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# An intronic polymorphic deletion in the *PTEN* gene: implications for molecular diagnostic testing

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**Background:** A cohort of 629 patients with suspected Bannayan–Riley–Ruvalcaba syndrome or Cowden syndrome was tested for mutations in the *PTEN* gene.

**Methods:** Dosage analysis of *PTEN* was carried out using a *PTEN*-specific multiplex ligation-dependent probe amplification (MLPA) kit, whereas point mutation analysis was performed using direct sequencing.

**Results:** Approximately 4% of the patients from the testing cohort were heterozygously deleted for the two MLPA probe-binding sites situated in intron 1. The same deletion was subsequently seen in ~3% of 220 normal controls, and in patients from the testing cohort with a causative mutation elsewhere in the *PTEN* gene. Sequencing of the variant revealed an 899 bp deletion, the 3' breakpoint of which is only 58 bp from the start of exon 2.

**Conclusion:** Although all evidence suggests that the 899 bp deletion is a polymorphism with no clinical effect, it removes the binding sites of almost all published *PTEN* exon 2 forward primers, resulting in allelic loss during PCR.

The *PTEN* tumour suppressor gene is involved in the phosphoinositol 3-kinase pathway and encodes a phosphatase with lipid and protein substrates, active in many pathways involved in cellular growth. The *PTEN* hamartoma tumour syndromes form a spectrum of autosomal dominantly inherited syndromes with variable clinical manifestations, the most commonly reported conditions being Cowden syndrome (CS) and Bannayan–Riley–Ruvalcaba syndrome (BRRS) (Arch *et al*, 1997; Liaw *et al*, 1997; Nelen *et al*, 1997), which have both been shown to be caused in part by germline *PTEN* mutations. Both are characterised by multiple hamartomas and have many overlapping features.

Dosage analysis is an important part of the *PTEN* screening strategy as large deletions are detected in ~10% of point mutation-negative BRRS patients and have been reported in point mutation-negative CS patients (Zhou *et al*, 2003; Orloff and Eng, 2008). The method of choice for this analysis is the Multiplex Ligation-dependent Probe Amplification (MLPA) assay (Schouten *et al*,

2002) and MRC-Holland (Amsterdam, The Netherlands) released a *PTEN*-specific kit (P225) in 2007. Analysis of the *PTEN* gene is made difficult by the existence of a highly homologous *PTEN* pseudogene, so for this MLPA kit many of the probes had to be situated in the *PTEN* introns, rather than the exons, in order to distinguish it from its pseudogene. The original P225 kit that was used in this study had three probes corresponding to exon 2 – two of them in intron 1 (with ligation sites at c.80–504 and c.80–219) and one in intron 2 (with a ligation site at position c.164 + 302).

In our study, MLPA dosage analysis of the *PTEN* gene was performed on a large cohort of patients with suspected BRRS or CS. A significant proportion of patients were found to be heterozygously deleted for just the two 'exon 2' probes that were located in intron 1. Subsequent testing was then performed in order to characterise the deletion, to determine its frequency in our test cohort and the general population, and to investigate its origins and any potential phenotypic effects.

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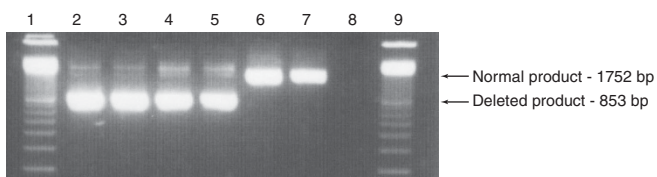
## MATERIALS AND METHODS

**Patients.** A cohort of 629 probands with suspected BRRS or CS was collected at the Wessex Regional Genetics Laboratory over a period of 19 years following referrals from local, national and international clinicians.

In order to provide a matched control cohort, 220 healthy controls were chosen and anonymised. These individuals were the normal partners who had been carrier-tested for autosomal recessive conditions in the Wessex laboratory.

**Multiplex ligation-dependent probe amplification analysis.** The MLPA was carried out according to the manufacturer's instructions with the P225 (version A1) *PTEN* kit on the cohort of 629 BRRS/CS probands. The MLPA PCR products (1 µl) were separated on an ABI3100 Sequencer and analysed using Genotyper (v3.7) software (Applied Biosystems, Foster City, CA, USA). *PTEN* intron/exon copy number was determined by exporting peak heights into an Excel spreadsheet, specifically designed to assess the ratio of each test peak relative to all other peaks for the given individual. Further, ratios of test:control peaks and control:control peaks for each sample were compared with normal individuals included in each run (Bunyan *et al*, 2004).

