Differential expression of novel naturally occurring splice variants of \textit{PTEN} and their functional consequences in Cowden syndrome and sporadic breast cancer

Shipra Agrawal\textsuperscript{1,3,4} and Charis Eng\textsuperscript{1,2,5,*}

\textsuperscript{1}Genomic Medicine Institute, Cleveland Clinic Lerner College of Medicine, 9500 Euclid Avenue, Mailcode NE-50, Cleveland, OH 44195, USA, \textsuperscript{2}Department of Genetics, Case Western Reserve University School of Medicine, Cleveland, OH, USA, \textsuperscript{3}Human Cancer Genetics Program, Comprehensive Cancer Center and \textsuperscript{4}Department of Molecular Virology, Immunology and Medical Genetics, The Ohio State University, Columbus, OH, USA and \textsuperscript{5}Cancer Research, UK Human Cancer Genetics Research Group, University of Cambridge, Cambridge, UK

Received December 14, 2005; Revised and Accepted January 18, 2006

\textit{PTEN}, a dual-phosphatase tumor suppressor, is inactivated in Cowden syndrome (CS), characterized by high risk of breast and thyroid cancer, and in variety of sporadic cancers. Despite the importance of alternative splicing, very limited information on its role in \textit{PTEN} and associated cancers is available. We identified eight novel \textit{PTEN} splice variants (SVs) that retained intron 3 regions (3a, 3b, 3c); intron 5 regions (5a, 5b, 5c); excluded part of exon 5 (DelE5) or all of exon 6 (DelE6), respectively. Analysis of SVs in 12 sporadic breast cancers revealed full-length (FL)-\textit{PTEN} transcript reduction in 10; SVs 3b, 3c and 5c not expressed in 7, 6 and 4, respectively, and under-expressed in the rest. In contrast, SV-5b was over-expressed in breast cancers. \textit{PTEN} SV analysis in 16 CS/CS-like patients and eight controls revealed that SV-5a is under-expressed and SV-3a over-expressed in the germline of CS/CS-like individuals when compared with controls. Although SV-5a expression decreased P-Akt level and cyclin D1 promoter activity, SVs 5b and 5c increased cyclin D1 promoter activity. Thus, SV-5a behaving like FL-\textit{PTEN} corroborates our observation that SV-5a is under-expressed in CS when compared with controls. Similarly, SV-5b functionally counters PTEN’s action and is over-expressed in sporadic breast cancers. Furthermore, we demonstrate that expression of these SVs is under the regulation of p53. Our observations suggest that differential expression of \textit{PTEN} and its SVs could play a role in the pathogenesis of sporadic breast cancers and CS, and may lend a novel way of making a rapid molecular diagnosis of CS without mutation analysis.

\textbf{INTRODUCTION}

\textit{PTEN} (Phosphatase and Tensin homolog, deleted on chromosome TEN) is a tumor suppressor on 10q23.3 that has been found to be mutated in many types of sporadic neoplasias and heritable disorders. Germline \textit{PTEN} mutations have been found in 85\% of Cowden Syndrome (CS; MIM 158350) and 65\% of Bannayan–Riley–Ruvalcaba Syndrome (BRRS; MIM 153480) probands (1–6). It is believed that CS is linked to 10q23 without genetic heterogeneity (3), thus suggesting that other defects involving \textit{PTEN} should be involved. CS is a difficult to recognize, multiple hamartoma syndrome characterized by a high risk of breast and thyroid carcinomas (7,8). Although somatic hemizygous deletions are noted in sporadic breast and thyroid cancers, somatic intragenic mutations are rare (8).

\textit{PTEN} functions as a tumor suppressor by eliciting apoptosis and G1 cell cycle arrest. It is a dual-specificity phosphatase that dephosphorylates both lipid and protein substrates. It is able to dephosphorylate phosphatidylinositol-3,4,5-triphosphate (PI-3,4,5-P3 or PIP3), the product of phosphatidyl inositol-3-kinase (PI3K). PIP3 activates

\*To whom correspondence should be addressed. Tel: +1 2164443440; Fax: +1 2166360655; Email: engc@ccf.org or spsmce@netscape.net

© The Author 2006. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oxfordjournals.org
phosphoinositide-dependent kinase (PDK1), which in turn activates Akt/PKB by phosphorylation. Inactivation of PTEN allows constitutive and unregulated activation of the Akt/PKB signaling pathway resulting in uncontrolled proliferation (9,10). PTEN also inhibits growth factor-induced Shc phosphorylation and suppresses the MAP kinase signaling pathway (11).

