Title: 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

Running Title: *PTEN* deletions and promoter mutations upregulate Akt in Cowden syndrome

Keywords: *PTEN*, deletion, promoter mutation, Cowden syndrome, Bannayan-Riley-Ruvalcaba syndrome

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Total Pages: 32 Tables: 1 Figures: 4

Abstract

Germline intragenic mutations in *PTEN*, a tumor suppressor gene located on 10q23.3 and encoding a lipid and protein-tyrosine phosphatase, are associated with 80% of patients with Cowden syndrome (CS) characterized by multiple hamartomas and an increased risk of breast, thyroid, and endometrial cancers, and 60% of patients with Bannayan-Riley-Ruvalcaba syndrome (BRRS) characterized by macrocephaly, lipomatosis, and speckled penis. Despite extensive PCR-based *PTEN* mutation analysis in these two syndromes, the underlying genetic causes remain to be determined in a considerable proportion of classic CS and/or BRRS without a PCR-detectable *PTEN* mutation. Because CS is believed to be without locus heterogeneity, we hypothesized that gross gene deletions and mutations in the *PTEN* promoter might alternatively account for a subset of apparently mutation-negative CS and/or BRRS patients. Applying real-time quantitative multiplex PCR and fluorescent-based semi-quantitative multiplex PCR assays, we identified three (2.5%) germline hemizygous *PTEN* deletions in a series of 122 apparently mutation-negative classic CS (N=96) or BRRS (N=26). Fine mapping of the region suggested that one deletion encompassed the whole gene, and the other two included exon 1 and encompassed exons 1 to 5 of *PTEN*, respectively. Two of the three deletion cases carried the diagnosis of BRRS and one CS. Of note, the BRRS patient with only exon 1 deletion had mild features. Mutation analysis of the *PTEN* promoter revealed nine cases (7.4%) harboring heterozygous germline mutations. All nine individuals with promoter mutations had classic CS, representing almost 10% of all CS subjects. Eight had breast cancers and/or benign breast tumors, but otherwise mild features. Western blot analysis for PTEN protein, obtained from one deletion positive and five *PTEN* promoter mutation positive patient lymphoblast cell lines, revealed a half reduction protein level and

multiple bands of immmunoreactive protein, respectively. In contrast, control samples contained only one reactive band of expected size. Further analysis revealed an elevated level of phosphorylated Akt in the five promoter mutation positive samples, compared to control cell lines, indicating that the PTEN protein present in the promoter mutation positive cases was not active. Our observations suggest that classic BRRS without PCR-detected intragenic *PTEN* mutations should be offered deletion analysis while classic CS without PCR-detectable *PTEN* mutation should be offered promoter analysis. Protein from promoter variant positive individuals should be subjected to Western blot for PTEN and P-Akt to confirm pathogenicity.

Introduction

Cowden syndrome (CS, OMIM 158350) is an autosomal dominant disorder that is characterized by multiple hamartomas affecting derivatives of all three germ layers and by an increased risk of breast, thyroid, and endometrial neoplasia (Eng 2000). *PTEN/MMAC1/TEP1* (OMIM 601728), a tumor suppressor gene located at 10q23.3, encodes a dual-specificity phosphatase which functions as a major 3'-phosphatase and antagonizes the phosphoinositol-3-kinase (PI3K)/Akt pro-apoptotic pathway (Nelen et al. 1996; Li and Sun 1997; Li et al. 1997; Steck et al. 1997; Maehama and Dixon 1998; Stambolic et al. 1998). Via this and PI3K/Akt-independent pathways, proper PTEN signaling leads to G1 cell cycle arrest and/or apoptosis (Furnari et al. 1998; Li and Sun 1998; Stambolic et al. 1998; Weng et al. 1999; Weng et al. 2001a; Weng et al. 2001b; Weng et al. 2001c; Weng et al. 2001d; Weng et al. 2002). When ascertained strictly by the International Cowden Consortium Operational Diagnostic Criteria, approximately 80% of CS have been found to carry germline mutations in *PTEN* (Liaw et al. 1997; Marsh et al. 1998). In addition, germline mutations of *PTEN* have been found in 60% of individuals with Bannayan-Riley-Ruvalcaba syndrome (BRRS, OMIM 153480) (Marsh et al. 1997; Marsh et al. 1998; Marsh et al. 1999). Subsequently, the clinical spectrum of disorders that are associated with germline *PTEN* mutations has expanded to include seemingly disparate syndromes, such as Proteus syndrome (OMIM 176920), Proteus-like syndromes, and VATER association with macrocephaly (Zhou et al. 2000b; Reardon et al. 2001; Zhou et al. 2001; Smith et al. 2002).

