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Different splicing defects lead to differential effects downstream of the lipid and protein phosphatase activities of PTEN

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***PTEN*, encoding a dual phosphatase tumor suppressor, is mutated in 85 and 65% of individuals with Cowden syndrome (CS) and Bannayan–Riley–Ruvalcaba syndrome (BRRS), respectively. Approximately 23 germline mutations in putative splice sites have been published, but resulting downstream outcome data are limited. We determined splicing defects in *PTEN* in 40 germline *PTEN* mutation positive cases and 33 mutation negative cases with classic CS, BRRS and CS- or BRRS-like features. Altered splicing was observed in 4/40 mutation positive probands and 2/33 mutation negative probands. We then sought to characterize the transcriptional and biochemical outcomes of the five distinct splice-site mutations, which led to the skipping of exon 3, 4 or 6. Two mutation negative BRRS patients also showed exon 3 skipping, and later, genomic sequencing revealed a mutation deep in intron 2. The splice-site mutations leading to the deletions of exon 3, 4 or 6 resulted in reduced dual phosphatase activities of PTEN. Deletion of exon 4 was associated with severely reduced lipid phosphatase activity, whereas exon 3 skipping resulted in markedly reduced protein phosphatase activity. In addition, exon 3 deleted transcript and protein were stable and localized to the nucleus more efficiently than the wild-type PTEN. In contrast, exon 4 skipping resulted in unstable transcripts and severely truncated unstable PTEN protein lacking its phosphatase domain. We have not only described for the first time, the effect of a deep intronic/branch-site mutation on exon skipping in *PTEN* but also found that different splice-site mutations resulting in the deletion of different exons lead to distinct outcomes.**

INTRODUCTION

PTEN encodes a tumor suppressor, which is ubiquitously expressed and inactivated in inherited cancer syndromes and in a variety of sporadic cancers. Germline intragenic mutations in *PTEN* are associated with 85% of individuals with classic Cowden syndrome (CS) and 65% of individuals with Bannayan–Riley–Ruvalcaba syndrome (BRRS) (1–5). CS is a complex, autosomal dominant disorder characterized by multiple hamartomas and carcinomas of the breast in 25–50% of CS females and of the thyroid in 10% of all CS patients (5,6). BRRS is a congenital disorder characterized by macrocephaly, hemangiomatosis, lipomatosis and a risk of cancer (7). The CS gene was mapped to 10q22–q23 (8) and identified as *PTEN*, a nine-exon gene predicted to result in a 1212 bp transcript and a 403 amino acid protein (9). PTEN functions as a tumor suppressor by eliciting apoptosis and/or G1 cell cycle arrest (10,11). It is a dual-specificity phosphatase that dephosphorylates both lipid and protein substrates (10,12,13). Phosphatase activity of PTEN is essential for tumor suppression. PTEN's lipid phosphatase activity dephosphorylates phosphatidylinositol-3,4,5-triphosphate (PI-3,4,5-P3 or PIP3),

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the product of phosphatidyl inositol-3-kinase. PIP3 activates phosphoinositide-dependent kinase, 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 (14,15). PTEN also inhibits growth factor-induced Shc phosphorylation and suppresses the MAP kinase (MAPK) signaling pathway (16). It is believed that the protein phosphatase activity suppresses MAPK signaling, thus when PTEN's protein phosphatase activity is suppressed, p42/44 MAPK levels are increased (15–18). Domain mapping of PTEN has been done to characterize different roles of the phosphatase domain, the PDZ domain, C2 domain, importance of its phosphorylated sites and cellular localization studies, although much remains to be understood in terms of its function (19–22).

Among the 105 different germline *PTEN* mutations reported for CS and BRRS as of the last review, 17 were found at the exon–intron boundary in either splice donor or acceptor sites (Supplementary Material, Table S1) (4). In addition, six other splice-site mutations have been reported in *PTEN* in CS/BRRS cases (summarized in Supplementary Material, Table S1). Thus, putative splice-site mutations may account for 22% of all the germline *PTEN* mutations reported for CS and BRRS. As summarized in Supplementary Material, Table S1, of the 23 germline putative splice-site mutations reported, 18 do not have data on the effect of the mutation on mRNA and none has data on the downstream biochemical outcome (1,2,4,23–29). Somatic mutations or deletions of *PTEN* have also been observed in many non-cultured sporadic neoplasias including colorectal cancer, glioblastoma and endometrial cancer (30–32). Also, rare somatic splice-site mutations in sporadic solid tumors have been reported as illustrated in Supplementary Material, Table S2 (33–37). Only in one case, the IVS7+7A>G mutation was shown to result in exon 7 skipping.

Besides mutations that obviously involve splice donor and acceptor sites, splicing defects can also be caused by the generation of cryptic splice sites due to mutations in other regions of the gene. In hereditary breast–ovarian cancer syndrome, at least one naturally occurring germline missense mutation within an exon in *BRCA2* has been shown to result in exon splicing and formation of truncated protein (38). Germline missense and nonsense mutations in the mismatch repair gene *MLH1* causing Lynch syndrome (HNPCC) have been reported to lead to skipping of exons (39). Such findings are important to mutation interpretation, as they imply that aberrant splicing could be associated with missense, nonsense or even with silent variants that do not lead to amino acid substitutions. Overall, however, little has been explored in this regard for heritable cancer syndromes and there is no information regarding downstream protein or functional outcome.

Altogether, there has been no data of how splice-site mutations and variants, which putatively alter splicing, affect the function of PTEN and downstream biochemical consequences. Therefore, in the present study, we sought to identify and characterize the transcriptional consequences and biochemical outcome of splice site and putative splice-site mutations and variants of unknown significance, in a consecutive series of classic CS/BRRS probands, comprising 40 with germline *PTEN* mutations and 33 who are mutation negative.