Findings emerging from the Human Genome Project have allowed us to understand that splicing and other mRNA processing mechanisms are crucial in regulating gene function. Many examples of alterations in splicing and differential expression of splice variants (SVs) have been reported in various sporadic cancers and also in other diseases (12–16). Despite the importance of mRNA level regulation, there is very limited information on its role in PTEN whether at the germline or somatic levels. Although a few SVs of PTEN have been described in human and Drosophila, there has been no report of association between the SVs and cancer (17–20). Recently, it has been shown that one of the three SVs of Drosophila Pten is enzymatically more active and its expression changes dramatically during Drosophila development compared with those of two isoforms (17). We have previously reported that splice aberrations of PTEN can be due to germline point mutations in canonical splice sites as well as those deep in an intron, and we have further shown that these lead to altered functional and biological outcomes (21). Therefore, in this present study, we sought to identify and characterize naturally occurring SVs of PTEN in CS/CS-like probands and sporadic breast cancers and assay their functional downstream consequences.

RESULTS

Identification of novel naturally occurring SVs of PTEN

Knowledge of naturally occurring SVs of PTEN in humans is lacking and until now, only two such SVs have been identified which are not associated with any mutation, and further, no functional significance has been attributed to them. These two previously described SVs are inclusion of parts of introns 5 and 8 immediately 3’ of exons 5 and 8, respectively, resulting in two alternative forms of PTEN transcript (18). To determine the presence and importance of further naturally occurring SVs of PTEN, we amplified the whole PTEN transcript and serial consecutive parts of it (Fig. 1A). Subsequent cloning and sequencing of additional RT-PCR amplicons revealed eight novel SVs of PTEN (Fig. 1B). Three of these (3a, 3b and 3c) include different segments of intron 3 immediately 3’ of exon 3; three others (5a, 5b and 5c) are the result of inclusion of different regions of intron 5 immediately 3’ of exon 5; DelE5 excludes part of exon 5 (nucleotides 261–337) and DelE6 excludes all of exon 6. Another SV, which included both intron 3 sequences common to SVs 3b and 3c was also observed. SV-5b is similar to the previously described variant that includes intron 5 in the sequence, but the intronic sequence included in our SV is longer than the one previously described (18).

Full-length (FL)-PTEN encodes a protein of 403 amino acids and all our novel SVs should result in premature termination resulting in truncated proteins. Although the SVs 3a, 3b, 3c and DelE5 result in severely truncated forms of PTEN (73–85 amino acids), the other SVs 5a, 5b, 5c and DelE6 result in longer proteins of 170–190 amino acids (Fig. 1B). Further, the protein predicted from these latter SVs would include the functional domain of PTEN, the dual phosphatase domain, thus most of our functional studies are focussed on these SVs (see what follows). SV-DelE5 was not pursued for functional, heritable or sporadic cancer study, as it was detected in only one of the several clones obtained.

Expression analysis of PTEN SVs in different normal tissues

PTEN is ubiquitously expressed and tissue-specific splicing may lead to differential expression of its SVs, which may suggest different roles for different SVs in different tissues. In order to screen for relative levels of FL-PTEN transcript and its SVs in different tissue types, we used the cDNA panel comprising different adult human tissues from Orgene Technologies and the MCF7 breast cancer cell line, using the quantitative SYBR green assay method. For each variant in all tissues, the experiment was performed three times in triplicates, normalized to corresponding levels in liver (liver was selected as all SVs were expressed in the liver) and the relative levels calculated (Fig. 2). FL-PTEN transcript was expressed as expected in all the tissues tested but at varying levels. There was significantly reduced expression in brain, heart and muscle tissues; moderate expression in kidney, liver, small intestine, lung, testis and ovary; and high expression in prostate, spleen and leukocytes (Fig. 2). Although SVs 3b, 5c and DelE6, showed differential expression patterns in different tissues, other SVs like 5a, 3a and 3c were not expressed at all in any normal tissue types tested but were detected in the MCF7 breast cancer cell line (Fig. 2). As mentioned subsequently, SV-3c was, however, detected, in normal breast tissue, which was not included in the tissue panel tested. There was high expression of FL-PTEN in leukocytes, but the SVs DelE6, 3b and 5c were expressed in reduced amounts. There was a significant reduction in the expression of the SVs 3b and 5c in prostate and spleen, whereas FL-PTEN transcript and the SV-DelE6 were highly expressed. In contrast, in the testis, SVs 3b and 5c had high levels of expression when compared with FL-PTEN transcript and SV-DelE6 both of which were reduced. Brain, heart and muscle tissues had particularly low levels of FL-PTEN transcript and its SVs.

Expression of FL-PTEN transcript and SVs in fetal and adult tissues

To explore the possibility of variable expression of FL-PTEN and its SVs in development, we measured their relative levels in fetal and adult tissues (muscle, testis, kidney, spleen, liver and brain). FL-PTEN transcript levels were higher in muscle, testis and brain fetal tissue when compared with their corresponding adult tissues, whereas in the spleen it was reversed with higher expression observed in adult tissue (Fig. 3). In kidney and liver tissues, FL-PTEN expression levels did not differ significantly between fetal and adult tissues.

