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Mutation Analysis of the Putative Tumor Suppressor Gene *PTEN/MMAC1* in Primary Breast Carcinomas

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Abstract

A novel gene was identified recently at chromosome 10q23, named *PTEN* or *MMAC1*, and based on several criteria it was designated as a potential human tumor suppressor gene. Loss of heterozygosity affecting this region of 10q is observed in several cancer types, especially glioblastoma, and inactivating mutations of the *PTEN/MMAC1* gene are found in some of these cancers as well as cell lines and xenografts. Breast cancer is among the tumor types in which mutations are documented, and germline mutations of the gene appear to be responsible for the rare autosomal dominant familial cancer syndrome known as Cowden disease, which includes breast cancer among its clinical features. To further determine the role that *PTEN/MMAC1* mutations may play in breast tumorigenesis, the entire coding region was screened for mutations in 54 unselected primary breast cancers. Two mutations were identified, a somatic 2-bp deletion in an apparently sporadic breast cancer, and a germ-line 4-bp deletion in a breast cancer patient with a clinical history consistent with Cowden disease. These data indicate that somatic mutations of *PTEN/MMAC1* occur in only a small fraction of primary breast cancers and confirm the role of this gene in the etiology of Cowden disease. Evidence is also presented suggesting that numerous polymorphisms and missense variants exist in the PTEN/MMAC1 transcript.

Introduction

A hallmark of tumor suppressor gene mutation is LOH[2] in a particular chromosomal region affecting the homologous wild-type allele. The existence of a putative tumor suppressor gene on chromosome 10q23 is implied by LOH that includes this region in most cases of glioblastoma multiforme (1–3), many cases of advanced prostate carcinoma (4–6), and in significant fractions of many other solid tumor types. The analysis of homozygous deletions affecting chromosome 10q23 in human tumor xenografts and cell lines led to the identification of a novel gene in this region designated *PTEN* (7) and *MMAC1* (8). Somatic loss of function mutations of this gene were identified in a number of glioma cell lines and primary tumors, four prostate cancer cell lines, one primary renal carcinoma, and a total of six breast cancer cell lines, xenografts, or primary tumors (7, 8). These findings, together with predicted protein sequence motifs suggesting its function as a protein tyrosine or dual-specificity phosphatase, support the classification of *PTEN/MMAC1* as a new tumor suppressor gene with potential involvement in a wide variety of human cancer types.

The existence of somatic mutations in breast cancers is of particular interest, as a rare autosomal dominant hereditary cancer syndrome known as Cowden disease, which includes breast cancer among its clinical manifestations, maps to chromosome 10q22–23 (9). The presence of *PTEN/MMAC1* germ-line mutations in four of five Cowden disease kindreds indicates that inherited mutations of this gene are likely responsible for this syndrome (10). Taken together, the above findings suggest that *PTEN/MMAC1* may play a significant role in breast tumorigenesis. However, initial reports suggest that the mutation frequency of *PTEN/MMAC1* may be higher in tumor cell lines and xenografts than in primary specimens, and only 14 primary breast cancers, all of which were preselected for 10q23 LOH, were examined for mutations (7, 8). The purpose of this study was to determine the extent of involvement of this gene in breast cancer by examining the entire *PTEN/MMAC1* coding region for mutations in a relatively large, unselected series of primary breast tumors.

