Report

Germline *PTEN* Promoter Mutations and Deletions in Cowden/Bannayan-Riley-Ruvalcaba Syndrome Result in Aberrant PTEN Protein and Dysregulation of the Phosphoinositol-3-Kinase/Akt Pathway

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Germline intragenic mutations in *PTEN* are associated with 80% of patients with Cowden syndrome (CS) and 60% of patients with Bannayan-Riley-Ruvalcaba syndrome (BRRS). The underlying genetic causes remain to be determined in a considerable proportion of classic CS and BRRS without a polymerase chain reaction (PCR)-detectable *PTEN* mutation. We hypothesized that gross gene deletions and mutations in the *PTEN* promoter might alternatively account for a subset of apparently mutation-negative patients with CS and BRRS. Using real time and multiplex PCR techniques, we identified three germline hemizygous *PTEN* deletions in 122 apparently mutation-negative patients with classic CS ($N=95$) or BRRS ($N=27$). Fine mapping suggested that one deletion encompassed the whole gene and the other two included exon 1 and encompassed exons 1–5 of *PTEN,* respectively. Two patients with the deletion were diagnosed with BRRS, and one patient with the deletion was diagnosed with BRRS/CS overlap (features of both). Thus 3 (11%) of 27 patients with BRRS or BRRS/CS-overlap had *PTEN* deletions. Analysis of the *PTEN* promoter revealed nine cases (7.4%) harboring heterozygous germline mutations. All nine had classic CS, representing almost 10% of all subjects with CS. Eight had breast cancers and/or benign breast tumors but, otherwise, oligo-organ involvement. PTEN protein analysis, from one deletion-positive and five *PTEN*-promoter-mutation–positive samples, revealed a 50% reduction in protein and multiple bands of immunoreactive protein, respectively. In contrast, control samples showed only the expected band. Further, an elevated level of phosphorylated Akt was detected in the five promoter-mutation–positive samples, compared with controls, indicating an absence of or marked reduction in functional PTEN. These data suggest that patients with BRRS and CS without PCR-detected intragenic *PTEN* mutations be offered clinical deletion analysis and promoter-mutation analysis, respectively.

Cowden syndrome (CS [MIM 158350]) is an autosomal dominant disorder characterized by multiple hamartomas affecting derivatives of all three germ layers and by an increased risk of breast, thyroid, and endometrial neo-

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plasia (Eng 2000). *PTEN/MMAC1/TEP1* (MIM 601728) is a tumor-suppressor gene located at 10q23.3, which antagonizes the phosphoinositol-3-kinase (PI3K)/Akt pathway (reviewed by Waite and Eng [2002]). Proper PTEN signaling leads to G1 cell–cycle arrest and/or apoptosis (reviewed by Waite and Eng [2002]). When ascertained strictly by International Cowden Consortium Operational Diagnostic Criteria, ~80% of patients with CS demonstrate germline *PTEN* mutations (Liaw et al. 1997; Marsh et al. 1998). In addition, ~60% of individuals with Bannayan-Riley-Ruvalcaba syndrome (BRRS [MIM 153480])—another autosomal dominant hamartoma syndrome characterized by a classic triad of macrocephaly, lipomatosis, and speckled penis—carry germline *PTEN* mutations (Marsh et al. 1997, 1998, 1999). Subsequently, the clinical spectrum of disorders associated with germline *PTEN* mutations has expanded to include seemingly disparate syndromes, such as Proteus syndrome (MIM 176920), Proteus-like syndromes, and VATER association with macrocephaly (Zhou et al. 2000*b,* 2001; Reardon et al. 2001; Smith et al. 2002).