SV-DelE6, like FL-PTEN transcript, was expressed at higher levels in fetal tissues when compared with their
corresponding adult counterparts. In contrast, the SV-3b was mostly unexpressed in the fetal tissues except in fetal spleen and liver. SV-5c showed similar levels in both adult and fetal liver, high expression in fetal muscle and no expression in fetal testis, kidney, spleen and brain. Interestingly, both SV-3b and SV-5c were expressed at high levels in adult testis but unexpressed in fetal testis (Fig. 3).

Expression analysis of PTEN SVs in normal breast tissues when compared with sporadic breast cancers

To study the differential expression of SVs of PTEN in sporadic breast cancers, we quantitated their levels in a panel of serial concentrations (1000×, 100×, 10×, 1×) of cDNA derived from 12 breast cancers and 12 normal breast tissues. At both 1000× and 100× concentrations, FL-PTEN transcript levels were reduced in 10/12 breast cancers when compared with the normal breast tissues (Fig. 4). The control GAPDH showed equal expression levels in normal and breast cancer tissues.

SV-5c was expressed in all the normal breast tissues, but not detected in four of the breast cancers even at 1000× concentration, and had reduced expression in the remaining eight breast cancers. Overall, SV-3b was expressed in all normal breast tissues and breast cancers at 1000× concentration (Fig. 4). However, at the 100× concentration, SV-3b was not expressed in one normal breast and in seven breast cancers tissues (Fig. 4). At the 1000× concentration, SV-3b
Figure 2. Expression pattern of FL-PTEN (PTEN) and its SVs in different normal adult tissues. Relative expression of FL-PTEN and its SVs as measured by quantitative SYBR green PCR in a panel of different adult normal tissues and MCF breast cancer line. The levels are normalized to one of the tissues, liver. SVs 5a, 3a and 3c were not expressed at all in any tissues except the MCF7 breast cell line, and thus those graphs are not included. The relatively large error bars in some of the graphs (SV-3b and SV-delE6) may be due to relatively low expression levels as well as the relatively small sample sizes.

Figure 3. Relative expression levels of FL-PTEN (PTEN) and SVs in fetal and corresponding adult tissues. The levels were measured using quantitative SYBR green PCR method and normalized to adult liver tissue. The SVs not illustrated in this figure were not expressed in any of these tissues. The relatively large error bars in some of the graphs (SV-3b and SV-delE6) may be due to relatively low expression levels as well as the relatively small sample sizes.
could be detected in nine normal tissues but in only six breast cancer tissues at reduced levels. In contrast, SV-5b was detected at a higher level in nine of the 12 breast cancer tissues compared with normal tissues. SVs 5a and 3a could not be detected well in either normal or breast cancer tissues.

Expression of PTEN SVs in the germline of CS probands
In order to get an indication whether SVs of PTEN are differentially expressed in CS when compared with normal controls, we quantitated the relative levels of expression of PTEN transcript SVs in 16 CS/CS-like cases (11 CS and five CS-like) without any evidence of germline mutations or deletions, and eight normal controls. All the cases and controls were selected after ruling out any mutation by DGGE and sequencing of the coding region, promoter region and canonical splice sites as well as 30–40 bp deep into the intronic region at either ends. Large gene deletion and rearrangement was also excluded. Among these mutation negative cases, SV-5a showed under-expression levels in these patients when compared with controls (Fig. 5A, \( P < 0.001 \)). SV-5b was somewhat under-expressed in patients (Fig. 5A, \( P < 0.0028 \)), whereas SV-3a was over-expressed in the patients compared with normal controls (Fig. 5A, \( P < 0.0025 \)). The ratio of SV-3a to SV-5a expresional levels was significantly higher in patients when compared with controls (Fig. 5B).

Regulation of expression of PTEN SVs
It is known that transcription and splicing are coordinated and linked events and they do not act independently. As p53 is a major transcription regulator of PTEN and because of some preliminary studies (Truong et al., 96th Annual AACR, 2005) showing its role in alternative splicing in other genes, we assessed the effect of p53 over-expression on relative expression levels of SVs of PTEN. mRNA extracted from MCF7 cell lines expressing normal or increased amounts of p53 was used for the detection of relative levels of different SVs of PTEN, using a quantitative SYBR green assay method. Using GAPDH expression levels as an internal control, we found a minimal reduction in expression of FL-PTEN transcript and variant DelE6 with constitutive p53 over-expression. When p53 was ectopically over-expressed, we found a reduction in expression of SVs 3a, 3b and 5a (Fig. 6). In contrast, over-expression of p53 resulted in significant induction of SVs 3c and 5c and a minimal induction of SV-5b (Fig. 6). This suggests that p53 may act on PTEN not only as a transcriptional regulator but also alter its splicing pattern.

