A Large Germline Deletion of the MEN1 Gene in a Family with Multiple Endocrine Neoplasia Type 1

Mari Kishi,1 Toshihiko Tsukada,1, 3 Satoko Shimizu,1 Hitoyasu Futami,1 Yukio Ito,2 Masako Kanbe,2 Takao Obara2 and Ken Yamaguchi1

1Growth Factor Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104 and 2Department of Endocrine Surgery, Tokyo Women’s Medical College, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162

Multiple endocrine neoplasia type 1 (MEN1) is a familial cancer syndrome inherited as an autosomal dominant trait. Various heterozygous germline mutations of the responsible gene, MEN1, have been identified within its exons in many, but not all, affected individuals. We here demonstrate, by DNA polymorphism analysis and gene dosage analysis with polymerase chain reaction (PCR), a large heterozygous germline MEN1 deletion in a kindred with MEN1, in whom no mutation could be detected in the PCR-amplified exons. The deletion spanned an at least 7 kb region containing the entire MEN1 gene. These findings indicate that a large germline deletion of the MEN1 gene, which escapes detection in PCR-based sequence analysis, should be considered as a potential cause of MEN1.

Key words: Multiple endocrine neoplasia type 1 — MEN1 — Tumor suppressor gene — Gene dosage — DNA testing

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant familial cancer syndrome characterized by parathyroid, enteropancreatic and pituitary tumors.1, 2) The responsible gene, MEN1, was recently identified on chromosome 11q13 by positional cloning.3) A heterozygous MEN1 germline mutation underlies an inherited predisposition to the development of MEN1-related tumors. Although the molecular function of the encoded protein, menin, is unknown,3) MEN1 is considered to be a tumor suppressor gene because loss of heterozygosity of the MEN1 locus frequently occurs in the MEN1-related tumors.4)

Previous studies have identified various MEN1 germline mutations within its exons in many affected individuals with MEN1.3, 5–8) Although several missense mutations and in-frame deletions have been identified, the detected germline mutations were mostly nonsense or frameshift mutations, which cause truncation of menin and, presumably, loss of function of the gene. These findings are consistent with a tumor suppressor function of this gene.

The previous studies also revealed that some individuals affected with MEN1 had no detectable MEN1 germline mutation.2–5, 7) Because these studies analyzed only protein-coding exons that were amplified by polymerase chain reaction (PCR), a mutation in another region of the gene or a large heterozygous deletion could have been missed. Alternatively, some of these mutation-negative individuals could have had a distinct syndrome caused by some other gene(s) that meets the formal definition of MEN1 as suggested previously,5) or could have had multiple independent tumors by chance in two or more MEN1-related endocrine tissues. Because identification of a MEN1 germline mutation enables presymptomatic recognition or early exclusion of this disease in individuals at risk, it is important to characterize MEN1 germline mutations which escape detection by PCR-based mutation screening.

We encountered a case of familial MEN1 with three affected individuals, two of whom (Fig. 1, patients a and c) were previously analyzed and exhibited no detectable MEN1 germline mutation in the protein-coding exons.7) We analyzed blood cell DNA of the third member of this family (Fig. 1, patient b) for MEN1 mutation after having obtained informed consent for genetic analysis. Nucleotide sequences of both transcribed and non-transcribed strands were determined by direct sequencing of the PCR product described previously.7) As expected, no germline mutation was detected in the MEN1 protein-coding region of this patient, but a previously described polymorphism A541T (GCA→ACA)11) at codon 541 in exon 10 was present as a single sequence without the wild-type codon (GCA) (data not shown). The MEN1 codon 541 of her son (Fig. 1, patient c) had only the wild-type (GCA) sequence (data not shown). These findings suggest that the both individuals had only a single allele of the MEN1 gene. Another affected member (Fig. 1, patient a) also had only the polymorphic sequence (ACA) (data not shown), which is compatible with MEN1 hemizygosity.

