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Mutation Analysis of the *PTEN/MMAC1* Gene in Japanese Patients with Cowden Disease

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Cowden disease (CD), also known as multiple hamartoma syndrome, is an autosomal dominant cancer syndrome associated with high risk of breast and thyroid cancer. Recently, germline mutations in *PTEN/MMAC1*, which has nine exons encoding a dual specificity phosphatase with homology to tensin and auxilin, have been identified on chromosome 10q23 in some 40 to 80% of CD patients. Our polymerase chain reaction amplification and sequence analysis of all coding regions identified five different mutations including four novel germline mutations among 5 of 12 unrelated Japanese CD patients. The novel findings included a missense mutation (G→T) at nucleotide 1004 in exon 8 resulting in an arginine-to-leucine change at codon 335 (R335L), two novel splice-site mutations (209+1delGT and 209+1delGTAA) in intron 3, and insertion of G at nucleotide 632 in exon 6 (632insG). We also detected a nonsense mutation (C→T) at nucleotide 697 producing R233X in exon 7, which has been reported previously. From reported phenotypic data concerning CD patients from five different families who had the R233X mutation, it may be suggested that R233X mutation correlates with macrocephaly. Although previous reports have implicated exon 5 as a "hot spot," we found no mutation in exon 5.

Key words: Cowden disease — *PTEN/MMAC1* gene — Germline mutations

Cowden disease (CD), also known as multiple hamartoma syndrome, is an autosomal dominant cancer syndrome carrying a high risk of tumors of the breast, thyroid, and skin.[1, 2] Recently, Nelen *et al*. have confirmed that the *PTEN* gene on chromosome 10q23 is the gene responsible for CD.[3] The same gene was isolated independently by Steck *et al*. and designated *MMAC1*; they suggested that it was a tumor suppressor gene, because coding-region mutations were observed in a number of cell lines or tumor specimens from gliomas and prostate, kidney, and breast carcinomas.[4] Subsequently, mutations have been identified in several specimens or cell lines, including those from glioblastoma, prostate carcinoma, endometrial carcinoma, and malignant melanoma.[5–9]

PTEN/MMAC1 comprises nine exons encoding a 403-amino-acid open reading frame. Sequence analysis of the open reading frame has demonstrated a protein tyrosine phosphatase domain sharing closest homologies with protein tyrosine phosphatases called dual-specificity phosphatases. These remove phosphate groups from tyrosine as well as serine and threonine.[4, 5] *In vitro*, *PTEN/MMAC1* has been shown to dephosphorylate substrates at tyrosine, serine, and threonine.[10]

Liaw *et al*. first identified germline mutations of the *PTEN/MMAC1* gene in four of five families with Cowden disease.[11] Subsequently about 60 mutations have been reported.[12–23] In this study we performed germline *PTEN/MMAC1* mutation analysis in Japanese CD patients and their relatives.

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MATERIALS AND METHODS

Patients CD was diagnosed using criteria formulated by the International CD Consortium.[3] Subjects were from 12 unrelated Japanese families, each including one of the CD patients.

DNA amplification Genomic DNA was isolated from whole blood of patients and their family members. Complete exons and their associated splice junctions were amplified by polymerase chain reaction (PCR). Primers used for PCR were as previously described.[4, 11] Reactions were carried out in 50 μl of 10 m*M* Tris-HCl (pH 8.9), containing 50-μM concentrations of each of four deoxynucleoside triphosphates, 80 m*M* KCl, 1.5 m*M* $MgCl_2$, and 40 pmol of each oligonucleotide. Two microunits of Tth DNA polymerase was used. Amplification was performed with 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. PCR products were loaded onto and run through 1% agarose gels. Appropriate bands were cut from the gel and were purified using microconcentrators (Microcon; Millipore, Bedford, MA).

