Mutational Spectrum of Multiple Endocrine Neoplasia Type 2 and Sporadic Medullary Thyroid Carcinoma in Taiwan

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Background/Purpose: Multiple endocrine neoplasia type 2 (MEN 2) is an autosomal dominant cancer predisposition syndrome, and >95% of MEN 2 patients carry rearranged during transfection (RET) proto-oncogene mutants. We aimed to elucidate the genotype and phenotype relationship of RET proto-oncogene mutations in Taiwanese subjects with medullary thyroid cancer (MTC).

Methods: We genotyped the MEN-2-associated germ-line mutations by PCR-based sequencing of the RET gene. DNA was extracted from a total of 69 members from eight unrelated families with individuals affected by MTC, and from seven sporadic cases of MTC.

Results: RET mutations were found in four MEN 2A families, all at codon 634 (one with C>R, two with C>F, and one with C>W). One MEN 2A patient carried a de novo mutation at codon 634 (C>R). In two families of MEN 2B, all carried the mutation at codon 918 (M>T). These two cases of MEN 2B were all de novo mutations. One family of familial MTC or unclassified MEN 2 carried the codon 620 (C>F) mutation. Among the seven sporadic cases of MTC, none was found to carry any mutation in hotspot exons. Only two non-synonymous variants (T278N/exon 4 and D489N/exon 7) were found in two cases. However, these two variants were not uncommon in our elderly population.

Conclusion: We found that all eight MTC patients with a family history or with the other phenotypes of MEN 2 had RET mutations, whereas no significant RET mutation was found in seven patients with isolated MTC without family history and other endocrine diseases. Molecular scanning of the RET gene in MEN 2 and MTC in Taiwanese patients probably should be limited to exons 10, 11 and 16, initially to be cost-effective. [J Formos Med Assoc 2009;108(5):402–408]

Key Words: human, multiple endocrine neoplasia type 2A, multiple endocrine neoplasia type 2B, mutation, RET protein, thyroid neoplasms

Medullary thyroid carcinoma (MTC) is a rare malignant thyroid tumor that arises from thyroid calcitonin-secreting C cells, and accounts for 5–10% of all thyroid cancers. Around 25% of MTC cases are hereditary.1 Usually, MTC is the earlier clinical phenotype and the most common cause of death in multiple endocrine neoplasia type 2 (MEN 2). The subtype, MEN 2A accounts for >80% of all MEN 2. The clinical features of MEN 2A include MTC, pheochromocytoma (50%), and parathyroid hyperplasia or adenoma (20–30%). MEN 2B is associated with early aggressive behavior of MTC, characterized by mucosal neuromas, intestinal ganglioneuromatosis and marfanoid
Taiwanese MEN 2 and MTC mutations

Methods

Subjects
We recruited 69 members from eight unrelated families with individuals affected by MTC, and seven cases of sporadic MTC. The diagnostic criteria of MTC were confirmed by documented histopathology. Some of the index cases had been reported previously. The control group consisted of 109 elderly people without a history of thyroid cancer (53 women, 56 men; mean age, 73.1 ± 10.0 years). Informed consent was obtained from all participants or their legal guardians. The study was approved by the Human Research Ethics Committee of the National Taiwan University Hospital.

DNA sequencing and genotyping
Genomic DNA was extracted from peripheral blood leukocytes using the Puregene DNA Purification Kit (Gentra Systems, Minneapolis, MN, USA), according to the manufacturer’s protocol. PCR amplification of the 20 exons of the RET gene was performed. Since exon 21 and parts of exon 20 were not translated, they were not included. Information on the primers and PCR protocols are as reported, with some modifications, and are available upon request. The PCR products were purified and sequenced using the ABI PRISM® BigDye™ Terminator Cycles Sequencing Kit and were analyzed by an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Exons 8, 10, 11 and 13–16 were sequenced in both directions. The sequences were compared with RET sequence data from NCBI Blast Human database (http://www.ncbi.nlm.nih.gov/BLAST/). If there was any uncertainty in the sequence data, they were inspected visually by two investigators. An independent genomic DNA sample from peripheral blood or buccal mucosa was obtained from any subjects who were found to carry a mutation in the first test. A second test was performed to confirm the presence of the mutations.

Furthermore, genomic DNA was obtained as a control population from 109 unrelated elderly

habitits. Approximately half of the MEN 2B cases develop pheochromocytoma but not hyperparathyroidism. Familial MTC (FMTC) accounts for 5–15% of patients who suffer from MTC alone without other endocrine manifestations.

MEN 2 is an autosomal dominant cancer predisposition syndrome that has been described for over 100 years. More than 90% of MEN 2 carriers eventually develop MTC. MEN 2 can be classified into three major subtypes: MEN 2A, MEN 2B and FMTC, depending on the occurrence of tissue-specific endocrine tumors, characteristic features and the number of affected family members.