To confirm the hemizygosity of the MEN1 locus in these patients, we estimated the MEN1 gene dosage by a refined method of double competitive PCR. This assay technique

3To whom correspondence should be addressed.
was a modification of the previously described quantitative PCR that employed two internal DNA standards, one for the gene of interest and the other for a reference gene (Fig. 2A).9, 10) The internal standard DNA served as a control for amplification efficiency in each PCR reaction. Because the two internal standards were mixed before they were added to genomic DNA samples, the reference gene served as an internal control for the ratio of the amount of the genomic DNA to that of the internal standard DNA, which may have varied among samples. The use of these multiple controls allowed comparison of data of genomic DNA samples from different sources, including normal subjects.9, 10) The modification included the use of internal standards that gave rise to PCR products highly homologous to and only 3-bp shorter or longer than those of their respective genomic target sequences. The sequence and size similarities between the standard and the genomic sequences are considered to be crucial for equal amplification efficiency of the two sequences. The use of an automated genetic analyzer enabled rapid separation and quantitation of these PCR products.

The internal standard for MEN1 was synthesized by PCR of a mutant MEN1 gene, K119del,7) which contained a 3-bp deletion at codon 119. PCR of this mutant gene with primers 2-5 (5′-GGTGTCTCCAGCGTGAGCTGGTG-3′) and 2-2 (5′-TCATGGATAAGATTCCCACCTACTGGG-3′) generated a 146-bp fragment, which was 3-bp smaller than the normal 149-bp PCR product that corresponds to the 115-bp exon 2 sequence and the 34-bp sequence of the downstream intron (Fig. 2A). We used the TR3 gene11) on chromosome 12q13 as a reference gene. The TR3 internal standard was synthesized by PCR of the murine homologue of TR3, nur77,12) which is 85% homologous to the human TR3 within the amplified region, with primers TR3hG (5′-CACTTCTCGCCCAGCCAGACTTA-3′) and TR3C (5′-ACCTTGAAAGACCCCGCTTTGAGCCTC-3′). This TR3 standard was 3-bp larger than the 401-bp human TR3 PCR product (Fig. 2A). Genomic DNA (50 ng) was coamplified in a 10 µl reaction mixture with fixed amounts (0.1–1 fg) of the two internal DNA standards by PCR using either MEN1-specific or TR3-specific primer pairs as described above. The PCR conditions were as follows: 1 min at 95°C for 1 cycle; 1 min at 95°C, 1 min at 64°C, 2 min at 72°C for 22 cycles; 5 min at 72°C for 1 cycle. One of the primers of each primer pair was labeled with fluorescein isothiocyanate (FITC) and the amount of each PCR product was quantitated as a fluorescence peak area (integrated fluorescence signal intensity) (Fig. 2B) using an automated genetic analyzer (ABI PRISM310, Perkin Elmer, Foster City, CA). Gene dose quotient, which represents the relative copy number of the MEN1 gene in the cells, was calculated from the amounts of the four PCR products: Gene dose quotient = (MEN1 PCR product/MEN1 internal standard PCR product) ÷ (TR3 PCR product/TR3 internal standard PCR product).
Germline Deletion of \(\textit{MEN1}\) Gene

The calculated gene dosage of the affected individuals in this family was approximately a half of those of the normal control and a patient previously shown to be heterozygous for a \(\textit{MEN1}\) point mutation (Table I). The results of this double competitive PCR assay were highly reproducible with the coefficient of variation being less than 14%. These findings confirmed the hemizygosity of the \(\textit{MEN1}\) gene in the affected individuals of this family.

To estimate the size of the deletion, we analyzed micro-satellite length polymorphism around the \(\textit{MEN1}\) locus using DNA markers, \(\textit{D11S4940}, \textit{D11S4946}\), and \(\textit{PYGM}\) (Fig. 3A).\(^{13,14}\) The \(\textit{MEN1}\) gene, transcribed in the direction from the telomere to the centromere, is located at most 200 kb centromeric to \(\textit{D11S4940}\) and at most 100 kb telomeric to \(\textit{PYGM}\).\(^{14}\) \(\textit{D11S4946}\) is located 0.5 kb upstream from the \(\textit{MEN1}\) transcription start point.\(^{3,14}\) Genomic DNA was amplified by PCR with a pair of primers, one of which was labeled with FITC. The primer sequences and PCR conditions were obtained from the Genome Database (http://gdbwww.gdb.org/). The size of the PCR product was estimated by electrophoresis using the automated genetic analyzer.