Sequence analysis We performed sequencing of all exons and their associated splice junctions by using a nonradioactive sequencing method (Sequencing High-Plus; Toyobo, Osaka). PCR product was added to the reaction mixture (17 μl) containing 5 pmol of each primer, 50 m*M* Tris-

HCl (pH 8.8), 1.5 to 4 m*M* $MgCl_2$, 10-μM concentrations of each of four dNTPs (7-deaza-dGTP, dATP, dTTP, and dCTP) and 4 units of ΔTth DNA polymerase. Aliquots (4 μl) were added to four tubes, each containing 2 μl of either 60 μM biotin-9-ddGTP, 90 μM biotin-9-ddATP, 900 μM biotin-9-ddUTP, or 600 μM biotin-9-ddCTP. The mixture was incubated for 30 cycles of 30 s at 95°C, 15 s at 58°C, and 4 min at 60°C using the Program Temp Control System PC-700 (ASTEC, Fukuoka). The reaction was terminated with 4 μl of stop solution (deionized formamide containing 0.1% bromophenol blue, 0.1% xylene cyanol, and 20 m*M* disodium EDTA, pH 7.0).

The sequencing reaction products (2 μl each) were electrophoresed on a 6% polyacrylamide gel and blotted onto

Table I. Summary of Patient Data and Description of Mutations

Patient	Sex	Age	Skin	Thyroid	Breast	CNS	Mutation and predicted effect
1	F	48	Trichilemmomas Acral keratoses Papillomatous papules Mucosal lesions	Follicular adenoma	Adenocarcinoma	–	CGA to CTA: R335L (exon 8)
2	F	38	Trichilemmomas Acral keratoses Papillomatous papules Mucosal lesions Fibromas	Goiter	–	–	632insG: Frameshift (exon 6)
3	M	40	Trichilemmomas Acral keratoses Papillomatous papules Mucosal lesions	–	–	Epilepsy	IVS3+1delGT: Splice-donor defect (IVS3)
4	F	62	Trichilemmomas Acral keratoses Papillomatous papules Mucosal lesions	Adenocarcinoma	Adenocarcinoma	–	IVS3+1delGTAA: Splice-donor defect (IVS3)
5	M	28	Trichilemmomas Acral keratoses Papillomatous papules Mucosal lesions	Follicular adenoma	Gynecomastia	Macrocephaly	CGA to TGA: R233X (exon 7)
6	M	41	Trichilemmomas Acral keratoses Papillomatous papules Mucosal lesions	Follicular adenoma	–	–	ND
7	M	42	Trichilemmomas Acral keratoses Papillomatous papules	–	–	–	ND
8	F	30	Acral keratoses Mucosal lesions Fibromas	Thyroiditis	–	Mental retardation	ND
9	F	50	Mucosal lesions	Goiter	Adenocarcinoma	–	ND
10	F	51	Trichilemmomas Acral keratoses	Follicular adenoma	Adenocarcinoma	–	ND
11	F	42	Trichilemmomas Acral keratoses Papillomatous papules Mucosal lesions Fibromas	Goiter	–	Macrocephaly	ND
12	F	55	Trichilemmomas Acral keratoses Papillomatous papules Mucosal lesions	Follicular adenoma	Multiple cysts	Lhermitte-Duclos Macrocephaly	ND

CNS, central nervous system; F, female; M, male; –, absent; ND, not detected; IVS, intervening sequence.