RESULTS

cDNA sequencing revealed splice defects due to splice-site mutations and branch-site mutations

mRNA was extracted from lymphoblastoid cell lines (LBL) from 12 normal controls, 40 CS/BRRS probands with germline mutations or variants in *PTEN*, including the promoter, and 33 CS/BRRS, belonging to 32 unrelated families, with no detectable germline mutations or variations within *PTEN* or its promoter. Before harvesting, half of the cells from each subject and each control were also treated with puromycin, so that unstable mRNA transcripts formed due to splicing defects leading to truncated proteins may be detectable, which would otherwise be lost by nonsense-mediated decay (NMD). The entire *PTEN* cDNA was sequenced for all the normal controls and for the mutation positive and negative CS/BRRS samples. Of the 40 samples with germline mutations or variants, five (12%) had splice-site mutations and four of these resulted in splice aberrations. Specifically, the splice-site mutations IVS3+1G>A and IVS3+5G>A in two different patient samples led to the splicing out of exon 3; IVS3−1G>A led to exon 4 exclusion and IVS6+1G>T led to the splicing out of exon 6 (Figs 1 and 2), whereas, surprisingly, we did not observe any splice defect in the sample harboring the IVS8+1G>A germline mutation (Table 1). Interestingly, among the 33 patients with no germline mutation or variation, two (6%) BRRS patients were found to have wild-type *PTEN* mRNA transcripts as well as transcripts with exon 3 skipping (Figs 1 and 2). Sequencing of germline DNA using primers that allowed viewing deeper into the intronic regions of these samples revealed mutations deep within intron 2, IVS2−38insG (Table 1). Sequencing of normal DNA panel of 50 controls did not reveal any such mutation.

Skipping of different exons result in different biochemical outcomes downstream of PTEN

The cell extracts from LBL samples derived from patients showing the splice aberrations described earlier and from normal controls were tested for PTEN protein levels and for its lipid and protein phosphatase activity by measuring their respective downstream products. P-Akt levels were analyzed as a direct indication of the lipid phosphatase activity of PTEN, whereas p42/44 P-MAPK levels were analyzed as an indicator of PTEN's protein phosphatase activity. Actin protein levels were used as internal controls.

The four patient samples resulting in transcripts with exon 3 deletion and one each for exon 4 and exon 6 deletion all showed reduced PTEN levels when compared with the three normal controls. When compared with controls, PTEN levels were reduced to 80% (20% reduction) in patients with exon 3 deletion. Notably, PTEN levels were reduced to less than half in samples whose transcripts exhibited exon 4 (37%