Polymorphic markers \(\textit{D11S4940}\) and \(\textit{PYGM}\) showed heterozygosity in the three affected individuals (Fig. 3B). In contrast, each individual showed only a single polymorphic length for \(\textit{D11S4946}\) (Fig. 3B). The length of this marker in the son (patient c) differed from that in his mother (patient b), excluding the possibility of homozygosity and indicating the hemizygous state of this marker site. The other affected member (patient a) also showed a single peak for this marker, which is also compatible with hemizygosity of the marker site. These findings, together with the findings of hemizygosity of codon 541 and the reduced gene dosage, indicate that the deletion was at least 7 kb in size and spanned the entire \(\textit{MEN1}\) gene, although we could not exclude the formal but unlikely possibility that the detected deletions were not contiguous. The deletion was estimated to be at most 300 kb in size, because heterozygosity of the \(\textit{D11S4940}\) and \(\textit{PYGM}\) sites was retained. Contig maps encompassing the \(\textit{MEN1}\) gene have been constructed in several laboratories,\(^{14,15}\) and the establishment of the fine physical map with these contigs should allow a more precise estimation of the deletion size even with a small amount of DNA extracted from clinical materials. Similar large germline deletions were previously described.

Table I. \(\textit{MEN1}\) Gene Dosage Determined by Double Competitive PCR

| DNA sample       | Gene dose quotient \(^a\) | Gene dose \(^b\) |
|------------------|---------------------------|-----------------|
| Patient a\(^c\)  | 0.51±0.04                 | 0.8             |
| Patient b\(^d\)  | 0.51±0.02                 | 0.8             |
| Patient c\(^e\)  | 0.56±0.04                 | 0.9             |
| \(\textit{MEN1}\) heterozygote\(^f\)| 1.02±0.07               | 1.7             |
| Normal control   | 1.22±0.09                 | 2.0             |

\(^a\) Gene dose quotient = (genomic \(\textit{MEN1}\) peak area/\(\textit{MEN1}\) standard peak area) ÷ (genomic \(\textit{TR3}\) peak area/\(\textit{TR3}\) standard peak area). The values are expressed as the mean and the standard error (\(n=3\)).

\(^b\) Gene dose was normalized to the normal control whose gene dose was 2, and was calculated using the following formula: Gene dose = (gene dose quotient of the subject DNA/gene dose quotient of the control DNA) × 2.

\(^c\) \(\textit{MEN1}\) patients illustrated in Fig. 1.

\(^d\) A familial \(\textit{MEN1}\) patient previously shown to be heterozygous for a \(\textit{MEN1}\) point mutation in exon 9.\(^7\)

![Fig. 3. DNA polymorphism analysis using polymorphic DNA markers. A, Schematic diagram of chromosome 11q13 illustrating the relative positions of three polymorphic markers (open bars). Positions of the \(\textit{MEN1}\) gene (closed box), primers used in the gene dosage assay (2-5 and 2-2) and codon 541 in exon 10 are also shown. The distance between \(\textit{D11S4940}\) and \(\textit{PYGM}\) is less than 300 kb. The \(\textit{MEN1}\) gene is transcribed in the direction from telomere (Tel) to centromere (Cen). B, Polymorphic PCR products arising from the marker sites shown above. The panels a, b and c show the electropherograms of the PCR products of the patients a, b and c illustrated in Fig. 1, respectively. Positions of the size markers are indicated by arrows with the marker size.](image-url)
for hereditary syndromes including neurofibromatosis type 2. Wilms’ tumors and hereditary non-polyposis colorectal cancer.

This is the first report of a large germline MEN1 deletion which could not be detected by PCR-based sequencing analysis. These findings indicate that the possibility of a large heterozygous deletion of the MEN1 gene should be considered when a negative result is obtained in PCR-based screening of the limited gene regions. This conclusion should be generally applicable to DNA testing of familial cancer syndromes caused by a tumor suppressor gene, and therefore, individuals suspected of having these syndromes should also be examined for a large germline deletion of the responsible genes.