Despite extensive *PTEN* mutation analysis in both the research and clinical setting in the two autosomal dominant hamartomatous syndromes, CS and BRRS, the underlying genetic causes remain undetermined in 20% and 40%, respectively, of

individuals with classic CS and/or BRRS, in whom no mutations have been detected by conventional mutation detection techniques, ie, PCR-based single-strand conformation polymorphism analysis (SSCP), denaturing gradient gel electrophoresis (DGGE) and direct DNA sequencing [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 PCR-based mutation detection strategies, and promoter mutations. To test our hypotheses, therefore, we used a combination of real-time quantitative multiplex PCR analysis, fluorescent-based semi-quantitative PCR assays and microsatellite analyses to define and characterize *PTEN* and regional deletions in 122 CS and BRRS probands 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.

Patients, Materials and Methods

Subjects and DNA Extraction

After informed consent, peripheral blood was obtained from 122 unrelated individuals, who were diagnosed with CS according to the diagnostic criteria set by the International Cowden Consortium (Eng 2000) and BRRS by the original clinical definition (Gorlin et al. 1992). In addition, these subjects were found to have no germline mutations in the *PTEN* coding region, exon-intron boundaries or flanking intronic regions by DGGE and direct sequencing, were analyzed for gene deletions, duplications and promoter mutations. Overall, 96 (79%) had classic CS, and 26 (21%) had BRRS. Clinical phenotypic details including organ-specific cancers and benign processes and PHTS-related features [detailed by (Marsh et al. 1998)], were available for all individuals and their families. Normal controls comprised commercially available pooled human genomic DNA (Promega, Madison, WI) and 186 healthy Caucasian blood donors. Germline DNA from peripheral blood leukocytes from cases and controls were extracted using standard techniques (Miller et al. 1988). In addition, remaining leukocytes from 32 normal controls and a subset of cases were EBV-transformed for lymphoblastoid cultures (Fukushima et al. 1992).

Multiplex Real-Time Quantitative PCR

The real-time quantitative PCR and analysis were carried out using the ABI 7700 Sequence Detector System (ABI/Perkin Elmer, Foster City, CA) as previously described (Sieber et al. 2002). Real-time quantitative PCR assay determines gene dosage (or copy number) by monitoring PCR amplification in real-time and using the 5’-nuclease activity of *Taq* DNA polymerase. *PTEN* exons 1 and 5 were chosen as targets for the real-time quantitative PCR assay, while the remaining exons were

analyzed by using a fluorescent-based semi-quantitative multiplex PCR approach as detailed below. Exon 8 of human *RET*, a proto-oncogene located at 10q11.2 which is not deleted or amplified in the germ-line of CS and/or BRRS patients, was chosen as internal control. The primers and probes were designed by using PRIMER EXPRESS software (Applied Biosystems). The *PTEN* exon 1 and exon 5 probes were labeled at the 5' ends with the reporter dye FAM and at the 3' end with the MGBNF quencher. The *RET* exon 8 probe was labeled at the 5' end with reporter dye VIC and at the 3' end with the MGBNF quencher. Primer and probe sequences are listed in Table 1. The real-time quantitative multiplex PCR assay was optimized according to the instructions in User Bulletin No.5 (Applied Biosystems, data not shown).

The assay was carried out in 96-well optical reaction plates (Applied Biosystems). Briefly, DNA aliquots of 15 ng/ul were prepared for PCR. Each experiment included triplicate patient samples, one normal control and one deletion control. Each 25-ul reaction contained 1 x TaqMan Universal PCR Master mix (Applied Biosystems), 300 nM *PTEN* exon 1 or exon 5 and 100 nM *RET* exon 8 forward primers, 900 nM *PTEN* exon 1 or 100 nM exon 5 and 300 nM *RET* exon 8 reverse primers, 200 nM *PTEN* exon 1 or exon 5 and *RET* exon 8 probes, and 30 ng DNA. The thermal cycling conditions were 50^{o}C for 2 min and 95^{o}C for 10 min, followed by 40 cycles of denaturing at 95^{o}C for 15 s and annealing and extension at 60^{o}C for 1 min.

