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Cancer Genetics Report

PTEN c.511C>T Nonsense Mutation in a BRRS Family Disrupts a Potential Exonic Splicing Enhancer and Causes Exon Skipping

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Bannayan–Riley–Ruvalcaba syndrome (BRRS) is an autosomal dominant disorder characterized by macrocephaly, intestinal hamartomatous polyps, lipomas and pigmented macules of the glans penis. We identified a Thai family affected with BRRS. In addition to typical manifestations of BRRS, the proband has a large hepatic AVM which is rarely found in BRRS. The molecular analysis revealed affected members were heterozygous for an exon skipping-associated nonsense mutation c.511C>T in the *PTEN* gene. The mutation was previously assumed to be deleterious by causing a change to a termination codon, Q171X. We, herein, found that another pathogenic effect was splicing related by disrupting a potential exonic splicing enhancer (ESE) and causing an entire exon 6 skipping. The results prompted us to investigate other reported missense/nonsense mutations in the *PTEN* gene. We found that they do not colocalize with ESE sites, suggesting that most of their pathogenic effects are not through ESE disruption.

Key words: Bannayan–Riley–Ruvalcaba syndrome – Cowden syndrome – nonsense mutations – exonic splicing enhancer

INTRODUCTION

Germline mutations in the tumor suppressor gene *PTEN* (phosphatase and tensin homologue deleted on chromosome 10) have been implicated in PTEN hamartoma tumour syndromes (PHTS) including Cowden syndrome (CS; MIM 158350), Bannayan–Riley–Ruvalcaba syndrome (BRRS; MIM 153480), Proteus syndrome and Proteus-like syndrome (1–4). Although the clinical manifestations of these four disorders differ significantly, all have increased likelihood of benign tumor growth. Of the four entities, CS and BRRS display the highest degree of clinical overlap. CS is an autosomal dominant disorder characterized by multiple hamartomas affecting derivatives of all three germ layers and an increased risk of breast, thyroid, and endometrial and other cancers (5–8). BRRS, a phenotypic variant of CS, is characterized by the cardinal features of macrocephaly, intestinal hamartomatous polyps, lipomas and pigmented macules of the glans penis. Other features in BRRS include Hashimoto's thyroiditis, vascular malformations, hemangiomas and mental retardation (9–11).

Approximately 60–65% of individuals with a clinical diagnosis of BRRS have a detectable *PTEN* gene mutation (1,12–14). The *PTEN* gene maps to 10q23.3 and encodes a dual-specificity phosphatase which antagonizes the phosphoinositol-3-kinase (PI3K)/Akt pathway leading to G1-cell-cycle arrest or apoptosis as well as inhibits cell spreading via the focal adhesion kinase pathway (15–19). Genotype–phenotype analyses revealed an association between the presence of *PTEN* mutation in BRRS and the development of lipomas and tumors of the breast (1). Individuals with BRRS and *PTEN* mutations are currently thought to have the same cancer risks as individuals with CS. Therefore, it has been suggested that individuals with BRRS and *PTEN* mutation should receive equal attention with respect to cancer surveillance (1).

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At least 173 different disease-causing mutations have been characterized in the *PTEN* gene (http://www.hgmd.cf.ac.uk). Of these, 75 are missense/nonsense mutations. Identical mutations have been identified in some families with Cowden syndrome and in others with BRRS. In addition, families whose members have overlapping features of both conditions have been identified (1,14,20). There is still no clear explanation for this phenomenon.

We present a Thai BRRS family with a germline mutation, c.511C>T (p.Q171X), in the *PTEN* gene. The mutation has never been reported in BRRS. In addition, our patient has a large AVM in the liver which is rarely found in BRRS. We also demonstrated that this nonsense mutation actually disturbed splicing presumably from disrupting a potential exonic splicing enhancer (ESE) causing skipping of the whole exon 6. In addition, we have found that other reported missense/nonsense mutations in the *PTEN* gene do not colocalize with ESE sites, suggesting that most of their pathogenic effects are not through ESE disruption.

PATIENTS AND METHODS

Clinical Report

A 48-year-old Thai male patient presented with a 5-day history of painless hematochezia. He denied any fever, nausea, vomiting, abdominal pain and weight loss. He rarely drinks or smokes. The gastroscopy revealed three medium size esophageal varices at distal esophagus, multiple polyps starting from hypopharynx to lower esophagus, in gastric remnant, duodenum and jejunum. Colonoscopy showed multiple sessile polyps in his entire colon and rectum (more than 100 polyps). Their sizes varied from 0.5–1.5 cm. The biopsy showed hamartomatous polyps.

His past medical history was notable for multiple episodes of gastrointestinal bleeding. When he was 15 years old, he was hospitalized for upper GI bleed. He underwent gastroscopy which revealed multiple gastric polyps. He also developed several subcutaneous nodules of various sizes on his trunk and extremities since age 10. He had undergone several surgical resections of subcutaneous lipomas.