The underlying genetic causes remain undetermined in 20% and 40%, respectively, of individuals with classic CS and BRRS in whom no mutations have been detected by conventional mutation-detection techniques (reviewed by Waite and Eng [2002]). Because CS is believed to be without genetic heterogeneity (Nelen et al. 1996), we hypothesized that apparently *PTEN*-mutation–negative CS and BRRS may be attributed to large gene rearrangements and deletions, which cannot be detected by conventional techniques, and promoter mutations. To test our hypotheses, therefore, we used a combination of real-time quantitative multiplex PCR analysis, fluorescent-based semiquantitative PCR assay, and microsatellite analysis to define and characterize *PTEN* and regional deletions in a large series of probands with CS and BRRS previously found not to have intragenic *PTEN* mutations. Further, deletion-negative samples were subjected to sequence analysis of the promoter region of *PTEN.* Finally, we biochemically characterized the potential pathogenicity of the deletion and promoter mutations.

After written informed consent was received, DNA from peripheral blood was obtained from 122 unrelated individuals diagnosed with CS ($N = 95$; 79%), according to the International Cowden Consortium diagnostic criteria (Eng 2000), or BRRS ($N = 27$; 21%), by clinical definition (Gorlin et al. 1992). Real-time quantitative PCR analysis was performed by use of the ABI 7700 Sequence Detector System (ABI/Perkin Elmer), as described elsewhere (Sieber et al. 2002). *PTEN* exons 1 and 5 were chosen as targets for the real-time quantitative PCR assay, whereas the remaining exons were analyzed by use of fluorescent-based semiquantitative multiplex PCR assay. Human *RET* exon 8 was chosen as the internal control. Primer and probe sequences are listed in table 1. The raw data obtained from real-time PCR was analyzed by use of the comparative C_T method (as described in User Bulletin No. 2, ABI/Perkin Elmer), with normalization to the internal control, *RET.* Samples without

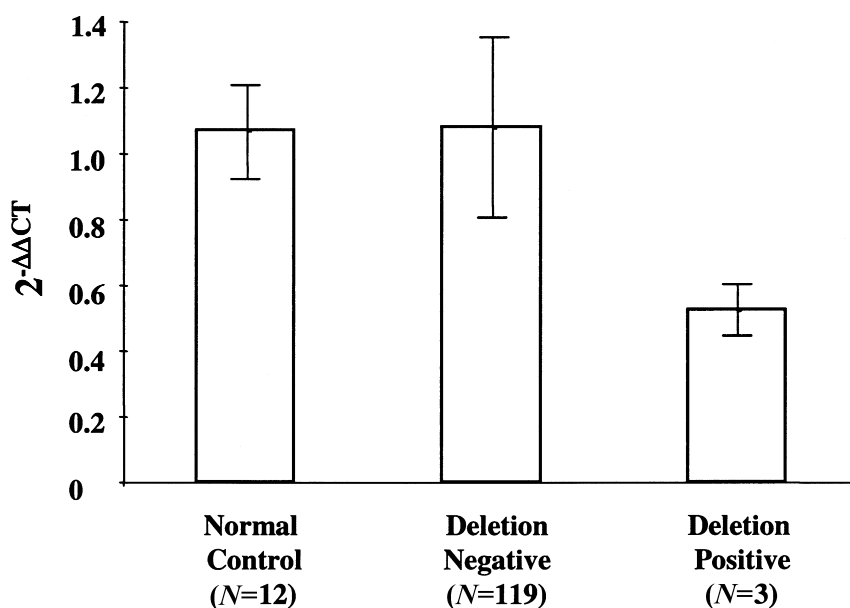

Figure 1 Real-time quantitative multiplex PCR results for 12 normal control subjects and 122 apparently mutation-negative individuals with CS and/or BRRS at *PTEN* exon 1. Normal control subjects showed $2^{-(\Delta\Delta CT)}$ values between 0.93 and 1.21. Patients with two copies of *PTEN* displayed values between 0.81 and 1.35, whereas patients with hemizygous deletions (one copy) had values between 0.45 and 0.60.

