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# *PTEN* mutation spectrum and genotype–phenotype correlations in Bannayan–Riley–Ruvalcaba syndrome suggest a single entity with Cowden syndrome

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**Germline mutations in the tumour suppressor gene *PTEN* have been implicated in two hamartoma syndromes that exhibit some clinical overlap, Cowden syndrome (CS) and Bannayan–Riley–Ruvalcaba syndrome (BRR). *PTEN* maps to 10q23 and encodes a dual specificity phosphatase, a substrate of which is phosphati-**

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**dylinositol 3,4,5-triphosphate, a phospholipid in the phosphatidylinositol 3-kinase pathway. CS is characterized by multiple hamartomas and an increased risk of benign and malignant disease of the breast, thyroid and central nervous system, whilst the presence of cancer has not been formally documented in BRR. The partial clinical overlap in these two syndromes is exemplified by the hallmark features of BRR: macrocephaly and multiple lipomas, the latter of which occur in a minority of individuals with CS. Additional features observed in BRR, which may also occur in a minority of CS patients, include Hashimoto's thyroiditis, vascular malformations and mental retardation. Pigmented macules of the glans penis, delayed motor development and neonatal or infant onset are noted only in BRR. In this study, constitutive DNA samples from 43 BRR individuals comprising 16 sporadic and 27 familial cases, 11 of which were families with both CS and BRR, were screened for *PTEN* mutations. Mutations were identified in 26 of 43 (60%) BRR cases. Genotype–phenotype analyses within the BRR group suggested a number of correlations, including the association of *PTEN* mutation and cancer or breast fibroadenoma in any given CS, BRR or BRR/CS overlap family ($P = 0.014$), and, in particular, truncating mutations were associated with the presence of cancer and breast fibroadenoma in a given family ($P = 0.024$). Additionally, the presence of lipomas was correlated with the presence of *PTEN* mutation in BRR patients ($P = 0.028$). In contrast to a prior report, no significant difference in mutation status was found in familial versus sporadic cases of BRR ($P = 0.113$). Comparisons between BRR and a previously studied group of 37 CS families suggested an increased likelihood of identifying a germline *PTEN* mutation in families with either CS alone or both CS and BRR when compared with BRR alone ($P = 0.002$). Among CS, BRR and BRR/CS overlap families that are *PTEN* mutation positive, the mutation spectra appear similar. Thus, *PTEN* mutation-positive CS and BRR may be different presentations of a single syndrome and, hence, both should receive equal attention with respect to cancer surveillance.**

## INTRODUCTION

Bannayan–Riley–Ruvalcaba (BRR) syndrome and Cowden syndrome (CS) are two hamartoma syndromes displaying both clinical and genetic overlap. Hamartomas are developmentally disorganized, benign growths and occur in multiple organ systems in CS including the breast, thyroid, skin, central nervous system and gastrointestinal tract (1–5). Trichilemmomas, benign tumours of the hair follicle infundibulum, as well as mucocutaneous papules are hallmark hamartomas of CS, occurring in 99% of CS patients (2–5), but are not considered to be true components of BRR. Other hamartomas occurring frequently in CS include breast fibroadenomas (70% of affected females), thyroid adenomas and multinodular goitre (40–60% of all CS patients), and gastrointestinal polyps (35–40% of affected individuals). Thirty-eight per cent of CS patients have megencephaly or macrocephaly. CS patients also have an increased lifetime risk of developing breast and thyroid cancer. Breast cancer develops in 25–50% of affected females and thyroid cancer in 3–10% of all affected individuals (4,5). Malignant and benign disease is also seen in the central nervous system in ~40% of cases (4). Lhermitte–Duclos disease (LDD), also a feature of CS, describes dysplastic gangliocytoma of the cerebellum which manifests as seizures, tremors and poor coordination (6–9).

BRR is characterized by the classic triad of macrocephaly, lipomatosis and pigmented macules of the glans penis (speckled penis) (10–12). Some features of BRR, such as pigmented macules of the glans penis and very early age of onset, have never been reported in CS. Other BRR component features such as macrocephaly, lipomatosis, haemangiomas, Hashimoto's thyroiditis, mental and developmental delay can be quite common in BRR but occur only in the minority of CS patients. It is of note that an increased risk of malignancy has not been formally documented in BRR.