Functional analysis of PTEN SVs
We functionally analyzed the PTEN SVs by measuring PTEN’s downstream readouts, i.e. the effect of expression of these SVs on phosphorylation of Akt (P-Akt) and cyclin D1 promoter reporter activity. Over-expression of FL-PTEN

Figure 4. Expression of PTEN and SVs in 12 normal and 12 sporadic breast cancer tissues using the human breast cancer rapid-scan panel. 1000× and 100× indicates the concentrations at which the cDNAs were initially diluted. SVs 5a and 3a could not be detected in these panels and hence were not included here. GAPDH is used as a control.
results in decreased P-Akt levels as expected. Similarly, over-expression of SV-5a resulted in a slight reduction in P-Akt, whereas over-expression of the other SVs had no effect on P-Akt levels (Fig. 7A). Expression of FL-PTEN or SV-5a was associated with decreased cyclin D1 promoter activity as measured by the reporter firefly luciferase, but over-expression of SV-5b and SV-5c resulted in increased cyclin D1 promoter activity (Fig. 7B). No effect of SV-DelE6 was observed on the cyclin D1 promoter. Thus, it appears that while SV-5a cooperates with FL-PTEN downstream of PTEN, the downstream effects of SVs 5b and 5c action may be counter-acting that of FL-PTEN. Of note, the proteins corresponding to SVs 5a, 5b, 5c and DelE6 seem to be very short-lived, as they could not be detected in the cell culture system expressing them. It is known that PTEN protein lacking the C-terminal is very short-lived and faintly detectable (21,22), thus even though all these SVs will be translated, they would be degraded much faster than the FL-PTEN. To verify that these SVs are indeed translated from this expression vector in which they were cloned, we detected their expression in a cell-free in vitro system using in vitro transcription and translation (TNT) systems, where the degradation of these proteins is uncoupled (Fig. 7C).

**DISCUSSION**

As PTEN is a key tumor suppressor exhibiting its function via lipid and protein phosphatase activities, effecting cell cycle arrest and apoptosis, its regulation is very essential. PTEN is mutated in 85% of CS cases and 65% of BRRS cases (1–6,8), but the etiology in mutation-negative CS/BRRS and especially CS-like cases remains to be understood. Recent studies have shed some light on the mechanisms of PTEN regulation including its cellular localization (23–26), phosphorylation and degradation pathway (22,27,28) and its interaction with other important tumor suppressors such as p53 (29–32). The present study suggests a novel RNA-based regulation pathway via generation of various SVs, which differ in their expression in normal tissues, in the germline of CS and in sporadic breast cancers, thus suggesting their potential pleiotropic role even during normal development and functioning.

Splicing defects in cancer-related genes have been found to be due to inherited or somatic mutations in cis-regulatory elements and also could be due to oncogenic signaling and variations in the composition, concentration, localization and activity of trans-acting regulatory factors. We have previously shown that inherited mutations in the splice site junctions as well as deep in the intron lead to aberrant splicing in PTEN (21). In this study, we have identified eight novel naturally occurring SVs of PTEN, six of which are inclusions of extra sequences from introns (3 and 5) and two are exclusion of exonic sequences (5 and 6). Interestingly, naturally occurring
variant transcript SV-DelE6 has also been previously described by us, being caused by an intronic splice site mutation in a CS patient (21). SV-5b is similar, but not identical, to the previously described splice variant PTEN-B (18): they both insert sequences from intron 5 starting at g.92602 of genomic sequence, but the PTEN-B insert ends at g.92861, whereas SV-5b extends a little longer through g.92928 bases. However, the predicted protein of 170 amino acids is the same because of the presence of a premature stop codon 5′ of g.92861.

