (grade G3) were diagnosed and histologically confirmed in 1997. One uncle (age 58 years, patient 1 in Fig. 4A) of the index patient had successfully undergone parathyroid surgery for HP in 1989. There is currently no clinical evidence for *MEN1* syndrome in any family member, and serum gastrin and prolactin levels are within normal ranges in all patients with HP. Studies were conducted in accordance with the Declaration of Helsinki, and informed consent was obtained from all patients. Clinical diagnoses were confirmed by histological evaluation of resected tumours in all cases.

Clinical laboratory analyses

Serum PTH (normal range 10–65 pg/ml) was measured as intact PTH with a chemiluminescence immunometric assay (Nichols Institute, San Juan Capistrano, CA, USA). Serum gastrin levels (normal range <100 pg/ml) were analyzed using a radioimmunoassay (ICN, Orangeburg, NY, USA), and serum prolactin (normal range 5–15 ng/ml, male) was measured with a time-resolved fluoroimmunoassay (Delfia; WallacOy, Turku, Finland).

Generation of DNA templates

From cryoconserved parathyroid tumour tissue and peripheral blood mononuclear cells, total RNA and genomic DNA was isolated using a guanidinium single-step technique (TRI reagent; MRC, Cincinnati, OH, USA). First strand complementary DNA was synthesized from 1–3 μ g RNA with MMLV reverse transcriptase after random hexamer priming (PCR Amp; Perkin Elmer, Weiterstadt, Germany). Genomic DNA from archival tumour samples was isolated by a standard phenol–ethanol

procedure from microdissected paraffin-embedded sections after xylene extraction. Control DNA (either *MEN1* 249 del4 or wild-type) used in single-strand conformational polymorphism-heteroduplex (SSCP/HD) analysis screening for *MEN1* mutation 249 del4 was cloned into the pGEM vector (Promega, Mannheim, Germany) after PCR amplification.

Polymerase chain reaction

For *MEN1* mutational analyses, six overlapping DNA segments covering the entire *MEN1* coding region were amplified by PCR from tumour or peripheral blood cDNA template. Oligonucleotide primer sequences and reaction conditions have been described (Karges *et al.* 1999). The following sense/antisense primer combinations were used as described (Ludwig *et al.* 1999), with numbers indicating the 5' nucleotide of primers in reference to *MEN1* genomic sequence (Genbank U93237): 2241/2561, 2508/4431, 4370/5346, 5210/7199, 6693/7734, and 7685/8084. To study the intronic mutation 654+3(A→G) leading to a partial deletion of exon 3 through alternative splicing, primers 4370 (Karges *et al.* 1999) and 5' TCCTGCCCATTTGGCTCAGC (intron 4) were used for genomic PCR and sequencing. The putative *MEN1* promoter region was amplified from genomic tumour DNA with primers 1234 (5' ATCGAG CCACTGCATTCCAG, sense) and 1758 (5' CGCAGA GCCTGGAAATAGTG, antisense). All amplifications were performed on a Perkin Elmer 9600 PCR system.

SSCP/HD analysis

DNA from all tumour samples was screened for *MEN1* mutations with two different SSCP/HD systems. *MEN1* RT-PCR fragments were analyzed by SSCP/HD electrophoresis on Clean Gel DNA-HP 10% and 15% acrylamide gels rehydrated with the SSCP/Delect buffer system (ETC, Kirchentellinsfurt, Germany). In addition, fragments were studied twice at different temperatures (4 °C and 12 °C) on a two buffer PAGE-based SSCP/HD system using 12% non-denaturing acrylamide gels (Liechti-Gallati *et al.* 1999). For both systems, horizontal Multiphor II electrophoresis chambers (Pharmacia, Freiburg, Germany) and conventional silver staining to visualize DNA bands were used. Out of a total of ten different mutations and polymorphisms described here, six and nine were detected using the commercial rehydrated system and the system described by Liechti-Gallati *et al.* (1999) respectively.

DNA sequencing

DNA was purified by column centrifugation (Qiaquick; Qiagen, Hilden, Germany) and cycle sequenced by

dideoxy chain termination (DNA Sequencing Kit; Perkin Elmer) on an automated fluorescence-based DNA sequencer (ABI 373A; Applied Biosystems, Warrington, UK). Sequencing primers for the *MEN1* coding sequence (CDS) have been shown above. For sequencing the *MEN1* promoter region (Vanbellinghen *et al.* 1999), primers 1234 (sense) and 1723 (5' GCGCTGTGGCTT CTGGGATC, antisense) were used. Sequencing of candidate DNA was performed in sense and antisense orientation and repeated until resolution of ambiguities. Sequences were compared with Genbank entries U93236 and U93237, and sequence variations were designated according to Beaudet & Tsui (1993).