His birth history was unavailable. The family history was significant for multiple subcutaneous masses in his mother and his second daughter. Even though his mother was noted to have multiple subcutaneous masses and now is 80 years old, she has never suffered from major illnesses. She was unavailable for evaluation. His 21-year-old elder daughter has not had any clinical features of BRRS. His second daughter is 17 years old. According to her mother, she was born at term after an uncomplicated pregnancy with birth weight 4000 g (>97th centile). Other birth parameters were unavailable. Her development was appropriate for age. She was diagnosed with hypothyroidism at age 9. She also developed multiple subcutaneous masses of various sizes on all four extremities and trunk since age 9. The proband also has 10 other siblings. They were noted to have no clinical features of BRRS and unavailable for evaluation. The pedigree of this family is shown in Fig. 1A.

At the first genetic evaluation of the proband, physical examination revealed a height of 1.75 m (25th centile), weight of 60 kg (15th centile) and head circumference of 61 cm (>97th centile). A small purplish nodule was noted at his right buccal mucosa. The thyroid gland was not enlarged on palpation. Abdominal examination revealed an old surgical scar with centrifugal superficial vein dilatation. His abdomen was moderately distended with positive shifting dullness. His liver and spleen were not enlarged. Multiple subcutaneous nodules of various sizes were seen on his trunk and all four extremities. Examination of the genital region showed pigmented macules of the glans penis (Fig. 1B and C).

Abdominal CT scan showed hypodensity lesion at left lobe of the liver, splenomegaly and ascites (Fig. 2A). Angiogram was performed and showed a large arteriovenous malformation at left hepatic lobe with enlarged left hepatic artery (Fig. 2B and C). A rapid draining into dilated portal vein was noted. Embolization of the feeding branches was performed three times with less than 20% residual shunt remained.

Physical examination of the proband's second daughter showed a height of 1.71 m (95th centile), weight of 63 kg (50th centile). There were no dysmorphic features.

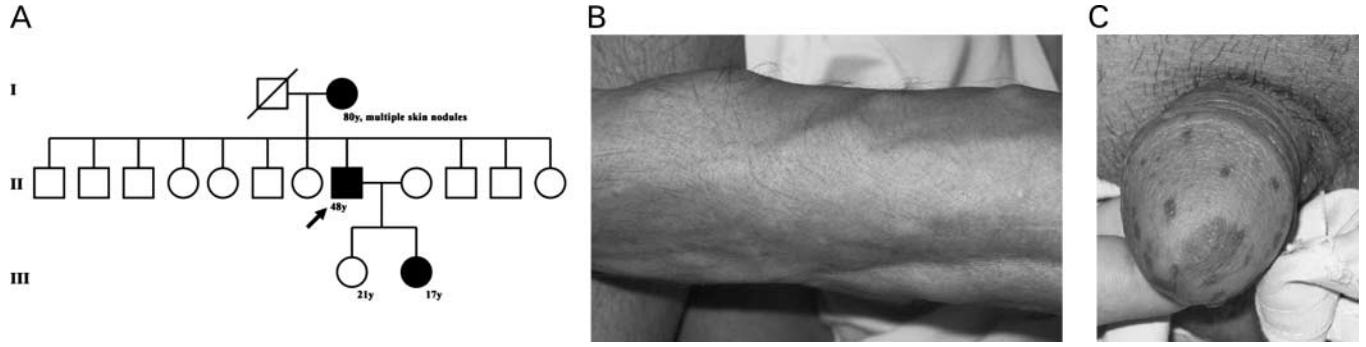

Figure 1. Pedigree of a BRRS family (A) and photograph of the proband showing multiple subcutaneous lipomas over the upper extremity (B) and pigmented macules of the glans penis (C). (Please note that a colour version of this figure is available as supplementary data at http://www.jjco.oxfordjournals.org)

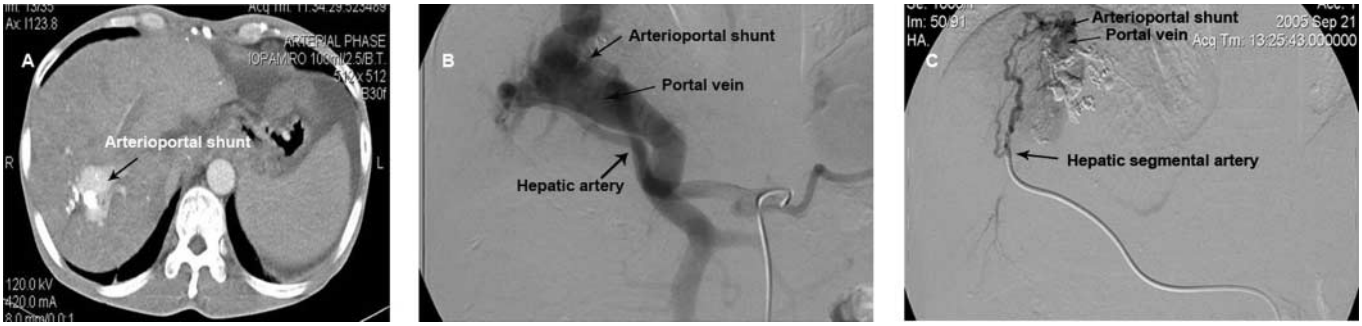

Figure 2. Imaging of the hepatic arteriovenous malformation in the proband. (A) Computed tomographic scan of the upper abdomen showing hepatic arterioportal shunt. (B, C) Angiogram of the hepatic arteriovenous malformation showing a large high flow arterioportal shunt from hepatic arteries to portal vein with marked enlargement of portal vein. (Please note that a colour version of this figure is available as supplementary data at http://www.jjco.oxfordjournals.org)

