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 germ-line 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 LOH2 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 DNA; 1.5 mM MgCl2; 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-32P-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 cyanole 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 (FMCI 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 8.0)-1 mM EDTA] buffer for 1 h at room temperature. One μl of the diluted 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 μl urea in TBE buffer. After
The first mutation was a 2-bp deletion affecting codon 385 (TAC(Tyr)→TGC(Cys)), resulting in a premature stop at codon 385 (Fig. 1B). This mutation was determined to be present in the germline 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 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.

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

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Nucleotide position</th>
<th>Primers</th>
<th>Primer sequence used to generate a product of the indicated size, listed in a 5'-to-3' orientation.</th>
<th>Product length</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(−25)−118</td>
<td>F: TTTCTTCTGACCGACGCTCC</td>
<td>R: CTCAGGACGACCCCTGATGCA</td>
<td>143</td>
</tr>
<tr>
<td>2</td>
<td>63−223</td>
<td>F: GAGCTTACGCTGCTGATATA</td>
<td>R: CCGCAGGACGACCCCTGATGCA</td>
<td>161</td>
</tr>
<tr>
<td>3</td>
<td>171−326</td>
<td>F: GAAGCTCAGCTGCTGATATA</td>
<td>R: CCGCAGGACGACCCCTGATGCA</td>
<td>156</td>
</tr>
<tr>
<td>4</td>
<td>277−429</td>
<td>F: CATACAACACACAGCTGATAG</td>
<td>R: GCCCGATTGTTAAATCTAGTAA</td>
<td>153</td>
</tr>
<tr>
<td>5</td>
<td>380−528</td>
<td>F: GAAGCTCAGCTGCTGATATA</td>
<td>R: CCGCAGGACGACCCCTGATGCA</td>
<td>149</td>
</tr>
<tr>
<td>6</td>
<td>467−626</td>
<td>F: GAGCTTACGCTGCTGATATA</td>
<td>R: CCGCAGGACGACCCCTGATGCA</td>
<td>160</td>
</tr>
<tr>
<td>7</td>
<td>567−713</td>
<td>F: GAAGCTCAGCTGCTGATATA</td>
<td>R: CCGCAGGACGACCCCTGATGCA</td>
<td>147</td>
</tr>
<tr>
<td>8</td>
<td>651−793</td>
<td>F: GAGCTTACGCTGCTGATATA</td>
<td>R: CCGCAGGACGACCCCTGATGCA</td>
<td>159</td>
</tr>
<tr>
<td>9</td>
<td>737−895</td>
<td>F: GAGCTTACGCTGCTGATATA</td>
<td>R: CCGCAGGACGACCCCTGATGCA</td>
<td>143</td>
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<tr>
<td>10</td>
<td>845−1066</td>
<td>F: GAGCTTACGCTGCTGATATA</td>
<td>R: CCGCAGGACGACCCCTGATGCA</td>
<td>147</td>
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<tr>
<td>11</td>
<td>920−1066</td>
<td>F: GAGCTTACGCTGCTGATATA</td>
<td>R: CCGCAGGACGACCCCTGATGCA</td>
<td>147</td>
</tr>
<tr>
<td>12</td>
<td>1024−1166</td>
<td>F: GAGCTTACGCTGCTGATATA</td>
<td>R: CCGCAGGACGACCCCTGATGCA</td>
<td>143</td>
</tr>
<tr>
<td>13</td>
<td>1097+(+36)</td>
<td>F: GAGCTTACGCTGCTGATATA</td>
<td>R: CCGCAGGACGACCCCTGATGCA</td>
<td>149</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Exon</th>
<th>Codon(s)</th>
<th>Nucleotide(s)</th>
<th>Mutation/variant</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td>66</td>
<td>9</td>
<td>385</td>
<td>1154–1155</td>
</tr>
<tr>
<td>B.</td>
<td>94</td>
<td>8</td>
<td>317−318</td>
<td>951–954</td>
</tr>
</tbody>
</table>

- **Mutations**
  - Exons 66 and 94
  - Nucleotide(s) 385 and 317–318
  - Mutation/variant del CT and del ACTT

- **mRNA sequence variants**
  - Codons 365, 407, 672
  - Nucleotide(s) TAC(Tyr), TGT(Cys), and TAC(Tyr)

- **Ambiguous nucleotides**
  - Codons 317–318
  - Nucleotide(s) GTG(Val)

- **Codon sequences**
  - Codons 317–318
  - Nucleotide(s) GTG(Val)

- **Amino acid sequences**
  - Amino acids 317–318
  - Amino acids Val–Val

- **Sequence analysis**
  - Using SSCP and sequencing analyses

- **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. 1A). 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. 1B). This mutation was determined to be present in the germline 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.

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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 3). 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.

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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).

References


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