Original articles

Novel germline mutations in the PTEN tumour suppressor gene found in women with multiple cancers

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Abstract

Germline mutations in PTEN can predispose people to Cowden syndrome (CS) and Bannayan-Ruvalcaba-Riley (BRR) syndrome, rare, autosomal dominantly inherited neoplastic disorders. To determine whether germline mutations in PTEN contribute to genetic predisposition to multiple primary tumours within the general population, we conducted a nested case-control study, among 32 826 members of the prospective Nurses’ Health Study cohort; cases were women with more than one primary tumour at different anatomical sites. We screened all nine exons of PTEN and flanking intronic splice sites for all 103 eligible cases using SSCP and sequencing. We observed two novel germ-line heterozygous missense mutations in exon 5 in five of the cases; three were V119L and two were V158L. Neither mutation was observed in 115 controls free of cancer. Both mutants showed partial tumour suppressor activity when compared to wild type PTEN when transfected into a PTEN null breast cancer cell line. The phenotype was cell line specific suggesting that genetic background affects growth suppression activity of the mutants. These data provide evidence that germline mutations in PTEN may be a more frequent predisposing factor for cancers in women than previously suggested.

Keywords: population based; tumour suppressor; multiple cancers; germline mutations

PTEN (MMAC1/TEP1), a tumour suppressor gene located on chromosome 10q23.3, encodes a 403 amino acid dual specificity phosphatase with homology both to the protein tyrosine phosphatase (PTP) family and to cytoskeletal proteins, tensin and auxin. PTEN has been shown to be somatically deleted or mutated in a fraction of breast cancers (4-6%), prostate cancers (35%), endometrial cancers (35-50%), glioblastomas (23-44%), and sporadic melanomas (43%), suggesting that it functions as a tumour suppressor gene. A large proportion of tumour associated PTEN mutations are found in exon 5, the region encoding the phosphatase domain. Mutations in PTEN identified in primary tumours, tumour lines, and in patients with the rare inherited cancer syndromes Cowden syndrome (CS) and Bannayan Ruvalcaba-Riley syndrome (BRR), result in ablation of phosphatase activity, showing that enzymatic activity is important for PTEN’s ability to function as a tumour suppressor.

Germline PTEN mutations have been identified as the cause of CS, which is characterised by benign adenomas and malignant neoplasms of the breast, thyroid, endometrium, and skin. In addition to benign breast disease, adenocarcinoma of the breast develops in approximately 30-50% of women with CS at a mean age of diagnosis 10 years younger than breast cancer in the general population; the lifetime risk of developing epithelial thyroid cancer is 10%. Endometrial carcinoma is part of the spectrum of CS. Inherited PTEN mutations have also been found in BRR, a rare autosomal dominant disorder characterised by microcephaly, vascular malformations, and benign neoplasms such as lipomas and intestinal hamartomatous polyps. Unlike CS, BRR patients are affected shortly after birth. In CS and BRR, 77% of all mutations are found in exons 5, 7, and 8; 43% of all mutations are concentrated in exon 5, which encodes the phosphatase core motif.

We set out to determine the frequency of germline mutations in PTEN in a population based series of women diagnosed with primary invasive cancer in more than one organ after enrolment in a cohort study. We performed mutational screening of all nine exons and flanking intronic splice sites by SSCP analysis and DNA sequencing. All novel variants that result in changes in the encoded protein were functionally characterised.

