MEN1 mutation analysis in Chinese patients with multiple endocrine neoplasia type 1

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Abstract

Multiple endocrine neoplasia type 1 (MEN1) is an inherited tumour syndrome characterized by the development of tumours of the parathyroid, anterior pituitary and pancreatic islets, etc. Heterozygous germ line mutations of MEN1 gene are responsible for the onset of MEN1. We investigated the probands and 31 family members from eight unrelated Chinese families associated with MEN1 and identified four novel mutations, namely 373_374ins18, 822delT, 259delT and 1092delC, as well as three previously reported mutations, such as 357_360delCTGT, 427_428delTA and R108X (CGA→TGA) of MEN1 gene. Furthermore, we detected a loss of heterozygosity (LOH) at chromosome 11q in the removed tumours, including gastrinoma, insulinoma and parathyroid adenoma from two probands of MEN1 families. RT-PCR and direct sequencing showed that mutant MEN1 transcripts remained in the MEN1-associated endocrine tumours, whereas normal menin proteins could not be detected in those tumours by either immunohistochemistry or immunoblotting. In conclusion, MEN1 heterozygous mutations are associated with LOH and menin absence, which are present in MEN1-associated endocrine tumours.

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Introduction

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant inherited disorder characterized by frequent occurrence of tumours of parathyroid, pancreatic islet and anterior pituitary, sometimes in combination with other rare tumours. The MEN1 gene was successfully identified in 1997, which is composed of ten exons spanning 2.8 kb that codes for a 610 amino acid nuclear protein, known as menin (Chandrasekharappa et al. 1997, Lemmens et al. 1997). Menin, which has no homology to any other known proteins, is considered to play a role in cell growth regulation, cell cycle, genome stability and synapse plasticity (Yang & Hua 2007). More than 400 germ line and somatic mutations in the MEN1 gene have been identified (http://uwcmml1s.uwcm.ac.uk/uwcm/mg/search/120173.html). However, more mutation detections favour in elucidating the correlation of genotype and phenotype (Kouvaraki et al. 2002, Wautot et al. 2002). The majority of MEN1 patients display heterozygous germ line mutations. MEN1-associated tumours generally have somatic deletions of the MEN1 locus and its surrounding regions in chromosome 11, known as loss of heterozygosity (LOH; Lubensky et al. 1996, Debelenko et al. 1997, Hessman et al. 2001). In the present study, we investigated MEN1 gene mutations in
eight Chinese MEN1 families, and analysed LOH on chromosomal 11q and menin expression in MEN1-associated tumours.

**Subjects and methods**

**Subjects**

The MEN1 probands (one male and seven females, age range 18–43 years) from eight unrelated families and 31 family members, including seven clinically symptomatic (three males and four females, age range 21–66 years) and 24 asymptomatic subjects (12 males and 12 females, age range 8–73 years), were investigated in the current study. The diagnosis of MEN1 was based on the presence of at least two of the three major endocrine lesions described in the syndrome, including tumours of the parathyroid gland, endocrine pancreas and anterior pituitary. Three representative MEN1 pedigrees were shown in Supplementary Fig. 1, which can be viewed online at http://erc.endocrinology-journals.org/supplemental/. Symptomatic subjects for MEN1 were defined as any of a variety of clinical manifestations, such as renal stones, acral growth, amenorrhea, hypoglycaemia and gastrointestinal bleeding that led them to seek medical attention. The clinical characteristics for all the probands and symptomatic subjects from the eight families were summarized in Table 1. The protocols for the study were approved by the Hospital Ethics Committee for Human Research, and informed consent was obtained from each individual.

Seven probands presented with primary hyperparathyroidism (87.5%), six with pancreatic lesions (75%), including five insulinomas and one gastrinoma, and five with pituitary adenoma (62.5%), including four prolactinomas and one mixed prolactinoma, and GH-secreting tumour. Interestingly, the thymic carcinoid, which is considered as an extremely rare manifestation (prevalence 3.1%) and occurred mostly in male heavy smokers (>95%; Ferolla et al. 2005), was detected in proband 6, a nonsmoking female patient (31 years of age). The patient died from metastasis of thymic carcinoid in 28 months after surgery, another subject in family 1 (father of proband 1) also died of the thymic carcinoid in 36 months after surgery, confirming that the thymic carcinoid may be an important cause of death in MEN1. Consequently, prophylactic thymectomy should be considered when neck surgery for primary hyperparathyroidism in MEN1 patients (Brandi et al. 2001, Ferolla et al. 2005) to be undertaken. It is also noted that proband 2 had six lesions of prolactinoma, gastrinoma, ovary teratoma, insulinoma, thyroid and parathyroid adenomas, which were diagnosed and operated at the age of 25, 28, 35, 36, 37 and 43 years respectively.