**The PCR and Sequencing analysis.** The PCR amplification of genomic DNA was performed using various combinations of primers from intron 1 and exon 2 of the *PTEN* gene to generate a fragment spanning the deletion breakpoint. A PCR product was generated using primers 5'-aaatcaactcaatcatgtgaaagc-3' (forward) and 5'-ggtgaccagcattttatggag-3' (reverse), which give a normal-sized product of 1752 bp and a smaller product of 853 bp (see Figure 1). Reactions were carried out in a 25 µl volume containing 50 ng DNA, 1 µM forward primer, 1 µM reverse primer, 2.5 µl 10 × reaction buffer (Perkin Elmer, Waltham, MA, USA), 0.16 mM dNTPs, 1.5 mM MgCl<sub>2</sub>, 0.5 u Taq polymerase (Taq Gold, Perkin Elmer). Cycling parameters were 94 °C for 12 min followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 90 s. The PCR



**Figure 1.** Agarose gel electrophoresis of the PCR products from the intron 1/exon 2 region. Lanes 1 and 9 = size markers (100bp ladder; Sigma, Gillingham, Dorset, UK), lanes 2–5 = individuals with the intron 1 deletion variant, lanes 6 and 7 = patients without the intron 1 deletion variant, lane 8 = no DNA control.

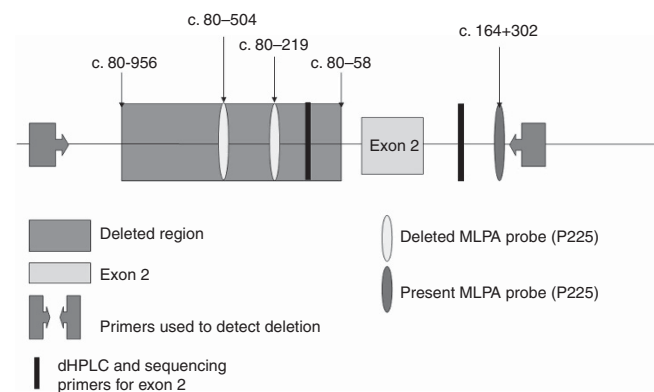
products were sequenced bidirectionally using the Big Dye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Warrington, UK) and analysed using Mutation Surveyor software version 3.2 (Soft Genetics, State College, PA, USA). All patients with the intron 1 deletion were sequenced.

Subsequent intron 1 deletion analysis of a cohort of 220 normal individuals was carried out using this PCR assay. All control individuals with the intron 1 deletion were also sequenced.

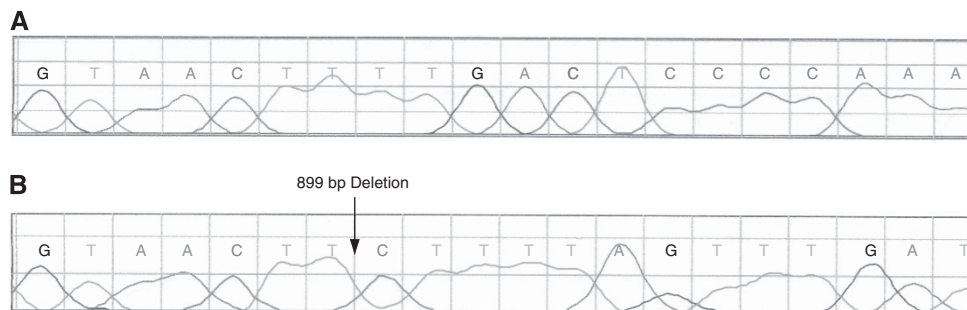
***PTEN* linkage.** We were able to perform haplotype analysis on 20 of the 26 deletion-positive patients from the BRRS/CS cohort to look for a common haplotype. This was done with the CA repeat markers D10S215 and D10S541, which closely flank the *PTEN* gene, using previously published primers and conditions (Feilotter *et al*, 1999). As the deletion-positive patients from the control group had been anonymised, these were not included in the linkage study.

## RESULTS

The MLPA analysis of the BRRS/CS cohort showed that 26 of the 629 patients (4%) had the intron 1 deletion (MLPA raw data not shown). Sequence analysis showed that this deletion was 899 bp in size and was situated 58 bp from the start of exon 2, c.80-956\_58del899 (HGVS nomenclature using RefSeq NM\_000314.4, see Figure 2). The deletion includes the ligation sites of the two intron 1 P225-A1 MLPA probes (see Figure 3). All 26 apparently unrelated patients had identical sequencing breakpoints. Twenty-five of these deletion-positive cases were of British origin and one was of Turkish heritage.



**Figure 3.** Schematic showing the position of the intron 1 deletion, the affected MLPA probe binding sites and the location of primer pairs within the *PTEN* gene.



**Figure 2.** Sequence chromatograms from a normal individual (A) and an individual with the intron 1 deletion variant (B). The position of the deleted bases is marked with an arrow.

Six of the 26 deletion-positive patients also had a causative point mutation or frameshift mutation elsewhere in the *PTEN* gene. In one of these six cases we know that the intronic deletion and the other point mutation (an exon 2 splice donor site mutation, c.164 + 1 G > A) are *in trans* as PCR amplification of exon 2 using a forward primer from within the intron 1 deletion solely amplified the allele containing the splice site mutation. Owing to the lack of parental samples, we were unable to determine phase in the other five cases.