Materials and methods

STUDY POPULATION

The Nurses’ Health Study is a prospective cohort study of 121 700 female registered
Germline mutations in PTEN

DNA, 1.5 mmol/l MgCl₂, 50 mmol/l KCl, 10 PCR mixture contained 100 ng of genomic DNA, and 1.5 U of Taq polymerase (Ampli-}

PCR samples that showed mobility shifts by SSCP analysis were amplified again using exon specific unlabelled primers and purified using the QIAquick PCR Purification Kit (Qiagen, Inc, Chadsworth, CA). Purified PCR products were sequenced directly using Big Dye Terminator cycle sequencing protocol (Perkin-Elmer), electrophoresed on 5% Long Ranger gels (FMC, Rockland, ME), and analysed on an ABI 377 automated DNA sequencer (Perkin-Elmer). Base calling of the sample files was done using the ABI sequence analysis software version 3.0. Factura v 2.0 and Sequence Navigator v 1.01 (Perkin-Elmer) were used to mark potential heterozygous positions and display them for evaluation. Heterozygotes were called at positions where the secondary peak's height was greater than or equal to 45-50% of the primary peak's height in both forward and reverse sequence reads. Where possible, restriction digestion with appropriate enzymes were performed to confirm the sequences.

DNA PREPARATION AND MUTATION SCREENING

Genomic DNA from each subject was prepared using a Qiagen QIAamp 96 Spin Blood Procedure (Qiagen Inc, Chadsworth, CA) for both cases and controls. We performed PCR-SSCP on all nine exons, including flanking intronic sequence, using primers based on previously described sequences. Each 50 µl of PCR mixture contained 100 ng of genomic DNA, 1.5 mmol/l MgCl₂, 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 8.3), 0.001% gelatin, 200 µmol/l dNTPs, 30 pmol fluorescent labelled primer, and 1.5 U of Taq polymerase (AmpliTaq, Perkin-Elmer Corp). Amplification was conducted using the following cycling parameters: an initial denaturation step at 95°C for five minutes, 35 cycles at 95°C for one minute, 54-58°C for one minute, 72°C for one minute, and a final extension at 72°C for five minutes. Target sequences were amplified using forward and reverse primers labelled with two different fluorescent dyes (6-Fam and Tet) at their 5' termini. PCR induced mutations. The mutated cDNAs were then subcloned as

SITE DIRECTED MUTAGENESIS

A full length PTEN cDNA2 in the pZErO™-2.1 vector (Invitrogen) was used to generate mutants. Mutants were constructed by standard oligonucleotide directed mutagenesis using Pfu polymerase (Stratagene). The PTEN V119L cDNA (M1) was created by PCR using primers 5'-GACAATCATCTTGCAATTCA CTGTAAAG-3' and 5'-GTCTCTGGTCCTTA GTTGGGAACTAAGGACCAGAGACAAAAAG TGCAAGATTGTCATCTTC-3'. Both constructs were confirmed by sequencing to rule out possible PCR induced mutations. The mutated PTEN cDNAs were then subcloned as NotI fragments into the expression vector pCEP4 for the colony suppression assay.

CELL LINES

Breast cancer cell lines MDA-MB-468, BT-549, and T47D were obtained from American Type Culture Collection. All media were supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 g/ml streptomycin sulphate.
Table 1  Descriptive characteristics of cases with invasive cancers at more than one anatomical site and control

<table>
<thead>
<tr>
<th></th>
<th>Cases (n=103)</th>
<th>Controls (n=115)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Family history</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any cancer among 1st degree relative</td>
<td>61.2%</td>
<td>55.7%</td>
</tr>
<tr>
<td>Mother</td>
<td>13.6%</td>
<td>9.6%</td>
</tr>
<tr>
<td>Sister</td>
<td>14.6%</td>
<td>8.7%</td>
</tr>
<tr>
<td>Colon cancer</td>
<td>20.3%</td>
<td>15.7%</td>
</tr>
<tr>
<td>Parents</td>
<td>10.7%</td>
<td>6.0%</td>
</tr>
<tr>
<td>Melanoma</td>
<td>4.9%</td>
<td>3.5%</td>
</tr>
<tr>
<td>Sibs</td>
<td>2.0%</td>
<td>1.7%</td>
</tr>
<tr>
<td><strong>Mean age at diagnosis of first cancer</strong></td>
<td>55.5 y</td>
<td>—</td>
</tr>
<tr>
<td><strong>Mean age at diagnosis of second cancer</strong></td>
<td>60.7 y</td>
<td>—</td>
</tr>
</tbody>
</table>

