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Inherited Mutations in PTEN That Are Associated with Breast Cancer, Cowden Disease, and Juvenile Polyposis

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Summary

PTEN, a protein tyrosine phosphatase with homology to tensin, is a tumor-suppressor gene on chromosome 10q23. Somatic mutations in PTEN occur in multiple tumors, most markedly glioblastomas. Germ-line mutations in PTEN are responsible for Cowden disease (CD), a rare autosomal dominant multiple-hamartoma syndrome. PTEN was sequenced from constitutional DNA from 25 families. Germ-line PTEN mutations were detected in all of five families with both breast cancer and CD, in one family with juvenile polyposis syndrome, and in one of four families with breast and thyroid tumors. In this last case, signs of CD were subtle and were diagnosed only in the context of mutation analysis. PTEN mutations were not detected in 13 families at high risk of breast and/or ovarian cancer. No PTEN-coding-sequence polymorphisms were detected in 70 independent chromosomes. Seven PTEN germ-line mutations occurred, five nonsense and two missense mutations, in six of nine PTEN exons. The wild-type PTEN allele was lost from renal, uterine, breast, and thyroid tumors from a single patient. Loss of PTEN expression was an early event, reflected in loss of the wild-type allele in DNA from normal tissue adjacent to the breast and thyroid tumors. In RNA from normal tissues from three families, mutant transcripts appeared unstable. Germ-line PTEN mutations predispose to breast cancer in association with CD, although the signs of CD may be subtle.

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Introduction

The protein tyrosine phosphatase and tensin homologue PTEN is a tumor suppressor of glioblastoma, breast cancer and prostatic cancer (Li and Sun 1997; Li et al. 1997; Steck et al. 1997), and malignant melanoma (Guldberg et al. 1997). The gene has at least three names: "PTEN" (*p*hosphatase with *ten*sin homology; Genbank accession number U93051), "MMAC1" (*m*utated in *m*ultiple *a*dvanced *c*ancers; Genbank accession number U92436), and "TEP1" (*T*GFB-regulated and *e*pithelial-cell–enriched *p*hosphatase; Genbank accession number U96180). In primary breast carcinomas, both somatic and germ-line PTEN mutations occur, albeit at low frequency (Rhei et al. 1997). Previous observations of loss of heterozygosity (LOH) at chromosome 10q23 in follicular thyroid cancer and endometrial cancers suggested that PTEN might act as a tumor suppressor for these cancers as well (Jones et al. 1994; Zedenius et al. 1995). In patients with Cowden disease (CD; MIM 158350 [Hanssen and Fryns 1995]), germ-line mutations in PTEN have been identified (Liaw et al. 1997; Nelen et al. 1997). Germ-line PTEN mutations are also associated with the closely related Bannayan-Zonana syndrome (Marsh et al. 1997).

In this report, we evaluate families with inherited predisposition to breast cancer and/or CD or related syndromes, for germ-line mutations in PTEN. We identify and characterize germ-line point mutations in PTEN in seven families, describe the cancer and other phenotypes associated with each of these mutations, and demonstrate somatic loss of the wild-type PTEN allele in the associated tumors.

Subjects and Methods

Family Ascertainment

Twenty-five families were included in this analysis. Five families (families 97, 903, 1085, 1130, and 1163;

Table 1

Phenotypes Associated with Inherited Mutations in PTEN

				Phenotype (Age [in Years] at Diagnosis)					
Mutation(s)	Effect	Family	Patient (Sex)	Skin Lesions	Breast	Endometrium	Thyroid	CNS	Gastrointestinal Tract
68 T→A	L22X	903	I-2 (F)	+	Ca (46)				
		903	II-1 (F)	+	Ca (24)		Fol Ca (18)		L Hem
328 C→T	Q109X	1130	I-2 (F)	+	Ca (34)				
565 A→T	R188X	1085	I-2 (F)	+	Bil Ca (31)		Fol Ad	Nf	G, D, C polyps
		1085	II-1 (F)	+	Ad (29)				
		1085	II-2 (F)	+					C polyps
697 C→T	R232X	97	II-1 (F)	+	Ca (27)				C polyps
1003 C→T	R334X	241	I-1 (M)	+				Macro	Sm Int Ca (34)
		241	II-2 (M)	+				Macro	C, Sm Int polyps
1003 C→T	R334X	227	II-1 (F)	+	DCIS (41)	Ca (42)	Fol Ad (42)	Macro	R Ca (42)
1028 T→A	V332E	1163	I-2 (F)		Ca (50)		Ad (49)	Neu (33, 34)	
and	and	1163	II-2 (F)	+	Ad (30)	Ca (48)	Fol Ad (32, 40)		G, C polyps (37, 38)
1039 T→C	F346L	1163	II-3 (F)		Atyp H (33)	Ca (42)	Fol Ad (33)		D polyps (31)
		1163	II-4 (M)					Pin (42)	C polyps
		1163	II-5 (F)		Ca (42)				
		1163	III-1 (F)		Ad (17)		Pap Ca (11)		