Intron 1 PCR analysis of the 220 normal controls showed that six individuals (3%) also had the 899 bp deletion, confirmed by sequencing to be identical to that seen in the BRRS/CS cohort. Although the control samples were anonymised before testing, all patients in that cohort were completely healthy at the time of DNA extraction with no known symptoms of BRRS or CS.

Haplotype analysis using markers D10S215 and D10S541 showed no evidence that the intron 1 deletion is a founder mutation. Allele sizes from the 20 apparently unrelated deletion-positive patients from the BRRS/CS cohort were extremely widespread. For these 20 individuals the deletion was found to be associated with a minimum of 11 haplotypes and a maximum of 19 (data not shown).

## DISCUSSION

There is no obvious mechanism that would cause the recurrence of this 899 bp deletion from intron 1 of the *PTEN* gene. The deleted segment has no large quasipalindromic sequences and no flanking repeats, whereas the MFOLD DNA folding prediction software ([www.mfold.rna.albany.edu/](http://www.mfold.rna.albany.edu/)) suggests that the breakpoints are not in exposed parts of hairpin loops, nor does the deleted segment form a hairpin loop itself.

Alamut mutation interpretation software ([www.interactive-biosoftware.com/alamut](http://www.interactive-biosoftware.com/alamut)) predicts the replacement of multiple essential splice elements and proposes a cryptic splice acceptor site at position c.80-50, but although multiple *PTEN* transcripts have been reported (Sharrard and Maitland, 2000; Sarquis *et al*, 2006), none of them report a loss of exon 2 or the inclusion of a larger exon 2 because of the use of this potential cryptic acceptor site. Other groups have studied *PTEN* RNA transcripts from individuals with this polymorphism and no effect on splicing was observed (Reviewer 3, personal communication).

As the deletion is present in healthy individuals, and in patients with a known causative *PTEN* mutation *in trans*, we can conclude that this variant has no phenotypic effect, but it does result in a potential problem for point mutation analysis if it is not detected. To allow the detection of possible splice site mutations and to get full exon coverage, it is common practice to site PCR primers at least 50 bp outside of exons. Most of the published *PTEN* exon 2 forward primers lie within the intron 1 deletion variant (e.g. Liaw *et al*, 1997; Marsh *et al*, 1997; Steck *et al*, 1997; Minaguchi *et al*, 2001; Silva *et al*, 2008), so these primers would only amplify one allele of exon 2 in a patient with the intronic deletion, and would also affect any method based on heteroduplex analysis.

The results from the linkage analysis were surprising. It was expected that the intron 1 variant would be associated with a common haplotype with the closely flanking markers D10S215 and D10S541, but the haplotypes in the 20 patients with the 899 bp deletion were essentially random. However, it is still possible that this is an extremely old founder mutation that has been through enough meioses to cause such variability in the flanking CA repeats. Mutations in several genes, e.g. *CFTR* and *BRCA1*, have been shown to persist for hundreds of generations (Morral *et al*, 1994; Neuhausen *et al*, 1996). The cross-over rates between the D10S215 and D10S541 markers and the *PTEN* gene are both ~1%

(Leal and Da-Silva, 1999), and CA repeat allele sizes have been previously shown to change meiotically (Kwiatkowski and Diaz, 1992). Although most of our cohort is of British origin, the intron 1 variant was seen in one patient of Turkish origin and was also seen in Canada (Jan Schouten, MRC-Holland, personal communication) and America (both African Americans and Caucasian Americans; Reviewer 3, personal communication), suggesting that this variant is present worldwide, supporting the theory that it may have existed for many generations.

There is a paucity of reported polymorphic intronic deletion variants so close to an exon. Curiously, the only other published example is also in the *PTEN* gene – a 39 bp deletion polymorphism in intron 7 in African American patients which has a 3' breakpoint 15 bp from the start of exon 8 (Zhou *et al*, 2002). Is this a quirk of the *PTEN* gene, or are other genes similarly affected but current methodology does not detect such variants? As most dosage detection techniques tend to focus on the exons, any intronic variability may go undetected.

The current commercial *PTEN* MLPA kit from MRC-Holland (P225 version B2) no longer detects the intron 1 variant as the two probes from the original version of the kit that were within the deleted area have now been moved, so it is important that another method is used to detect this variant. Any patients that were previously screened for point mutations in exon 2 of the gene using a forward primer from within the potentially deleted intron 1 region should now be retested in light of this finding. Here we provide a simple PCR-based test for the deletion variant that will easily detect any deletion carriers that will need to be re-sequenced with a new forward primer that lies within 58 bases of exon 2. In our cohort of BRRS/CS patients, exon 2 point mutations account for 11% of all *PTEN* mutations (10 out of 89 positive patients), therefore a significant number of mutations may have been missed due to non-amplification of an allele during PCR.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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