**Combinations of cancers**

- Breast and endometrial: 16% (17)
- Breast and colon: 12% (13)
- Breast and melanoma: 8.5% (9)
- Colon and endometrial: 7.7% (8)
- Lung and breast: 5.8% (6)
- Breast and ovarian: 4.8% (5)
- Endometrial and ovarian: 3.9% (4)
- Melanoma and colon: 1.9% (2)

**Results**

In the overall cohort, 103 women were diagnosed with histologically confirmed primary invasive cancer in at least two different organs between 1976 and 1996 and had given a blood sample in 1989-1990.

Comparisons of cases and controls with respect to year of birth (for which they were matched), family history, and the average age at diagnosis among cases and combinations of cancer sites among cases are presented in table 1. Cases and controls were similar in terms of age and ethnicity; of the 94 cases with known ethnicity 89 were white, reflecting the ethnic distribution of the cohort, four were Asian, and one was Hispanic. The mean age at first cancer diagnosis was 55.5 years and 60.7 years for the second cancer; the majority of women were diagnosed with breast cancer as either their first or second cancer. The average age at diagnosis was 54.0 years for the first cancer and 58.7 years for the second cancer for the five women with PTEN variants. The most common cancer combinations are described in table 1. The tumour spectrum observed may be biased away from the more rapidly fatal cancers, as to be included in the study women diagnosed between 1976 and 1989 had to be alive in 1989 in order to give a blood sample. In comparison to control women without diagnosed cancer, women with multiple cancers had a slightly higher frequency of family history for all types of cancers, but this difference was not statistically significant.

**Mutational Analysis**

Genotyping using SSCP and sequencing of all nine exons of the PTEN tumour suppressor gene among the cases showed germline heterozygote mutations only in exon 5; no mutations were found in exons 1-4 or 6-9. Five missense mutations (three G→C at codon 119 and two G→C at codon 158) (fig 1) and one silent variant were found in exon 5 among the 103 cases using precast 8-16% Tris-glycine gels (Novex) and transferred onto polyvinylidene difluoride membranes (Immobilin-P, Millipore) for western blotting. Membranes were blocked with Tris-buffered saline containing 0.05% Tween and 5% skim milk and then blotted with anti-PTEN rabbit polyclonal antibody CS486. Blots were developed with horseradish peroxidase conjugated secondary using the enhanced chemiluminescence system (Amersham).

**Colon Suppression Assay**

The generation of pCEP4-PTEN and pCEP4-PTEN-C124S has been described previously.

Three µg of pCEP4, pCEP-PTEN, pCEP4-PTEN-C124S, pCEP4-PTEN-V119L, or pCEP4-PTEN-V158L were transfected into cells using Fugene 6 (Boehringer-Mannheim). The constructs were transiently transfected into human breast cancer cell lines MDA-MB-468, BT-549, and T47D. The MDA-MB-468 and BT-549 lines contain truncating mutations for PTEN which are functionally inactive, whereas the T47D cell line expresses wild type PTEN. Hygromycin was added to the culture medium after 24 hours to begin selection. Cells were selected over a period of two to four weeks and stained with crystal violet. All experiments were done in triplicate and means were averaged. A two tailed t test was used to test for significant differences between means.

**Immunodetection of PTEN Protein**

For the generation of cell lysates, cells were washed in cold PBS and lysed by incubation at room temperature in a buffer containing 125 mmol/l Tris-HCl (pH 6.8), 10% 2-mercaptoethanol, 4% SDS, and 20% glycerol. Samples were resolved by SDS-PAGE using precast 8-16% Tris-glycine gels (Novex) and transferred onto polyvinylidene difluoride membranes (Immobilin-P, Millipore) for western blotting. Membranes were blocked with Tris-buffered saline containing 0.05% Tween and 5% skim milk and then blotted with anti-PTEN rabbit polyclonal antibody CS486. Blots were developed with horseradish peroxidase conjugated secondary using the enhanced chemiluminescence system (Amersham).