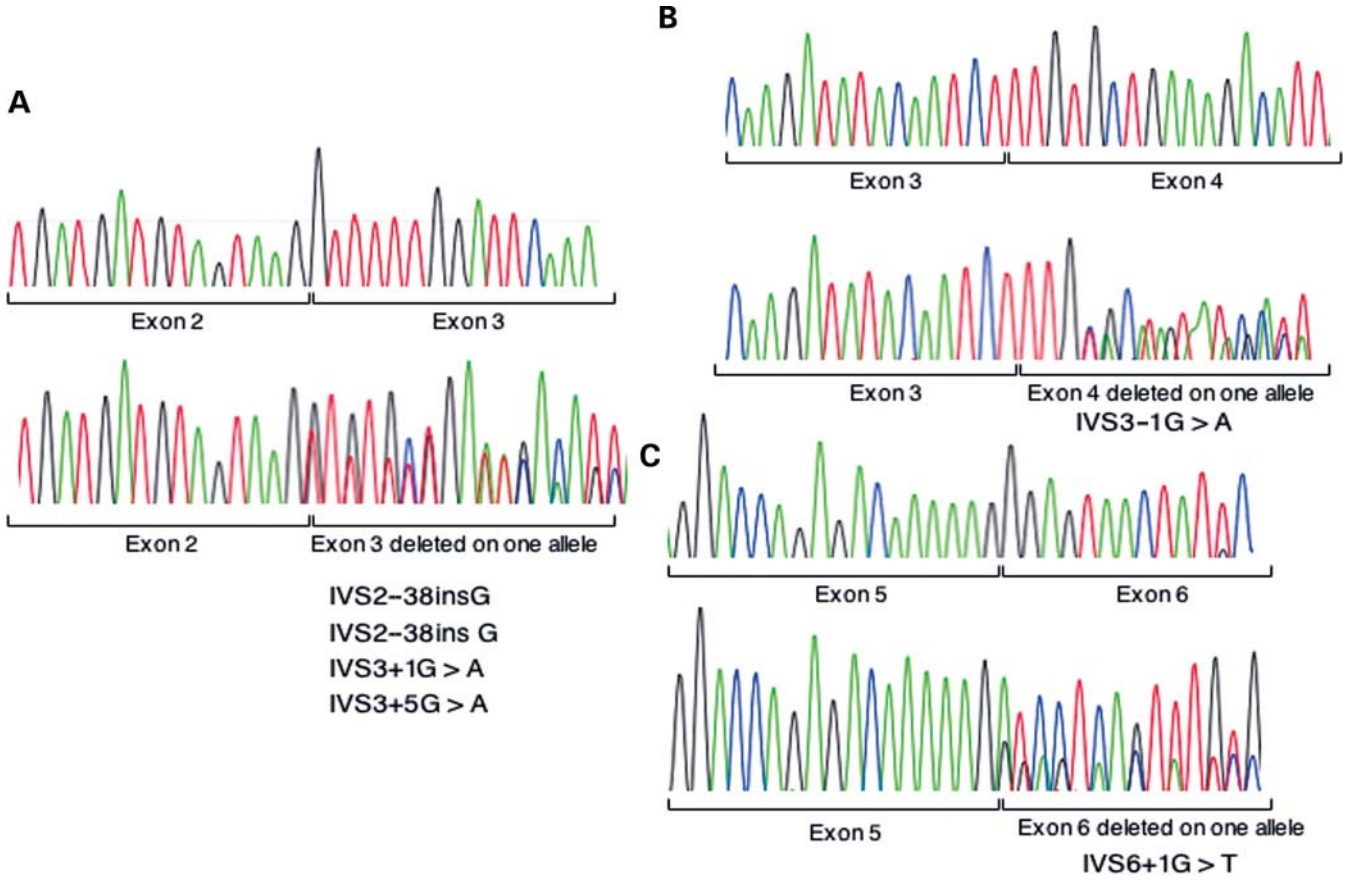

Figure 1. Chromatograms showing skipping of exons 3 (**A**), 4 (**B**) and 6 (**C**). (A) Germline mutations IVS2−38insG, IVS3+1G>A and IVS3+5G>A result in skipping of exon 3. Because exon 3 is skipped, the normal sequence from exon 2 to exon 3 (top panel) becomes the mutant sequence showing the heterozygous mutant sequence of exon 2 proceeding to that of exon 4 (bottom panel). (B) Exon 4 skipping due to IVS3−1G>A mutation results in heterozygous presence of transcripts with the wild-type sequence and one where sequence of exon 3 is juxtaposed against those of exon 5 (bottom panel). (C) Exon 6 skipping due to the IVS6+1G>T mutation leads to a transcript where exon 5 is juxtaposed against exon 7 (bottom panel, heterozygous pattern).

reduction) and exon 6 (42% reduction) skipping (Fig. 3). P-Akt levels were markedly elevated in the sample with the exon 4 deleted transcript, suggesting loss of PTEN lipid phosphatase activity, as probed by antibodies recognizing phosphorylated Akt at either the Ser473 or the Thr308 position (Fig. 3). In contrast, all four samples that have exon 3 deleted transcripts, irrespective of mutation type or status, showed highly increased p42/44 P-MAPK levels, which suggest inactive protein phosphatase activity (Fig. 3). The p42/44 P-MAPK levels were also increased in the samples with transcripts that have skipping of exon 4 and exon 6, respectively, although levels were not as high as in the sample with exon 3 deleted transcript. Thus, the marked upregulation of the Akt pathway manifested by increased P-Akt in the samples with exon 4 deleted transcript suggests significant reduction in PTEN's lipid phosphatase activity. In contrast, the marked increased in P-MAPK in all four samples with exon 3 deleted transcripts reflects significantly reduced protein phosphatase activity, without much effect on the lipid phosphatase activity (same P-Akt levels as controls). Interestingly, the sample with exon 6 deleted transcript showed only a slight decrease in lipid phosphatase activity, but the protein phosphatase activity appeared to be more adversely affected.

PTEN protein lacking exon 3 localizes to the nucleus more efficiently than wild-type PTEN

PTEN phosphorylation is known to play a role in its activity and stability (40). We tested the phosphorylation status of PTEN in the patient samples in the nuclear and the cytoplasmic fractions. We did not observe any significant differences in the phosphorylation status of PTEN in these cases (Fig. 4A). PTEN has been shown to traffic in and out of the nucleus related to the cell cycle (41) and has been recently shown to contain bipartite nuclear localization signal-like sequences which are necessary for MVP-mediated nuclear import (42). Therefore, to determine the effect of the splicing defects removing exon 3, 4 or 6, PTEN protein levels were measured in nuclear and cytoplasmic fractions of these patient samples and controls. Oct-1 and HSP-90α/β were used as controls for the clarity of the nuclear and cytoplasmic extracts. Nuclear PTEN protein from patient samples with the exon 3 deleted transcripts was relatively low in three of the four samples (Fig. 4A, lanes 4–6). Specifically, nuclear to cytoplasmic ratios of PTEN protein were reduced in samples whose transcripts had exon 3 skipping when compared with those of wild-type cells or even