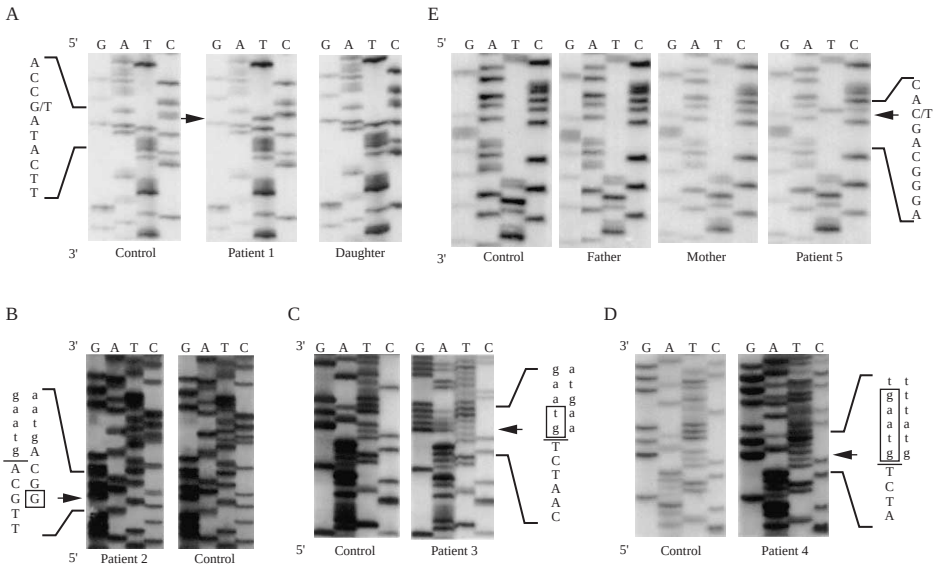

Fig. 1. Sequence analysis of the *PTEN/MMAC1* gene in Japanese patients with Cowden disease and their families. Partial sequences of the exons and exon-intron boundaries show A, G-to-T transversion at nucleotide 1004 (R335L); B, insertion of G at nucleotide 632 (632insG); C, deletion of GT of the splice-donor site of exon 3 (IVS3+1delGT); D, deletion of GTAA of the splice-donor site of exon 3 (IVS3+1delGTAA); and E, C-to-T transversion at nucleotide 697 (R233X). Relatives of patients 1 and 5 show only the wild-type allele.

a dried, positively charged nylon membrane (Imaging High-Chemilumi; Toyobo) according to the manufacturer's instructions. The chemiluminescent detection reaction was performed using a horizontal rolling apparatus (rolling mixer; Toyobo) at room temperature according to the manufacturer's instructions.[24)]

RESULTS

Constitutive DNA was collected from 12 affected members and from nonaffected members of CD families to be analyzed for *PTEN/MMAC1* mutations. We identified five different mutations including four novel germline mutations of the *PTEN/MMAC1* gene among 5 of 12 CD patients (Table I).

In patient 1, we detected a G-to-T substitution resulting in Arg-to-Leu alteration at codon 335 (R335L) in exon 8. To determine whether the missense mutation at codon 335 might represent a common polymorphism, we sequenced exon 8 in 50 unrelated Japanese individuals and found no evidence of this mutation in 100 alleles. Thus, this variant is likely to be a germline mutation. We also sequenced exon 8 in the proband's daughter, who did not have any clinical features of CD, but the mutation was not detected (Fig. 1A).

In patient 2, direct sequence analysis of the PCR-amplified genomic DNA product containing exon 6 revealed an insertion of C at nucleotide 632 in codon 211 (632insC; Fig. 1B). This mutation leads to a frame-shift and premature termination of the protein at codon 242, the 31st codon after the frame-shift; this is expected to be highly deleterious to the function of the protein.

We detected two novel splice-site mutations among patients 3 and 4. In patient 3, the first and second nucleotides (GT), at the splice-donor site of exon 3 were deleted (Fig. 1C). In patient 4, the first to the fourth nucleotides (GTAA), were deleted at the same splice-donor site (Fig. 1D). Using mismatch primers, restriction fragment length polymorphism analysis of 100 alleles from 50 unaffected subjects was performed; we could not identify the mutant allele in any individual (data not shown). Although we could not examine the effect of these mutations on RNA processing, they would lead either to exon omission or to insertion of intron 3 into the transcript.

In patient 5, a C-to-T substitution produced a nonsense alteration at codon 233 (R233X) in exon 7, which has been previously reported. Examinations of genomic DNA from the proband's unaffected parents failed to demonstrate this missense mutation, which suggested that the mutation arose as a *de novo* event in the *PTEN/MMAC1* gene in the patient (Fig. 1E).

We also fully sequenced all coding regions of the *PTEN/MMAC1* gene in the remaining seven patients; no mutations were detected.