Importantly, we found that these SVs are differentially expressed in a heritable breast cancer syndrome, CS and in sporadic breast cancers compared with normal controls and normal tissues, respectively. These SVs may exert their effect at several different levels. It is altogether possible that various SVs can regulate FL-PTEN at the transcription level. Alternatively, but not in a mutually exclusive manner, SV-PTEN proteins can functionally modulate PTEN function at the protein level. Thus, differential expression of these SVs that are translated to SV-PTEN proteins can result in signaling consequences. For example, we found that SV-5a behaved functionally like PTEN by decreasing cyclin D1 promoter activity and also by reducing phosphorylation of Akt, whereas SVs-5b and -5c seemed to act in opposition to FL-PTEN signaling by increasing cyclin D1 promoter activity. SV-5a downstream signaling behaving like FL-PTEN corroborates our observation that SV-5a is under-expressed in CS/CS-like probands when compared with controls. Similarly, the observation that SV-5b behaves functionally to counter PTEN’s action corroborates our observation that SV-5b is over-expressed in sporadic breast cancers. In a follow-up large-scale molecular epidemiologic study, we found that SV-5b over-expression occurs in CS and CS-like cases, who are positive for germline PTEN mutation, not observed in normal controls nor mutation-negative CS/CS-like individuals (M. Sarquis and C. Eng, manuscript in preparation), corroborating our functional data here. Other tumor suppressors like FHIT and TSG101 have also been found to have a number of SVs without any mutations in the splice sites (33). These SVs are detected in various human tumors like gastric, cervical, thyroid and testicular germ cell tumors for FHIT and breast, ovary, prostate, thyroid, cervical cancers and AML for TSG101 (33). Interestingly, inspite of the presence of these SVs, no alternative FHIT protein isoforms have been described and it is possible that the deregulation of splicing may serve as an alternative mechanism of inactivation of the tumor suppressor by altering the levels of functional mRNA. Alterations in splicing and differential expression of SVs have been reported for many other genes involved in various cancers and also in other diseases. For example, dysregulation of alternative splicing of BRCA1 has been indicated to be involved in tumor formation in the breast and the ovary as studied in their respective cell lines (12). There is an increased expression of a splice variant of CTLA-4 in T-cells, which may regulate the development of T-cell-mediated autoimmune diseases (14). Alternative SVs of MDM2 are known to exist and it has recently been described that lung carcinomas are linked with MDM2 alternative splicing (15). Recently, it has been

Figure 7. Functional analysis of SVs of PTEN. (A) P-Akt levels were measured in stable cell lines generated, expressing either the wild-type FL-PTEN, SVs 5a, 5b, 5c and DelE6 and control vector. (B) The consequence of expression of PTEN or its SVs on cyclin D1 promoter activity was measured by luciferase activity. MCF7 cells were transiently co-transfected with vectors containing FL-PTEN or each of the SVs and the cyclin D1 reporter promoter. (C) Protein products of PTEN and SVs 5a, 5b, 5c and DelE6 and vector (containing LacZ) as detected by the TNT (in vitro-coupled TNT) reaction. Protein products made are of right size corresponding to each SV.
found that an alternatively spliced p53 isoform, Deltap53, exerts its transcriptional activity independent of p53 and forms an essential element of the ATP-intra-S phase checkpoint (16).

Splice site selection under normal physiological conditions is regulated in the developmental stage in a tissue type-specific manner by changing the concentrations and the activity of splicing regulatory proteins (33). Expression of PTEN and its SVs was found to vary in different tissue types. Such tissue specific splicing leading to the generation of unique alternatively spliced variants has also been found in other systems like the transcription factor constitutive androstane receptor and pregnane X receptor (34,35). Interestingly, we also found that PTEN is expressed differently in fetal and corresponding adult tissues. Moreover, SVs 3b and 5c were particularly under-expressed in most of the fetal tissues. This observation may shed some light on the role and regulation of PTEN, as it pertains to some developmental disorders like BRRS, which is characterized by excessive growth before and after birth (8).

p53 is known to interact and regulate PTEN at the transcription as well as protein level. Here, we found that p53 has an effect on the expression of SVs of PTEN and thus may serve as an added regulatory mechanism. Recently, it has been demonstrated that PTEN regulates p53 stability and in turn regulates its own transcription (Tang and Eng unpublished data, 2005). Our finding, combined with this observation suggests that this auto-regulatory loop might also work in altering the expression of SVs of PTEN. Another molecule in the loop, MDM2, has been shown to have its alternative splicing highly correlated with the presence of TP53 mutations, which is suggestive of a mechanism by which mutational inactivation of p53 causes expression of a modified MDM2 protein (36). By speculation, another autoregulatory loop involving SAM68 could be working in effecting PTEN splicing. It is known that SAM68 alters the splicing of a gene like CD44, which is dependent on its phosphorylation by ERK (37). As ERK’s phosphorylation and activity is regulated by PTEN, the possibility of SAM68 affecting PTEN splicing would make a feedback loop dependent on PTEN’s phosphatase activity.

In the present study, we have identified SVs of PTEN that are expressed differentially in heritable and sporadic cancer and controls by exerting their effect at possibly functional or regulation level. This is a major step forward in understanding the mechanism of PTEN gene regulation and its inactivation in the inherited cancer syndromes, even in the absence of identifiable gene mutation. Such a discovery is also particularly helpful in aiding the approach of targeting anti-apoptotic SVs to lower the apoptotic threshold of a tumor cell and therefore increase the efficacy of chemotherapy drugs. Identification of these new SVs may also provide a rapid method for the detection of SNPs across the whole genome, which is important as most of the pharmacogenomics analyses focus primarily on the identification of SNPs. Overall, the SVs of PTEN described here would not only provide newer and more specific ways of diagnosing, but also may serve as potential therapeutic/preventative targets.