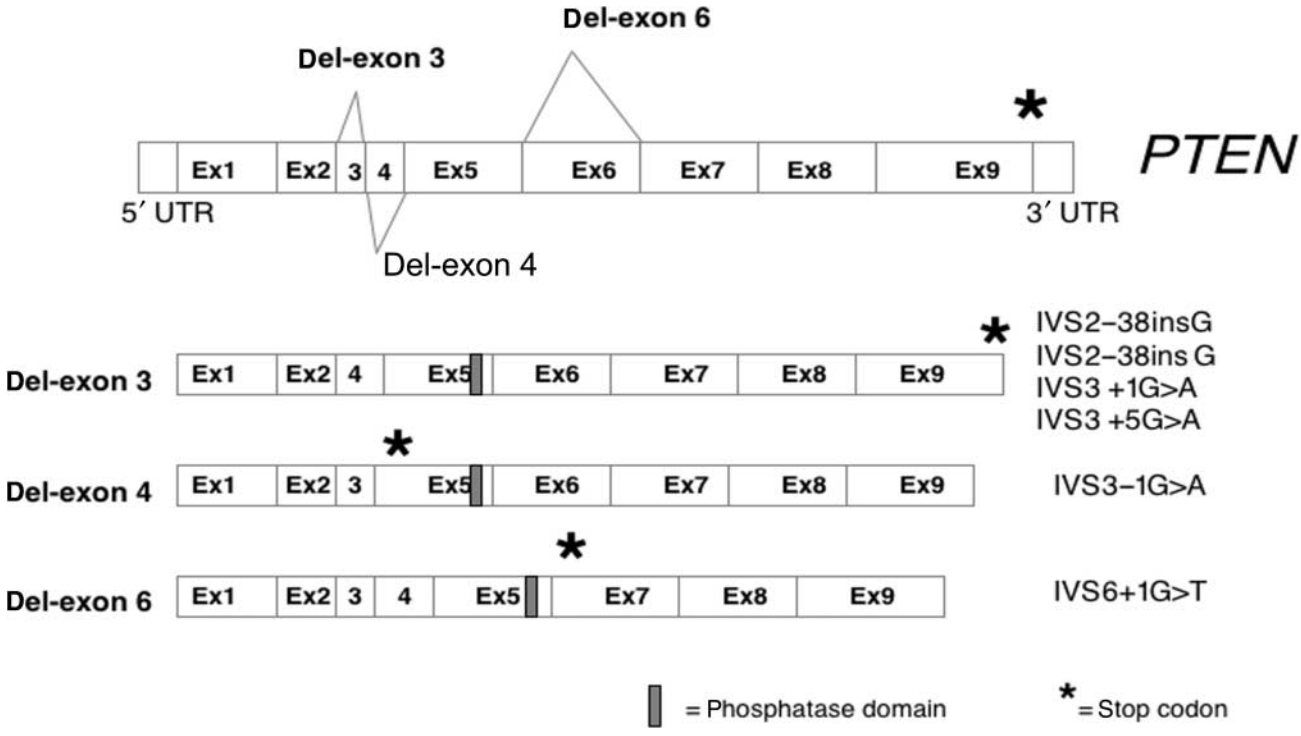

Figure 2. Schematic diagram showing the exons skipped due to germline mutations IVS2−38insG, IVS3+1G>A, IVS3+5G>A, IVS3-1G>A and IVS6+1G>T. Transcripts with exon 3 skipping results in a slightly shortened protein, missing only the amino acids encoded by exon 3, but which maintains its reading frame. Exon 4 skipping leads to a prematurely truncated protein missing the phosphatase domain including the phosphatase core motif as well as the C-terminal tail. Exon 6 skipping results in prematurely truncated protein which still contains the phosphatase core motif but lacks the C-terminal tail.

Table 1. List of splice site and intronic mutations in our study

Mutations in present study	mRNA change
IVS3+1G>A	Exon 3 skipping
IVS3+5G>A	Exon 3 skipping
IVS3−1G>A	Exon 4 skipping
IVS6+1G>T	Exon 6 skipping
IVS8+1G>A	No change
IVS2-38insG	Exon 3 skipping
IVS3−39A>G	No change
IVS1+35C>T	No change
IVS4−29insT	No change

to those from samples with exons 4 and 6 deleted transcripts. To explore this further, we expressed the exon 3 deleted *PTEN* transcript and the wild-type construct in MCF 7 cells and checked the nuclear and cytoplasmic fractions for the ratio of these exogenously expressed proteins. Although the wild-type PTEN localized in the nucleus similar to its endogenous counterpart, the exogenously expressed exon 3 deleted PTEN localized more efficiently to the nucleus when compared with the endogenous wild-type PTEN (Fig. 4B). We have not been able to separately identify the wild-type and exon 3 deleted PTEN in the patient samples, because of very small molecular weight difference, but it may be inferred that the majority of the nuclear PTEN in such patients is exon 3 deleted PTEN.

Transcripts with skipping of exon 4 or 6 undergo NMD when compared with transcripts with exon 3 skipping, which are stable

To determine the differential stabilities of transcripts which have exon 3, 4 or 6 spliced out in the patient samples, the cDNA from these patients was amplified with the primers flanking the respective spliced out exons and the resultant PCR product compared with the wild-type transcript level (Fig. 5A). All four patient samples containing mutations resulting in exon 3 skipping gave rise to similar levels of spliced transcript as well as wild-type mRNA (wild-type/exon 3 deleted ratio ∼1.3), irrespective of exposure to puromycin or not, suggesting that the exon 3 deleted mRNA is stable and not subjected to NMD (Fig. 5A and B). The exon 4 deleted transcript in the patient sample with the mutation IVS3−1G>A was, in contrast, very unstable, with the wild-type transcript being expressed 2.5 times more than the exon 4 deleted transcript. Treatment of cells with puromycin reduced this ratio to 1.3, suggesting the instability of exon 4 transcripts due to the NMD pathway. Similarly, puromycin treatment reduced the ratio of wild-type to exon 6 spliced out transcript in the patient sample with the IVS6+1G>T mutation to half than when not treated, suggesting the instability of exon 6 deleted mRNA transcript as well (Fig. 5A and B).

PTEN protein stability related to specific splice defects

Constructs containing the wild-type and exon 3, 4 or 6 deletions of *PTEN* were transiently transfected into MCF7 cells

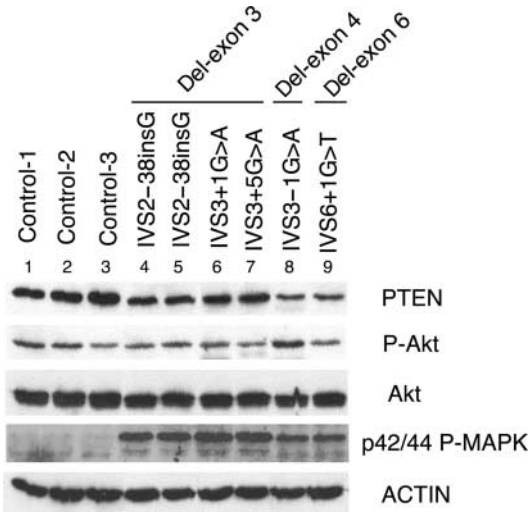

Figure 3. Western blot of protein lysates from the patient samples containing transcripts with skipping of exons 3, 4 and 6 (lanes 4–9) and from normal controls (lanes 1–3). PTEN protein levels are reduced in all patient samples whose transcripts have skipped exons, but this is more pronounced in samples with exon 4 or exon 6 deleted transcripts (lanes 8 and 9). Markedly elevated P-Akt levels are seen in patients whose transcripts have exon 4 deletion (thus indicating loss of PTEN lipid phosphatase activity) (lane 8). Markedly increased p42/44 P-MAPK levels are observed in all four patients whose transcript have exon 3 deletion (loss of PTEN protein phosphatase activity) (lanes 4–7). p42/44 P-MAPK levels are also increased in samples whose transcripts show skipping of exon 4 (lane 8) and exon 6 (lane 9) when compared with controls (lanes 1–3). Actin levels are used as standard control.