Materials and Methods

Fifty-four primary breast carcinomas, representative for grade, stage, and histological subtype, were obtained as fresh-frozen biopsy specimens from patients diagnosed at the Memorial Sloan-Kettering Cancer Center from 1988 through 1991. Total RNA was prepared from pulverized tissue specimens using the TRIzol phenol and guanidinium isothiocyanate reagent (Life Technologies, Inc.). First-strand cDNA was synthesized from 1 μg of total RNA using random hexamer primers and Superscript II MMLV-H− reverse transcriptase (Life Technologies). Mutation screening was performed by SSCP analysis of the entire *PTEN/MMAC1* coding region using PCR primers designed to amplify 13 overlapping products from cDNA (Table 1). Each PCR was carried out in a volume of 20 μl containing 50 ng of cDNA; 1.5 mM $MgCl_2$; 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 200 μM each of dATP, dGTP, and dTTP; 20 μM dCTP, 1 μCi of [α-^{32}P]dCTP (6000 Ci/mmol; DuPont NEN Research Products); each primer at 0.8 μM; and 1 unit of Taq polymerase (Perkin-Elmer). Amplification was performed in a Perkin-Elmer 9600 thermal cycler for 35 cycles consisting of 20 s at 94°C, 20 s at 55°C, and 30 s at 72°C. PCR products were diluted 1:10 in denaturing loading buffer [95% formamide, 10 mM EDTA (pH 8.0), 0.02% xylene cyanol FF, and 0.02% bromphenol blue], heated at 94°C for 5 min, and placed on ice, and 4 μl were subjected to electrophoresis. Gels for SSCP analysis consisted of 0.5× mutation detection enhancement solution (FMC BioProducts) and 0.6× TBE buffer, and were run in 0.6× TBE buffer at 6–8 W for 16 h at room temperature. Gels were dried and exposed to Hyperfilm MP autoradiography film (Amersham Corp.) for 4–16 h.

For sequence analysis, variant and wild-type bands were excised from SSCP gels following autoradiography and suspended in 50 μl of TE [10 mM Tris (pH 8)-1 mM EDTA] buffer for 1 h at room temperature. One μl of the eluted DNA sample was used as a template for PCR amplification under conditions identical to those described above for SSCP analysis, except that all deoxynucleotide triphosphates were at 200 μM and radiolabeled dCTP was omitted. Each PCR product was electrophoresed in its entirety in NuSieve 3:1 agarose (FMC BioProducts), visualized with ethidium bromide, excised from the gel, and purified using the Qiaex II gel extraction kit (Qiagen). Two ng of each DNA template were sequenced using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham). All products were sequenced in both directions, using one or the other PCR primer. Following the sequencing reactions, 6 μl of each termination reaction were added to 4 μl of stop solution and heated to 70°C for 10 min, and 4 μl were loaded into each gel lane. Sequencing gels consisted of 6% polyacrylamide and 7 M urea in TBE buffer. After

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[2] The abbreviations used are: LOH, loss of heterozygosity; SSCP, single-strand conformation polymorphism; TBE, Tris-borate EDTA.

Table 1 *Primers for the PCR amplification of cDNA for PTEN/MMAC1 analysis*

Product No.	Nucleotide position[a]	Primers[b]	Product length
1	(−25)–118	F: TTTCTTCAGCCACAGGCTCC	143
		R: CTGCAGGAAATCCCATAGCA	
2	63–223	F: CGACTTAGACTTGACCTATA	161
		R: GTCTTTCAGCACAAAGATTG	
3	171–326	F: GGATTCAAAGCATAAAAACC	156
		R: TCAAGATCTTCACAAAAGGG	
4	277–429	F: CATAACCCACCACAGCTAGA	153
		R: GCCCCGATGTAATAAATATG	
5	380–528	F: GAAAGGGACGAACTGGTGTAA	149
		R: ATACACATAGCGCCTCTGACT	
6	467–626	F: GGGAAGTAAGGACCAGAGACA	160
		R: CCGCCACTGAACATTGGAATA	
7	567–713	F: ACCAGTGGCACTGTTGTTTCA	147
		R: AACTTGTCTTCCCGTCGTGT	
8	651–793	F: CTGCCAGCTAAAGGTGAAGA	143
		R: GCATCTTGTTCTGTTTGTGG	
9	737–895	F: CGTTACCTGTGTGTGGTGATA	159
		R: CTTGATCACATAGACTTCCA	
10	845–1006	F: GACCAGAGGAAACCTCAGAA	162
		R: ATCGGTTGGCTTTGTCTTTA	
11	920–1066	F: GCGTGCAGATAATGACAAGG	147
		R: TTGACGGCTCCTCTACTGTT	
12	1024–1166	F: AAGGTGAAGCTGTACTTCAC	143
		R: TTCTCTGGATCAGAGTCAGTG	
13	1097–(+36)	F: CACCAGATGTTAGTGACAATG	149
		R: CATGGTGTTTTATCCCTCTTG	

[a] Based on GenBank accession no. U92436, with position 1 corresponding to the first base of the start codon, and position +1 corresponding to the first base of the stop codon.
[b] PCR primer sequences used to generate a product of the indicated size, listed in a 5′-to-3′ orientation. F, forward; R, reverse.