### Materials and Methods

#### Patients

A series of 16 unrelated probands with CS meeting the individual or family diagnostic criteria of the International Cowden Consortium (2) who were germline PTEN mutation negative (including no mutations in the promoter region and no large rearrangements) were analyzed after obtaining informed consent under IRB approved protocols from the respective institutions.

#### PCR, cloning and sequencing

Using the RNeasy kit from Qiagen (Valencia, CA), total RNA was extracted from a normal control lymphoblastoid cell line treated with puromycin as described previously (21). After DNase digestion, cDNA was prepared using superscript reverse transcriptase II (Invitrogen, Carlsbad, CA) and random hexamers. PCR was performed using primers specific for FL-PTEN transcript (1F-9R) as well as a series of primer pairs spanning three exons at a time, across all the nine exons (Table 1). When multiple amplicons resulted, each band was gel-purified using the Gel purification Qiagen kit and further subjected to PCR with the respective primer pair combinations to enrich the products. The PCR products were cloned in pCR TOPO II vector (Invitrogen) and the resultant colonies were picked and cultured. The DNA extracted from these cultures, using Qiagen DNA miniprep kit, was sequenced using the M13 universal forward and reverse primers. Sequencing was performed using dGTP technology and the ABI 3730 analyzer (Applied Biosystems, Perkin-Elmer Corp., Norwalk, CT) according to the manufacturer’s recommendation. The Sequencer software package (version 4.2, GeneCodes Corp., Ann Arbor, MI) was used for sequence analysis.

---

**Table 1. Primers used for identification of SVs**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1F</td>
<td>TGAAAAACGAGCGCCATGCACCACATCTCAAAAGAGATCG</td>
</tr>
<tr>
<td>9R</td>
<td>TGATCAAGGTCATTGTGACTAACA</td>
</tr>
<tr>
<td>5’UTR-F</td>
<td>CCTACGGCCAGGCTCCCCACAGAC</td>
</tr>
<tr>
<td>2R</td>
<td>TTGTCTCCTGTATACGCCCTTCAA</td>
</tr>
<tr>
<td>1F</td>
<td>TGATCAGCGCCATGCTT9GAGCCGTATACAGGA</td>
</tr>
<tr>
<td>3R</td>
<td>AAAGGATATTGTGCAACTCTGC</td>
</tr>
<tr>
<td>3F</td>
<td>TGATTCAAAGCATAAAACCAC</td>
</tr>
<tr>
<td>5R</td>
<td>CAGGAAACAGCTATGACCGTGGTATGGCTTCTTCAA</td>
</tr>
<tr>
<td>4F</td>
<td>TGTAAACGCGGCCAGTCAAGATATA</td>
</tr>
<tr>
<td>4R</td>
<td>CAGGAAACAGCTATGACCGTGGTATGGCTTCTTCAA</td>
</tr>
<tr>
<td>5’UTR-R</td>
<td>CAGTTTATCAAGGTATTTTCA</td>
</tr>
</tbody>
</table>

---

**MATERIALS AND METHODS**

**Patients**

A series of 16 unrelated probands with CS meeting the individual or family diagnostic criteria of the International Cowden Consortium (2) who were germline PTEN mutation negative (including no mutations in the promoter region and no large rearrangements) were analyzed after obtaining informed consent under IRB approved protocols from the respective institutions.

**PCR, cloning and sequencing**

Using the RNeasy kit from Qiagen (Valencia, CA), total RNA was extracted from a normal control lymphoblastoid cell line treated with puromycin as described previously (21). After DNase digestion, cDNA was prepared using superscript reverse transcriptase II (Invitrogen, Carlsbad, CA) and random hexamers. PCR was performed using primers specific for FL-PTEN transcript (1F-9R) as well as a series of primer pairs spanning three exons at a time, across all the nine exons (Table 1). When multiple amplicons resulted, each band was gel-purified using the Gel purification Qiagen kit and further subjected to PCR with the respective primer pair combinations to enrich the products. The PCR products were cloned in pCR TOPO II vector (Invitrogen) and the resultant colonies were picked and cultured. The DNA extracted from these cultures, using Qiagen DNA miniprep kit, was sequenced using the M13 universal forward and reverse primers. Sequencing was performed using dGTP technology and the ABI 3730 analyzer (Applied Biosystems, Perkin-Elmer Corp., Norwalk, CT) according to the manufacturer’s recommendation. The Sequencer software package (version 4.2, GeneCodes Corp., Ann Arbor, MI) was used for sequence analysis.
cDNA constructs of PTEN and its SVs were also made in the mammalian expression vector pcDNA4/HisMax TOPO (Invitrogen). cDNA derived from a normal control lymphoblastoid cell line was amplified using 5′-UTR-forward primer which would include the start codon for PTEN and the 3′-UTR-stop reverse primer for wild-type PTEN and reverse primers which would include the stop codon for each of the different SVs (Table 2). The resulting products were cloned into the pcDNA4 vector using the TOPO TA expression kit (Invitrogen). The positive clones were checked for sequence integrity by direct nucleotide sequencing.