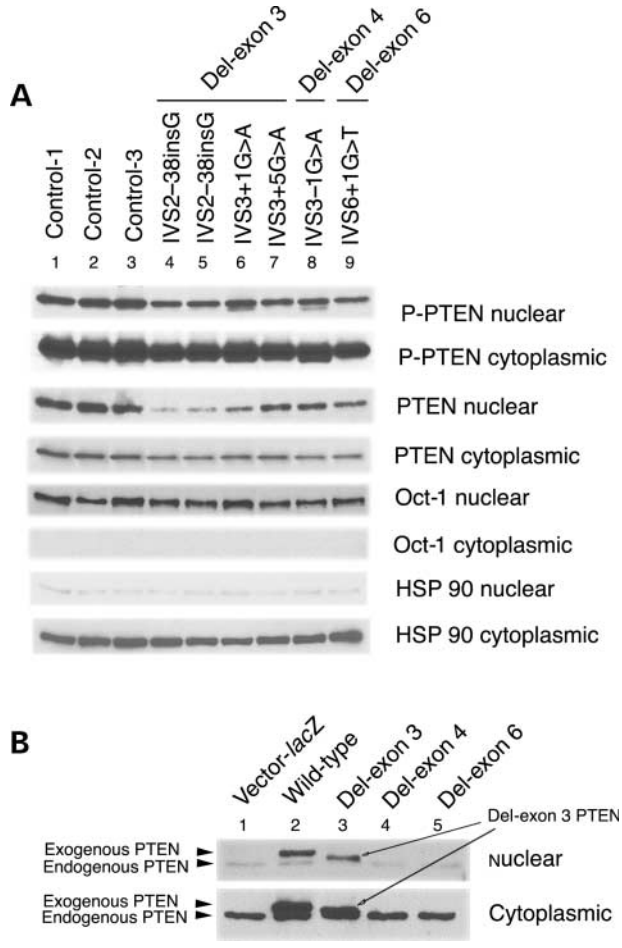

Figure 4. Phosphorylation status and cellular localization of PTEN (**A**) Phosphorylation status and levels of PTEN in the nuclear and cytoplasmic compartments of patient samples whose transcripts lack exons 3, 4 and 6 (lanes 4–9) and the normal controls (lanes 1–3). Phosphorylation status does not change with the skipping of exons 3, 4 or 6 (lanes 4–9) when compared with normal controls (1–3) in either nuclear fraction or cytoplasmic fraction. However, total nuclear/cytoplasmic ratio of PTEN is decreased in the samples whose transcripts lack exon 3 (lanes 4–7) when compared with controls (lanes 1–3). Oct-1 and HSP-90α/β were used as controls for confirmation of the purity of nuclear and cytoplasmic extracts. (**B**) Transient expression in MCF-7 cells reveals localization in the nuclear and cytoplasmic compartments of the Vector (lane 1), wild-type PTEN (lane 2), exon 3 (lane 3), exon 4 (lane 4) and exon 6 (lane 5) deleted PTEN. Note that the endogenous PTEN is the lower band and the exogenously expressed PTEN is the upper band. Note that in the exon 3 deletion sample, wild-type PTEN is markedly decreased in the nucleus and exon 3 deleted PTEN accumulates better.

and checked for PTEN protein expression. Deletion of exon 3 maintains the PTEN open reading frame with the deletion of 15 amino acids encoded by exon 3 (Fig. 2). However, exon 4 or 6 deletion disrupts the reading frame and leads to the production of truncated proteins (Fig. 2). These truncated proteins resulting from exon 4 or 6 skipping were undetectable in the cell culture system in transiently transfected cells, whereas proteins resulting from the exon 3 deletion transcript is expressed well as detected by the anti-Xpress antibody against the N-terminal tag (Fig. 6A). These observations reflect differential protein stability unrelated to NMD of transcript because all mRNA transcripts of the transiently expressed constructs were stable for all three different exon deletion types and the wild-type (Fig. 6B). To show that the protein is being encoded by the exon 3, 4 or 6 deleted constructs but degraded in the cellular system at different rates, we expressed them in a cell-free system. The exon 3, 4 or 6 deleted proteins were expressed efficiently, corresponding to the estimated molecular weights of the truncated proteins in cell-free rabbit reticulocyte transcription coupled translation system (Fig. 6C).

DISCUSSION

In the present study, we have characterized the effects of different splice-site mutations in 73 CS/BRRS, CS-like and BRRS-like patients, comprising 40 *PTEN* mutation positive and 33 mutation negative, at both the transcriptional and biochemical levels. What was immediately interesting was the identification of two splice alterations in samples from germline *PTEN* mutation negative individuals. Subsequent sequencing in the deep intronic regions revealed a IVS2−38insG. A splicing-related site typically is located 20–50 nt in an intron upstream from a splice acceptor site, also called branch site, and is also known to be necessary for proper processing of introns in addition to the splicing junctions. The branch site has the consensus sequence (C/U)N(C/U)U(A/G)A(C/U) where the emboldened A residue is invariant and conserved in all genes and N is any nucleotide (43). Intron 2 of *PTEN* gene contains this conserved branch sequence (UGUUAAU), which extends from IVS2−33 to