The raw data obtained were analyzed using the comparative C_T method (as described in User Bulletin No.2). After normalization against the internal control, *RET*, the *PTEN* copy number of a given patient was determined by comparison to a normal calibrator. *PTEN* copy number was reflected by a $2^{-(\Delta\Delta CT)}$ value, where $\Delta\Delta C_T = (C_T PTEN\text{-}C_T RET)_{sample} - (C_T PTEN\text{-}C_T RET)_{normal}$. The ΔC_T value, represented the

mean C_T value of each sample-triplicate, where C_T was defined as the cycle number during the exponential phase at which the amplification plot crossed a fixed normalized emission intensity threshold (average 0.05). Samples without *PTEN* deletions were expected to give $2^{-(\Delta\Delta CT)}$ 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.

Semi-quantitative Fluorescent Multiplex PCR

Short fragments (<300 bp) of *PTEN* exons 2, 3, 4, 6, 7, 8, 9 and *RET* exon 8, as an internal control, were simultaneously PCR amplified, using 6-FAM labeled primers (Table 1). Three multiplex PCRs were necessary to cover all of the exons. PCR was performed in a final volume of 25 ul containing 100 ng genomic DNA, 0.2-1 uM of each primers, 1x Qiagen HotStartTaq multiplex PCR buffer and 0.5x Q-solution (Qiagen, Valencia, CA). After a denaturation and 'hot-start' step of 15 min at 95^{o}C, there were 21 cycles of 30 s at 95^{o}C, 90 s at 55^{o}C and 90 s at 72^{o}C, followed by a final extension of 10 min at 72^{o}C. PCR products were electrophoresed on a semi-automated sequencer (ABI-3700, Applied Biosystems), data were analyzed using the Gene Scanner Version 3.7 (Applied Biosystems). The peak height of the corresponding fragment was compared between each sample and normal control in the fluorescent multiplex PCR assay. A 0.5 reduction of the peak(s) indicates an hemizygous deletion of the corresponding exon(s), whereas exonic duplication results in a 1.5 increase of the peak height. Each positive sample was re-analyzed in at least three independent experiments.

Microsatellite and SNP Analysis

Genotypes were determined by using nine polymorphic marker loci as described previously (Marsh et al. 1999; Zhou et al. 2000a) (Fig. 2). Markers AFMa086wg9, IVS+4 109 ins/del TCTTA and IVS8+32 G/T are intragenic to *PTEN*, D10S215, D10S1765 and D10S541 are within 500 Kb up- or downstream of *PTEN*. Markers D10S573, D10S532 are located about 3 cM upstream of *PTEN*, and marker D10S583 lies about 7 cM downstream of *PTEN*.

PTEN Promoter Mutation Analysis

Primers were designed to amplify the full length of the *PTEN* promoter region between –1344 bp and –745 bp upstream of the translation start codon (Sheng et al. 2002) (Table 1). PCR was carried out in 25-ul reactions containing 1 x Qiagen HotStartTaq Master buffer, 1 x Q-solution (Qiagen, Valencia, CA), 0.4 nM of froward and reverse primers. After a denaturation and 'hot-start' step of 15 min at 95^{o}C, the PCR consisted of 35 cycles of 30 s at 94^{o}C, 45 s at 65^{o}C, and 45 s at 72^{o}C, followed by a final extension of 10 min at 72^{o}C. After pre-sequencing treatment with restriction enzymes Exon I and SAP (USB, Cleveland, OH), PCR products were directly sequenced on both strands using the big Dye Terminator Kit (Applied Biosystems) and a model 377 automated sequencer (Applied Biosystems)**.**

Cellular Protein Extraction

Lymphoblast cell lines were generated by immortialization of patient and control lymphocytes with Cylclosporin-A, and Epstein-Barr virus. Cells were maintained in RPMI containing 20% FBS and passaged as needed. Approximately 10 million cells were pelleted by centrifugation (800 x g, 10 min, 4˚C), the resulting

pellet was then washed twice with ice cold PBS, followed by centrifugation and resuspended in mammalian lysis buffer (Pierce, Rockford, IL) containing 500 mM PMSF, 5 μg/ml each of leupeptin, aprotinin, and pepstatin, 2 mM sodium orthovanadate, 50 mM NaF and 10 mM β−glycerophosphonate. Insoluble cellular material was removed by centrifugation at 4˚C. Protein concentration was determined by the bicinchoninic acid method with bovine serum albumin as a standard (Smith et al. 1985).