Table 1. Summary of genetic features of the proband

Characteristics	Variable
Ethnicity	Thai
Gene	*PTEN*
GenBank accession No.	U93051, U96180, U92436
Chromosome assignment	10q23
Type of DNA variant	Germline nonsense mutation
Mutation	A C → T substitution in exon 6 at nucleotide 511 resulting in a change from glutamine to a stop codon (Q171X) as well as a partial exon 6 skipping causing a frameshift with a stop codon 9 amino acids downstream
Allelic frequency	–
Method of mutation detection	Direct sequencing

The thyroid gland was not enlarged. Multiple subcutaneous nodules of various sizes were seen on her trunk and bilateral forearms and legs. Endoscopy and colonoscopy were performed and revealed multiple small sessile polyps (0.3–0.5 cm in size) in esophagus, duodenum, terminal ileum, sigmoid colon and rectum. Genetic features of BRRS and the proband are summarized in Table 1.

PTEN Gene Analysis

After informed consent was received, 3 ml of peripheral blood from the patient and his two daughters were obtained. Total RNA was isolated from white blood cells using QIAamp® RNA blood mini kit (Qiagen, Valencia, CA, USA). Reverse transcription was performed using ImProm-II™ reverse transcriptase (Promega, Madison, WI, USA), according to the manufacturer's instructions. PCR amplification of the *PTEN* cDNA exons 1–9 was performed using primers PTENF1 and PTENR1 as shown in Table 1. We used 2 μl of first-strand cDNA, 1 × PCR buffer (Promega, Madison, WI, USA), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM of each primer and 0.1 U Taq DNA polymerase (Promega) in a total volume of 20 μl. PCR products were treated with ExoSAP-IT (USP Corporation, Cleveland, OH, USA), according to the manufacturer's recommendations, and sent for direct sequencing at the Macrogen Inc., Seoul, Korea. Genomic DNA of the proband and his daughters was extracted from peripheral blood lymphocytes using standard techniques. The 3′ end of exon 5, the whole exon 6, and the whole exon 7, including flanking intronic sequences of the *PTEN* gene were amplified using primers PTENEx5-F, PTENEx5-R, PTENEx6-F, PTENEx6-R, and PTENEx7-F, PTENEx7-R respectively as shown in Table 2. PCR was performed in 20 μl, containing 2 μl of genomic DNA, 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM of each primer and 0.1 U Taq DNA polymerase. The products were directly sequenced as above. RTPCR was performed using primers PTENF2, PTENR2 to amplify the *PTEN* cDNA exons 5–8. All the PCR conditions were shown in Table 2.

Study of Association of Pathogenic Missense/Nonsense Mutations and ESE Sites in the *PTEN* Gene

Published data on pathogenic missense/nonsense mutations of the *PTEN* gene were used. We searched the coding regions of the *PTEN* gene for the presence of ESE motifs with ESEfinder software (http://rulai.cshl.edu/tools/ESE/) (21). To lower the number of false-positive results, we used a more stringent than recommended threshold value of 3.0 for all four types of ESE motifs as recommended in a previous study (22). ESEs in exon/exon boundaries were excluded as these sequences were separated by an intron. For an association study of nonsense mutations and ESE sites, we calculated the percentages of sequences that were potential ESEs, which provided us with the proportion of nonsense mutations expected to be in ESE motifs. We, then, classified reported nonsense mutations into those in ESE motifs and those do not. Comparison of the observed and expected frequencies of the mutations in ESE sites was performed using standard χ^2 and

Table 2. Primers and PCR conditions

Primers	Sequences	Annealing temperatures (°C)
PTENF1	5′-AAGTCCAGAGCCATTTCCAT-3′	61
PTENR1	5′-GACACAATGTCCTATTGCCA-3′	
PTENF2	5′- TGAAGACCAT AACCCACCAC-3′	55
PTENR2	5′-CCTTGTCATTATCTGCACGC-3′	
PTENEx5-F	5′-GATCTTGACCAATGGCTAAG-3′	53
PTENEx5-R	5′-CACAATGTATATACACATACATC-3′	
PTENEx6-F	5′-ATGTTCTTAAATGGCTACGAC-3′	55
PTENEx6-R	5′-AACCCATTGCTTTTGGCTTC-3′	
PTENEx7-F	5′-GATTGCAGATACAGAATCCAT-3′	55
PTENEx7-R	5′-TAATGTCTCACCAATGCCAG-3′	

P-value by a program available at http://www.unc.edu/~preacher/. For the association study of missense mutations and ESE sites, we performed similar analysis.