Table 2 *Summary of PTEN/MMAC1 mutations and sequence variants*

Tumor No.	Exon	Codon(s)	Nucleotide(s)	Mutation/variant
Mutations				
66	9	385	1154–1155	del CT (somatic)
94	8	317–318	951–954	del ACTT (germline)
mRNA sequence variants				
	3	68	203	TAC(Tyr)→TGC(Cys)
	4	81	242	TTT(Phe)→TCT(Ser)
	4	83	248	TGC(Cys)→TAC(Tyr)
	4/5	86	258	GCA(Ala)→GCG(Ala)
	5	135	405	ATA(Ile)→ATT(Ile)
	5	136	407	TGT(Cys)→TAT(Tyr)
	6	177	531	TAT(Tyr)→TAC(Tyr)
	6	182	544	TTA(Leu)→GTA(Val)
	6	186	556	CTG(Leu)→GTG(Val)
	6	208	623	GGC(Gly)→GTC(Val)
	7	224	672	ATA(Ile)→ATG(Met)

electrophoresis at 70 W for 2–3 h, gels were fixed in 10% methanol/10% acetic acid, dried, and subjected to autoradiography as above for 16 h.

Genomic DNA was prepared from fixed and embedded tissue specimens as described previously (11) or from fresh frozen tissues using standard procedures (12). Mutation screening of *PTEN/MMAC1* was performed exactly as above using genomic DNA as template for SSCP analysis and intron-based PCR primers as described (8). Mutations identified by analysis of tumor cDNA were confirmed by SSCP and sequence analyses of genomic DNA from each patient, and mutations were classified as germ line or somatic based on the presence or absence of the mutation in corresponding normal, uninvolved tissue specimens.

Results and Discussion

Mutation screening of the entire coding region of *PTEN/MMAC1* revealed mutations in 2 of 54 (4%) primary breast cancers (Table 2). The first mutation was a 2-bp deletion affecting codon 385 (TCT/GAT→TGA/T), resulting in a premature stop codon at the same location (Fig. 1*A*). This mutation was determined to be somatic in origin, occurring in a stage II infiltrating lobular carcinoma from a 71-year-old patient with no family history of cancer. The second mutation was a 4-bp deletion affecting codons 317 and 318 (GTA/CTT→GT), resulting in a premature stop at codon 319 (Fig. 1*B*). This mutation was determined to be present in the germ line of a 32-year-old patient with a medical history of Cowden disease. In addition to multiple trichilemmomas, oral papillomatosis, and gastrointestinal polyps, she had bilateral, stage II, moderately differentiated infiltrating ductal carcinomas. Subsequent to the breast cancer diagnoses, she developed endometrial cancer at age 34 and had a family history of breast cancer. Both of the above mutations were characterized by SSCP and sequencing analyses of cDNA and genomic DNA from the respective breast tumors. No additional mutations were identified in this series of breast cancers using either cDNA or genomic DNA to screen for sequence variants.

In addition to these mutations, a number of polymorphisms and missense variants were observed in breast tumor cDNA samples (Table 2). The variants were present in exons 3–7 and appeared to be relatively common, given that they occurred to some extent in most of the tumors examined. It is not clear why these mRNA variants were not noted in the first three reports on the characterization of the *PTEN/MMAC1* gene (7, 8, 10).