Western Blot Analysis

Proteins (30 μg) were separated by 10% SDS-PAGE using the Laemelli method (Laemmli 1970), after electrophoresis, proteins were electrophoretically transferred to nitrocellulose (Towbin et al. 1979). Nitrocellulose blots were blocked by incubation for 1 hr in 5% milk in TBS-T (10 mM Tris pH=7.0, 100 mM NaCl and 0.1% Tween 20). Blots were then incubated for 2 hr, at room temperature with either anti-PTEN or an antibody raised against the Ser 473 phosphorylated Akt (P-Akt) (Santa Cruz, Santa Cruz, CA). All primary antibodies were diluted 1:1000 in 3% BSA. After the primary incubation, the blots were subjected to six 10-min washes and then incubated for 1 hr with the appropriate HRP-conjugated secondary antibody at a 1:5000 dilution. Blots were washed again, and visualized by enhanced chemiluminesence according to manufacturer's instruction.

Results

Using the combination of real-time quantitative multiplex PCR and fluorescent multiplex PCR assays, we analyzed 122 DNA samples from individuals with classic CS and/or BRRS with no apparent germline intragenic *PTEN* mutation detected by PCR-based techniques. We found 3 (2.5%) individuals harboring hemizygous germline deletions encompassing all or part of *PTEN*. The $2^{-(\Delta\Delta CT)}$ values ranged from 0.45 to 0.60 for the three patients with deletions, from 0.81 to 1.35 for the deletion negative cases, and from 0.93 to 1.21 for the 12 normal individuals tested (Fig. 1). One sample (1397-1) showed deletion of all nine *PTEN* exons, suggesting that the deletion encompassed the entire gene. The other two samples (141-2 and 1621-1) had deletions encompassing exon 1 to 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 as well, and found no deletions except for the ones detected by real-time quantitative multiplex PCR, thereby confirming the sensitivity of fluorescent multiplex PCR assay.

To assess the extent of the *PTEN* germline deletions, three polymorphic markers intragenic to *PTEN* and six polymorphic markers flanking the 10q23.3 region were genotyped (Fig. 2). All 3 deletion patients were apparently homozygous (suggesting hemizygosity) at markers most close 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 real-time quantitative PCR assays, and suggesting a 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 exon 1 to exon 5. In the third case (1621-1), while the homozygosity spanned from D10S215 to IVS8+32, deletion was only found at exon 1 by real-time quantitative PCR assay, suggesting that the three intragenic markers are truly homozygous. All these deletions likely extend at least 50 kb upstream of the translational start site (D10S1765).

Germline DNA from the remaining 119 patients without deletions were sequenced for mutations in the 600-bp full promoter region of *PTEN*. Nine patients (9/119, 7.6%) were found to have heterozygous sequence variants within the *PTEN* promoter region (Fig. 3). None of these promoter sequence variants were found among 186 normal Caucasian controls, suggesting 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), suggesting that they were indeed polymorphisms.

To functionally assess the *PTEN* promoter point mutations, cellular protein was isolated from lymphoblastoid cells lines available from five cases with promoter mutations and subjected to Western blot analysis. Figure 4 shows that PTEN protein was recognized in control as well as case samples with promoter sequence variations. Control samples and a sample from a *PTEN* mutation negative CS patient displayed a single immunoreactive protein of the correct size. Interestingly, samples from patients carrying the promoter mutations showed a decrease in immunoreactive PTEN of the correct molecular weight (Fig.4A, open arrowhead), concordant with a dramatic increase of a slightly lower immunoreactive band (Fig. 4A, closed arrowheads). This lower band is visible in the *PTEN* mutation negative CS sample and in an occasional

control, however, not to the same extent as the promotor mutation samples and never with a loss of immunoreactivity of PTEN. Three of the five promoter mutation positive cases had a laddering effect with several immunoreactive bands recognized at both lower and higher molecular weights (Fig. 4A, astericks). We have analyzed 32 control samples and 23 *PTEN* mutation negative samples and have not observed this laddering effect (data not shown). These data strongly suggest that the lower molecular weight immunoreactive band and the laddering effect that we observe are specific and related to the promoter mutations in these patients.