Table 1

Probes and Primers Used for Real-Time Quantitative Multiplex PCR, Fluorescent Semiquantitative Multiplex PCR Assay, and Promoter Sequencing

Primer/Probe	Sequence (5′→3′)	Amplicon Size (bp)
PTEN E1:		
Forward	GAGGATGGATTCGACTTAGACTTGA	
Reverse	CCCACGTTCTAAGAGAGTGACAGAA	85
Probe	FAM-CCTGTATCCATTTCTG-MGBNF	
PTEN E5:		
Forward	CCTACTTGTTAATTAAAAATTCAAGAGTTTTTT	
Reverse	GTGGGTTATGGTCTTCAAAAGGATA	98
Probe	FAM-TGTGCAACTGTGGTAAA-MGBNF	
RET E8:		
Forward	GTCCTGTGCAGTCAGCAAGAGA	
Reverse	CCACTCACACCTGCCTGTTG	79
Probe	VIC-CCTCACACTCCAGCCG-MGBNF	
PTEN E2:		
Forward	FAM-GTTTGATTGCTGCATATTTCAG	163
Reverse	TGAAATAGAAAATCAAAGCATTC	
PTEN E3:		
Forward	FAM- AAAATCTGTCTTTTGGTTTTTC	178
Reverse	TTGCAAGCATACAAATAAGAA	
PTEN E4:		
Forward	FAM-CATTATAAAGATTCAGGCAAT	205
Reverse	GACAGTAAGATACAGTCTATC	
PTEN E5:		
Forward	FAM-CTTTTTACCACAGTTGCACA	282
Reverse	GGAAAGGAAAAACATCAAAA	
PTEN E6:		
Forward	FAM-CCTGTTAAAGAATCATCTGGA	120
Reverse	AAGGATGAGAATTTCAAGCA	
PTEN E7:		
Forward	FAM- AGGCATTTCCTGTGAAATAA	172
Reverse	TTGATATCACCACACACAGG	
PTEN E8:		
Forward	FAM-CTCAGATTGCCTTATAATAGTC	245
Reverse	TCTGAGGTTTCCTCTGGTC	
PTEN E9:		
Forward	FAM-TCATATTTGTGGGTTTTCATT	260
Reverse	TCATGGTGTTTTATCCCTCT	
RET E8:		
Forward	FAM-CTGTGACCCTGCTTGTCT	135
Reverse	CACTCACACCTGCCTGTT	
Promoter:		
Forward	GCGTGGTCACCTGGTCCTTT	683
Reverse	GCTGCTCACAGGCGCTGA	

PTEN deletions were expected to yield $2^{-(\Delta\Delta CT)}$ values close to 1, whereas samples with hemizygous deletion or duplication were expected to give $2^{-(\Delta\Delta CT)}$ values close to 0.5 or 1.5, respectively. Positive results were controlled on at least three independent experiments. We found three (2.5%) individuals who harbored hemizygous germline deletions encompassing all or part of *PTEN*. The $2^{-(\Delta\Delta CT)}$ values ranged 0.45–0.60, for the three patients with deletions; 0.81–1.35, for the deletion-negative cases; and 0.93–1.21, for the 12 normal individuals tested (fig. 1). One sample (1397-1) showed deletion of all nine *PTEN* exons, which suggests that the deletion encompassed the entire gene. The other two samples (141-2 and 1621-1) had deletions encompassing exons 1–5 and exon 1 only, respectively. No sample was found to have exonic duplication. Further, we included a fragment of *PTEN* exon 5 in the fluorescent multiplex PCR assay and found no deletions except for the ones detected by real-time quantitative multiplex PCR, thereby confirming the sensitivity of fluorescent semiquantitative multiplex PCR assay.

To assess the extent of the *PTEN* germline deletions, three polymorphic markers intragenic to *PTEN* and five polymorphic markers flanking the 10q23.3 region were

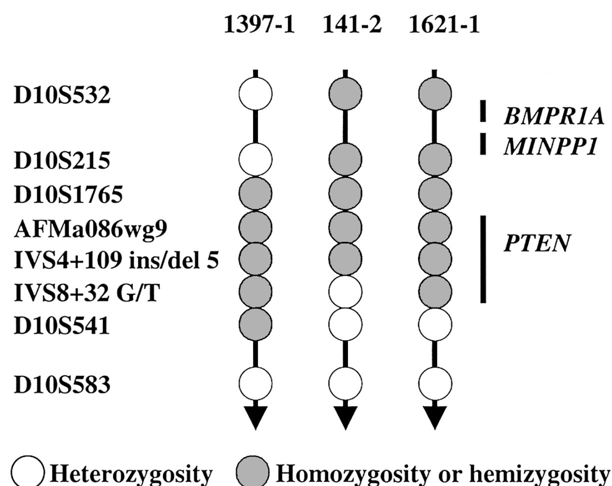

Figure 2 Genotyping results for the three patients with BRRS and/or CS with hemizygous *PTEN* deletions.