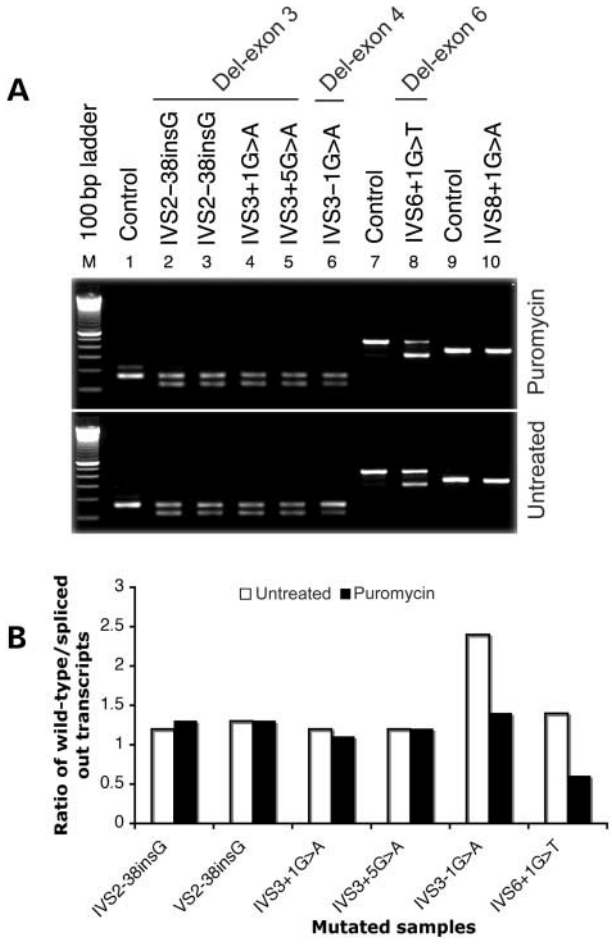

Figure 5. RT–PCR of wild-type and exon deleted transcripts in the normal control and patient samples. (**A**) cDNA from the normal control and patient samples whose transcript contain exon deletions due to splicing defects/mutations were PCR amplified using primers exon2F–exon5R (for exons 3 and 4 deletions; lanes 1–6); exon 5F–exon 7R (for exon 6 deletion; lanes 7–8) and exon 7F–exon 9R (for IVS8+1G>A mutation; lanes 9–10). The amplicons made at the exponential phase of PCR were then separated on 1% agarose gel. (**B**) Ratio of the wild-type/defective spliced out transcripts in patients with mutations resulting in transcripts with skipped exon 3, 4 or 6 in the presence or in the absence of puromycin.

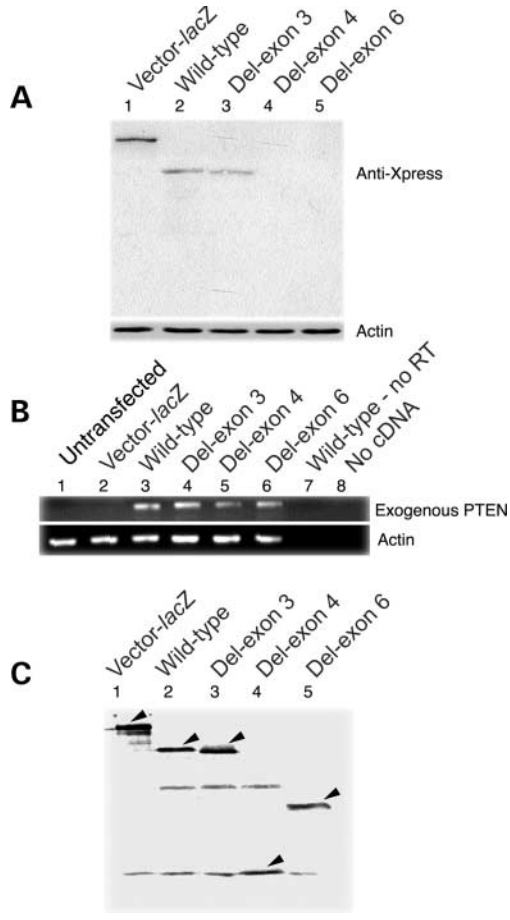

Figure 6. Expression and stability of the wild-type PTEN and the exon 3, 4 or 6 deleted proteins. (**A**) MCF 7 cells were transiently transfected with the vector control, wild-type *PTEN* and exons 3, 4 and 6 deleted *PTEN*. The exogenously expressed protein was probed with the anti-Xpress antibody against the N-terminal tag. (**B**) After transient transfection in MCF 7 cells with the vector control, wild-type *PTEN* and exon 3, 4 or 6 deleted *PTEN*, total RNA was extracted and subjected to RT–PCR using one of the primers in the vector (T7) and one in exon 2 of *PTEN* to detect only the exogenously expressed mRNA transcripts of *PTEN* (wild-type or exon deleted). (**C**) To verify that the exons 3, 4 and 6 deleted transcripts will encode a protein, the respective constructs driven by the T7 promoter were expressed in rabbit reticulocyte cell-free system using a transcription-coupled translation system. The bands indicated by arrows is the expressed product in each lane corresponding to *lac* Z (lane 1), wild-type PTEN (lane 2), exon 3 deleted PTEN (lane 3), exon 4 deleted PTEN (lane 5) and exon 6 deleted PTEN (lane 5).

IVS2−39. The IVS2−38insG mutation will thus disrupt the lariat formation and proper splicing, leading to the skipping of exon 3 in the cases harboring this mutation. In contrast, we did not observe splice defect due to an obvious splice-site mutation IVS8+1G>A. The majority of introns has GT at the 5′ splice site and AG at the 3′ splice site, also known as GT-AG rule. It is known that the mutation of the first nucleotide from G to A of a major-class intron inhibits the second step of splicing *in vitro* and many natural mutations giving rise to various genetic diseases consist of such a mutation (44). However, now widespread existence of many other types of intron ends has been established, of which AT-AC ends are most common and they are known to be processed by the minor splicing pathway. Other natural intron boundaries have also been identified, namely AT-AA and AT-AG, and it is also observed that a mutation in one nucleotide can suppress a mutation at the other end. It has also been reported that the minor splicing pathway, which recruits U12 SnRNAs and plays a role in the processing of the 5′-AT and AC-3′ introns, could also be involved in 5′-AT and AG-3′ introns. A few naturally occurring introns have 5′-AT and AG-3′ ends, and they are believed to be spliced by the minor splicing pathway (44). Intron 38 of the Myosin VIIa gene, intron 2 of CACNL1A1 and intron 24 of SCN10A, all have 5′-AT and AG-3′ ends and are processed by the minor pathway (44). The IVS8+1G>A mutation would change the intron ends from 5′-GT-AG-3′ to 5′-AT-AG-3′, and we speculate on the possibility that this might recruit the U12 mediated minor splicing pathway machinery for its processing. As the U12 SnRNPs are very much less abundant than the U2 SnRNPs

(involved in the major pathway), it is still possible that the overall message from the IVS8 variation is decreased, thus still leading to the pathogenicity of such a mutation.