Note.—Ad = adenoma; Atyp H = atypical hyperplasia; Bil = bilateral; C = colon; Ca = adenocarcinoma; D = duodenal; DCIS = ductal carcinoma in situ; Fol = follicular; G = gastric; Hem = hemangioma; L = liver; Macro = macrocephaly; Neu = malignant neuroma; Nf = neurofibroma; Pap = papillary; Pin = pineal-gland tumor; R = renal cell; and Sm Int = small intestine. A plus sign (+) denotes a chronic condition.

table 1) were ascertained for co-occurrence of breast cancer and CD in the same family. Four other families (including family 227; table 1) were ascertained for co-occurrence of breast and thyroid tumors without definite diagnosis of CD, although the proband of family 227 was subsequently evaluated as having features of the syndrome (table 1). Thirteen families at high risk of breast, ovarian, and/or prostate cancer but with no detected signs of CD were also included. At least one affected member in each of these 13 families had wild-type sequences at BRCA1 and BRCA2; in 2 families, linkage of cancer predisposition to markers flanking PTEN yielded small positive LOD scores (1.3 and 1.2).

Three other families with diagnoses of CD were included. Family 241 (table 1) was diagnosed with both juvenile polyposis syndrome (MIM 174900) and CD. In another CD family, individuals had macrocephaly, skin lesions, and mental retardation but no cancer, in three generations. In a third CD family, affected individuals had thyroid adenomas, intestinal polyps, neurofibromas, lipomas, and colon cancer, at ages 48 and 68 years.

Of the families described above, seven (including 903, 1085, 1130, and 1163) were evaluated clinically at the Norwegian Radium Hospital. Three families (including 227 and 241) were evaluated clinically at the University of Washington hospitals. The other families were from our series of families at high risk of breast and ovarian cancer. Protocols for human subjects were approved by the institutional review boards at the University of Washington, the Norwegian Radium Hospital, and other collaborating institutions, as appropriate.

Lymphoblast cell lines were prepared (Henle and Henle 1970; Raskind et al. 1984), and DNA was extracted from cell lines and from whole blood, by use of a high–salt-extraction method (Gentra Systems).

Genotyping Families and Tumors

Markers D10S583, D10S215, D10S541, D10S573, and D10S564 were used to determine haplotypes for pedigree analysis and for evaluation of LOH in tumors. Primer sequences for these markers are available on-line from the GenBank (http://www.ncbi.nlm.nih.gov/genbank/query_form.html). Markers were typed by use of PCR conditions and electrophoresis protocols described in the work of Friedman et al. (1994).

Dissection of Tumor Blocks

Paraffin-embedded tissue samples were cut into 5-micron sections for staining with hematoxalin and eosin.

Table 2

Primers That Amplify PTEN cDNA

Exons	Primer		Alignment to Published cDNA Sequences (nt)		Size (nt)
	Forward	Reverse	U93051 (PTEN)	U92436 (MMAC)	
1–5	5′-CCCAGACATGACAGCCATCATC-3′	5′-GTGGTGGGTTATGGTCTTCAAAAG-3′	1–289	1028–1323	296
4–6	5′-CTGAAAGACATTATGACACCGCC-3′	5′-GTCTCTGGTCCTTACTTCCCCATAG-3′	215–486	1249–1520	272
5–7	5′-GGAAAGGGACGAACTGGTGTAATG-3′	5′-AGCTGGCAGACCACAAACTGAG-3′	379–659	1413–1693	281
6 and 7	5′-CAATGTTCAGTGGCCGAACTTG-3′	5′-TTTTCTGAGGTTTCCTCTGGTCC-3′	611–866	1645–1900	256
7–9	5′-CAAAGTAGAGTTCTTCCACAAACAG-3′	5′-TAGCCTCTGGATTTGACGG-3′	759–1078	1763–2112	320

