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 germline 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 diagnosed cancer ($p = 0.02$). 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 auxilin. *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%), and sporadic melanomas (23–44%), 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 resulted 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 served as positive controls1 for the SSCP analysis where available.

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 Range 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 digests 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, Chadoworth, 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.2 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 mol/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’ ends. Exon 5 was amplified into two overlapping products for SSCP analysis.

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 CTGTAAGG-3’ and 5’-CAGTGAATTGC TGCAAGATTGTCATCTTC-3’. The V158L cDNA (M2) was created using primers 5’-GGGGAACTAAGGACCAGAGACAAAAAGTGCAAGATTGTCATCTTC-3’ and 5’-GTCTCTGGTCCTTAGTTTCTGTAAGG-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>Family history</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>Parents</td>
<td>2.0%</td>
<td>1.7%</td>
</tr>
<tr>
<td>Mean age at diagnosis of first cancer</td>
<td>60.7 y</td>
<td></td>
</tr>
<tr>
<td>Mean age at diagnosis of second cancer</td>
<td>55.5 y</td>
<td></td>
</tr>
<tr>
<td>Percentage of all women</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breast</td>
<td>70.8% (73)</td>
<td></td>
</tr>
<tr>
<td>Endometrial</td>
<td>35.0% (36)</td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>27.0% (28)</td>
<td></td>
</tr>
<tr>
<td>Melanoma</td>
<td>13.5% (14)</td>
<td></td>
</tr>
<tr>
<td>Ovarian</td>
<td>8.7% (9)</td>
<td></td>
</tr>
<tr>
<td>Combinations of cancers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breast and endometrial</td>
<td>16% (17)</td>
<td></td>
</tr>
<tr>
<td>Breast and colon</td>
<td>12% (13)</td>
<td></td>
</tr>
<tr>
<td>Breast and melanoma</td>
<td>8.5% (9)</td>
<td></td>
</tr>
<tr>
<td>Colon and endometrial</td>
<td>7.7% (8)</td>
<td></td>
</tr>
<tr>
<td>Lung and breast</td>
<td>5.8% (6)</td>
<td></td>
</tr>
<tr>
<td>Breast and ovarian</td>
<td>4.8% (5)</td>
<td></td>
</tr>
<tr>
<td>Endometrial and ovarian</td>
<td>3.9% (4)</td>
<td></td>
</tr>
<tr>
<td>Melanoma and colon</td>
<td>1.9% (2)</td>
<td></td>
</tr>
</tbody>
</table>

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 (Immobolin-I, 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).

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 and transferred onto polyvinylidene difluoride membranes (Immobolin-I, 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).

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Colon Suppression Assay

The generation of pCEP4-PTEN and pCEP4-PTEN-C124S has been described previously.21 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.21 The MDA-MB-468 and BT-549 lines contain truncating mutations of PTEN whereas the T47D cell line expresses wild type PTEN.1 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.

Figure 1  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.

### STRUCTURAL AND PHYLOGENETIC ANALYSIS OF VARIANT RESIDUES

Sequence alignments showed that the valine at position 119 (M1) is conserved in PTEN.

<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 breast cancer</td>
<td>Bilateral ovarian cancer</td>
</tr>
<tr>
<td>4</td>
<td>119 GTT→CTT</td>
<td>119 Val→Leu</td>
<td>Endometrial cancer</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>119 GTT→CTT</td>
<td>119 Val→Leu</td>
<td>Invasive ductal breast cancer</td>
<td>Prostate</td>
</tr>
</tbody>
</table>

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

![Figure 2](http://jmg.bmj.com/37.5.336/figure2.png)
homologues and other phosphatases down to yeast and the valine at position 158 (M2) is conserved in PTEN homologues among vertebrates. In the PTEN structure, both mutant residues are located in the catalytic domain, about 7 angstroms and 9 angstroms, respectively, from residues involved in the phosphatase catalytic site as defined by a match to the PROSITE database entry PS00383. Furthermore, the residual mutated in M1 is inaccessible to solvent. The residue mutated in M2 is within 5 angstroms of residues 14, 15, and 159. These residues are all conserved among proteins sharing at least 30% sequence identity with PTEN, as reported in the HSSP database entry corresponding to the PTEN structure (1D5R). 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 active site, and the exclusion of leucine from these positions, 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-Ins (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. This is similar to more recent estimates of penetrance of mutations in BRCA1 derived population based studies.

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...
Germline mutations in PTEN

surprising as identical mutations in PTEN have been found in both CS and BRR.23 Modifier
genes 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.27–29 Though the heterozygote mice all showed an increase in tumour incidence and hyperplo-
iferative lesions of the intestines, the spectrum of other hyperplenerative and neoplastic disor-
ders differed dramatically between lines.

Estimates of the frequency of CS range from 1 in 1 000 00027 to 1 in 250 000.28 Among people
with a clinical diagnosis of CS, the PTEN mutation prevalence ranges from 13 to
80%.17–11 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.26 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 thought to have clinical CS. In a series of 64
non-CS cases ascertained with the minimum

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row, Lisa Li, Gary Gearn, and Michael L Fitzgerald for their technical assistance, and Barbara Egan for disease follow up
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the participants in the Nurses’ Health Study for their dedication and commitment.

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