**Mutation analysis**

DNA was extracted from peripheral blood leukocytes and from the removed tumour tissues using DNeasy Tissue Kit (Qiagen). Exons 2–10 of MEN1 gene were amplified by PCR in a volume of 50 μl mixture containing 1.5 mM MgCl2, 10 mM dNTP, 0.2 μg genomic DNA, 20 μmol of each primer and 2.5 U Taq DNA polymerase (Sangon, Shanghai, China). The primer sequences were previously reported (Tanaka et al. 1998), except for the primer pair amplifying exon 8 (forward 5′-agagaccacagtctcaca-3′, reverse 5′-ggacagagtggagctc-3′). Amplification was performed with preheating at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 45 s, annealing at 55–62 °C for 45 s and extension at 72 °C for 1 min. The PCR products were purified using a gel extraction kit (Qiagen) and sequenced in both sense and antisense directions on the ABI 3700 Sequencer (Perkin–Elmer PE Applied Biosystems, Foster City, CA, USA). The obtained sequences were compared with the reference sequences of the MEN1 gene (U93237).

**LOH**

LOH analysis was performed using DNA from MEN1-associated endocrine tumour tissues and corresponding peripheral leukocytes. Eighteen short tandem repeat (STR) markers were used to determine the scope of LOH in chromosomal arm 11q (Table 2). DNA fragments were labelled with fluorescent dye using M13 universal tailed primer method. PCR products were analysed using Beckman–Coulter CEQ 8800 sequencer (Beckman–Coulter, Fullerton, CA, USA). Data collection and analysis were performed with Fragment Analysis Module (Beckman–Coulter). LOH is defined as positive if the allele peak ratio is >1.5.

**RT-PCR**

RNA was prepared using TRIzol reagent (Invitrogen). Total RNA (2 μg) was heated at 70 °C for 5 min and placed on ice for 5 min. A mixture of 5 μl M-MLV RT 5× reaction buffer, 10 mM of each of the four dNTPs, 200 U M-MLV transcriptase enzyme, 25 pg/μl random primer and 20 U RNase inhibitor (all from Promega) was added to each sample, followed by incubation at 40 °C for 60 min and 70 °C for 15 min. cDNA (2 μl) products were amplified with 1 U Ampli Taq Gold (PE Applied Biosystems), together with β-actin as an internal control. The specific primers for MEN1 that spanned exons 2 and 3 (cDNA411 to 699), and the
Table 1: Clinical manifestations and *MEN1* mutations in eight families with MEN1

<table>
<thead>
<tr>
<th>Family</th>
<th>Subject</th>
<th>Age</th>
<th>Sex</th>
<th>Parathyroid</th>
<th>Pituitary</th>
<th>Pancreas</th>
<th>Others</th>
<th>Mutation</th>
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<tr>
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<td>Insulinoma</td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td>1–3</td>
<td>38</td>
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<td></td>
<td></td>
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<td>Thymic carcinoid</td>
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<tr>
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<td>1–4</td>
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<td></td>
<td></td>
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<tr>
<td>2</td>
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<td>43</td>
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<td></td>
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<td>18</td>
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<td></td>
<td>Arterial hyperplasia</td>
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<td>Insulinoma</td>
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<td></td>
<td>Pancreas tumour</td>
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<tr>
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<td>HPT</td>
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<td>Dermatofibroma</td>
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<td>PRL†</td>
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<td>HPT</td>
<td></td>
<td>Prolactinoma</td>
<td></td>
<td>1092delC(^b)</td>
</tr>
</tbody>
</table>

PTH†, hyperparathormonemia only; HPT, hyperparathyroidism, hyperparathormonemia plus hypercalcemia; PRL†, hyperprolactinemia only; prolactinoma, hyperprolactinemia plus pituitary adenoma.

\(^a\) Asymptomatic carrier.

\(^b\) Novel mutation.
sequences of the primers for MEN1 amplification are as follows: forward 5'-GAC CTG TCC CTC TAT CCT CG-3' and reverse 5'-TGA CCT CAG CTG TCT GCT CC-3'. Reactions were carried out in the PTC-225 DNA Engine Tetrad (MJ Research Inc., Waltham, MA, USA). Preheating was performed at 95 °C for 8 min, followed by denaturation at 95 °C for 45 s, annealing at 58 °C for 45 s and extension at 72 °C for 1 min for 30 cycles. The PCR products were loaded onto 1.5% agarose gels for electrophoresis and visualized by ethidium bromide under u.v. light.

**Immunohistochemistry**

Formalin-fixed and paraffin-embedded sections were deparaffinized in xylene and rehydrated through descending ethanol concentration to distilled water. Endogenous peroxidase was quenched by incubating the slides in 3% H2O2 for 10 min at room temperature. Primary antibody against C19 of menin protein (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added after dilution at 1:200, and then the slides were incubated at 4 °C overnight. Next, the sections were covered with biotinylated secondary antibody and incubated for 30 min at room temperature. After the sections were finally incubated with ABC complex/HRP for 30 min at room temperature, they were left to develop with 3-3'-diaminobenzidine (DAB). As soon as the sections were developed, the slides were immersed in distilled water.