![Figure 1](https://example.com/figure1.jpg) Sequence electrophoretograms of PTEN variants found in women with multiple primary tumours. Direct sequence analysis of DNA amplified from the region of exon 5 which contained mutations. (A-C) Arrows point to the heterozygote, G/C, (shown in the reverse complement) mutation found at codon 158 (GTA/CTA). This mutations creates a DdeI restriction site. (B) DNA sequence of homozygous wild type.
studied (table 2). For the G→C at codon 158 that creates a DdeI restriction site, RFLP analysis confirmed the sequencing results. To determine whether the mutations might represent common polymorphisms, we screened all 115 controls for exon 5 using SSCP and sequencing, and found no mutations (p=0.02 by Fisher’s exact test).

Three women (samples 3, 4, 5) had a Val→Leu substitution at codon 119 (M1) (within the phosphatase domain) and two women (samples 1 and 2) had a Val→Leu substitution at codon 158 (M2) (C-terminal to the phosphatase domain) (table 2). One woman (sample 2) had an additional silent variant at codon 130. Four of the five women (samples 1, 2, 4, and 5) with an exon 5 mutation had developed breast cancer. Endometrial cancer was diagnosed in two of the three women with mutations in codon 119; one of these women (sample 3) also had ovarian cancer, and another (sample 4) had a history of lung adenocarcinoma and ovarian teratoma. Samples 2 and 5 were the only two women with a family history of cancer in a first degree relative (table 2). The women are self-identified whites; further details of ancestry in the five women with the PTEN variants is unknown.

Cowden syndrome was not mentioned in any of the medical records; in particular, there was no mention of CS associated skin lesions or hamartomas. Although two of the women reported benign colon polyps and one reported benign uterine fibroids, from the limited information available, they did not meet the criteria for diagnosis of Cowden syndrome, although we cannot exclude this diagnosis as we were not able to conduct a standardised physical examination.

### Table 2: Summary of PTEN variants in women with multiple primary tumours

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mutation</th>
<th>Amino acid change</th>
<th>Cancer diagnosis</th>
<th>Family history*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>158 GTA→CTA</td>
<td>158 Val→Leu</td>
<td>Invasive ductal breast cancer</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>158 GTA→CTA</td>
<td>158 Val→Leu</td>
<td>Lower genital tract cancer</td>
<td>Colon cancer</td>
</tr>
<tr>
<td>3</td>
<td>119 GTT→CTT</td>
<td>119 Val→Leu</td>
<td>Invasive ductal breast cancer</td>
<td>Endometrial cancer</td>
</tr>
<tr>
<td>4</td>
<td>119 GTT→CTT</td>
<td>119 Val→Leu</td>
<td>Lung adenocarcinoma</td>
<td>Bilateral ovarian cancer</td>
</tr>
<tr>
<td>5</td>
<td>119 GTT→CTT</td>
<td>119 Val→Leu</td>
<td>Invasive ductal breast cancer</td>
<td>Endometrial cancer</td>
</tr>
</tbody>
</table>

*Family history cancer of female breast, colon, prostate, endometrial, ovarian, or melanoma.