RESULTS

RNA was prepared from leukocytes of the proband and reverse transcribed. The cDNAs were then sequenced. The chromatogram showed two transcripts with different height. The proband was heterozygous for a mutation in the *PTEN* gene causing a skipping of the whole 142-bp exon 6 (Fig. 3B). This skipping is predicted to result in alterations starting from codon 165 and leading to a frameshift with a stop codon at position 174. The premature termination codon (PTC) usually triggers nonsense-mediated mRNA decay. This mechanism is likely to result in a reduction of PTC-harboring mRNA in our patient as shown in Fig. 3A and B. The genomic DNA of the relevant regions of the proband and his affected daughter was amplified by PCR and directly sequenced. The sequences revealed that they were heterozygous for c.511C>T presumably producing a stop codon (p.Q171X) (Fig. 3D). We further went through all the rest of the sequenced cDNA. The corresponding mutation found in the genomic DNA was also identified as a small peak in the chromatogram with the other two transcripts, the wild type transcript and the degraded frameshift cDNA resulting from an exon 6 skipping (Fig. 3E). No other mutations were identified in *PTEN* cDNA and genomic DNA of the proband. RT-PCR to amplify exon 5 to exon 8 revealed two bands of different sizes and amount in the proband (Fig. 3A). A reduced amount of exon-skipped transcript harboring PTC is most likely due to an incomplete penetrance of the exon 6 skipping as well as nonsense-mediated mRNA decay. RNA and genomic DNA of the unaffected elder daughter were also included in the study (Fig. 3C and F).

Based on the assumption that mutation-associated exon skipping has been mostly associated with ESE disruption, we investigated whether the c.511C>T mutation lies in and abrogates a high score ESE motif in the region encompassing the mutated residue. We analyzed the wild-type and mutant *PTEN* exon 6 sequences with two available ESE-prediction softwares, ESEfinder (21) and RESCUE-ESE (23). The results revealed that, although slightly reducing the score of an SRp40 motif from 4.6 to 4.3 without falling below the threshold value, the c.511C>T mutation eliminates a potential ESE of an SF2/ASF motif from 3.53 to 0.582 (Fig. 4).

Potential ESE motifs found in the *PTEN* gene are listed in Table 3. After excluding ESEs in exon/exon boundaries, we found potential ESE motifs encompassing 390 bp out of the 1212 bp (32.2%) of *PTEN* coding region. Of the 28 reported nonsense mutations, nine are in ESE (32.1%). Using the χ^2 test, we have found that nonsense mutations do not colocalize with ESEs ($\chi^2 = 0$, df = 1, $P = 0.99$). For analysis of missense mutations, of the 47 reported pathogenic missense mutations, 15 are in ESE (31.9%). Using the χ^2 test, we have also found that missense mutations do not colocalize with ESEs ($\chi^2 = 0.001$, df = 1, $P = 0.97$).

DISCUSSION

Our patient has clinical features including vascular malformation suggestive of BRRS. Hemangiomas and AVMs are recognized features of BRRS. There are occasional reports of large visceral AVMs. However, there has been no prior report of BRRS with AVMs in the liver. The pathogenesis of the AVM in this syndrome is still under investigation. There is growing evidence that PTEN is involved in modulating angiogenesis. PTEN is expressed in vascular smooth muscle cells and has an antiangiogenic effect (24,25). It has been shown that PTEN down-regulates new vessel formation through suppression of vascular endothelial growth factor (VEGF) expression (26). These findings suggest that loss of PTEN function leads to dysregulation of