genotyped as described elsewhere (Marsh et al. 1999; Zhou et al. 2000*a*) (fig. 2). Markers AFMa086wg9, IVS4+109 ins/del TCTTA (IVS4+109), and IVS8+32 G/T (IVS8+32) are intragenic to *PTEN,* whereas D10S215, D10S1765, and D10S541 are ≤500 Kb up- or downstream of *PTEN.* Markers D10S532 and D10S583 are located ~3 cM upstream and 5 cM downstream of *PTEN,* respectively. All three patients with deletions were apparently homozygous (which suggests hemizygosity) at markers closest to or within the *PTEN* gene (D10S1765, AFMa086wg9, IVS4+109, and IVS8+32), consistent with the hemizygous deletions already identified by real-time quantitative multiplex PCR and fluorescent multiplex PCR assays. In one case (1397-1), "homozygosity" spanned from the upstream marker D10S1765 to D10S541, downstream of *PTEN,* concordant with whole-gene deletion detected by the quantitative PCR assays, and suggesting the deletion which also includes all of the *PTEN* promoter and likely all of the 3′ UTR. The second deletion case (141-2) showed "homozygosity" at D10S215, D10S1765, AFMa086wg9, and IVS4+109 and heterozygosity at IVS8+32, consistent with partial *PTEN* deletion, encompassing exons 1–5. In the third case (1621-1), although the homozygosity spanned from D10S215 to IVS8+32, deletion was detected only at exon 1 by real-time quantitative PCR assay, which suggests that the three intragenic markers are truly homozygous. All these deletions likely extend to at least 50 kb upstream of the translational start site (D10S1765). To investigate whether these three deletions include another gene close to the 10q23.3 region, we analyzed *MINPP1,* located ~500 kb upstream of *PTEN,* using semiquantitative duplex PCR assay; no evidence of involvement of this gene was found, thus also excluding the relevant juvenile polyposis gene *BMPR1A* (upstream of *MINPP1*) as part of the deletion (fig. 2).

Germline DNA from the remaining 119 patients without deletions was subsequently subjected to sequence analysis for mutations in the 600-bp full-promoter region of *PTEN.* Primers were designed to amplify the full promoter region between −1344 bp and −745 bp upstream of the translation start codon (Sheng et al. 2002) (table 1). Ten heterozygous sequence variants within the *PTEN* promoter region were found in nine patients with CS (9/119, 7.6% of total; 9/97, 10% of CS) (fig. 3). None of these promoter sequence variants were found among