LOH studies

For LOH studies, polymorphic microsatellite markers flanking the *MEN1* locus on chromosome 11q13 were analyzed. Markers PYGM and D11S4908 (Manickam *et al.* 1997) were amplified by PCR using Cy5 labelled oligonucleotides and studied by laser-induced fluorescence after electrophoresis on the ALF sequencing system (Pharmacia). After signal quantification (area under the curve) using Fragment Manager software (Pharmacia), LOH was scored as a significant decrease ($\geq 75\%$) of the fluorescence signal of one allele. Markers were considered informative if two different alleles were distinguishable in DNA of control tissue from that individual.

Statistical analysis

For comparison of patient characteristics, the Mann-Whitney U test (rank-sum test) was used. The χ^2 test was used to compare categorical values. The level of statistical significance was set at $P=0.05$. Analyses were performed with the help of the SPSS software package.

Results

MEN1 mutations in patients with sporadic HP

Parathyroid tumours of 31 unrelated patients from Southern Germany with sporadic primary HP (Table 1) were analyzed by SSCP/HD and subsequent DNA sequencing of candidate mutants. Parathyroid tissue from patients with secondary HP ($n=16$) due to chronic renal failure were used as controls, and all samples were analyzed in blinded fashion.

Overall, *MEN1* mutations were detected in 6 of 31 (19.3%) tumours from patients with primary HP, but in none with secondary HP (Table 2). Mutations were distributed over the entire *MEN1* coding region and included missense (E378V, $n=1$), nonsense (Q584X, $n=1$), frameshift (468 insT, $n=1$) and deletion (194 del26,

Table 2 MEN1 mutations and polymorphisms in parathyroid adenomas from patients with primary and secondary HP

HP	Total no. of patients	Patients with MEN1 mutation*		Patients with MEN1 polymorphism
		CDS	Promoter	
Primary	31 (100%)	6 (19.3%)†	0	3 (9.6%)
Secondary	16 (100%)	0	ns	4 (25%)

*All mutations identified were somatic.

†One patient had unilateral adrenal adenoma and primary hyperaldosteronism, without evidence for sporadic or familial MEN1 syndrome or genomic MEN1 mutation.
ns, not studied.

$n=1$) mutations. In two unrelated patients, cDNA sequencing showed the deletion of a 105 bp segment (nucleotides 550–654) constituting the 3' moiety of exon 3, compatible with the presence of an mRNA splice variant. Alternatively, the deletion might represent a genomic 105 bp loss located adjacent to an exon/intron boundary. To resolve this issue, genomic tumour DNA was amplified and sequenced, revealing an intronic mutation (654+3A→G) located at the 5' splice junction of intron 3 in both cases. No deletion was found at the genomic DNA level, indicating that the partial loss of exon 3 represents a MEN1 mRNA transcript variant generated by alternative splicing.

MEN1 mutations detectable in tumour tissue may result from MEN1 germ line mutations (e.g. in familial MEN1 syndrome), or they may reflect somatic MEN1 mutations undetectable in healthy tissue. We therefore analyzed peripheral blood cDNA of MEN1 mutation-positive patients side by side with tumour cDNA. In patients with familial MEN1 syndrome and familial HP (Fig. 1), both characterized by MEN1 germ line mutations, mutant SSCP patterns were observed in tumour and peripheral blood DNA. In contrast, patients with sporadic HP (Fig. 1 and data not shown) had a mutant MEN1 SSCP pattern in tumour tissue and a wild-type pattern in control tissue, indicating that their MEN1 mutations were of somatic origin, i.e. restricted to the parathyroid tumour.

MEN1 promoter analysis

Disruption of promoter activity by mutation of regulatory elements is a common motif to abrogate tumour suppressor function in various human neoplasias. We therefore studied the 5' flanking region (490 bp) of the MEN1 gene containing the putative MEN1 promoter, including a 184 bp region located immediately upstream of the MEN1 gene which has been shown to be critical for *in vitro* MEN1 transcriptional activity (Vanbellingen *et al.* 1999). Genomic DNA from 31 primary HP tumours was amplified by PCR and sequenced using a nested oligonucleotide located within the MEN1 5' untranslated (non-coding) region

(Fig. 2). Sequence information was obtained from 29 tumours. In none of these tumours was divergence from the wild-type sequence identified, affecting regulatory

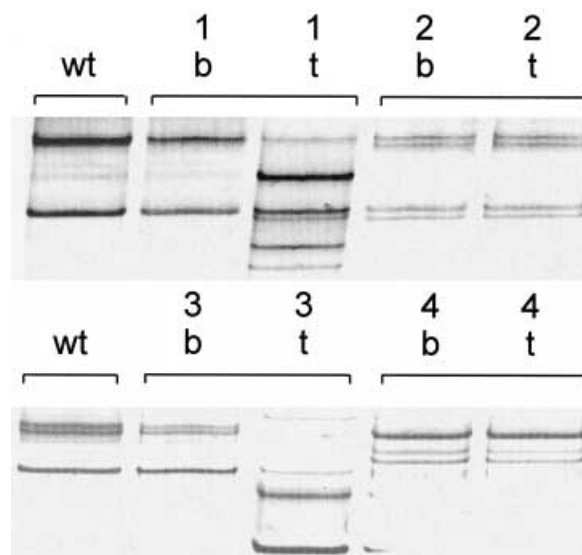


Figure 1 SSCP analysis of MEN1 RT-PCR fragments generated from parathyroid tumour (t) and peripheral blood (b) RNA from patients with sporadic HP (lanes 1 and 3), familial HP (lanes 2), MEN1 syndrome (lanes 3), and blood RNA from healthy control (lanes wt). The following MEN1 mutations are shown: somatic 194 del26 (1), germ line 249 del4 (2), somatic 654+3 A→G (3), and germ line 678 del6 (4).