We have demonstrated the usefulness of gene dosage assay by the use of refined double competitive PCR for detection of a large heterozygous gene deletion. This assay yielded reproducible results and allowed the detection of gene deletion where a specific polymorphic DNA marker was not available. Because this quantitative assay does not require comparison between multiple individuals in a pedigree, it can be used even for individuals whose kindred is not available for genetic analysis. This technique will, therefore, be useful in a clinical setting as a reliable detection method of a large germline deletion of tumor suppressor genes. The possible existence of such a deletion has generally been neglected because of the lack of appropriate technology to detect it.

We thank Drs. Misao Ohki and Fumie Hosoda, Radiobiology Division, National Cancer Center Research Institute, for valuable advice. This work was supported in part by Grants-in-Aid from the Ministry of Health and Welfare, Japan, for Cancer Research (6-29 and 9-32) and for the Second-Term Comprehensive 10-Year Strategy for Cancer Control, and by a Grant-in-Aid for Cancer Research from Foundation for Promotion of Cancer Research.

(Received September 26, 1997 / Revised October 27, 1997 / Accepted October 30, 1997)

REFERENCES

1) Yamaguchi, K., Kameya, T. and Abe, K. Multiple endocrine neoplasia type 1. *Clin. Endocrinol. Metab.*, 9, 261–284 (1980).

2) Trump, D., Farren, B., Wooding, C., Pang, J. T., Besser, G. M., Buchanan, K. D., Edwards, C. R., Heath, D. A., Jackson, C. E., Jansen, S., Lips, K., Monson, J. P., O’Halloran, D., Sampson, J., Shale, S. M., Wheeler, M. H., Zink, A. and Thakker, R. V. Clinical studies of multiple endocrine neoplasia type 1 (MEN1). *Q. J. Med.*, 89, 653–669 (1996).

3) Chandrasekharappa, S. C., Guru, S. C., Manickam, P., Olufemi, S.-E., Collins, F. S., Emmert-Buck, M. R., Debelenko, L. V., Zhuang, Z., Lubensky, I. A., Liotta, L. A., Crabtree, J. S., Wang, Y., Roe, B. A., Weisemann, J., Boguski, M. S., Agarwal, S. K., Kester, M. B., Kim, Y. S., Heppner, C., Deng, G., Yu, M. and Smith, H. S. An improved method of competitive PCR for detection of a large heterozygous gene deletion. *Science*, 276, 404–407 (1997).

4) Larsson, C., Skogseid, B., Öberg, K., Nakamura, Y. and Nordenskjöld, M. Multiple endocrine neoplasia type 1 gene maps to chromosome 11 and is lost in insulinares. *Nature*, 332, 85–87 (1988).

5) Agarwal, S. K., Kester, M. B., Debelenko, L. V., Heppner, C., Emmert-Buck, M. R., Skarulis, M. C., Doppman, J. L., Kim, Y. S., Lubensky, I. A., Zhuang, Z., Green, J. S., Guru, S. C., Manickam, P., Olufemi, S.-E., Liotta, L. A., Chandrasekharappa, S. C., Collins, F. S., Heppner, A. M., Burns, A. L. and Marx, S. J. Positional cloning of the gene for multiple endocrine neoplasia-type 1. *Science*, 276, 1169–1175 (1997).

6) The European Consortium on MEN1. Identification of the multiple endocrine neoplasia type 1 (MEN1) gene. *Hum. Mol. Genet.*, 6, 1177–1183 (1997).

7) Shimizu, S., Tsukada, T., Futami, H., Ui, K., Kameya, T., Kawanaka, M., Uchiyama, S., Aoki, A., Yasuda, H., Kawano, S., Ito, Y., Kanbe, M., Obara, T. and Yamaguchi, K. Germline mutations of the MEN1 gene in Japanese kindreds with multiple endocrine neoplasia type 1. *Jpn. J. Cancer Res.*, 88, 1029–1032 (1997).