MEN 2 is caused by mutations in the RET (REarranged during Transfection) proto-oncogene. More than 95% of MEN 2 patients carry RET mutations. The RET proto-oncogene is located on chromosome 10q11.2 and comprises 21 exons, and encodes a trans-membrane tyrosine kinase receptor. It is involved in the ligand-mediated RET signaling pathway of the glial cell line-derived neurotrophic factor family in neural crest tissues. RET mutation is found in approximately one per 30,000 in the population. Over 90% of MEN 2A and FMTC families have missense mutations in one of the six conserved cysteine residues at codons 609, 611, 618, 620 (in exon 10), 630 or 634 (in exon 11) in the extracellular cysteine-rich region. Among these cysteine codons, the most frequent mutation is at codon 634 (63–87%), particularly with C634R and C634Y (50%) in MEN 2A. All mutations are thought to induce ligand-independent constitutive activation of the receptor and subsequent signaling. Approximately 95% of the MEN 2B patients carry the M918T (exon 16) missense mutation, whereas the others harbor a mutation at codon 883 (exon 15) in the tyrosine kinase domain. These may interfere with ATP binding, and therefore alter the substrate specificity of the RET substrate-recognition pocket of the catalytic core, which leads to ligand-independent activation or modification of kinase activity.

The aim of this study was to elucidate the RET mutations in Taiwanese subjects with MTC, and to evaluate the potential value of genetic diagnosis in our population.
subjects without MTC, to check the allele frequency of T278N and D489N. The PCR products underwent one additional denaturation step at 94 °C for 5 minutes, followed by ramping to 25 °C over 70 minutes to allow the formation of homo- or heteroduplexes, before being subjected to denaturing HPLC (DHPLC) analysis. DHPLC was performed on the WAVE DNA fragment analysis system equipped with a DNASeP column that used a UV-C scanner to detect eluted DNA (Transgenomic, Crewe, UK), as described previously.23 DNA sequencing performed on PCR products showed unidentifiable mobility and conformational change on the chromatogram.

Results

This series is the largest genetic study of MTC patients in Taiwan. There were 69 subjects from eight unrelated Taiwanese families with MTC, and seven sporadic cases of MTC. They were tested for MEN-2-associated mutations by PCR-based sequencing of the RET gene exons 8, 10, 11 and 13–16, for which, hotspot mutations have been documented. RET mutations were found in four MEN 2A families (A–D), all at codon 634 (one with C>R, two with C>F, and one with C>W; Table 1). One 23-year-old woman (family H), initially classified as sporadic MTC, was lost to follow-up. According to her mother, she received adrenalectomy a few years before in the USA. Therefore, she should probably be reclassified as MEN 2A. This is a de novo mutation of codon 634 (C>R). In two families with MEN 2B (F and G), all carried a mutation at codon 918 (M>T; Table 1). Interestingly, these two cases of MEN 2B both had de novo mutations. The mutation was not detected in their parents or siblings. In family E, there were two family members with MTC and three asymptomatic gene carriers (aged < 50 years) who had not yet manifested other endocrine disorders (Table 1). The patients were categorized as FMTC or unclassified MEN 2, based on rigorous criteria,2,6 and persistent follow-up will be needed to evaluate the risk of pheochromocytoma. In this family, a mutation was found at codon 620 (C>F). All of the mutations were heterozygous and the characteristics of the families are summarized in Table 1.

Among the seven sporadic cases of MTC, none was found to carry any mutation after examining the seven exons that harbored the hotspots. We further extended the sequence analysis to the entire RET coding region beyond the hotspot regions in the remaining seven subjects. We found five exonic single nucleotide polymorphisms (SNPs) (A45A/exon 2, A432A/exon 7, P679P/exon 11, L769L/exon 13, and S904S/exon 15)