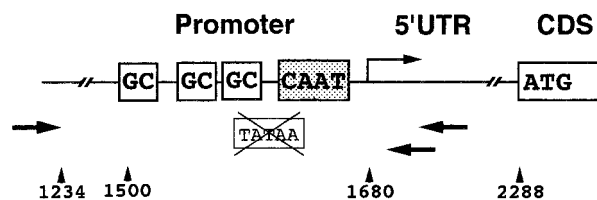


Figure 2 Schematic illustration of MEN1 promoter analysis, with putative promoter containing CAAT and GC elements, but no TATAA box. Primers used for MEN1 promoter amplification and sequencing in HP patients are depicted as horizontal bars. Positions are according to GenBank U93237.

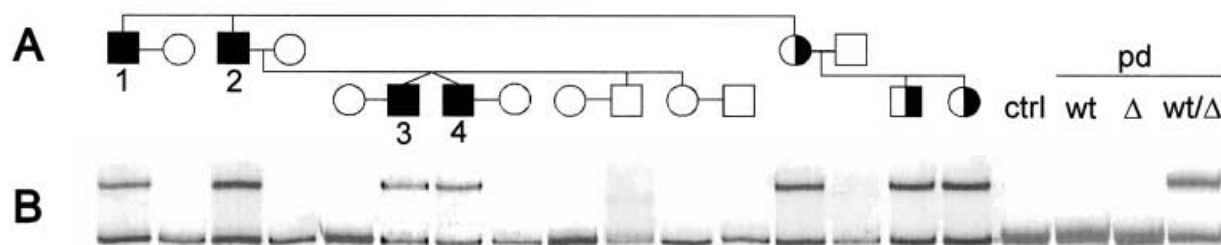


Figure 3 Mutational analysis of kindred with familial isolated hyperparathyroidism. (A) Family pedigree. Squares, males; circles, females; solid symbols, HP confirmed; open symbols, healthy individuals; semi-solid symbols, healthy carrier of *MEN1* mutation. Patients are numbered as in the text. (B) SSCP/HD analysis for *MEN1* mutation 249 del4 in peripheral blood cDNA. Mutation carriers are characterized by heteroduplex formation (upper band), while the lower band represents homoduplex found in healthy family members and unrelated healthy control individual (ctrl). Three lanes on the right show control fragments generated from wild-type (wt) and mutated (Δ) *MEN1* DNA template cloned into plasmids (pd). wt/ Δ denotes 1:1 mixture of mutated and wild-type product.

elements of transcription (e.g. CAAT box, GC boxes), or other regions within the putative *MEN1* promoter.

LOH of the *MEN1* locus in sporadic HP

Loss of tumour suppressor function of the *MEN1* gene product (menin) in affected neoplastic tissues would require the inactivation of both *MEN1* wild-type alleles. We therefore extended our analysis to study the LOH in tumour DNA from patients with sporadic HP and somatic *MEN1* mutations. Using PCR microsatellite markers PYGM (centromeric) and D11S4908 (telomeric) flanking the *MEN1* locus, informative amplifications from peripheral blood and parathyroid tumour DNA were obtained in all cases. LOH of the *MEN1* locus was found in all parathyroid tumours from these *MEN1* mutation-positive patients (fluorescence curves not shown). Together with the somatic mutations described above, these findings indicate that similar to parathyroid adenomas associated with the familial *MEN1* syndrome (Ludwig *et al.* 1999), bi-allelic loss of wild-type *MEN1* expression may be found in sporadic HP, though at lower prevalence. In our studies, LOH analysis was not performed in HP patients without somatic *MEN1* mutations, as peripheral blood DNA was not obtained from these individuals.

Clinical aspects of *MEN1* mutation status

Of adenomas associated with primary HP, 19.3% (6 of 31) had somatic *MEN1* mutations. Clinical features of patients with such *MEN1* somatic mutations were not significantly different from those without, including age at diagnosis, gender, serum calcium and serum PTH levels (Table 1). Likewise, the number of adenomas resected upon surgery was not different in both groups (Table 1), and clinical histological assessment after light microscopy showed no difference in tumours with or without *MEN1* mutation (not shown).