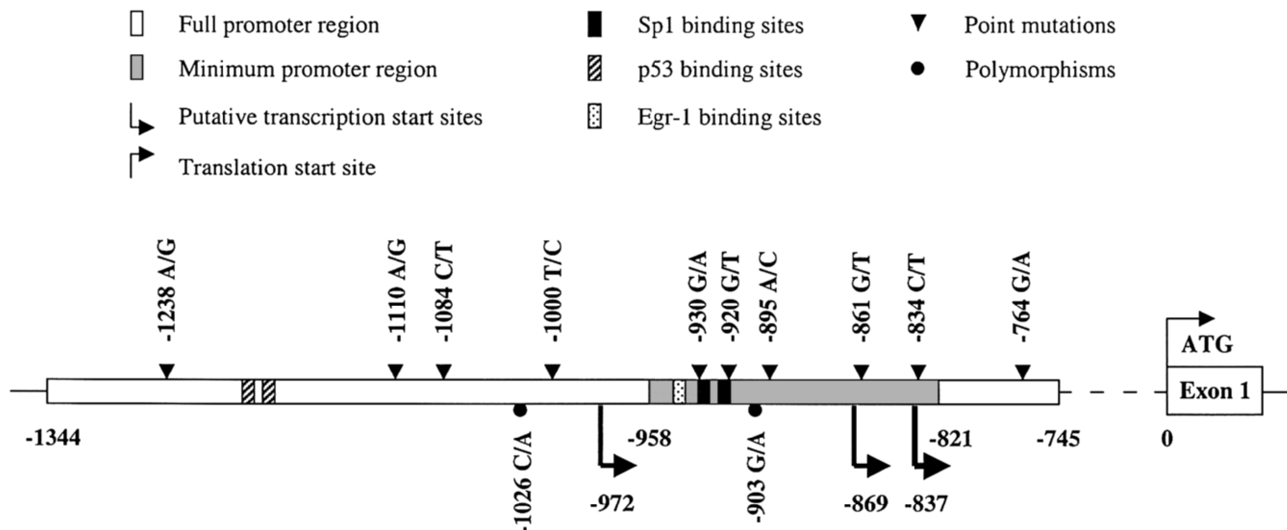

Figure 3 Germline *PTEN* promoter mutations and polymorphisms found in probands with CS

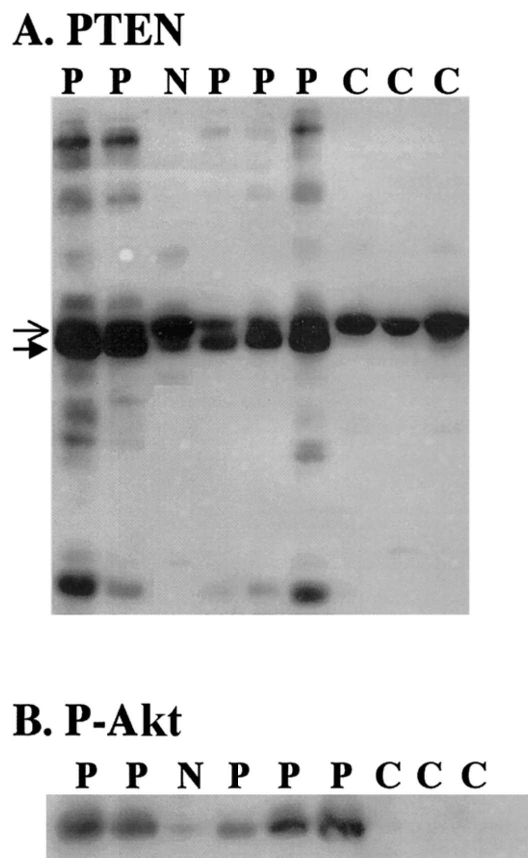

Figure 4 Aberrant PTEN protein species and increased phosphorylation of Akt in promoter-mutation samples. Samples from patients with promoter mutations (P), a patient who is *PTEN*-mutation negative (N), and normal control subjects (C) were analyzed. *A,* Western analysis for PTEN protein. Open arrows indicate the expected molecular weight of PTEN; closed arrows indicate the slower migrating band. *B,* Western analysis for P-Akt.

186 normal white control subjects (372 chromosomes), which suggests that the former are likely pathogenic. Two other sequence variants (−903 G/A and −1026 C/A) were present in both patients and normal control individuals with similar allele frequencies (data not shown), which suggests that they were indeed polymorphisms.

To functionally assess the *PTEN*-promoter-point mutations, cellular protein from five case subjects with promoter mutations was obtained during Trizol isolation of RNA and was subjected to western blot analysis (Waite and Eng 2003). Control samples and a sample from a patient with CS who was negative for the *PTEN* mutation display a single immunoreactive protein of the correct size (fig. 4). It is interesting that samples from patients carrying the promoter mutations showed a decrease in PTEN protein of the correct molecular weight (fig. 4*A, open arrowhead*), concordant with a dramatic increase of a slightly lower band (fig. 4*A, closed arrowhead*). This lower band is visible in the *PTEN*-mutation–negative CS sample and in an occasional control sample but not to the same extent as the promoter-mutation samples and never with a loss of the correct PTEN band. Three of the five promoter-mutation–positive cases had a laddering effect, with several bands recognized at both lower and higher molecular weights (fig. 4*A*), which has not been observed in 32 control samples or 23 *PTEN*-mutation–negative samples (data not shown). These data strongly suggest that the lower molecular weight band and the laddering effect are specific and related to the promoter mutations in these patients.