Figure 2 PTEN expression and growth suppression in breast cancer cell lines. (A, C) Detection of PTEN protein after transfection. Western analysis using anti-PTEN or anti-β-tubulin antibodies on total cell lysates from the PTEN null cell lines, BT-549 (A) and MDA-MB-468 (C) after transfection. Arrows indicate the position of PTEN and β-tubulin. Cells were transfected with empty pCEP4 vector (Vector) and pCEP4 vectors containing wild type PTEN (WT), PTEN-C124S (C124S), PTEN-V119L (V119L), and PTEN-V158L (V158L). Approximately equal amounts of lysate are present in all lanes as indicated by the β-tubulin expression. (B, D) Measurement of colony suppression by PTEN. Cells were transfected with pCEP4 based constructs (vector, wild type, C124S, V119L, V158L) that contain the hygromycin resistance cassette or the vector CMV-β-galactosidase (mock) which lacks the hygromycin resistance cassette and grown with media in the presence of 200 μg/ml of hygromycin. After 14 days no cells were present in the mock flasks. Large colonies grew in flasks transfected with pCEP4 vector and C124S. For BT-549 (B), V119L and V158L colonies were appreciably larger than wild type but smaller than the colonies seen with vector and C124S. For MDA-MB-468 (D), V119L and V158L colonies were similar in size and number to wild type. Colonies from vector and C124S were much more numerous and larger than the colonies of V119L, V158L, and wild type.
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In this same multiple alignment, the variant leucine amino acid is not found at either position 119 or 158. Instead, only valine, isoleucine, and threonine are found at position 119, while lysine, valine, isoleucine, and arginine are found at position 158. Given the buried disposition of residue 119, the proximity of the mutant residues to the PTEN active site, and the exclusion of leucine from either position in the phylogenetic comparisons, the mutations, especially V119L, might be disruptive to PTEN structure or function or both.

**TUMOUR SUPPRESSOR ACTIVITY**

To investigate the possibility that these new variants may impair the tumour suppressor function of PTEN, we examined these mutants in a standard colony suppression assay using the selection agent hygromycin and vectors expressing wild type PTEN (pCEP4-PTEN), phosphatase dead PTEN (pCEP4-PTEN-C124S), and the mutants M1 (pCEP4-PTEN-V119L) and M2 (pCEP4-PTEN-V158L). All transfected cell lines expressed comparable levels of wild type and mutant PTEN as detected by immunoblotting (fig 2, panels A and C). Examination of colony formation in MDA-MB468 (fig 2, panel D) and T47D (data not shown) showed that M1 and M2 mutants formed fewer colonies than vector alone (p<0.01) but were not statistically different from wild type PTEN (table 3). Colonies formed by cells transfected with the empty vector pCEP4 were numerous and large, comparable to those formed by cells expressing the functionally inactive PTEN construct pCEP4-PTEN-C124S. Colony formation was inhibited in those cell lines expressing the wild type PTEN construct, pCEP4-PTEN. The M1 and M2 expressing cells did form colonies in cell line BT-549 (fig 2, panel B), though significantly (p<0.001) fewer in number and not as robust compared to the colonies formed with the vector alone or with the PTEN-C124S mutant, but clearly different (p<0.001) from those expressing wild type PTEN (p<0.001) (table 3).

**Discussion**

We report two novel functional germline mutations within exon 5 of the tumour suppressor gene, PTEN. To our knowledge, this is the first population based study that describes germline PTEN mutations in women with multiple primary tumours at various sites. To date, these specific mutations have not been observed in published Cowden pedigrees. The mutations we observed occur in evolutionarily conserved positions. Results from the colony suppression assay show that the mutations impair the tumour suppressor activity of PTEN. The fact that the mutants V119L and V158L give an intermediate phenotype suggests that these mutants may have partial activity or altered function. Previously, PTEN protein with the CS mutation, G129E, has been shown to have normal phosphatase activity against protein substrates both in vitro and in cell lines but has no phosphatase activity against Ptd-In(3,4,5)P3, suggesting that these two activities have different protein sequence requirements. Evidence from the colony suppression assay suggests that we have identified hypomorphic alleles that alter but do not abolish PTEN’s function. Also, colony formation was cell line specific; colonies formed only with BT-549, suggesting that a specific genetic/cellular milieu is required to elicit a phenotype with these mutations. The implication is that germline mutations in PTEN may not always result in a cancer phenotype, but instead may require a specific genetic background for complete penetrance. In contrast to other tumour suppressor genes, such as BRCA1 where the penetrance in breast cancer pedigrees is high (up to 85%) for developing breast cancer, women affected with CS have only a 30-50% lifetime risk of developing breast cancer.25 This is similar to more recent estimates of penetrance of mutations in BRCA1 derived population based studies.26 27 First degree family history of cancer was only present in two of the five women with PTEN variants, consistent with a relatively low penetrance of these specific mutations. The fact that the same mutations were found among women with different tumours is not