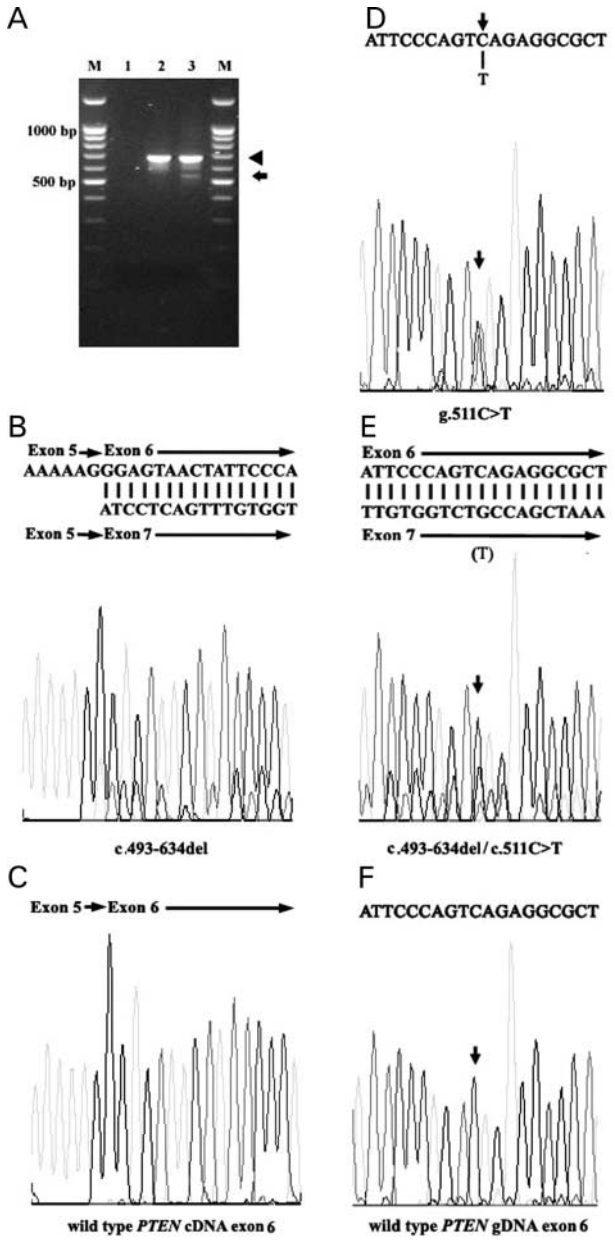

Figure 3. The *PTEN* gene analysis. (A) RT-PCR using primers PTENF2 and PTENR2 to amplify exons 5–8 of the *PTEN* cDNA. M: 100-bp marker. The 500 and the 1000-bp bands were indicated. 1, no cDNA; 2, unaffected control; 3, proband. There was an expected 671-bp band (arrowhead) from both control and the proband. A 529-bp product with less intensity was also found in the proband (arrow). The mutation found in the proband is likely to cause skipping of the whole 142-bp exon 6. (B) Partial sequences of the cDNA of the proband showing two transcripts with different height. The higher representing the wild type transcript whereas the lower representing the transcript with skipping of the entire exon 6. (C) Partial sequences of the cDNA of the unaffected elder daughter. (D) gDNA of the proband showing a C → T substitution in exon 6 at nucleotide 511 (arrows). (E) The further sequences of the cDNA of the proband showing the corresponding nucleotide substitution found in the gDNA as indicated by the arrow in addition to the wild type exon 6 transcript and the degraded exon-skipped transcript. (F) gDNA of the unaffected elder daughter showing only C in exon 6 at nucleotide 511 (arrow). All the shown chromatograms were in the 5′ to 3′ direction. (Please note that a colour version of this figure is available as supplementary data at http://www.jjco.oxfordjournals.org)

angiogenesis, a possible mechanism underlying AVM in this syndrome.

His cDNA was sequenced and revealed that he was heterozygous for a mutation in the *PTEN* gene causing a skipping of the whole 142-bp exon 6 (c.493-634del). In order to identify the basis of exon skipping, we amplified and sequenced the gDNA of the proband and his affected daughter from the 3′ end of exon 5 to the 3′ end of exon 7. To our surprise, we observed no mutation in the studied *cis*-acting consensus elements including flanking intronic sequences as well as a possible branch site in intron 5 (TTTCAAT) known to be involved in RNA splicing. However, a C → T substitution was found in exon 6 at nucleotide 511, which is expected to change a glutamine to a stop codon (Q171X) leading to the truncation of 233 amino acids from the PTEN protein. This corresponding nonsense mutation with very low magnitude was identified in the chromatogram of the sequenced cDNA suggesting a nonsense-mediated mRNA decay. This nucleotide substitution also caused a partial exon 6 skipping from a mutation causing ESE disruption. The fact that we found the transcript with exon 6 skipping resulting in frameshift leading to truncated protein product in this patient suggested another pathogenic effect of g.511C>T (Q171X). A reduced amount of the exon-skipped transcript harboring PTC is likely due to nonsense-mediated mRNA decay. Here we showed that ESE disruption causing an exon 6 skipping might be one of the pathogenic effects of c.511C>T mutation in the BRRS family. Further studies to show the c.511C>T mutation actually disrupts an SF2/ASF motif by using a mini gene construct and RNA-protein binding experiments will provide a strong evidence supporting this finding.

ESEs are discrete, degenerative motifs of 6–8 nucleotides located inside exons. The study of normal splicing suggests that most exons contain at least one functional ESE site. ESEs are required for definition and/or efficient splicing of the exons in which they reside. ESEs are target sequences for the family of conserved essential splicing factors—the serine/arginine-rich (SR) proteins (27). Nucleotide substitution in ESEs can result in decreased binding of SR proteins or other splicing factors to the ESE, leading to a failure to recognize the sequence as exon by the spliceosome and to exon skipping. Splicing signals are a frequent target of mutations in genetic diseases and cancer. It has been estimated that at least 15% of point mutations that result in a human genetic disease cause RNA splicing defects (28). We analyzed the wild type and mutant *PTEN* exon 6 sequences with ESEfinder software and found that the c.511C>T mutation eliminated the potential ESE of SF2/ASF motif. This result suggested that the c.511C>T mutation disrupted an ESE motif and caused an entire exon 6 skipping. This skipping is predicted to result in alterations starting from codon 165 and leading to a frameshift, causing a degradation of PTC-harboring mRNA.