Germline mutations in the tumour suppressor *PTEN* have been found in 13–81% of CS patients (13–16) and 57–60% of BRR cases (16,17). *PTEN* maps to 10q23 and encodes a dual-specificity phosphatase which has homology to the focal adhesion molecules tensin and auxilin (18–21). A classic phosphatase (PTPase) core motif is encoded within exon 5 of this gene. One of PTEN's major endogenous substrates is phosphotidylinositol 3,4,5-triphosphate [Ptd-Ins(3,4,5)$P_3$], a phospholipid in the phosphatidylinositol 3-kinase (PI-3 kinase) pathway (22–24) which previously has been established to be important in cell growth signalling (25). In this pathway, PTEN may act as a 3-phosphatase to dephosphorylate Ptd-Ins(3,4,5)$P_3$ to Ptd-Ins(4,5)$P_2$. Mutant or decreased PTEN leads to the accumulation of Ptd-Ins(3,4,5)$P_3$, which is required for activation of protein kinase B (PKB)/Akt, a known cell survival factor (22–24,26).

In the present study, we have performed *PTEN* mutation analysis in 43 unrelated BRR cases, including a subset of 11 families with both BRR and CS, and compared these data with those found in a previous study of 37 CS families (16). Genotype–phenotype correlations were sought both within the BRR group and between the CS and BRR groups in an attempt to identify trends unique to either group or alternatively which support the hypothesis that BRR and CS are different presentations of the same syndrome.

## RESULTS

### *PTEN* mutation scanning in BRR and BRR/CS overlap families

Constitutive DNA from 43 unrelated patients with BRR (32 with BRR alone and 11 with both BRR and CS) were screened for germline *PTEN* mutations using denaturing gradient gel electrophoresis (DGGE). DGGE variants were then sequenced. Germline *PTEN* mutations, including point missense or nonsense mutations, insertions, deletions, deletion–insertions, splice site mutations, gross deletion and a balanced translocation were identified in 60% (26 of 43) of BRR cases (Table 1). Where possible, the screening of both affected and unaffected relatives showed that these mutations segregated

with the disease phenotype. These mutations were observed along the entirety of the gene, with the exception of exons 1, 4 and 9 (Fig. 1A), a very similar mutation spectrum to that found in 37 CS families (16) (Fig. 1B). No significant difference was observed between the presence of mutations in the apparently sporadic cases of BRR (seven of 16, 44%) compared with that in familial cases of BRR (19 of 27, 70%) ($P$ = 0.113). Ten of the 11 (91%) BRR/CS overlap families were shown to have germline *PTEN* mutation.

Four nonsense point mutations were common to both CS and BRR or BRR/CS overlap families. These were Q110X (one CS, one BRR/CS), R130X (two CS, two BRR/CS, one BRR), R233X (two CS, two BRR) and R335X (one CS, one BRR/CS). Interestingly, while both gross deletion, 46XY, del(10)(q23.2–q24.1), and balanced translocation, 46XY, t(10;13)(q23.2;q33), were observed in BRR, such gross genetic abnormalities were not seen in CS.

### Genotype–phenotype analyses within the BRR and BRR/CS overlap cohort

A number of genotype–phenotype associations were identified within and between the BRR and BRR/CS groups. Compared with the BRR-only group, the distinguishing features in the 11 BRR/CS families were a minimum of one individual with BRR, and an individual(s) with either cancer or breast fibroadenomas in conjunction with trichilemmomas or papillomatous papules [i.e. a CS (4) rather than BRR phenotype]. Within the BRR and BRR/CS group, the presence of a germline *PTEN* mutation was associated with lipomatosis ($P$ = 0.028, Table 2). It is of note that in the combined BRR and BRR/CS group, the presence of a *PTEN* mutation was associated with the presence of either cancer or breast fibroadenoma (i.e. traditionally, a CS phenotype) in any given family ($P$ = 0.014). Similarly, a correlation between truncating *PTEN* mutations and the presence of cancer or breast fibroadenoma was identified ($P$ = 0.024, Table 2).

Correlations were not identified between the presence of a *PTEN* mutation and intestinal polyps, speckled penis or café-au-lait spots, thyroid involvement or haemangiomas ($P$ = 0.154–1.000). With the exception of cancer and breast fibroadenomas (see above), no additional correlations could be made between phenotype and mutation position (5' of or within the PTPase core motif versus 3' of the core motif) or type (truncating or non-truncating) in the mutation-positive BRR and BRR/CS cases ($P$ = 0.166–1.000).