| Family | Subtype | Phenotypes other than MTC | Proband age of diagnosis | Codon | Base substitution | Amino acid change | No. of carriers | Total no. of available members [Ref.]
<table>
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<tbody>
<tr>
<td>A</td>
<td>MEN 2A</td>
<td>Hyperparathyroidism</td>
<td>27</td>
<td>634</td>
<td>TGC→CGC</td>
<td>Cys→Arg</td>
<td>3</td>
<td>17 [18]</td>
</tr>
<tr>
<td>B</td>
<td>MEN 2A</td>
<td>Pheochromocytoma</td>
<td>30</td>
<td>634</td>
<td>TGC→TTC</td>
<td>Cys→Phe</td>
<td>3</td>
<td>5 [17]</td>
</tr>
<tr>
<td>C</td>
<td>MEN 2A</td>
<td>Pheochromocytoma</td>
<td>36</td>
<td>634</td>
<td>TGC→TTC</td>
<td>Cys→Phe</td>
<td>12</td>
<td>18 [18]</td>
</tr>
<tr>
<td>D</td>
<td>MEN 2A</td>
<td>Pheochromocytoma</td>
<td>36</td>
<td>634</td>
<td>TGC→TGG</td>
<td>Cys→Trp</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>E</td>
<td>FMTC/ unclassified</td>
<td>Nil</td>
<td>40</td>
<td>620</td>
<td>TGC→TTC</td>
<td>Cys→Phe</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>F</td>
<td>MEN 2B</td>
<td>Mucosal neuroma</td>
<td>15</td>
<td>918</td>
<td>ATG→ACG</td>
<td>Met→Thr</td>
<td>1</td>
<td>5 [19]</td>
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<tr>
<td>G</td>
<td>MEN 2B</td>
<td>Mucosal neuroma</td>
<td>12</td>
<td>918</td>
<td>ATG→ACG</td>
<td>Met→Thr</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>H</td>
<td>MEN 2A?</td>
<td>Pheochromocytoma*</td>
<td>23</td>
<td>634</td>
<td>TGC→CGC</td>
<td>Cys→Arg</td>
<td>1</td>
<td>5 [17]</td>
</tr>
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</table>

*This sporadic MTC patient was lost to follow-up, and the information was provided by her mother.
and two non-synonymous variants (T278N/exon 4 in case 7 and D489N/exon 7 in case 3) (Table 2). Interestingly, these two non-synonymous variants, T278N in exon 4 (rs35118262) and D489N in exon 7 (rs9282834), had variable allele frequencies in different populations. By DHPLC analysis, we found the allele frequencies of these two SNPs were 0.037 and 0.037 in 109 elderly Taiwanese subjects without a history of thyroid cancer. Therefore, they do not seem to be potential genetic modifiers. All of the intronic SNPs have been documented in the Ensembl data base (Table 2).

Discussion

The EUROMEN Study Group found an age-dependent progression of early MTC depending on the specific RET codon. In subjects with codon 634 mutants, the mean age at diagnosis was 10 years, the average interval from tumor development to nodal metastasis was 6.6 years, and the cumulative risk rose steadily from 14 years of age onwards.11,24 Skinner et al provided at least 5 years of follow-up data to suggest that an MEN 2A asymptomatic carrier who underwent total thyroidectomy before 8 years of age could be cured.25 The consensus of the 7th International MEN Workshop advocated stratification of the medical management of hereditary MTC into three levels according to gene-based information. Level 3 (highest risk): children with MEN 2B or RET codon 883, 918, or 922 mutation have the most aggressive MTC and should undergo prophylactic thyroidectomy within the first 6 months, preferably within the first month of life. Level 2 (high risk): children with any RET codon 609, 611, 618, 620, 630, or 634 mutation should undergo thyroidectomy by age 5 years. Level 1 (least high risk): children with RET codon 768, 790, 791, 804, and 891 mutations may be operated on later, or opt for periodic pentagastrin-stimulated calcitonin testing. Nevertheless, the recommendations about central lymph node dissection at initial thyroidectomy are controversial, and a more individualized approach may be needed among the MEN 2 variants.6,26 This recommendation highlights the need for

<table>
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<tr>
<th>Case no.</th>
<th>Exonic SNP Codon/exon*</th>
<th>Intronic SNP SNP ID: Allele*</th>
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<tbody>
<tr>
<td>1</td>
<td>A45A/exon 2, A432A/exon 7, L769L/exon 13</td>
<td>rs35906041: CC &gt;-, rs2742243: T &gt; C, rs3026750: G &gt; A, rs34827976: C &gt; T, rs2477237: C &gt; A, rs17028: C &gt; T</td>
</tr>
<tr>
<td>2</td>
<td>A45A/exon 2, A432A/exon 7, L769L/exon 13, S904S/exon 15</td>
<td>rs35906041: CC &gt;-, rs2742243: T &gt; C, rs3026750: G &gt; A, rs34827976: C &gt; T</td>
</tr>
<tr>
<td>3</td>
<td>A45A/exon 2, A432A; D489N/exon 7, L769L/exon 13</td>
<td>rs12246855: A &gt; G, rs12246856: A/C, rs35906041: CC &gt;-, rs2742243: T &gt; C, rs3026750: G &gt; A, rs34827976: C &gt; T, rs2477237: G &gt; A, rs17028: C &gt; T</td>
</tr>
<tr>
<td>4</td>
<td>A45A/exon 2, A432A/exon 7, L769L/exon 13</td>
<td>rs2742243: T &gt; C, rs3026750: G &gt; A, rs34827976: C &gt;-, rs2477237: G &gt; A, rs17028: C &gt; T</td>
</tr>
<tr>
<td>6</td>
<td>A432A/exon 7</td>
<td>Nil</td>
</tr>
<tr>
<td>7</td>
<td>A45A/exon 2, T278N/exon 4†, A432A/exon 7, L769L/exon 13, S904S/exon 15</td>
<td>rs2435351: G &gt; A, rs35906041: CC &gt;-, rs2742243: T &gt; C, rs3026750: G &gt; A, rs34827976: C &gt;-, rs2477237: G &gt; A, rs17028: C &gt; T</td>
</tr>
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DNA-based testing and effective clinical intervention for possible cure of MEN 2 carriers. For routine genetic testing, recent consensus guidelines suggest that only a limited number of mutations, which involve RET exons 10, 11 and 13–16, should be analyzed, and these account for >95% of MEN 2 carriers.\(^6\) Some studies have shown more complex genetic variations, with high incidence (10–40%) occurring throughout the RET gene outside the hotspot exons.\(^{27,28}\) However, the clinical significance of some of the novel variants remains uncertain.\(^{29}\)