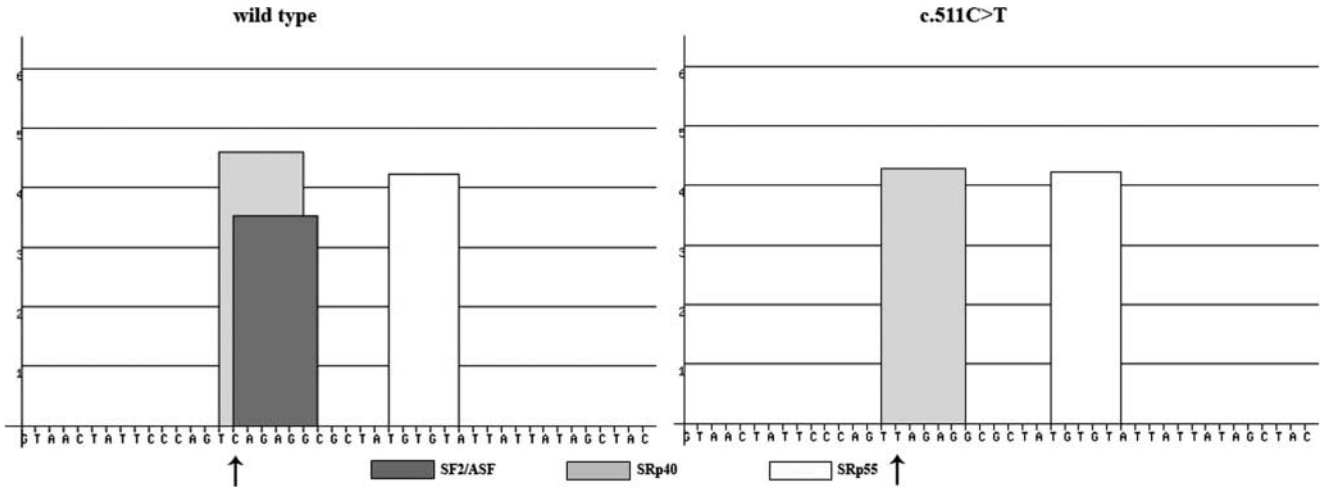

Figure 4. Effect of the c.511C>T mutation on SR proteins matrix score identified by ESEfinder software. The c.511C>T mutation eliminates the potential ESE of SF2/ASF motif from 3.53 to 0.582 and slightly reduces the score of an SRp40 motif from 4.6 to 4.3 without falling below the threshold value. The SC35 and SRp55 motifs are unchanged. The arrows indicate C in the wild type and T in the mutant.

At least 173 different mutations have been reported in the *PTEN* gene. Of these, 28 are nonsense mutations. Most mutation-screening studies are only conducted on genomic DNA. Q171X has already been reported one time in Cowden disease on genomic DNA (http://www.hgmd.cf.ac.uk), so the skipping of exon 6, which is the main pathogenic effect of this mutation, was not investigated. To our knowledge, the c.511C>T mutation is the first nonsense mutation in the *PTEN* gene causing exon skipping by disrupting ESE in patients affected with BRRS.

Nonsense mutations can induce the skipping of constitutive exons and one of the possible mechanisms of nonsense-associated altered splicing is the ESE disruption model. A putative role for ESE disruption in nonsense-associated exon skipping has been previously reported in a few disease-causing genes including *DMD*, *BRCA1* and *NF1* (28). Even though nonsense mutations always produce truncated nonfunctional proteins which are deleterious enough to cause diseases regardless of their location with respect to ESEs, it would be interesting to investigate if there is association between reported nonsense mutations in the *PTEN* gene and ESE sites. In addition it has been demonstrated that missense mutations in some cancer predisposition genes, *hMSH2* and *hMLH1* preferentially locate in ESE motifs (22). There is still no report that correlates specific point mutations in *PTEN* coding region with the skipping of the exon harboring the mutation. This prompted us to additionally investigate the possibility of reported missense mutations in the *PTEN* gene being pathogenic because of disrupting ESEs.

We have found that nonsense mutations do not colocalize with ESEs. This result suggests that most of the nonsense-associated exon skipping is not a consequence of ESE disruption. Three other possible mechanisms, e.g. nuclear scanning, indirect nonsense-mediated mRNA decay, or secondary-structure disruption model have been proposed (28). Recent data have also indicated that single-base changes can create negative elements, a splicing silencer (29,30). It is also possible the softwares do not pick up all potential ESE sites. Additional work is needed to better identify the relevant mechanism and machinery responsible for nonsense-associated exon skipping. For analysis of missense mutations, we have also found that missense mutations do not colocalize with ESEs, similar to the studies found in some previously reported diseases, such as metachromatic leukodystrophy (31). This result suggests that pathogenic effects of the majority of missense mutations in the *PTEN* gene are not splicing-related but through other mechanisms, e.g. structural changes and RNA instability. Since *PTEN* encodes a dual-specificity phosphatase which might be sensitive to structural changes, missense mutations causing alterations in amino acids may be as deleterious as those disrupting ESEs.

In summary, we identified a Thai family with Bannayan–Riley–Ruvalcaba syndrome with an additional rare feature, large AVMs in the liver. The molecular analysis revealed an exon skipping-associated nonsense mutation c.511C>T (p.Q171X) in the *PTEN* gene. This mutation has been previously reported in CS, but not in BRRS. The nonsense mutation was predicted to be pathogenic resulting in a truncated protein product. However, we demonstrated here that it also disturbed splicing presumably from disrupting a potential ESE causing skipping of the whole exon 6. This is the first nonsense-mediated exon skipping in the *PTEN* gene being deleterious possibly from disrupting an ESE. The association study between reported pathogenic nonsense/missense mutations and ESE sites, however, revealed that the mutations do not colocalize with ESE sites suggesting that most of their pathogenic effects are through other mechanisms. It would be interesting to investigate the consequence of each *PTEN*