### Genotype–phenotype analyses between the CS, BRR/CS and BRR groups

Prior to making comparisons across the three groups, four hypotheses were generated. The first hypothesis was that there would be a statistically significant difference in the frequency of *PTEN* mutations in CS patients compared with BRR patients. The mutation frequencies in the CS only series (81%, $n$ = 37) and the CS/BRR overlap cases (91%, $n$ = 11) were similar and, hence, were grouped together to test our first hypothesis. A significant difference was observed in mutation frequency between the grouped CS plus BRR/CS families (83%, $n$ = 48) and the entire BRR-only set (50%, $n$ = 32) ($P$ = 0.002, Table 3). Similarly, the mutation frequency of the combined CS plus BRR/CS group was also different from that of the familial BRR-only set ($P$ = 0.041, Table 3).

The second and third hypotheses were based on genotypic differences between CS and BRR, i.e. that there would be different mutational spectra (truncating versus non-truncating, and 5' versus 3' of the core motif) between these two syndromes. Independently of the patient groupings, i.e. whether the BRR/CS patients were analysed as a separate group or included with familial BRR, familial and sporadic BRR or CS alone, the mutational spectra based on position and type of mutation were not significantly different ($P$ > 0.05, Table 3).

The fourth hypothesis relates to mutations within the core motif. Of the 26 mutations identified in BRR patients, only three cases (two BRR/CS and one BRR family) representing 12% of mutations identified were located in the PTPase core motif. All three of these families had the nonsense mutation R130X. In the previous CS study, 23% of mutations, both nonsense (five) and missense (two) point mutations, were found in the core motif (Fig. 1B). Although the numbers were small, the difference between point missense and nonsense mutations in the core motif in CS versus BRR was not found to be significant ($P$ = 0.167, Table 3).

### Hemizygosity analysis in *PTEN* mutation-negative BRR and BRR/CS

Rare BRR patients have been found to have gross deletion of 10q23 including *PTEN*. Thus, all *PTEN* mutation-negative probands were analysed for the possibility of germline whole gene deletion. In order to assess for hemizygosity at this locus, three flanking dinucleotide repeat markers and five intragenic polymorphic markers were used to amplify constitutive DNA from the affected individuals (Fig. 2A). Heterozygosity at a particular marker was used as the exclusion criterion for hemizygosity. The order of the five intragenic markers in relation to each other has not been clarified. Two of these polymorphic markers, IVS4+109insTCTTA and IVS8+32T/G, have been identified previously in intervening sequences (IVS) 4 and 8, respectively (27,28). The microsatellite marker AFMa086wg9 had been mapped to IVS2 (20). Two additional intragenic microsatellite markers, *D10S2491* and *D10S2492*, were isolated and noted to be within *PTEN* (29) although their precise location with respect to the three previous markers was unknown. To determine more precisely where these markers are, genomic DNA from the glioblastoma cell line A172, which had been shown to contain only the first two *PTEN* exons (20), was utilized as a template. Using previously described PCR conditions (30,31), only *D10S2491* was able to be amplified from A172, placing this marker 5' of, or centromeric to, AFMa086wg9. Hence, *D10S2492* must be located 3' of IVS2 but centromeric to the 3' end of *PTEN* although its precise location could not be determined with the resources available (data not shown). Thus, the most likely order of these *PTEN* intragenic markers is *D10S2491*–AFMa086wg9–IVS4+109insTCTTA–*D10S2492*/IVS8+32T/G.

Using this panel of eight polymorphic markers, both flanking and within the *PTEN* locus, we found that all of the 17 *PTEN* mutation-negative BRR cases displayed retention of heterozygosity in at least one of these markers, and 13 of 17 (76%) also showed retention of heterozygosity in at least one intragenic marker (Fig. 2A).