MEN1 gene polymorphisms in HP

A considerable rate of variant *MEN1* SSCP patterns was detected not only in tumours from patients with primary HP, but also secondary HP. All variant RT-PCR fragments were characterized by sequencing. In addition to the mutations described above, *MEN1* polymorphisms R171Q ($n=1$) and 435C/T ($n=2$) were identified in three tumours from patients with primary HP (Table 2). Similarly, polymorphisms were found in 4 of 16 secondary HP tumours (435C/T, $n=3$; 1254 C/T, $n=1$). Polymorphisms were characterized by a double peak in sequencing reactions, suggesting allelic heterozygosity. Except for R171Q, which has been described as a common polymorphism found in 1.4% of healthy individuals (Chandrasekharappa *et al.* 1997), these nucleotide substitutions are silent, i.e. they do not lead to amino acid exchanges of the *MEN1* gene product. Peripheral blood DNA was not studied in these patients. Overall, the 435C/T polymorphism was identified in 5 of 47 individuals analyzed as well as in the human endocrine tumour cell line LCC (W Karges, unpublished observations), thus representing a common polymorphism in our population.

MEN1 mutation 249 del4 and LOH (11q13) in fiHP

In the index patient of a large family (Fig. 3A) with fiHP, parathyroid tumour RNA was analyzed by SSCP/HD electrophoresis. RT-PCR segment 2508/4431 corresponding to *MEN1* exons 1 (partial), 2 and 3 (partial) showed an aberrant band shift pattern (Figs 1 and 3B). DNA sequencing revealed a 4 bp deletion in exon 2 (249 del4) resulting in a frame shift and a premature stop codon 96 nucleotides further downstream (Fig. 4). When complementary DNA from peripheral blood of this patient was analyzed, an aberrant SSCP pattern was found (Fig. 1), caused by germ line *MEN1* mutation 249 del4 as confirmed by sequencing (not shown).

The *MEN1* mutation 249 del4 does not create or delete endonuclease restriction sites, and efforts using

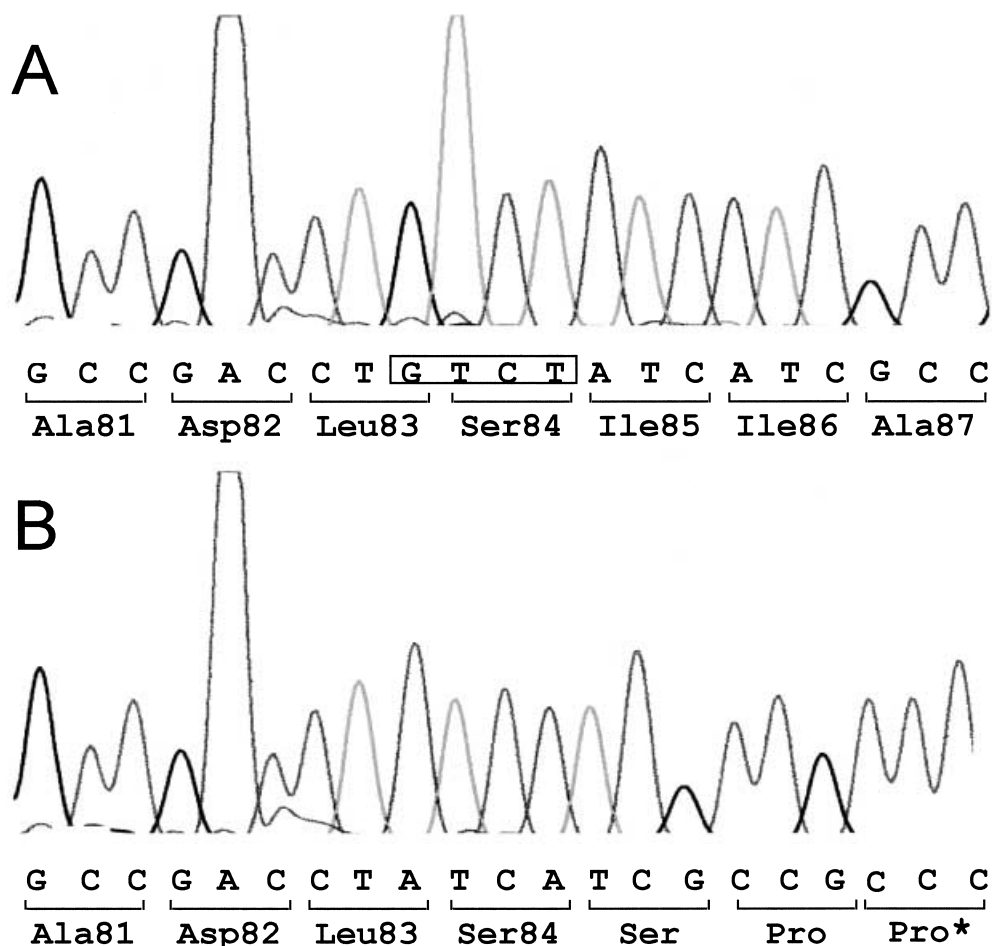


Figure 4 Detection of *MEN1* mutation 249 del4 fiHP. Pherogram of *MEN1* (A) wild-type DNA sequence and (B) mutated fiHP patient DNA sequence, characterized by 249 del4 frameshift mutation followed by premature stop codon (*).