**Western blot**

The cell lysates were extracted from parathyroid adenoma, insulinoma and gastrinoma with lysis buffer (RIPA, 1×PBS, 1% NP40, 5 mM EDTA, 1 mM sodium orthovanadate, 1% phenylmethylsulphonyl fluoride and complete protease inhibitor cocktail). The protein concentrations were determined with DC Protein Assay Reagent (Bio-Rad Laboratories). Cell lysates (25 µg) were loaded onto SDS/PAGE. The protein bands were stained with Ponceau solution and transferred to Hybond-ECL nitrocellulose membrane (Amersham Biosciences). The membrane was blocked with a 5% nonfat milk at 4 °C overnight and incubated with anti-menin antibody (1:750) at room temperature for 2 h. Menin expression was visualized with ECL plus kit (Amersham Biosciences). MEN1+/+ and MEN1−/− mouse embryonic fibroblasts (MEF) cell lysates were loaded as positive and negative control respectively.
Results and discussion

MEN1 mutations

Germ line mutations of the MEN1 gene were screened in 39 individuals from eight families. Seven different types of germ line mutations were identified, of which four mutations were novel ones (373ins18, 822delT, 259delT and 1092delC; Fig. 1), and the other three (357_360delCTGT, 427_428delAT and R108X (CGA > TGA)) had previously been reported. Mutation R108X (CGA > TGA) was identified in families 5 and 7. Five deletion mutations caused open reading frames shift, one nonsense mutation produced an earlier stop codon, and the 373ins18 mutation caused six amino acid in-frame insertion. In these eight MEN1-associated families, MEN1 germ line mutations were detected in 15 symptomatic subjects, as well as in three asymptomatic subjects (carriers) with an average age of 16.3 years (range 7–25 years of age).

Most MEN1 gene mutations have been reported in coding sequences (Kouvaraki et al. 2002, Wautot et al. 2002). The novel mutation 373ins18, causing six

Figure 1 Subclone sequencing of MEN1 gene. Four novel mutations 373ins18, 822delT, 259delT and 1092delC in the probands of families 3, 4, 6 and 8 respectively were shown in A, B, C and D respectively.
amino acid in-frame insertion at position 373 and being situated in a putative interacting domain for Smad3 binding, possibly affects Smad3 signalling (Kaji et al. 2001), which is a crucial player in transforming growth factor-β (TGF-β) signalling pathway. In vitro study has confirmed that amino acid substitution in Smad3-binding domains of menin blocked Smad3-mediated transcriptional activation in the TGF-β pathway (Kaji et al. 2001). Blockage of TGF-β signalling may disrupt cell stability, drive cells towards inappropriate growth and ultimately result in tumour formation (Sowa et al. 2004).

**LOH analysis in MEN1-associated tumours**

LOH was performed for the removed MEN1-associated tumours from families 2 and 4. An entire heterozygous deletion of chromosome 11q was identified in gastrinoma, insulinoma and parathyroid adenoma from the probands of families 2 and 4 using 18 STR markers spanning 115.4 Mb (Fig. 2). These findings supported the ‘two hits’ hypothesis in the pathogenesis of MEN1-associated tumours (Knudson & Strong 1971).

**Analysis of MEN1 expression in MEN1-associated tumours**

Using RT-PCR (Fig. 3A) and direct sequencing (data not shown), we had demonstrated that mutant MEN1 transcripts could be identified in gastrinoma, insulinoma and parathyroid adenoma from the probands of families 2 and 4. Moreover, normal menin expression was absent in those tumours determined by immunoblotting (Fig. 3B) and immunohistochemistry (Fig. 3C), which is consistent with LOH analysis, although we could not detect the prematurely truncated menin by immunoblotting due to the limitation of a C19 antibody against menin used in the present study. It indicates that a complete inactivation of the normal MEN1 gene occurs in MEN1-associated tumours.

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**Figure 2** LOH assay for MEN1-associated insulinoma and gastrinoma from the proband of family 2. STR markers D11S2002 and D11S2006 flanking MEN1 gene were displayed in A and B respectively. PBMC, peripheral blood mononuclear cells. The arrows indicate the allele lost.

**Figure 3** (A) RT-PCR for MEN1 gene of the MEN1-associated tumours from the probands of families 2 and 4. (B) Immunoblotting for menin of MEN1-associated tumours from the probands of families 2 and 4. Menin expression is absent in those tumours. (C) Immunohistochemistry for menin expression of MEN1-associated tumours from the proband of family 2. Menin staining is negative in the tumour cells and positive in the surrounding capsule tissues. The amplification is 400x. Ins, insulinoma; Gas, gastrinoma; Par, parathyroid adenoma; Pan, normal pancreas; ++/+, MEN1 positive MEF cell lysates; −/−, MEN1 negative (knock-out) MEF cell lysates.
In conclusion, we have identified seven MEN1 germ line mutations in eight Chinese families with MEN1, of which four mutations have not previously been reported. The complete inactivation of the MEN1 gene and subsequent absence of normal menin expression are present in MEN1-associated tumours.

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