Because immunoreactive PTEN of the correct molecular weight was present in the samples containing *PTEN* promoter mutations, albeit to a lower extent, we decided to determine if this protein, or the presumably non-full-length proteins (laddering effect), was active. PTEN anatagonizes the PI3K/Akt pathway by decreasing phosphatitidylinsositol (3,4,5) triphosphate levels [reviewed by (Waite and Eng 2002)]. This reduction ultimately leads to reduced Akt phosphorylation [reviewed by (Waite and Eng 2002)]. Thus, when PTEN is active, the levels of Akt phosphorylation are low. When PTEN is absent or inactive, the levels of phosphorylated Akt increases. Figure 4B shows that in control and *PTEN* mutation negative samples that the level of phosphorylated Akt (detected by an anti-phospho-Akt antibody) is low to nondetectable, indicating active PTEN function. In contrast, the level of phosphorylated Akt in the samples from cases containing promoter mutations are dramatically elevated. These data indicate that the PTEN protein produced has inactive lipid phosphatase activity.

All nine individuals with germline *PTEN* promoter mutations appear to have mild phenotypic features, and non-multi-organ involvement. Interestingly, the BRRS subject with exon 1 and upstream deletion also posseses a relatively mild phenotype,

with only macrocephaly and lipomas. Although based on small sample size, there is a trend towards gastrointestinal hamartomatous polposis in individuals with deletions compared to the 119 CS or BRRS without deletions (P=0.1, Fisher's 2-tailed exact test).

Discussion

Based on PCR-based mutation scanning technologies, 80% and 60% of classic CS and BRRS individuals, respectively, have been found to harbor germline intragenic mutations in *PTEN*. All PCR-based technologies are limited by their inability to detect neat-single-exon deletions, large deletions and rearrangements [reviewed by (Eng et al. 2001); Eng, 2001 #119]. Therefore, using a combination of different technologies, we have found three of 122 CS/BRRS individuals previously without intragenic *PTEN* mutations to have germline deletions encompassing part or all of *PTEN*. 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 one of the three deletion positive patient's lymphoblastoid cell lines revealed about 50% reduction in PTEN protein level, consistent with hemizygousity of *PTEN* (Waite and Eng, unpublished data). Two of these probands have gastrointestinal polyposis, one of whom has CS. This is unusual as classic CS patients do not become symptomatic from hamartomatous polyps because these polyps are characteristically diminutive (Weber et al. 1998). While our patients' deletions are not cytogenetically obvious, at least three other unrelated CS or BRRS patients have been reported previously 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. Overall, therefore, at least five BRRS probands have been found to have deletions of or encompassing *PTEN*.

Until now, the *PTEN* promoter has not been examined in patients with CS and/or BRRS. However, *in vitro* work has shown that activated PPARγ, p53, and EGR1 have been shown to up-regulate PTEN transcription (Patel et al. 2001;

Stambolic et al. 2001; Virolle et al. 2001), suggesting that changes to the promoter sequence may indeed result in changes to PTEN protein structure, levels and function. The genomic characterization of the *PTEN* promoter (Sheng et al. 2002) prompted us to scan for possible mutations in our apparently mutation-negative CS and/or BRRS patients. Among 119 mutation-negative or deletion negative CS or BRRS cases, nine CS probands were found to carry germ-line heterozygous point mutations in the promoter that were absent in 372 control chromosomes. Of significance, all nine individuals with *PTEN* promoter mutations had a diagnosis of classic CS, yet had relatively mild phenotypic features and oligo-organ involvement [<4 organ involvement: see (Marsh et al. 1998) for classification]. Interestingly, one deletion positive proband with exon 1 and upstream involvement had similarly mild features and two-organ involvement.

Of the nine patients with 10 promoter mutations (one case had two different sequence variants), five were localized to the minimum *PTEN* promoter region (-958 to –821), two of which (-920 G>T, -930 G>A) are predicted to alter two putative Sp1 transcription factor binding sites (Fig. 3). Further, protein analysis revealed a reduced expression of wildtype PTEN, a strong lower molecular weight immunoreactive band, and a laddering effect of protein immunoreactive with a specific monoclonal antibody against human PTEN, suggesting that these point nucleotide substitutions are functionally significant and thus represent promoter mutations. These data suggest that the promoter variats may result in alternative start sites yielding PTEN protein of various sizes (Fig. 4A). 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. 4A). Although the transcriptional regulation of *PTEN* is only now beginning to be elucidated, we suspect that these variants would

alter *PTEN* transcription resulting in impaired protein expression. The presence of some wildtype protein together with PTEN proteins of various sizes might be posulated to result in the milder phenotype associated with these promoter variants. Another possibility is that the PTEN protein formed is also somehow 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. The precise mechanism for the appearance of the both the lower and higher molecular weight immunologically reactive PTEN species remains to be investigated.