With the H- and E-stained slide as a guide, normal and tumor cells were microdissected from adjacent 10-micron sections that had also been deparaffinized. Genomic DNA was extracted from the microdissected cells by use of the manufacturer's suggested protocol (Gentra Systems).

Sequencing Genomic DNA, for PTEN Mutations

To analyze patient DNA for mutations in the PTEN gene, nested PCR products corresponding to each of the nine exons were amplified. PCR primers are as described by Steck et al. (1997). PCR products were purified by centrifugation through Sephacryl 300 (Sigma) in 96-well plates (Nalge Nunc International). Purified PCR products were quantified by visual inspection following electrophoresis through 2% agarose gels and ethidium bromide staining. Patient samples were sequenced by use of Energy Transfer Dye Primers (Amersham). The sequencing products were resolved on an ABI377 fluorescent DNA sequencer. Base calling of the trace files was done by use of the ABI sequence-analysis software version 3.0. PTEN-coding sequence and flanking splice junctions were sequenced from 70 independent normal chromosomes.

Transcript Analysis

Isolation of poly(A)$^+$ RNA was performed by use of oligo-dT cellulose in a high-salt environment (Sambrook et al. 1989). Patient and control mRNA was reverse transcribed with random hexamers (Amersham) and Superscript Moloney murine leukemia virus RTase (Gibco-BRL), by use of standard procedures. Table 2 indicates the primer pairs used to amplify cDNA, the exons amplified by each pair, and the sizes of amplified products.

PCR was performed for 35 cycles, at 94°C for 15 s, 55°C for 15 s, and 72°C for 1 min, and the products were purified by centrifugation through Sephacryl-300 columns (Sigma). Purified PCR products were cycle sequenced by use of dye-terminator chemistry (Perkin Elmer–ABI). Sequencing products were resolved on 4% LongRanger acrylamide gels (FMC), with an ABI-377 fluorescent sequencer.

The existence of a pseudogene similar to PTEN complicates mutation analysis of PTEN cDNA. We used cDNA to evaluate stability of mutant PTEN transcripts but not as the template for original mutation detection. Variants detected in cDNA can be inferred to be from the PTEN gene if they are also observed in genomic DNA amplified with PTEN-specific genomic primers.

Results

Mutations in Families

Mutations in the PTEN-coding sequence were identified in the seven families illustrated in figure 1. All seven mutations involved single-nucleotide substitutions. Six were nonsense mutations leading to immediate stops in exons 1 and 5–8. One variant comprised two missense mutations in exon 9, 1028 T→A (V342I) and 1039 T→C (F346L), on the same parental chromosome. No polymorphisms in the PTEN-coding sequence were detected in any of 70 unrelated chromosomes.

Germ-line mutations in PTEN and their associated phenotypes are indicated in table 1 and figure 1. Multiple invasive cancers occurred in the seven families with germ-line mutations. Among 10 women >30 years of age who had confirmed or probable germ-line mutations, there have been eight breast cancers, three endometrial cancers, one thyroid cancer, one malignant neuroma, and one renal-cell carcinoma. Of the two adult men with germ-line mutations, one died of intestinal cancer at age 35 years, and the other was diagnosed with brain cancer at age 42 years.