allele-specific PCR for mutation screening were inconclusive. We therefore used SSCP/HD to screen peripheral blood cDNA of the entire family (Fig. 3B). In the father (patient 2) and one uncle (patient 1), both affected by HP, the 249 del4 mutation was identified and confirmed by sequencing. In the mutation-positive identical twin (patient 4), the diagnosis of mild HP had been established in 1999, while currently the other 249 del4 mutation carriers have normal calcium and PTH levels.

Genomic DNA from parathyroid tumour and control tissues (thyroid, peripheral blood) for LOH studies was available from all three individuals with established fiHP who had undergone surgery. Microsatellite markers PYGM (centromeric) and D11S4908 (telomeric) were informative in all individuals, showing allelic heterozygosity in all control tissues as indicated by the presence of two major microsatellite peak clusters (Fig. 5). In contrast, in parathyroid tumour tissue from fiHP patients 1

and 2 (Fig. 5), only one allelic peak cluster was detectable, while the second allele was absent (arrows), indicating a loss of heterozygosity for the *MEN1* locus in these tumours. Identical results were obtained in patient 3 (curves not shown).

These findings suggest that, as in *MEN1*-associated HP adenoma (Ludwig *et al.* 1999), a bi-allelic loss of *MEN1* wild-type gene expression caused by germ line *MEN1* mutation and a subsequent LOH is critically involved in tumour development in fiHP in this family. No clinical or laboratory findings suggesting additional endocrine dysfunction was present in individuals with HP (currently aged 58, 60 and 33 years), thus making *MEN1* syndrome very unlikely. However, the index patient and his father were affected by non-endocrine neoplasias (acute myelogenous leukaemia (AML) and adenocarcinoma of the lung respectively), and we attempted to evaluate the potential role of the *MEN1* tumour suppressor in these cancers.

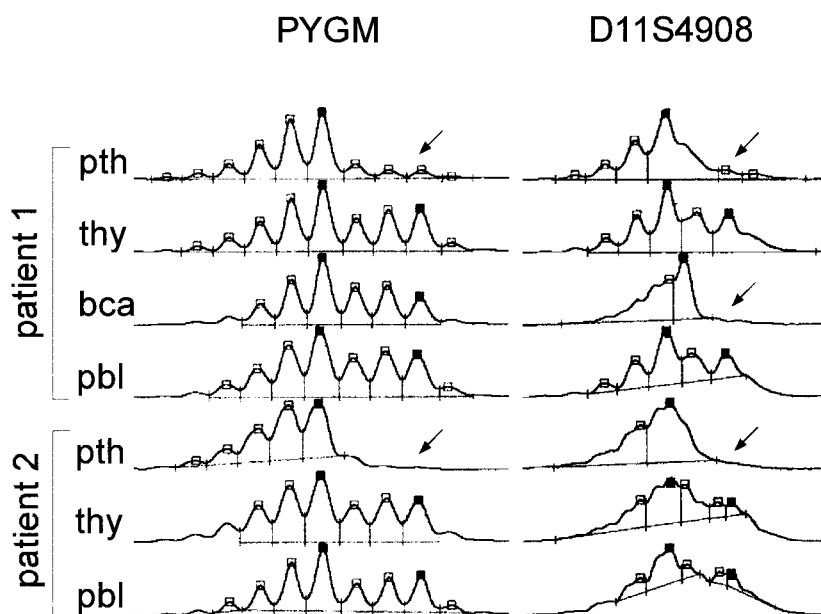


Figure 5 Fluorescent microsatellite analysis to detect *MEN1* LOH in tissue from patients with fHP. Original graphs of dinucleotide markers PYGM (left) and D11S4908 (right) are shown. Tissues studied are: parathyroid adenoma (pth), thyroid (thy), metastasis of bronchial carcinoma (bca), and peripheral blood (pbl). Both allelic peak clusters consist of one major peak (■) and several minor peaks (□). LOH is characterized by the absence of the second allele (peak cluster), indicated by arrows.

We reasoned that the *MEN1* germ line mutation provides a heterozygous *MEN1* gene 'knockout' in these patients, so that LOH of 11q13 might completely abrogate gene expression. Bone marrow DNA was obtained for analysis from the index patient at the time of diagnosis of AML, but the fraction of tumour cells (myeloblasts) in the sample as determined by bone marrow cytology was only 40%, with non-neoplastic cell prevailing. Thus LOH analysis was considered unpromising, and had to be abandoned.