Table 3. ESE motifs found in the *PTEN* gene

SF2/ASF Motifs				SC35 Motifs				SRp40 Motifs				SRp55 Motifs			
Position in open reading frame		Motif	Score	Position in open reading frame		Motif	Score	Position in open reading frame		Motif	Score	Position in open reading frame		Motif	Score
Left	Right			Left	Right			Left	Right			Left	Right		
50	56	AAGAGGA	3.2	108	115	ATTTCCTG	3.3	2	8	TGACAGC	4.7	114	119	TGCAGA	4.3
136	142	TACAGGA	3.0	133	140	GTATACAG	3.1	35	41	ACAAAAG	3.1	179	184	AGCATA	3.3
287	293	CACAGCT	3.1	325	332	GACCAATG	4.3	135	141	ATACAGG	5.0	247	252	TGCAGA	4.3
384	390	GGGACGA	4.3	364	371	ATTCACTG	3.0	175	181	TCAAAGC	3.9	287	292	CACAGC	3.3
443	449	CACAAGA	4.1	450	457	GGCCCTAG	3.7	192	198	TTACAAG	5.4	357	362	TGCAGC	4.7
511	517	CAGAGGC	3.5	457	464	GATTTCTA	4.1	228	234	TGACACC	4.4	408	413	TGCATA	5.2
586	592	CACAAGA	4.1	477	484	GACCAGAG	3.7	286	292	CCACAGC	5.1	419	424	TACATC	4.9
693	699	CACACGA	6.6	566	573	GACCAGTG	4.7	302	308	TCAAACC	3.6	522	527	TGTGTA	4.2
733	739	CAGCCGT	5.0	615	622	GTTCAGTG	3.8	327	333	CCAATGG	4.2	924	929	TGCAGA	4.3
848	854	CAGAGGA	5.7	649	656	GTCTGCCA	3.6	366	372	TCACTGT	3.7	1036	1041	TACTTC	3.2
999	1005	CAACCGA	3.3	689	696	GACCCACA	3.7	418	424	TTACATC	3.1	1187	1192	AGCATA	3.3
1057	1063	GAGCCGT	3.4	845	852	GACCAGAG	3.7	436	442	TTAAAGG	4.4				
1070	1076	CAGAGGC	3.5	1074	1081	GGCTAGCA	3.7	442	448	GCACAAG	3.3				
1097	1103	CACCAGA	3.1	1086	1093	AACTTCTG	3.3	461	467	TCTATGG	3.5				
1100	1109	CAGATGT	3.5	1143	1150	CACCACTG	3.6	510	516	TCAGAGG	4.6				
1160	1166	CAGAGAA	3.2	1183	1190	GATCAGCA	3.0	568	574	CCAGTGG	4.0				
								585	591	TCACAAG	5.8				
								617	623	TCAGTGG	4.4				
								658	664	CTAAAGG	4.1				
								683	689	ATTCAGG	3.9				
								692	698	CCACACG	5.7				
								705	711	AGACAAG	3.9				
								730	736	CCTCAGC	4.1				
								774	780	CCACAAA	3.0				
								847	853	CCAGAGG	4.2				
								933	939	TGACAAG	4.9				
								1010	1016	TTTCTCC	3.6				
								1040	1046	TCACAAA	3.4				
								1069	1075	CCAGAGG	4.2				
								1140	1146	TGACACC	4.4				
								1145	1151	CCACTGA	3.3				
								1199	1205	TTACAAA	3.1				
								1201	1207	ACAAAAG	3.1				

mutation found using genomic DNA at the transcript level. The differences in the expression level of various transcripts might lead to modification of the phenotype. These findings could have implications to help explain a still unclear genotype-phenotype correlation in the PTEN hamartoma tumor syndrome.