cDNA panels

Panels of cDNAs from different adult tissues [multiple choice human set 1 (brain, heart, kidney, spleen, liver, leukocytes) and multiple choice human set 2 (small intestine, muscle, lung, prostate, testis, ovary)] and fetal tissues [multiple choice human set 1 (brain, heart, kidney, spleen, liver, leukocytes) and fetal tissues [multiple choice human set 2 (small intestine, muscle, lung, prostate, testis, ovary)] were purchased from Origene Technologies Inc. (Rockville, MD).

These first strand cDNAs were synthesized from poly A+ RNA using an oligo(dT) primer. The human breast cancer rapid-scan panel containing first strand cDNA from 12 normal breast tissues and 12 breast cancers comprising 11 invasive adenocarcinomas and one carcinoma in situ was also obtained from Origene Technologies Inc. (Rockville, MD). Using oligo(dT) primers and MMLV reverse transcriptase, first-strand cDNA was synthesized from the poly A+ RNA. The first-strand cDNAs from each tissue was subjected to normalization to ensure an equivalent control reverse transcript. Each cDNA was serially diluted in water to a series of four concentrations (labeled 1000, 100, 10 and 1), with the lowest concentration (1x) being approximately 1 pg. The diluted cDNAs were subsequently arrayed in a 96-well PCR plate.

Real-time PCR using SYBR green quantitative assay

Quantitative RT-PCR was performed using the BioRad iCycler PCR machine. Each PCR reaction contained 0.5 μl of cDNA template and primers at a concentration of 100 nM in a final volume of 25 μl with 12.5 μl of SYBR Green Reaction Mix (Bio-Rad Inc., Hercules, CA). cDNAs from different adult and fetal tissues, CS, CS-like or normal control lymphoblastoid cell lines, which were described previously (21), and those derived from cell lines expressing p53 in pNTAP vector (Stratagene, La Jolla, CA) or an empty vector were used for assay. The primer pairs used were for the amplification of the FL-PTEN transcript or its SVs and an internal control, GAPDH (Table 3). The reaction was carried out for 42 cycles with the following conditions: 95°C × 30 s; annealing at 59°C × 30 s and extension at 72°C × 30 s. A melting curve was obtained starting at 95°C with 10 s increments of 0.5°C for 80 cycles. Each PCR reaction generated only the expected amplicon as shown by the melting temperature profiles of the final products and by gel electrophoresis. Standard curves were performed using cDNA to determine the linear range and PCR efficiency of each primer pair. Reactions were done in triplicate and relative amounts of cDNA were normalized to GAPDH transcript.

Transfection and generation of stable cell lines

MCF7 breast cancer cells were maintained in Dulbecco’s minimum essential media (Gibco-Invitrogen) with 10% FBS and the antibiotics Penicillin and Streptomycin and grown at 37°C with 5% CO2. The cells were checked for sensitivity to the antibiotic Zeocin by plating them at 25% confluency and then replenishing the selective media every 3–4 days at different concentrations of Zeocin (0–1000 μg/ml). For generation of stable lines, the cells were seeded at 50–80% confluency in six-well plates one day prior to transfection. One microgram plasmid DNA was transfected in each well using DMRIE reagent (Invitrogen) in Opti-MEM media (Gibco-Invitrogen) according to the users’ manual. After 24 h, fresh medium was added to the cells. After 48 h, the