However, a biopsy sample of a hepatic metastasis of the undifferentiated adenocarcinoma of the lung was available from the father; on histological examination this was free of contaminating normal tissue. Microsections of the tissue were used for microsatellite PCR without formal DNA extraction. There was a significant LOH for marker D11S4908 (telomeric), with a microsatellite fluorescence pattern as observed in parathyroid tissue, indicating the loss of the wild-type allele (Fig. 5, arrow). However, for the centromeric marker PYGM, allelic heterozygosity was retained (Fig. 5). In this tumour, direct analysis of the *MEN1* region containing the 249 del4 mutation by PCR amplification and fragment size analysis was not successful, so that no further evidence for LOH of the *MEN1* gene locus could be obtained. Taken together, these ambiguous results are compatible with the involvement of *MEN1* gene in non-endocrine tumours, but further studies (e.g.

in non-endocrine tumours from *MEN1* patients) are required for a clear understanding of this issue.

Discussion

Familial clustering has frequently helped to elucidate molecular genetic mechanisms of disease, in a cognitive process sequentially involving linkage analysis, mapping, identification and functional characterization of pathogenically relevant genes. Hereditary tumour syndromes caused by germ line mutations of tumour suppressor genes or proto-oncogenes have served as role models for phenotypically similar or related sporadic neoplasias. For instance, medullary thyroid carcinoma (MTC) in familial *MEN2* syndrome is caused by activating germ line mutations of the *RET* receptor tyrosine kinase proto-oncogene, while sporadic MTC is frequently characterized by somatic *RET* mutations restricted to neoplastic tissue (Ponder 1999).

Primary hyperparathyroidism seems to be another good example of this theme. In this work, we show that a considerable proportion (19.3%) of patients with sporadic HP carry mutations of the *MEN1* tumour suppressor gene in their parathyroid adenomas. *MEN1* somatic mutations in sporadic parathyroid adenomas were randomly distributed over a wide range of the *MEN1*

coding sequence, while the putative *MEN1* promoter, including a 184 bp region critical for *MEN1* transcriptional activity (Vanbellingham *et al.* 1999), was not affected. We observed various types of mutations, with minor (E378V) or striking (Q584X, 468 insT, 194 del26) changes in *MEN1* protein sequence. Neither type of *MEN1* mutation (genotype) nor the overall mutation status (positive vs negative) was correlated with the clinical phenotype, but the overall number of patients studied was limited.

The fact that in familial *MEN1* syndrome, minor germ line modifications of *MEN1* protein structure are associated with a neoplastic phenotype (Chandrasekharappa *et al.* 1997, The European Consortium on *MEN1* 1997, Agarwal *et al.* 1999), as well as the rigid conservation of the molecule during evolution (Stewart *et al.* 1998, Bassett *et al.* 1999, Karges *et al.* 1999) would indirectly suggest that even subtle structural changes are sufficient for a loss of tumour suppressor function in HP adenomas. *MEN1* mutations were not found in secondary HP with functional parathyroid hyperplasia due to chronic renal failure, excluding the possibility that parathyroid hyperactivation by itself was responsible for the observed genetic changes.

The observed mutations were accompanied by a deletion of the remaining *MEN1* wild-type allele through loss of heterozygosity for 11q13, indicating that a loss of *MEN1* tumour suppressor gene expression is causative in the development of sporadic HP tumours, similar to HP adenomas associated with the familial *MEN1* syndrome. However, four of five sporadic HP adenomas did not show abnormalities of the *MEN1* gene (coding sequence or promoter, Table 2). These findings, together with similar observations reported earlier (Heppner *et al.* 1997, Carling *et al.* 1998, Farnebo *et al.* 1998) illustrate that other molecular mechanisms must be involved in the tumorigenesis of sporadic HP adenomas, either functionally linked to the *MEN1* tumour suppressor, or unrelated to the *MEN1* system. In this context, further characterization of *MEN1* intracellular binding partners or downstream activation molecules (Agarwal *et al.* 1999) may provide candidate proteins targeted by somatic genetic events during HP tumour development.

Familial HP is rare and may be associated with distinct molecular defects (e.g. mutations of the calcium sensor) or clinical characteristics (e.g. jaw tumours) (Zahrani & Levine 1997). Familial isolated HP due to parathyroid adenoma may be difficult to distinguish from incipient *MEN1* syndrome. In the family described here, no signs, symptoms or other findings of *MEN1*-associated endocrine neoplasia other than HP were found in patients up to 60 years of age. The *MEN1* germ line frameshift mutation (249 del4) associated with the HP phenotype in these individuals has not been described in *MEN1* patients, but functionally similar *MEN1*-associated mutations (i.e. frameshift mutations leading to a premature protein

truncation) have been found in its proximity in the 3' moiety of exon 2 (Chandrasekharappa *et al.* 1997). After initial negative reports (Tanaka *et al.* 1998), other families with fiHP and *MEN1* germ line mutations have been described (Teh *et al.* 1998, Fujimori *et al.* 1998, Poncin *et al.* 1999).