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References

1. Marsh DJ, Kum JB, Lunetta KL, Bennett MJ, Gorlin RJ, Ahmed SF, et al. PTEN mutation spectrum and genotype-phenotype correlations in Bannayan–Riley–Ruvalcaba syndrome suggest a single entity with Cowden syndrome. *Hum Mol Genet* 1999;8:1461–72.
2. Zhou XP, Marsh DJ, Hampel H, Mulliken JB, Gimm O, Eng C. Germline and germline mosaic PTEN mutations associated with a Proteus-like syndrome of hemihypertrophy, lower limb asymmetry, arteriovenous malformations and lipomatosis. *Hum Mol Genet* 2000;9:765–8.
3. Zhou X, Hampel H, Thiele H, Gorlin RJ, Hennekam RC, Parisi M, et al. Association of germline mutation in the PTEN tumour suppressor gene and Proteus and Proteus-like syndromes. *Lancet* 2001;358:210–1.
4. Smith JM, Kirk EP, Theodosopoulos G, Marshall GM, Walker J, Rogers M, et al. Germline mutation of the tumour suppressor PTEN in Proteus syndrome. *J Med Genet* 2002;39:937–40.
5. Starink TM, van der Veen JP, Arwert F, de Waal LP, de Lange GG, Gille JJ, et al. The Cowden syndrome: a clinical and genetic study in 21 patients. *Clin Genet* 1986;29:222–33.
6. Hanssen AM, Fryns JP. Cowden syndrome. *J Med Genet* 1995;32:117–9.
7. Nelen MR, Padberg GW, Peeters EA, Lin AY, van den Helm B, Frants RR, et al. Localization of the gene for Cowden disease to chromosome 10q22–23. *Nat Genet* 1996;13:114–6.
8. Eng C. Will the real Cowden syndrome please stand up: revised diagnostic criteria. *J Med Genet* 2000;37:828–30.
9. Bannayan GA. Lipomatosis, angiomatosis, and macrencephalia. A previously undescribed congenital syndrome. *Arch Pathol* 1971;92:1–5.
10. Zonana J, Rimoin DL, Davis DC. Macrocephaly with multiple lipomas and hemangiomas. *J Pediatr* 1976;89:600–3.
11. Gorlin RJ, Cohen MM, Jr., Condon LM, Burke BA. Bannayan–Riley–Ruvalcaba syndrome. *Am J Med Genet* 1992;44:307–14.
12. Marsh DJ, Dahia PL, Zheng Z, Liaw D, Parsons R, Gorlin RJ, et al. Germline mutations in PTEN are present in Bannayan–Zonana syndrome. *Nat Genet* 1997;16:333–4.
13. Marsh DJ, Coulon V, Lunetta KL, Rocca-Serra P, Dahia PL, Zheng Z, et al. Mutation spectrum and genotype-phenotype analyses in Cowden disease and Bannayan–Zonana syndrome, two hamartoma syndromes with germline PTEN mutation. *Hum Mol Genet* 1998;7:507–15.
14. Pilarski R, Eng C. Will the real Cowden syndrome please stand up (again)? Expanding mutational and clinical spectra of the PTEN hamartoma tumour syndrome. *J Med Genet* 2004;41:323–6.
15. Cantley LC, Neel BG. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proc Natl Acad Sci USA* 1999;96:4240–5.
16. Gu J, Tamura M, Yamada KM. Tumor suppressor PTEN inhibits integrin- and growth factor-mediated mitogen-activated protein (MAP) kinase signaling pathways. *J Cell Biol* 1998;143:1375–83.
17. Myers MP, Stolarov JP, Eng C, Li J, Wang SI, Wigler MH, et al. P-TEN, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase. *Proc Natl Acad Sci USA* 1997;94:9052–7.
18. Tamura M, Gu J, Matsumoto K, Aota S, Parsons R, Yamada KM. Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. *Science* 1998;280:1614–7.
19. Weng L, Brown J, Eng C. PTEN induces apoptosis and cell cycle arrest through phosphoinositol-3-kinase/Akt-dependent and -independent pathways. *Hum Mol Genet* 2001;10:237–42.
20. Waite KA, Eng C. Protean PTEN: form and function. *Am J Hum Genet* 2002;70:829–44.
21. Cartegni L, Wang J, Zhu Z, Zhang MQ, Krainer AR. ESEfinder: A web resource to identify exonic splicing enhancers. *Nucleic Acids Res* 2003;31:3568–71.
22. Gorlov IP, Gorlova OY, Frazier ML, Amos CI. Missense mutations in hMLH1 and hMSH2 are associated with exonic splicing enhancers. *Am J Hum Genet* 2003;73:1157–61.
23. Fairbrother WG, Yeh RF, Sharp PA, Burge CB. Predictive identification of exonic splicing enhancers in human genes. *Science* 2002;297: 1007–13.
24. Deleris P, Bacqueville D, Gayral S, Carrez L, Salles JP, Perret B, et al. SHIP-2 and PTEN are expressed and active in vascular smooth muscle cell nuclei, but only SHIP-2 is associated with nuclear speckles. *J Biol Chem* 2003;278:38884–91.
25. Su JD, Mayo LD, Donner DB, Durden DL. PTEN and phosphatidylinositol 3′ -kinase inhibitors up-regulate p53 and block tumor-induced angiogenesis: evidence for an effect on the tumor and endothelial compartment. *Cancer Res* 2003;63:3585–92.
26. Koul D, Shen R, Garyali A, Ke LD, Liu TJ, Yung WK. MMAC/PTEN tumor suppressor gene regulates vascular endothelial growth factor-mediated angiogenesis in prostate cancer. *Int J Oncol* 2002;21:469–75.
27. Hastings ML, Krainer AR. Pre-mRNA splicing in the new millennium. *Curr Opin Cell Biol* 2001;13:302–9.
28. Cartegni L, Chew SL, Krainer AR. Listening to silence and understanding nonsense: exonic mutations that affect splicing. *Nat Rev Genet* 2002;3:285–98.
29. Disset A, Bourgeois CF, Benmalek N, Claustres M, Stevenin J, Tuffery-Giraud S. An exon skipping-associated nonsense mutation in the dystrophin gene uncovers a complex interplay between multiple antagonistic splicing elements. *Hum Mol Genet* 2006;15:999–1013.
30. Kashima T, Manley JL. A negative element in SMN2 exon 7 inhibits splicing in spinal muscular atrophy. *Nat Genet* 2003;34:460–3.
31. Shotelersuk V, Desudchit T, Tongkobpetch S. ASA E382K disrupts a potential exonic splicing enhancer causes exon skipping, but missense mutations in ASA are not associated with ESEs. *Int J Mol Med* 2004;14:683–9.