<table>
<thead>
<tr>
<th>Splice variant</th>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1F</td>
<td>CAGCCATCATCAAAGAGATCG</td>
</tr>
<tr>
<td></td>
<td>2R</td>
<td>TTGTTCTGTATACGCCTTCAA</td>
</tr>
<tr>
<td>SV-3a</td>
<td>1F</td>
<td>CAGCCATCATCAAAGAGATCG</td>
</tr>
<tr>
<td></td>
<td>2R</td>
<td>CTTTCAGCAATAAATCTTCT</td>
</tr>
<tr>
<td>SV-3b</td>
<td>1F</td>
<td>CAGCCATCATCAAAGAGATCG</td>
</tr>
<tr>
<td></td>
<td>2R</td>
<td>CTGGTACCTGTTCAACTCAA</td>
</tr>
<tr>
<td>SV-3c</td>
<td>1F</td>
<td>CAGCCATCATCAAAGAGATCG</td>
</tr>
<tr>
<td></td>
<td>3R</td>
<td>CGAGTACCTGTTAAGTCCAA</td>
</tr>
<tr>
<td>SV-5a</td>
<td>4F</td>
<td>TCTTGTGCTAGAAAGACATT</td>
</tr>
<tr>
<td></td>
<td>5R</td>
<td>GAGGCTCTCGTCTGGATTAA</td>
</tr>
<tr>
<td>SV-5b</td>
<td>4F</td>
<td>TCTTGTGCTAGAAAGACATT</td>
</tr>
<tr>
<td></td>
<td>5R</td>
<td>CGCCTGCGCCTCCCAAGT</td>
</tr>
<tr>
<td>SV-5c</td>
<td>4F</td>
<td>TCTTTTTGCTAGAAAGACATT</td>
</tr>
<tr>
<td></td>
<td>5R</td>
<td>GCCCTCTACAGGAGCTAGCAT</td>
</tr>
<tr>
<td>SV-De6</td>
<td>5-7F</td>
<td>AGGACCAAGAGCAAAAAAGT</td>
</tr>
<tr>
<td></td>
<td>8R</td>
<td>CTGTCCTGGTATGAGAAT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GAPDH-F</td>
<td>CCAATTTCCAGAGGCGAGA</td>
</tr>
<tr>
<td></td>
<td>GAPDH-R</td>
<td>AAATGAGCCCAAGCCTTCT</td>
</tr>
</tbody>
</table>
cells were split and plated in a 96-well plate at ~25% confluency in fresh medium containing 400 μg/ml Zeocin. The cells were fed with the selective medium every 3–4 days until foci were identified and there were no cells left in the wells without any vector (negative control). The wells containing the foci were expanded and grown in selective medium for further analysis.

Luciferase activity assay

MCF7 cells were grown and co-transfected as described earlier with the pcDNA4/HisMax TOPO constructs of PTEN or its SVs and firefly luciferase reporter vector driven by the cyclin D1 promoter (−1750CD1-Luc (38)) or pBluescript plasmid as a control. Renilla luciferase vector was also co-transfected as an internal control. Serum was added back to the medium after 12 h, cells harvested after 24 h and luciferase activity was detected by the dual luciferase reporter assay system (Promega Inc., Madison, WI) as described by the manufacturer.

SDS–PAGE and western blotting

Cells from the stable MCF7 cell lines expressing PTEN, SVs or vector were harvested and washed once with ice-cold PBS. Whole cell extracts were prepared using MPER (Pierce Biotechnology Inc., Rockford, IL) reagent containing protease inhibitor cocktail (Sigma, St Louis, MO). Protein concentrations were determined using BCA (Pierce Biotechnology Inc.) with BSA as a standard.

SDS–PAGE and western blot were performed according to procedures recommended by the Bio-Rad Protein III system (Bio-Rad Inc.). Antibodies against P-Akt (Ser 473) and actin were purchased from Cell Signaling Co. (Beverly, MA). The monoclonal antibody 6H2.1 raised against the last 100 C-terminal amino acids of PTEN was used to recognize the wild-type PTEN (39). Anti-Xpress antibody was purchased from Invitrogen. For western blot, 20 μg of protein was fractionated through 10% SDS–polyacrylamide gels and transferred to nitrocellulose membranes (S&S Inc., Keene, NH) using the Trans-Blot Cell system (Bio-Rad Inc.). After transfer, nitrocellulose membranes were blocked for 1 h with 5% milk in 0.1% Tris-buffered saline containing 0.1% Triton X-100 (TBST) and incubated with primary antibody overnight. Membranes were washed four times with TBST after the primary and secondary incubations. Blots were probed with horsedaradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG secondary antibody (Promega Inc.) for 2 h at room temperature. Proteins were detected using ECL substrate (Amersham Biosciences Inc., Chicago, IL) on film and quantified using ImageQuant software (version 5.1).

In vitro TNT

One microgram of each pcDNA4/HisMax TOPO constructs of PTEN or its SVs was used for the in vitro-coupled TNT reaction. Protein expression in rabbit reticulocyte lysate system was carried out in the presence of [35S]methionine (Amersham, Piscataway, NJ) as described by the manufacturer. The protein products were resolved by 15% SDS–PAGE.

ACKNOWLEDGEMENTS

We would like to thank Dr Michael C. Ostrowski for his continuing support and Dr Richard G. Pestell for the cyclin D1 full-length promoter. We are grateful to Drs Kristin A. Waite and Frank Weber for critically reviewing the manuscript. This work was funded, in part, by the American Cancer Society (RSG-02-151-01-CCE) and the Doris Duke Distinguished Clinical Scientist Award (both to C.E.).

Conflict of Interest statement. None declared.

REFERENCES