It is currently unclear why identical germ line mutations of tumour-associated genes may cause different phenotypes in individual pedigrees, e.g. *RET* mutation (codon 634) causing either *MEN2A* or isolated familial MTC in different families (Ponder 1999). In analogy to *MEN2*/familial MTC, one could envision *MEN1* and a subset of familial isolated HP as one disease entity, with fiHP as a 'minor' variant of classic *MEN1*. The demonstration of a common hereditary basis and similar somatic genetic events in tumour tissue in *MEN1* and fiHP as demonstrated in our study would support this view.

We observed a predisposition for non-endocrine tumours in *MEN1* mutation carriers in this family, and it is tempting to speculate that a loss of *MEN1* tumour suppressor function (initiated by the germ line 'first hit') may have contributed to tumour development. Microsatellite analyses were indeed compatible with a loss of heterozygosity for the *MEN1* locus in metastatic bronchial carcinoma in a *MEN1* mutation carrier, but our studies were clearly limited by restricted availability of suitable tumour specimens and small patient numbers. Thus, with the involvement of RB as a central element in some non-hereditary neoplasia in mind (Haber & Fearon 1998), further studies are required to assess the potential role of the *MEN1* gene in non-endocrine tumours.

In sporadic gastrinomas or other gastroenteropancreatic (GEP) tumours, a significant rate of somatic *MEN1* mutations has been found (Toliat *et al.* 1997, Zhuang *et al.* 1997), similar to the rate in sporadic HP parathyroid adenomas described here and by others (Heppner *et al.* 1997, Carling *et al.* 1998, Farnebo *et al.* 1998). Moreover, in patients with GEP tumours, a high prevalence of *MEN1* germ line mutations has been observed, commonly interpreted as evidence for clinically occult or incipient *MEN1* syndrome. We did not observe any *MEN1* germ line mutations in our sporadic HP patients, supporting the notion that truly sporadic primary HP is far more common than *MEN1*-associated HP. In contrast to sporadic GEP tumours, where *MEN1* genotyping is recommended particularly in young individuals, genetic screening for *MEN1* germ line mutations in patients with sporadic primary HP should therefore not be advocated on a routine basis. However, in the presence of additional diagnostic criteria (age ≤ 35 years, positive family history, multifocal or recurrent HP, other endocrine neoplasia), *MEN1* genotyping, though currently cumbersome, should be performed in these patients to rule out *MEN1* syndrome or familial isolated HP.

Acknowledgements

This work was supported in part by grants of the Else Kroener-Fresenius Stiftung and the Deutsche Forschungsgemeinschaft, SFB 518 (to W K and B O B).