We next investigated if the PTEN protein produced in these patients was active. PTEN antagonizes the PI3K/Akt pathway by decreasing phosphatidylinositol-3,4,5 triphosphate levels, which results in decreased Akt phosphorylation (reviewed by Waite and Eng [2002]). Thus, active PTEN results in low Akt phosphorylation, and deficient PTEN results in increased Akt phosphorylation. Figure 4*B* shows that, in control and *PTEN*-mutation–negative samples, the level of phosphorylated Akt (detected by an antiphospho-Akt antibody) is low to undetectable, which indicates active PTEN function. In contrast, the level of phosphorylated Akt in the samples from cases carrying promoter mutations are dramatically elevated (fig. 4*B*). These data indicate that the PTEN protein produced has nonfunctional lipid phosphatase activity.

It is interesting to note that PTEN is a dual-substrate phosphatase that dephosphorylates both lipid and protein substrates (reviewed by Waite and Eng [2002]). At this time, we can accurately assess only the lipid phosphatase activity by monitoring the levels of Akt phosphorylation. Although our lab has shown that the protein phosphatase activity of PTEN regulates the down regulation of the mitogen-activated protein kinase pathway (Weng et al. 2001), we have found that the level of activation of this pathway varies considerably, even in normal controls (K. A. Waite and C. Eng, unpublished observations). Therefore, we are unable to reliably investigate changes in the protein phosphatase activity of PTEN that may arise from various *PTEN* mutations. It is interesting to postulate that various degrees of changes in both the lipid and protein phosphatase activities may play a role in the wide range of clinical manifestations of CS and BRRS.

There is little doubt that all three deletions are functionally deleterious, as all three likely include the promoter as well as all or part of *PTEN.* Protein analysis on the lymphoblastoid cell lines of one of the three deletion-positive patients revealed ~50% reduction in PTEN protein level, consistent with hemizygosity of *PTEN* (K. A. Waite and C. Eng, unpublished data). All three patients had a diagnosis of BRRS or BRRS/CS overlap. Two of these probands have gastrointestinal polyposis. Although our conclusion is based on a small sample size, there is

a trend toward gastrointestinal hamartomatous polyposis in individuals with deletions, compared with the 119 patients with CS or BRRS without deletions ($P = .1$; Fisher's two-tailed exact test). Although our patients' deletions are not cytogenetically obvious, at least three other unrelated patients with CS or BRRS have been reported elsewhere to have deletions or rearrangements in the *PTEN* region detected by cytogenetics (Arch et al. 1997; Tsuchiya et al. 1998; Marsh et al. 1999; Ahmed et al. 2000). All three with cytogenetically detected *PTEN* deletion or rearrangements carried the clinical diagnosis of BRRS. Over all, therefore, at least five probands with BRRS have been found to have deletions of or encompassing *PTEN*. Given that hemizygous *PTEN* deletions detected in probands with BRRS encompassed from single exon to whole gene, it is very likely that certain "neat" single-exon deletions—such as the one in patient 1621–1, involving only exon 1—could be missed by use of FISH technique with application of a designed probe.

Until now, the *PTEN* promoter had not been examined in patients with CS and/or BRRS. However, in vitro work has shown that activated PPARγ and p53 result in up-regulated PTEN transcription (Patel et al. 2001; Stambolic et al. 2001; Virolle et al. 2001), which suggests that alterations of the promoter sequence could result in changes to PTEN protein structure, levels, and function. Among 119 mutation-negative or deletion-negative CS or BRRS cases, nine probands with CS were found to carry germline heterozygous point mutations in the promoter. Of significance, all nine individuals with *PTEN*-promoter mutations had a diagnosis of classic CS yet had relatively mild phenotypic features, as operationally defined by oligo-organ involvement (involvement of fewer than four organs; see Marsh et al. [1998] for classification) (table 2). It is interesting that one deletion-positive proband with exon 1 and upstream involvement had similarly mild features, with only two-organ involvement, macrocephaly, and lipomas. The deletion encompassing exons 1–5 in patient 141-2 was also present in a sibling, 141–1, both of whom have features of both CS and BRRS. It would be interesting to see whether these promoter mutations are also present in affected relatives of familial cases. Collection and analysis of parental DNA of these case subjects with promoter mutations are ongoing.