These data confirm the role of germ-line *PTEN/MMAC1* mutations in the etiology of Cowden disease and indicate that somatic mutations of *PTEN/MMAC1* are involved in only a small fraction of sporadic breast carcinomas. Previous suggestions that this gene is involved in breast tumorigenesis were founded on the identification of six breast cancers harboring mutations (7, 8); however, two of these six were tumor xenografts, and two were cell lines (7). The two primary cancers with mutations were from a group of 14 tumors that had been preselected for LOH affecting the 10q23 region (8). Thus, *PTEN/MMAC1* mutations may be more common in gliomas and prostate cancers, or alternatively, occur at a later stage in tumor progression or metastasis, as suggested by the somewhat higher incidence in cell

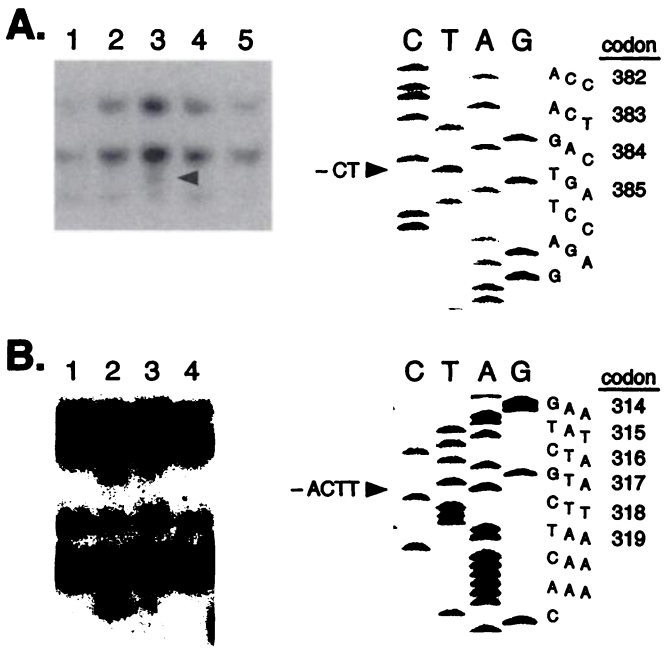

Fig. 1. *A*, detection of somatic 1154delCT mutation by SSCP and sequence analyses. *Left*, autoradiogram of SSCP gel containing PCR product 13 shows band of altered mobility *(Lane 3)*. The altered band was excised from the gel and sequenced. *Right*, autoradiogram of sequencing gel reveals a 2-bp deletion in codon 385, resulting in a premature stop (TGA) at the resultant codon 385. *B*, detection of germ-line 951delACTT mutation by SSCP and sequence analyses. *Left*, autoradiogram of SSCP gel containing PCR product 11 shows bands of altered mobility *(Lane 2)*. The altered bands were excised from the gel and sequenced. *Right*, autoradiogram of sequencing gel reveals a 4-bp deletion in codons 317–318, resulting in a premature stop (TAA) at resultant codon 319.

lines and xenografts. All of the breast cancers included in our study were primary site tumors, most of which were early stage (I or II).

The observations reported here raise several questions regarding the possible relationship of *PTEN/MMAC1* to breast and other cancers. First, it will be important to further characterize the mRNA missense variants described in this report and the role, if any, that these variants may play in breast tumorigenesis. Second, the fact that early-onset endometrial cancer occurred in the patient with Cowden disease is noteworthy. At least one allelotype analysis of endometrial cancers has shown a high frequency of LOH on chromosome 10q, with a minimal region of deletion defined at 10q23–q26, which includes the *PTEN/MMAC1* locus (13). The possible role of *PTEN/MMAC1* mutations in sporadic endometrial tumorigenesis should be studied. Finally, it should be emphasized that the PCR-based mutation screen in this study was designed to detect small frameshift and point mutations and would not have detected large genomic deletions encompassing the *PTEN/MMAC1* gene. Two breast tumor xenografts were reported to contain such large deletions (7), raising the possibility that a larger fraction of sporadic breast cancers may be affected by *PTEN/MMAC1* mutations than our results suggest, if genomic deletion is a common mechanism for its mutational inactivation. Such a mechanism appears to be relevant to the involvement of the *CDKN2* (p16/MTS1) tumor suppressor gene in many human cancers (14–16).

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