Table II. Phenotypes Reported with R233X Mutation in CD Patients

Patient designation	Sex	Skin	Thyroid	Breast	CNS	Reference
C	F	Trichilemmoma	Multinodular Goiter	–	Macrocephaly	Liaw *et al*. (1997)
II-1	F	+	–	Adenocarcinoma	–	Lynch *et al*. (1997)
CDbn	M/F	+	+ (benign)	+ (males only)	U	Marsh *et al*. (1998)
CDsi	M/F	+ (benign, malignant)	+ (benign)	+ (benign)	Macrocephaly	Marsh *et al*. (1998)
5	M	Trichilemmoma Acral keratoses Papillomatous papules Mucosal lesions	Follicular adenoma	Gynecomastia	Macrocephaly	This study

CNS, central nervous system; F, female; M, male; +, present; –, absent; U, undocumented.
Skin abnormalities include trichilemmomas, acral keraroses, papillomatous papules, and mucosal lesions.

DISCUSSION

To date, more than forty cases of CD have been reported in Japan.[25] Common manifestations of Japanese CD patients are mucocutaneous lesions (observed in 100%), thyroid lesions (68%), breast lesions (50% of affected females), and gastrointestinal lesions (90%). In Western countries, although mucocutaneous lesions (90–100%), thyroid lesions (50–67%), and breast lesions (76% of affected females) are seen as frequently as in Japan, gastrointestinal lesions (40%) are seen less frequently than in Japan.[26] The reason is considered to be that lesions in the digestive tract are often silent clinically and that intestinal explorations have been conducted in only a small portion of patients.[27] Probably for the same reason, central nervous system (CNS) involvement (e.g. macrocephaly and/or Lhermitte-Duclos disease) is less prevalent in Japan than in Western countries.

PTEN/MMAC1 mutations have been reported in five of six Japanese patients with CD.[18, 28] Among 12 Japanese CD patients, we detected five different mutations in five individuals, including four novel germline mutations (one missense mutation, two splice-site mutations, and a single base-pair insertion) (Table I).

Marsh *et al*. have suggested possible phenotype-genotype associations between breast involvement (unaffected, benign or malignant) and the presence of a detectable mutation, as well as an independent association between the number of organ sites involved and the position and nature of the mutation.[16] However, Nelen *et al*. recently reported that associations of *PTEN* mutations and the occurrence of malignant breast disease could not be confirmed.[22] Although sample size was small, we also could not detect obvious genotype-phenotype associations of the kind that Marsh *et al*. had suggested.

Exon 5, coding for the active site and flanking amino acids, has been suggested as a "hot spot" for mutations in patients with CD.[12] Of the 57 mutations previously reported, 20 have been in exon 5. However, in our Japanese cases we detected no mutation in exon 5.

In one patient we also detected an R233X mutation in exon 7, previously reported by Liaw *et al*.,[11] Lynch *et al*.,[15] and Marsh *et al*.[16] This alteration occurs in a potential tyrosine phosphatase-acceptor motif.[4] From reported phenotypic data concerning CD patients from five different families who had the R233X mutation (Table II), no obvious genotype-phenotype associations have emerged with respect to skin, breast and thyroid involvement. However, as regards CNS involvement, macrocephaly was seen in three of five patients (one of unknown status). It is possible that R233X mutation correlates with macrocephaly. Confirmation of this idea awaits further functional and molecular epidemiological studies of *PTEN/MMAC1*.

We detected *PTEN/MMAC1* mutations in 5 of 12 CD patients (42%), while previous reports have claimed that germline mutations involving this gene were detected in about 40 to 80% of CD patients.[11, 12, 16, 18, 22] As no mutation was detected in 7 of our 12 Japanese CD patients, CD in these individuals might have resulted from either changes in the introns (untranslated regions) or changes in regulatory regions of the *PTEN/MMAC1* gene.[11] Alternatively other influences such as methylational silencing may have altered the expression level of the gene.[12, 16] Finally CD may prove to be a genetically heterogenous disease, as Tsou *et al*. have inferred from their linkage data.[14]

To confirm phenotype-genotype associations in CD patients and to determine the genetic mechanisms underlying CD in patients with no demonstrated coding-region mutation, further functional and molecular epidemiologic studies of the *PTEN/MMAC1* gene and perhaps others should be performed.

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