Of the 10 promoter mutations (one patient had two different sequence variants), 5 were localized to the minimum *PTEN* promoter region (−958 to −821), 2 of which (−920G→T and −930G→A) are predicted to alter two putative Sp1 transcription factor–binding sites (fig. 3). Further, protein analysis revealed a reduced expression of wild-type PTEN, a strong lower–molecular-weight immunoreactive band, and a laddering effect of protein immunoreactive with a specific monoclonal antibody against human PTEN, which suggests that these point nucleotide substitutions are functionally significant and, thus, represent promoter mutations. These data suggest that the promoter variants may result in alternative start sites that yield PTEN protein of various sizes (fig. 4*A*).

It is interesting to note that the two samples with mutations at the two putative Sp1 binding sites were the two with doublet PTEN-immunoreactive bands but no laddering effect (fig. 4*A*). Although the transcriptional regulation of *PTEN* is only now beginning to be elucidated, we suspect that these variants would alter *PTEN* transcription, which would result in impaired protein expression. The presence of some wild-type protein, together with PTEN proteins of various sizes, might be postulated to result in the milder phenotype associated with these promoter variants. It is also possible that such mutations result in posttranslational modifications, which could result in altered mobility during SDS-PAGE. Another possibility is that the PTEN protein formed is altered at the protein level, which results in targeted degradation, and it is the degradation of PTEN protein that we are observing. The mechanisms of PTEN degradation are only now being understood (Vazquez et al. 2000; Torres and Pulido 2001; Waite and Eng 2003). Improper PTEN degradation could also result in impaired protein expression. Further analysis, as patient sample material

Table 2

Family-as-Unit Clinical Features of Probands Positive for *PTEN* Promoter Mutation

Multiorgan[a]	Breast Cancer	Thyroid Cancer	Uterine Cancer	Mutation
No	No	No	No	−1000TC, −1238A→G
No	Yes	No	No	−1110A→G
No	No	No	No	−1084C→T
No	No	Yes	No	−930G→A
No	Yes	No	No	−920G→T
No	Yes	No	No	−895A→G
Yes	Yes	Yes	No	−861G→T
No	Yes	Yes	No	−834C→T
No	Yes	No	No	−764A→G

[a] Multiorgan involvement operationally defined as at least five organs involved, as detailed by Marsh et al. (1998).

becomes available, will be necessary to determine the exact mechanism of the laddering effect.

Regardless of the mechanism of lower molecular weight proteins, we have demonstrated that the PTEN protein species produced in these promoter-mutation–positive patients is deficient. The levels of phosphorylated-Akt were significantly higher in samples haboring promoter sequence variants, compared with control subjects and *PTEN*-mutation–negative samples (fig. 4*B*), which indicates an increase in the activity of the pro-proliferative PI3K/Akt pathway.

On the basis of our observations that 11% of individuals with features of BRRS were found to have a deletion, it may be prudent to offer deletion analysis to patients with BRRS and patients with CS/BRRS with gastrointestinal polyposis but without PCR-based intragenic *PTEN* mutations. Further, our ~10% promoter-mutation frequency among probands with CS previously found to be PCR-mutation negative and deletion negative does suggest that promoter analysis might be useful in the clinical setting. That these promoter variants are deleterious and likely causative of CS has been demonstrated by aberrant PTEN protein bands on western blot, which resulted in activation of the pro-proliferative Akt pathway. Indeed, western analyses should be considered a useful molecular diagnostic adjunct to determine functionality of promoter variants.

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Electronic-Database Information

The URL for data presented herein is as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for CS, BRRS, PS, and *PTEN/MMAC1/TEP1*)

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