References

- Agarwal SK, Kester MB, Debelenko LV, Heppner C, Emmert-Buck MR, Skarulis MC & Doppman JL 1997 Germline mutations of the *MEN1* gene in familial multiple endocrine neoplasia type 1 and related states. *Human Molecular Genetics* **6** 1169–1175.
- Agarwal KS, Guru SC, Heppner C, Erdos MR, Collins RM, Park SY, Chandrasekharappa SC, Collins FC, Spiegel AM *et al.* 1999 Menin interacts with the AP1 transcription factor JunD and represses JunD activated transcription. *Cell* **96** 143–152.
- Bassett JHD, Rashbass P, Harding B, Forbes SA, Pannett AAJ & Thakker RV 1999 Studies of the murine homolog of the multiple endocrine neoplasia type 1 (*MEN1*) gene, *men1*. *Journal of Bone and Mineral Research* **14** 3–10.
- Beaudet AL & Tsui LC 1993 A suggested nomenclature for designating mutations. *Human Mutation* **2** 245–248.
- Carling T, Correa P, Hessman O, Hedberg J, Skogseid B & Lindberg D 1998 Parathyroid *MEN1* gene mutations in relation to clinical characteristics of nonfamilial primary hyperparathyroidism. *Journal of Clinical Endocrinology and Metabolism* **83** 2960–2963.
- Chandrasekharappa SC, Guru SC, Manickam P, Olufemi S-E, Collins FS, Emmert-Buck MR, Debelenko LV, Zhuang Z, Lubensky IA, Liotta LA *et al.* 1997 Positional cloning of the gene for multiple endocrine neoplasia-type 1. *Science* **276** 404–407.
- Cryns VL, Rubio M-P, Thor AD, Louis DN & Arnold A 1994a p53 abnormalities in human parathyroid carcinoma. *Journal of Clinical Endocrinology and Metabolism* **78** 1320–1324.
- Cryns VL, Thor A, Xu H-J, Hu S-X, Wierman ME & Arnold A 1994b Loss of the retinoblastoma tumor-suppressor gene in parathyroid carcinoma. *New England Journal of Medicine* **330** 757–761.
- Farnebo F, Teh BT, Kytölä S, Svensson A, Phelan C & Sandelin K 1998 Alterations of the *MEN1* gene in sporadic parathyroid tumors. *Journal of Clinical Endocrinology and Metabolism* **83** 2627–2630.
- Fujimori M, Shirahama S, Sakurai A, Hashizume K, Hama Y, Ito K, Shingu K, Kobayashi S, Amaro J & Fukushima Y 1998 Novel V184E *MEN1* germline mutation in a Japanese kindred with familial hyperparathyroidism. *American Journal of Medical Genetics* **16** 221–222.
- Haber DA & Fearon ER 1998 The promise of cancer genetics. *Lancet* **351** (Suppl II) 1–8.
- Hakim JP & Levine MA 1994 Absence of p53 point mutations in parathyroid adenoma and carcinoma. *Journal of Clinical Endocrinology and Metabolism* **78** 103–106.
- Heppner C, Kester MB, Agarwal SK, Debelenko LV, Emmert-Buck MR, Guru SC, Manickam P, Olufemi SE, Skarulis MC, Doppman JL *et al.* 1997 Somatic mutation of the *MEN1* gene in parathyroid tumours. *Nature Genetics* **16** 375–378.
- Karges W, Maier S, Wissmann A, Dralle H, Dosch HM & Boehm BO 1999 Primary structure, gene expression and chromosomal mapping of rodent homologs of the *MEN1* tumor suppressor gene. *Biochimica et Biophysica Acta* **1446** 286–294.
- Larsson C, Calender A, Grimmond S, Giraud S, Hayward NK, Teh B *et al.* 1995 Molecular tools for presymptomatic testing in multiple endocrine neoplasia type 1. *Journal of Internal Medicine* **238** 239–244.
- Liechti-Gallati S, Schneider V, Neeser D & Kraemer R 1999 Two buffer PAGE system-based SSCP/HD analyses: a general protocol for rapid and sensitive mutation screening in cystic fibrosis and other human genetic diseases. *European Journal of Human Genetics* **7** 590–598.
- Ludwig L, Schleithoff L, Kessler H, Wagner PK, Boehm BO & Karges W 1999 Loss of wild-type *MEN1* gene expression in multiple endocrine neoplasia type 1-associated parathyroid adenoma. *Endocrine Journal* **46** 539–544.
- Manickam P, Guru SC, Debelenko LV, Agarwal S, Olufemi SE, Weisemann J, Boguski MS & Crabtree JS 1997 Eighteen new polymorphic markers in the multiple endocrine neoplasia type 1 (*MEN1*) region. *Human Genetics* **101** 102–108.
- Poncin J, Abs R, Velkeniers B, Bonduelle M, Abramowicz M, Legros JJ, Verloes A, Meurisse M, Van Gaal L, Verellen C *et al.* 1999 Mutation analysis of the *MEN1* gene in Belgian patients with multiple endocrine neoplasia type 1 and related diseases. *Human Mutation* **13** 54–60.
- Ponder BAJ 1999 The phenotypes associated with *ret* mutations in the multiple endocrine neoplasia type 2 syndrome. *Cancer Research* **59** (Suppl) 1736s–1742s.
- Stewart C, Parente F, Piehl F, Farnebo F, Quincey D & Silins G 1998 Characterization of the mouse *Men1* gene and its expression during development. *Oncogene* **17** 2485–2493.
- Tanaka C, Yoshimoto K, Yamada S, Nishioka H, Ii S, Moritani M, Yamaoka T & Hakura M 1998 Absence of germ-line mutations of the multiple endocrine neoplasia type 1 (*MEN1*) gene in familial pituitary adenoma in contrast to *MEN1* in Japanese. *Journal of Clinical Endocrinology and Metabolism* **83** 960–965.
- Teh BT, Esapa CT, Houlston R, Grandell U, Farnebo F, Nordenskjöld M, Pearce CJ, Carmichael D, Larsson C & Harris PE 1998 A family with isolated hyperparathyroidism segregating a missense *MEN1* mutation and showing loss of wild-type alleles in the parathyroid tumors (Letter). *American Journal of Human Genetics* **63** 1549–1554.
- The European Consortium on MEN1 1997 Identification of the multiple endocrine neoplasia type 1 (*MEN1*) gene. *Human Molecular Genetics* **6** 1177–1183.
- Toliat M-R, Berger W, Ropers HH, Neuhaus P & Wiedenmann B 1997 Mutations in the *MEN1* gene in sporadic neuroendocrine tumours of gastroenteropancreatic system. *Lancet* **350** 1223.
- Vanbellinghen J-F, Beckers A & Poncin J 1999 Preliminary characterization of the promoter and cis-regulatory elements of the *MEN1* gene. *7th International MEN Workshop*, Gubbio, Italy, 30 June 1999.
- Zahrani AA & Levine MA 1997 Primary hyperparathyroidism. *Lancet* **349** 1233–1238.
- Zhuang Z, Vortmeyer AO, Pack S, Huang S, Pham TA & Wang C 1997 Somatic mutations of the *MEN1* tumor suppressor gene in sporadic gastrinomas and insulinomas. *Cancer Research* **57** 4682–4686.

Received 27 September 1999

Accepted 7 March 2000