Nonsense mutation 697 C→T in individual 9701 is de novo. Parentage in family 97 was verified by multiple markers on chromosome 10 (fig. 1). Both parents have wild-type sequence at bp 697 (fig. 2). It is not clear in which parental chromosome the new mutation occurred. Nonsense mutation R232X was observed previously in

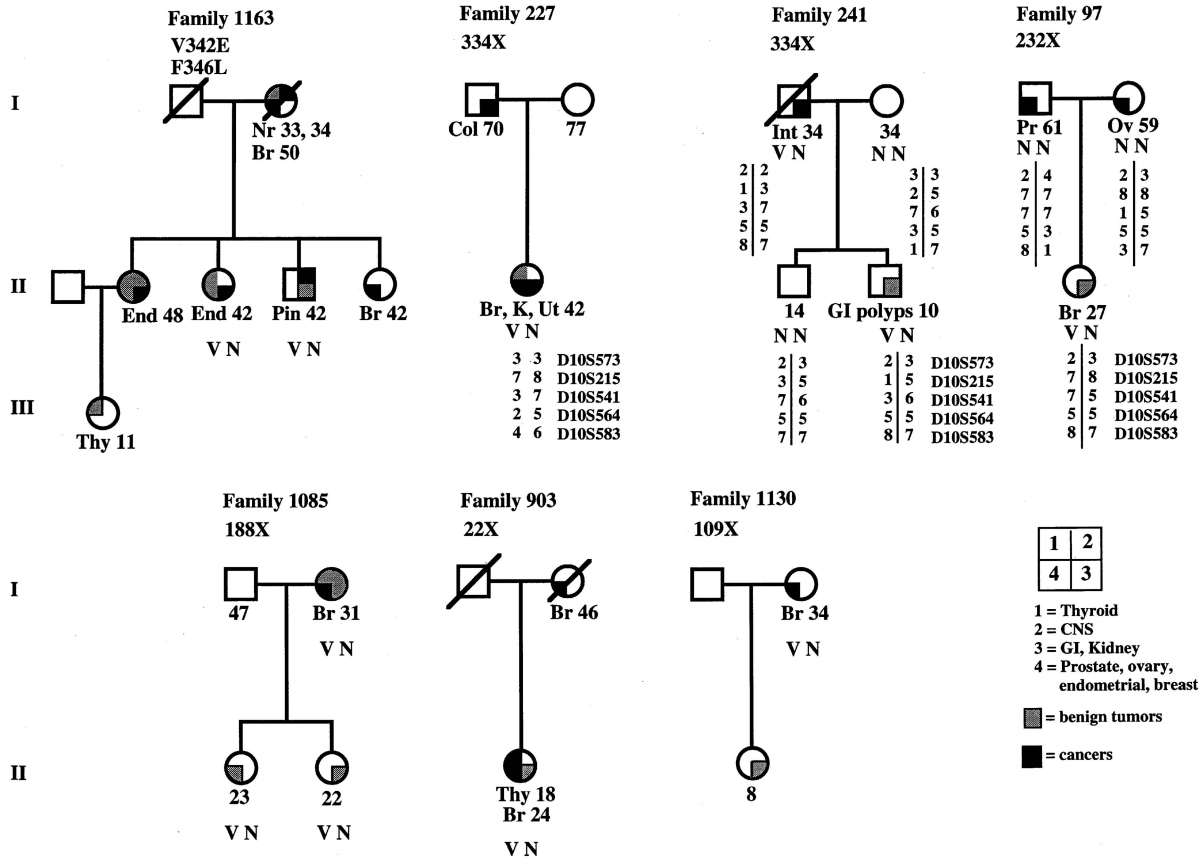

Figure 1 Cancers and noninvasive lesions in families with PTEN germ-line mutations. Symbols are divided into segments on the basis of organ site, with blackened quadrants representing cancers and with gray-shaded quadrants representing noninvasive tumors. PTEN mutations are indicated above each family, and the heterozygous variant (VN) or homozygous normal (NN) genotype for each tested individual is shown below the symbol representing that individual. Haplotypes comprising five markers flanking PTEN are shown for families 227 and 241, to demonstrate the independence of the 1003 C→T mutation. PTEN genotypes and marker haplotypes for family 97 indicate that the PTEN mutation in the daughter is de novo.

a CD patient (Liaw et al. 1997) and occurs in a potential tyrosine phosphate–acceptor motif (Steck et al. 1997).

The paired missense mutations in family 1163 occurred in both affected relatives for whom DNA was available. The wild-type transcript was present, but the mutant transcript was absent, in RNA prepared from whole blood of the affected individuals (fig. 2). Hence the two missense mutations are likely to be on the same chromosome. Mutation 1028 T→A changes the exon 9 splice acceptor from tagGT to tagGA and so may lead to aberrant splicing of this last exon. The instability could be due to the missense mutations or to aberrant splicing.