**Table 3: Mean colony counts and size for phosphatase inactive PTEN, mutant PTEN, and wild type PTEN constructs transfected into PTEN null breast cancer cell lines**

<table>
<thead>
<tr>
<th>Transfected PTEN constructs</th>
<th>Cell line BF549*</th>
<th>Cell line MDA-MB-468*</th>
<th>Colony size</th>
<th>Cell line BF549</th>
<th>Cell line MDA-MB-468</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector only (control)</td>
<td>275 (22.7)†‡</td>
<td>97.7 (10.9)†‡</td>
<td>Large</td>
<td>Large</td>
<td></td>
</tr>
<tr>
<td>PTEN mutant C124S (inactive)</td>
<td>278.3 (5.6)†‡</td>
<td>85 (9.3)†‡</td>
<td>Large</td>
<td>Large</td>
<td></td>
</tr>
<tr>
<td>PTEN mutant V119L (M1)</td>
<td>185 (9.3)†‡</td>
<td>10.3 (4.4)</td>
<td>Intermediate</td>
<td>Small</td>
<td></td>
</tr>
<tr>
<td>PTEN mutant V158L (M2)</td>
<td>195 (10.7)†‡</td>
<td>11.7 (2.4)</td>
<td>Intermediate</td>
<td>Small</td>
<td></td>
</tr>
<tr>
<td>Wild type PTEN</td>
<td>61.7 (2.9)</td>
<td>6.7 (2.9)</td>
<td>Small</td>
<td>Small</td>
<td></td>
</tr>
</tbody>
</table>

*Breast cancer PTEN null cell line.
†Statistically significant compared to wild type PTEN (p<0.001).
surprising as identical mutations in PTEN have been found in both CS and BR. Modifiers or stochastic effects may be responsible in part for the different phenotypes occurring in the cases with identical mutations (V119L samples 3, 4, 5 and V158L samples 1, 2).

Recent analyses of different lines of mutant mice suggest that genetic background can significantly affect PTEN phenotypes. Though the heterozygote mice all showed an increase in tumour incidence and hyperproliferative lesions of the intestines, the spectrum of other hyperproliferative and neoplastic disorders differed dramatically between lines.

Estimates of the frequency of CS range from 1 in 1 000 00017 to 1 in 250 000. Among people with a clinical diagnosis of CS, the PTEN mutation prevalence ranges from 13 to 80%.17 The contribution of germline mutations in PTEN towards genetic predisposition to cancers within the general population is for the most part unknown. A recent study of early onset breast cancer patients reported germline missense mutations in two of 60 patients; one of the two mutations was in exon 5.26 Rhei et al reported one germline mutation in 54 breast cancer patients, and this single patient was thought to have clinical CS. In a series of 64 non-CS cases ascertained with the minimum number of breast cancer patients, and this single patient was found to have a germline mutation in PTEN. In addition to breast and thyroid cancers, this person had endometrial cancer.27 We report a PTEN mutation prevalence of 5% in a series of 103 women with a clinical diagnosis of CS and Bannayan-Zonana syndrome, two hamartoma syndromes with germline PTEN mutation. Hum Mol Genet 1998;7: 507-15.


Sander C, Schneider R. Database of homology-derived protein structures and the structural meaning of sequence alignment. Proteins 1999:35:56-68.