8) Aoki, A., Tsukada, T., Yasuda, H., Kayashima, S., Nagase, T., Ito, T., Suzuki, T., Matsuoka, S., Kuwabara, N., Yoshimoto, K. and Yamaguchi, K. Multiple endocrine neoplasia type 1 presented with manic-depressive disorder: a case report with an identified MEN1 gene mutation. *Jpn. J. Clin. Oncol.*, 27, 419–422 (1997).

9) Deng, G., Yu, M. and Smith, H. S. An improved method of competitive PCR for quantitation of gene copy number. *Nucleic Acids Res.*, 21, 4848–4849 (1993).

10) Celi, F. S., Cohen, M. M., Antonarakis, S. E., Wertheimer, E., Roth, J. and Shuldiner, A. R. Determination of gene dosage by a quantitative adaptation of the polymerase chain reaction (gd-PCR): rapid detection of deletions and duplications of gene sequences. *Genomics*, 21, 304–310 (1994).

11) Chang, C., Kokontis, J., Liao, S. S. and Chang, Y. Isolation and characterization of human TR3 receptor: a member of steroid receptor superfamily. *J. Steroid Biochem.*, 34, 391–395 (1989).

12) Hazel, T. G., Nathans, D. and Lau, L. F. A gene inducible by serum growth factors encodes a member of the steroid and thyroid hormone receptor superfamiliy. *Proc. Natl. Acad. Sci. USA*, 85, 8444–8448 (1988).

13) Heppner, C., Kester, M. B., Agarwal, S. K., Debelenko, L. V., Emmert-Buck, M. R., Guru, S. C., Manickam, P., Olufemi, S.-E., Skarulis, M. C., Doppman, J. L., Alexander, R. H., Kim, Y. S., Sagar, S. K., Lubensky, I. A., Zhuang, Z., Liotta, L. A., Chandrasekharappa, S. C., Collins, F. S., Spiegel, A. M., Burns, A. L. and Marx, S. J. Somat
Germline Deletion of *MEN1* Gene

14) Guru, S. C., Olufemi, S.-E., Manickam, P., Cummings, C., Gieser, L. M., Pike, B. L., Bittner, M. L., Jiang, Y., Chinault, A. C., Nowak, N. J., Brzozowska, A., Crabtree, J. S., Wang, Y., Roe, B. A., Weisemann, J. M., Boguski, M. S., Agarwal, S. K., Burns, A. L., Spiegel, A. M., Marx, S. J., Flejter, W. L., de Jong, P. J., Collins, F. S. and Chandrasekharappa, S. C. A 2.8-Mb clone contig of the multiple endocrine neoplasia type 1 (*MEN1*) region at 11q13. *Genomics*, 42, 436–445 (1997).

15) Kitamura, E., Hosoda, F., Fukushima, M., Asakawa, S., Shimizu, N., Imai, T., Soeda, E. and Ohki, M. A 3-Mb sequence-ready contig map encompassing the multiple disease gene cluster on chromosome 11q13.1-q13.3. *DNA Res.*, 4, 281–289 (1997).

16) Watson, C. J., Gaunt, L., Evans, G., Patel, K., Harris, R. and Strachan, T. A disease-associated germline deletion maps the type 2 neurofibromatosis (NF2) gene between the Ewing sarcoma region and the leukemia inhibitory factor locus. *Hum. Mol. Genet.*, 2, 701–704 (1993).

17) Davis, L. M., Stallard, R., Thomas, G. H., Couillin, P., Junien, C., Nowak, N. J. and Shows, T. B. Two anonymous DNA segments distinguish the Wilms' tumor and aniridia loci. *Science*, 241, 840–842 (1988).

18) Nyström-Lahti, M., Kristo, P., Nicolaides, N. C., Chang, S.-Y., Aaltonen, L. A., Moisio, A.-L., Järvinen, H. J., Mecklin, J.-P., Kinzler, K. W., Vogelstein, B., De la Chapelle, A. and Peltomäki, P. Founding mutations and Alu-mediated recombination in hereditary colon cancer. *Nat. Med.*, 1, 1203–1206 (1995).