Nonsense mutation 1003 C→T appeared in both individual 22701 and family 241, as independent mutations. The haplotype constructed from D10S573, D10S215, D10S541, D10S564, and D10S583 in family 241 (fig. 1) shares no alleles with the comparable haplotype in patient 22701. Data from LOH studies of tumor specimens further suggests that allele 7 at D10S541 is on the haplotype with the mutation in patient 22701 (see below).

No germ-line mutations in the PTEN-coding sequence were detected in two families with CD but no early-onset cancers. Nor were germ-line PTEN mutations detected in any of the 13 breast cancer families that have wild-type sequence at BRCA1 and BRCA2. With the screening method that we employed, genomic deletions of an exon or more would not have been detected. However, large deletions do not appear to account for a substantial proportion of germ-line PTEN mutations, although these are common as somatic alterations (Li et al. 1997; Steck et al. 1997).

LOH in Tumors

Biopsy specimens from renal-cell carcinoma, uterine carcinoma, breast ductal carcinoma in situ (DCIS), and thyroid adenoma of patient 22701 were evaluated for

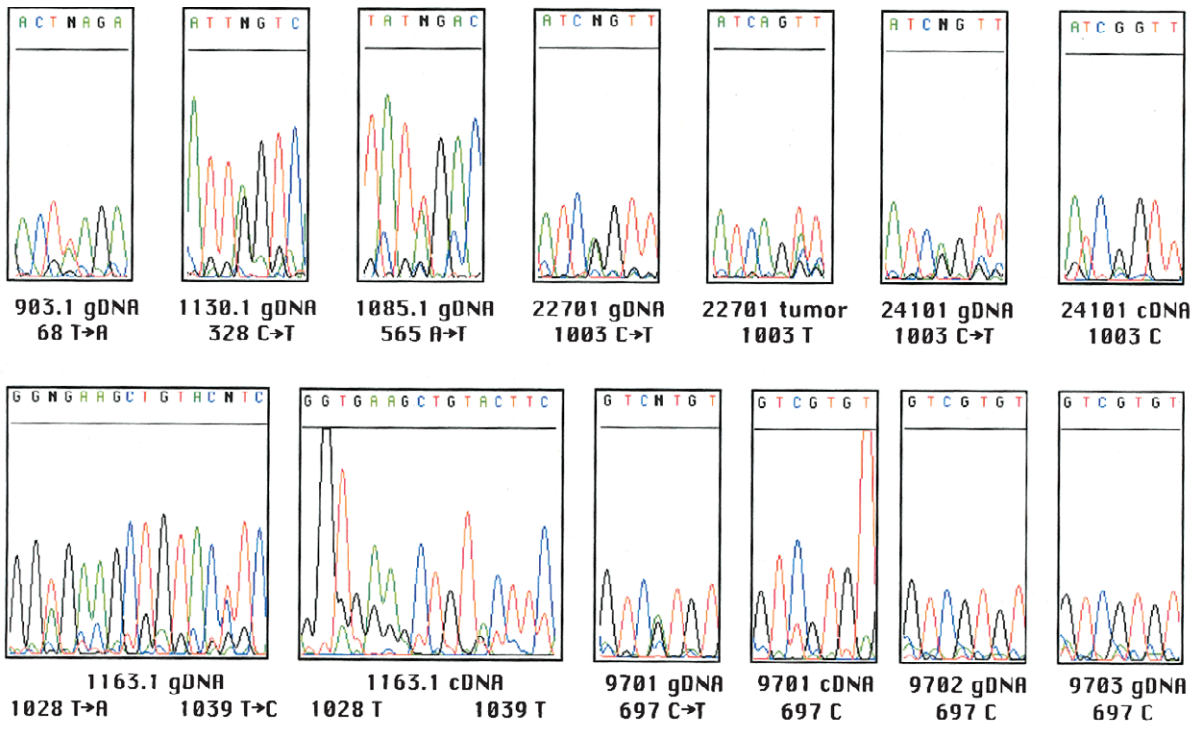

Figure 2 PTEN sequences from genomic DNA (gDNA), tumor DNA, and constitutional cDNA, from families with inherited mutations in this tumor-suppressor gene. Heterozygosity for inherited mutations in genomic DNA is illustrated for probands from seven families. Sequences may be either sense or antisense; mutations are indicated for the sense strand. Sequence "22701-tumor" reflects hemizygosity for the mutant PTEN allele in DNA from the renal-cell carcinoma of patient 22701. Sequences "24101 cDNA," "1163.1 cDNA," and "9701 cDNA" reflect the presence of only the wild-type transcript in normal cells from individuals with mutations, consistent with instability of the mutant transcripts. Sequences of genomic DNA from 9702 and 9703, parents of 9701, are homozygous normal, indicating that the mutation in individual 9701 is de novo.

LOH at PTEN bp 1003 and at markers D10S215, D10S541, and D10S564. All tumors revealed both LOH at the three markers and hemizygosity for the mutant allele at the altered site (fig. 2). Loss of the normal allele and retention of the mutant sequence support the hypothesis that PTEN acts as a tumor suppressor. Normal tissue surrounding the renal-cell carcinoma and the uterine carcinoma were heterozygous at bp 1003, as expected. Interestingly, DNA from apparently normal tissue adjacent to the DCIS and adjacent to the thyroid adenoma was clearly hemizygous, retaining only wild-type sequence.

Loss of Mutant Transcripts from Patient mRNA

For families 97, 241, and 1163, cDNA was prepared from lymphoblast poly(A)$^+$ RNA, in order to test the stability of mutant and wild-type transcripts. As shown in figure 2, mutant transcripts were not detected in RNA by dye-terminator sequencing. Loss of mutant transcripts from patients with nonsense mutations is consistent with degradation of these transcripts through a nonsense-mediated pathway (Decker and Parker 1994; Maquat 1996).