It has been well documented that many pathogenically truncated transcripts are subjected to NMD (45,46). All our altered *PTEN* transcripts result in shortened transcripts, yet, we have shown that only the mutations resulting in skipping of exons 4 and 6 in the transcripts exhibited NMD. In contrast, the three different mutations resulting in shortened but in-frame transcripts (exon 3 skipping due to IVS2−38insG, IVS3+1G>A and IVS3+5G>A) yielded stable transcripts not subjected to NMD. Interestingly, within each sample with exons 3 and 4 deleted transcripts, even in the presence of puromycin, the wild-type transcript is expressed more than the spliced out transcript (1.3 ratio). Notably, the splice-site mutations (IVS3+1G>A and IVS3+5G>A) and the branch-site mutations (IVS2−38insG) led to the splicing defects of exclusion of exon 3 to the same extent as seen by the ratio of wild-type to the exon 3 deleted transcript in all cases being similar.

In addition to transcript alterations, we have demonstrated that these different transcript aberrations result in different downstream outcomes. PTEN is a dual-specificity phosphatase with major lipid and protein phosphatase activities, which play key roles in growth suppression (12,15,17,22). Structure–function analysis has revealed that the phosphatase core motif is encoded within exon 5 but much of the N-terminal half is required for full phosphatase function. This is consistent with our observation that a transcript with exon 4 skipping, which results in a protein lacking the phosphatase core motif and part of the phosphatase domain, leads to a dramatic reduction in lipid phosphatase activity. PTEN protein resulting from transcripts with exon 4 or 6 skipping will also lack the C-terminal tail. It has been shown that the deletion of the C-terminal tail results in PTEN protein instability and increased degradation by the ubiquitin-proteosome pathway and so very small amounts of truncated protein can be detected (19,47,48). Thus, for mutations resulting in prematurely truncated protein, the mechanism of inactivation is haploinsufficiency via at least two mechanisms: the first is NMD of the mutant transcript and the second is by increased degradation of whatever small amounts of mutant PTEN protein are produced. Interestingly, exon 3 deleted transcript yields a protein with severely reduced protein phosphatase activity as manifested by the activation of MAPK but both transcript and protein are stable. Although the reading frame is maintained, as is the phosphatase core motif, it is possible that the slightly shortened protein cannot physically exercise its protein phosphatase function. Being stable, the exon 3 deleted mutant protein could act in a dominant negative manner. Another aspect of this dominant negative mechanism of inactivation of such proteins is impairment of PTEN nuclear import most likely due to competition of the mutant with wild-type PTEN. Initially, our observation of decreased nuclear PTEN given a single mutation was puzzling in light of our own observations that two NLS-like sequences are required to bind MVP for nuclear import (42). Our observations in this context suggest that PTEN protein lacking exon 3 may interact with MVP in an irreversible manner and thus compete with wild-type PTEN for its shuttle to the nucleus. Because protein phosphatase activity is more impaired than that of lipid phosphatase, this may give us a clue that nuclear localization plays a role in the inhibition of MAPK activation.

Our observations regarding splicing alterations resulting from some germline *PTEN* mutations suggest that altered splicing with differential downstream functional outcomes can also explain the pathogenesis of CS/BRRS. Notably, our data also suggest that the mechanism of PTEN inactivation resulting from altered splicing involves 'double jeopardy'. For example, transcripts encoding prematurely truncated protein are subjected to NMD and the few transcripts not degraded result in protein which lack at least one of its important functions (e.g. phosphatase activity) and which are subjected to degradation because it lacks the C-terminal tail. Thus, we believe that mutation in *PTEN*, whether germline or somatic, does not merely lead to a single downstream inactivation outcome but several outcomes which result in PTEN dysfunction by two or more mechanisms. On the basis of our observations, therefore, we can postulate that the different outcomes downstream of a mutation, especially resulting in altered splicing, may help to modulate the protean manifestations of CS/BRRS. Further investigation involving a large series of these subjects will be required to rigorously confirm our data presented here.

MATERIALS AND METHODS

Lymphoblastoid cell cultures

Seventy-three consenting subjects; 40 *PTEN* mutation positive and 33 mutation negative ascertained using the International Cowden Consortium clinical diagnostic criteria (49) by R.P. and C.E., and 12 normal control samples were accrued in accordance with protocols approved by the Human Subjects Protection Committees of The Ohio State University and participating institutions. These CS/BRRS families were meticulously characterized and no obvious differences in phenotype was noted between mutation positive and negative probands/families. Among the 40 cases with mutations/variants, three were silent or variants of unknown significance, six missense, nine nonsense, six in the promoter, two insertions, eight deletions, two (one of them also had a silent double mutation) had variants in the deep intronic regions and five splice-site mutations (affecting nucleotides within 5 bp of the exon–intron boundaries). Normal controls were operationally defined as without documented major illnesses, no CS/BRRS phenotype by the diagnostic criteria (5) and the absence of germline *PTEN* mutations. Peripheral blood leucocytes were obtained and transformed by EBV using standard procedures (50). These LBL were grown in RPMI 1640 media (Gibco-Invitrogen, Carlsbad, CA, USA) with 15% FBS and the antibiotics penicillin (100 U/ml) and streptomycin (100 μg/ml) with 5% CO_2 at 37°C. Where applicable, cells were treated with 100 μg/ml puromycin (Sigma, St Louis, MO, USA) for 6 h at 37°C before harvesting.

mRNA extraction, RT–PCR and sequencing

10^7 cells were harvested with or without puromycin treatment and total mRNA extracted using the RNAeasy kit from Qiagen

(Valencia, CA, USA), according to the manufacturer's recommendations. One microgram mRNA was treated with 2 U amplification grade DNase (Invitrogen, Carlsbad, CA, USA), which was subsequently inactivated by the addition of 1 μl of 25 mM EDTA and heating at 65°C for 5 min. cDNA was prepared using superscript reverse transcriptase II (Invitrogen) and random hexamers. For sequencing of the *PTEN* cDNA, the entire transcript was amplified using forward (5′UTRF) and reverse (9R) primers (Table 2). Sequencing was performed using dGTP technology and the ABI 3730 analyzer (Applied Biosystems, Perkin-Elmer Corp., Norwalk, CT, USA) according to the manufacturer's recommendation. The Sequencher software package (version 4.2, GeneCodes Corp., Ann Arbor, MI, USA) was used for sequence analysis.