In this study, we found that all eight MTC patients with a family history or other characteristic features of MEN 2 had RET germ-line mutations. Moreover, all of the mutations in our hereditary MEN 2A/FMTC cases were confined to exon 10 or 11 of the RET extracellular cysteine-rich region. Two MEN 2B had codon 918 mutations. Among the seven isolated MTC patients without family history and other endocrine disorders, we did not identify any mutation in hotspot exons or any significant mutations in the exons, other than the hotspots reported previously.\(^8,30\) Furthermore, we found two non-synonymous variants, T278N (rs35118262) and D489N (rs9282834), in two cases of sporadic MTC. Their allele frequencies were approximately 4% in Taiwanese elderly subjects without a history of thyroid cancer, and therefore, did not appear to play a role in MTC. Our findings suggest that, in Taiwanese patients with MTC, the mutation spectrum of the RET proto-oncogene is limited. For subjects with MEN 2A or FMTC, molecular scanning of exons 10 and 11 is essential and probably adequate. For MEN 2B, examination of exon 16 is essential and probably adequate. Based on our data, these investigations should be cost-effective. If no mutation is found, the search probably can be extended to the other hotspot exons and beyond. For sporadic MTC without family history or other endocrine disorders, molecular scanning of exon 11 is essential and probably adequate. However, this and further searches in hotspot exons and beyond may not be fruitful, according to our results. The present survey coincides with previous studies in other Asian countries.\(^{31–34}\) Consistent with the current consensus guidelines, molecular scanning for MTC, with or without MEN manifestations and family history, should be limited to exons 10, 11 and 16, and then extended to the other hotspot exons if necessary. Although comprehensive sequencing of genome is feasible technically, it may not be financially cost-effective to extend the search for mutations beyond these regions.

Specific RET mutations in patients with MEN 2 have been shown to predict the phenotypic expression of the disease and MTC aggressiveness, and therefore, to provide prognostic information to guide prophylactic thyroidectomy and screening for pheochromocytoma.\(^{26,35,36}\) This is especially valuable in children and young adults.\(^{37,38}\) However, the current recommendations are all based on observations in Caucasian populations. Since the genetic background of Asian populations is different, the natural course of various RET mutant carriers may be different.\(^{31,33,39–42}\) Our clinical observations showed that long-term survival was not uncommon with a persistent and indolent course of hereditary MTC, even in the absence of systemic treatment. Some of the MEN 2A cases had been followed regularly for >10 years and had persistent hypercalcitoninemia. One man died of sepsis and acute renal failure aged 71 years, and one woman died of pneumonia aged 46 years. None of the other family members directly died of MTC. More data from our own population are required before an evidence-based recommendation can be implemented. The age of onset for the mutation at codon 918 appears to be earlier than for mutations at the other codons. Our case number is still too small to correlate the clinical course with the genetic test results. Nevertheless, international guidelines should be used before that.

At present, cancer-prevention strategies for mutant gene carriers involve the prenatal diagnosis or preimplantation genetic diagnosis and assisted reproductive techniques to prevent recurrence of the mutant gene in future generations. This offers families another choice to fight against this notorious hereditary disease, especially for those who are stratified into the level 3 (highest risk)
genotype.\textsuperscript{43,44} MEN 2 provides a fascinating example for early prevention and cure of cancer. It also provides important insight into the psychosocial issues and genetic counseling in cancer therapy. We propose that, for the subjects with manifestations of MEN 2A or FMTC, exons 10 and 11 should be examined first. For those with manifestations of MEN 2B, exon 16 should be examined first. For sporadic MTC without family history and other endocrine disorders, sequencing exon 11 should be carried out first.

\section*{Acknowledgments}

We thank all the MTC patients and their families who agreed to participate in this study. We are grateful to the physicians who contributed their patients and medical support. This work was supported in part by Ching-Shing Medical Foundation to Dr Wei-Shiu Yang.

\section*{References}