Discussion

The genetics of germ-line mutations in PTEN is consistent with its somatic genetics and biochemistry, all of which indicate that the gene is a tumor suppressor for breast and other cancers (Li and Sun 1997; Li et al. 1997; Liaw et al. 1997; Steck et al. 1997). The PTEN gene has distinctive features at the levels of cells, families, and species. First, the gene is highly conserved. Human and mouse amino acid sequences are >97% identical (Steck et al. 1997). The gene is also highly conserved within humans.

Second, consequences of even minimal mutations may be profound, even at the level of transcription. Nonsense-mediated mRNA degradation leading to transcript instability has been characterized in several species (Leeds et al. 1991; Pulak and Anderson 1993; Cui et al. 1995). Nonsense-mediated mRNA degradation may

play a role in the reduced mRNA expression of disease-related nonsense mutations (Dunn et al. 1989; Lim et al. 1992; Friedman et al. 1994; Menon and Neufeld 1994). The absence of mutant transcripts in cDNA from affected individuals with PTEN germ-line nonsense mutations suggests that the PTEN mutant transcript may be degraded by a nonsense-mediated pathway.

Third, a high fraction of tumors have deleted the gene and some flanking sequence. The phenotypes associated with either somatic or germ-line mutations are highly variable. In the same person, one germ-line mutation and several somatic mutations may lead to tumors in multiple organ systems. The multiple hamartomas in various organs suggest expression in early development and tissue differentiation. A gene critical to early development could well be highly conserved among species.

Lhermitte-Duclos disease is observed in some families with CD and is associated with severe neurological symptoms (Lhermitte and Duclos 1920; Albrecht et al. 1992). None of the patients with detected PTEN mutations in our series were diagnosed with Lhermitte-Duclos disease. Because mutations in these families were found throughout the gene, and because mutations in families with Lhermitte-Duclos disease have been observed in exons 2 and 5–7 and in intron 4 (Liaw et al. 1997; Nelen et al. 1997), it appears unlikely that mutations causing Lhermitte-Duclos disease cluster in any one region of PTEN.

Fourth, a high proportion of observed mutations are new, rather than persisting over several generations or recurring as founder mutations in individuals who are not closely related. PTEN mutations that were observed in more than one family are independent events. These characteristics may reflect mutation and selection interacting in a particularly dramatic way for an important gene. PTEN may be vulnerable to the entire range of types of mutations, but the gene may be so functionally constrained and so ubiquitously expressed that essentially no alterations are benign.

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