Table 2. List of primers used in the study

Primer	Sequence
5′UTRF	CTTCAGCCACAGGCTCCCAGAC
9R	GATCAGGTTCATTGTCACTAACA
2F	ACTTGAGGACGTATACAGGA
5R	GTGGGTTATGGTCTTCAAAA
4F	TCTTTGTGCTGAAAGACATT
7R	CTGGCAGACCACAAACTGAGGAT
7F	TCCACAAACAGAACAAGATG
9R	TGATCAGGTTCATTGTCACTAACA
5′UTR-start[a]	GGCTCCCGGGCCATGACAGCCATCATCAAAG
3′UTR-stop[b]	CTTGATCATATGAAATTCAGACTTTTGTAATTTG
T7	TAATACGACTCACTATAGGG
2R	TTGTTCCTGTATACGCCTTCAA

[a]Primer contains the start codon ATG and *Sma* I restriction site.
[b]Primer contains the stop codon TGA and *Nde* I restriction site.

SDS–PAGE and western blotting

10^7 lymphoblastoid cells were harvested and washed once with ice cold PBS. Whole cell extracts were prepared using MPER (Pierce Biotechnology Inc., Rockford, IL, USA) reagent containing protease inhibitor cocktail (Sigma). Transfected cells were harvested after washing with ice cold PBS and whole cell extract and nuclear and cytoplasmic extracts were prepared using MPER and nuclear and cytoplasmic extraction reagent protein isolation kit (Pierce Biotechnology Inc.,), respectively. Protein concentrations were determined using BCA (Pierce Biotechnology Inc.,) with BSA as a standard.

SDS–PAGE and western blot were performed according to the procedures recommended by the Bio-Rad Protein III system (Bio-Rad, Inc.). Antibodies against P-PTEN (Ser380), Akt, P-Akt (Ser473), P-Akt (Thr308), p42/44 P-MAPK and actin were purchased from Cell Signaling Co. (Beverly, MA, USA). The monoclonal antibody 6H2.1 raised against the last 100 C-terminal amino acids of PTEN was used to recognize the wild-type PTEN (11). Anti-Xpress antibody was purchased from Invitrogen and Oct-1 and HSP-90α/β antibody was purchased from Santa Cruz (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). For western blot analysis, 20 μg of protein was fractionated through 10% SDS–PAGE and transferred to nitrocellulose membranes (S&S Inc., Keene, NH, USA) using the Trans-Blot Cell system (Bio-Rad Inc., Hercules, CA, USA). After transfer, nitrocellulose membranes were blocked for 1 h with 5% milk in TBST (0.1% Tris-buffered saline containing 0.1% Triton X-100) and incubated with primary antibody overnight. Membranes were washed four times with TBST after the primary and secondary incubations. Blots were probed with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG secondary antibody (Promega Inc. Madison, WI, USA) for 2 h at room temperature. Proteins were detected using ECL substrate (Amersham Biosciences Inc., Chicago, IL, USA) on film and quantified by using ImageQuant software (version 5.1).

Semi-quantitative RT–PCR

cDNA from the normal control and the patient samples with exon skipping due to splicing defects/mutations were PCR amplified using primers (Table 2) 2F-5R (when exons 3 and 4 were involved); 5F-7R (for exon 6 deletion) and 7F-9R (for IVS8+1G>A mutation) using HotstarTaq master mix (Qiagen). PCR was performed in a Peltier Thermal Cycler-200 (MJ Research, Bio-Rad Inc.,) by initial activation at 95°C for 15 min, followed by 32 cycles of 95°C for 30 s, 52°C for 30 s, 73°C for 30 s, followed by final extension at 72°C for 20 min. Products were analyzed on a 1% agarose gel and quantified for the ratio of normal allele over spliced out allele by using ImageQuant software (version 5.1).

Cloning

PTEN cDNA constructs which are wild-type, and which have deletions of exon 3, 4 or of exon 6 were made in the mammalian expression vector pcDNA4/HisMax TOPO (Invitrogen). cDNA from normal control and respective patient samples were amplified using 5′-UTR-start forward primer which would include the start codon and a 3′-UTR-stop reverse primer which would include the stop codon (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.

Transient transfection of MCF-7 breast cancer line

MCF7 breast cancer cells were maintained in Dulbeco's mimimum essential media (Gibco-Invitrogen) with 10% FBS and the antibiotics penicillin and streptomycin and grown at 37°C with 5% CO_2. The cells were seeded at 50–80% confluency in six well plates 1 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 respective users' manuals. Sera were added back to the cells, 6 h after transfection. Cells were harvested 48 h after transfection and used for total RNA and protein extraction and analysis as described earlier. Expression of the mRNA transcripts from the transfected constructs was determined by RT–PCR using primers (Table 2) T7 (in the vector sequence) and 2R (in the exon 2 of the *PTEN* gene) to identify the exogenously expressed transcripts only. Actin gene was amplified as an internal

control using the primers supplied by Ambion Inc. (Austin, TX, USA).

In vitro transcription and translation

To check whether the *PTEN* mRNA transcripts with exon 3, 4 or 6 deletion encode a protein, *in vitro*-coupled transcription and translation (TNT) was carried out using the rabbit reticulocyte lysate system (Promega Inc.,). One microgram of each pcDNA4/HisMax TOPO constructs of the mutants or the wild-type was used for the TNT. Protein expression in rabbit reticulocyte lysate system was carried out in the presence of [^{35}S]methionine (Amersham, Piscataway, NJ, USA) as described by the manufacturer. The protein products were resolved by 15% SDS–PAGE.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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