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 not active. The levels of phosphorylated-Akt were significantly higher in samples haboring promoter sequence variants compared to controls and *PTEN* mutation negative samples (Fig. 4B), indicating an increase in the activity of the pro-proliferative PI3K/Akt pathway.

It is interesting to note that PTEN is a dual-substrate phosphatase dephosphorylating both lipid and protein substrates [reviewed by (Waite and Eng 2002)]. At this time, we can only accurately assess the lipid phosphatase activity, by monitoring the levels of Akt phosphorylation. While our lab has shown that the protein phosphatase activity of PTEN regulates the down regulation of the mitrogen-activated protein kinase pathway (Weng et al. 2001d), we have found that the level of activation of this pathway varies considerably even in normal controls (Waite and Eng, unpublished observations). Therefore, we are unable to reliably investigate changes in PTEN's protein phosphatase activity 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.

Overall, our data demonstrating a ~2.5% germline *PTEN* deletion frequency amongst PCR-defined *PTEN* mutation negative CS/BRRS might suggest that deletion analysis has no place in the clinical setting. However, based on our observations and those in the literature, it may be prudent to offer deletion analysis, both microscopic and submicroscopic, in BRRS patients and CS patients with gastrointestinal polyposis without PCR-based intragenic *PTEN* mutations. Further, our ~10% promoter mutation frequency amongst CS probands previously found to be PCR-mutation negative and deletion negative does suggest that promoter analysis might be useful in the clinical setting when PCR-based methods yield negative results. That these promoter variants are deletrious and likely causative of CS have been demonstrated by aberrant PTEN protein bands on Western blot resulting in activation of the pro-proliferative Akt pathway. Indeed, Western analyses should be considered as a useful molecular diagnostic adjunct to determine functionality of promoter variants.

Acknowledgments

We are grateful to our patients and families, and their clinicians, who have continued to participate in our research. We thank Susan Whitman and Tamara Vukosavljevic (Real-time PCR Core Facility at the Comprehensive Cancer Center, The Ohio State University) for their excellent technical support, and Micheala Aldred for donating anonymous DNA samples from normal controls. This work is partially funded by the American Cancer Society (RSG-02-151-01-CCE to CE), Susan G. Komen Breast Cancer Research Foundation (BCTR 2000 462 to CE) and National Cancer Institute (P30CA16058 to The Ohio State University Comprehensive Cancer Center). CE is a recipient of the Doris Duke Distinguished Clinical Scientist Award.

Electronic Database Information

Accession numbers and URLs for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/

(for CS [MIM 158350], BRRS [MIM 153480], PS [MIM 176920], and *PTEN/MMAC1/TEP1* [MIM 601728])

Microsatellite marker loci within or flanking *PTEN*,

http://www.ensembl.org/Homo_sapiens/

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Table 1. Probes and primers used for real-time quantitative multiplex PCR and fluorescent multiplex PCR assays

Primer / Probe	Sequence (5' to 3')	Amplicon size (bp)
PTEN E1 Forward	GAGGATGGATTCGACTTAGACTTGA	85
Reverse	CCCACGTTCTAAGAGAGTGACAGAA	
Probe	FAM-CCTGTATCCATTTCTG-MGBNF	
PTEN E5 Forward	CCTACTTGTTAATTAAAAATTCAAGAGTTTTTT	98
Reverse	GTGGGTTATGGTCTTCAAAAGGATA	
Probe	FAM-TGTGCAACTGTGGTAAA-MGBNF	
RET E8 Forward	GTCCTGTGCAGTCAGCAAGAGA	79
Reverse	CCACTCACACCTGCCTGTTG	
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	

Figure Legends

Figure 1 Real-time quantitative multiplex PCR results for 12 normal controls and 119 apparently mutation-negative individuals with CS and/or BRRS at *PTEN* exon 1. Normal controls 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 only one copy had values between 0.45 and 0.60.

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

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

Figure 4 Aberrant immunoreactive PTEN species and increased phosphorylation of Akt in promotor mutation samples

30 mg of protein from lymphoblast cell lines, generated from control or patient blood, was separated on a 10% SDS-PAGE gel and subjected to western analysis as described in the Methods. Samples from patients with promotor mutations (P), a patient who is PTEN mutation negative (N) and normal controls (C) were analyzed. A. Western analysis for PTEN protein. Open arrows indicate the expected molecular weight of PTEN, closed arrows indicate the slower migrating immunoreactive band, and astericks indicate bands of aberrant molecular weight. B. Western analysis for P-Akt.