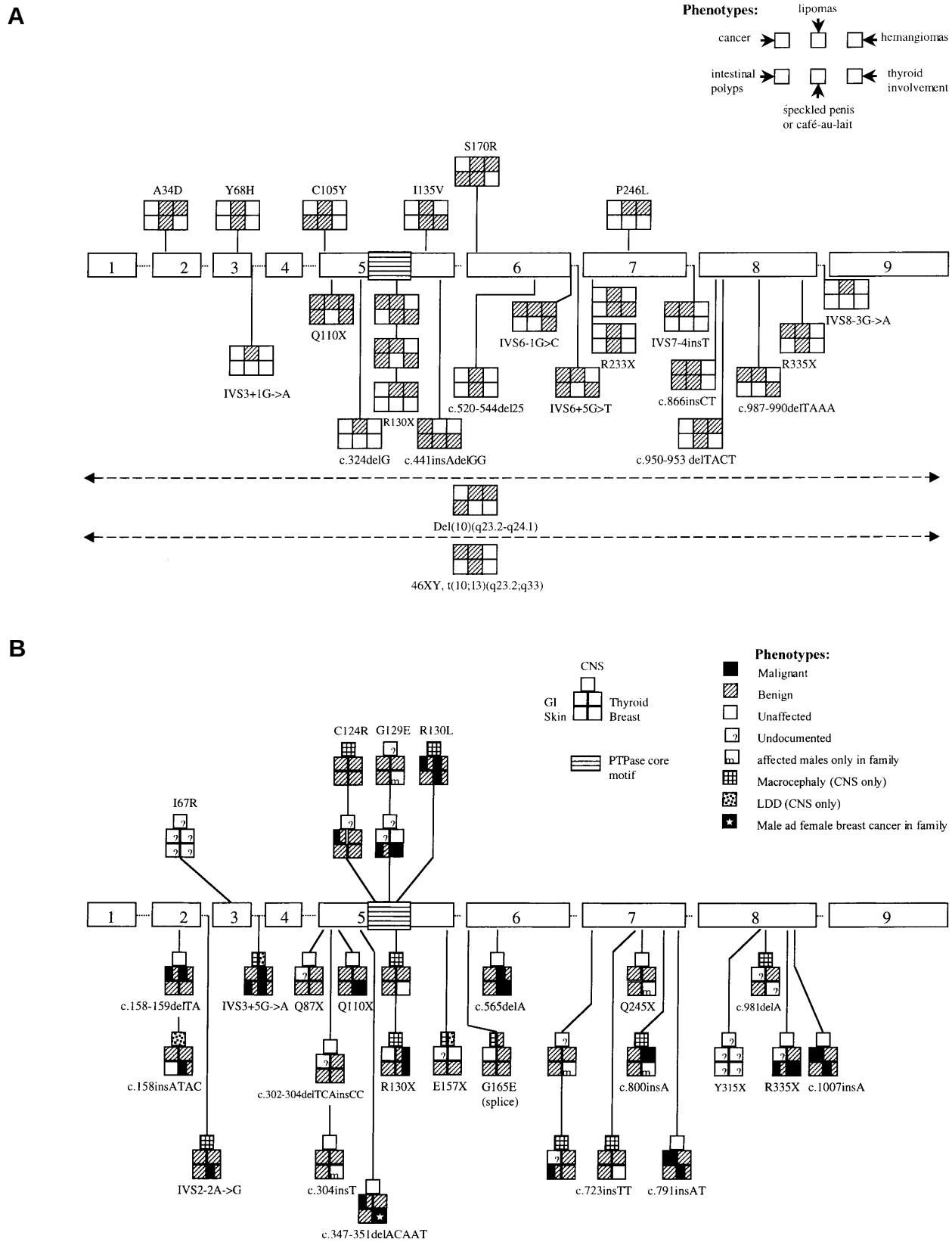


**Figure 1.** (**A**) *PTEN* mutations in BRR and BRR/CS overlap families. The PTPase core motif is indicated in exon 5. Truncating-type mutations are recorded in the lower portion of the figure and missense mutations in the upper portion. The presence of a phenotype is indicated by a shaded box in one of six positions as described in the figure. (**B**) *PTEN* mutations in CS families. Truncating-type mutations are presented in the lower portion of the figure and missense in the upper portion.

**Table 1.** *PTEN* mutations in BRR and BRR/CS overlap patients

| Family identifier | Phenotype | Familial/sporadic | Mutation | Exon/IVS |
|---|---|---|---|---|
| Ban1 | BRR | sporadic | A34D | exon 2 |
| Ban2 | BRR | sporadic | Y68H | exon 3 |
| Ban3 (BSN[a]) | BRR | sporadic | IVS3+1G→A | IVS3 |
| Ban4 | BRR | sporadic | C105Y | exon 5 |
| B/CS1 | BRR/CS | familial | Q110X | exon 5 |
| Ban5 | BRR | familial | c.324delG | exon 5 |
| B/CS2 | BRR/CS | familial | R130X | exon 5 |
| B/CS3 | BRR/CS | familial | R130X | exon 5 |
| Ban6 (B322/B383[a]) | BRR | familial | R130X | exon 5 |
| Ban7 | BRR | familial | I135V | exon 5 |
| B/CS4 | BRR/CS | familial | c.441insAdelGG | exon 5 |
| Ban8 | BRR | familial | S170R | exon 5 |
| Ban9 | BRR | familial | c.520–544del25 | exon 6 |
| B/CS5 | BRR/CS | familial | IVS6–1G→C | IVS6 |
| Ban10 | BRR | familial | IVS6+5G→T | IVS6 |
| Ban11 | BRR | familial | R233X | exon 7 |
| Ban12 | BRR | sporadic | R233X | exon 7 |
| Ban13 (B172/B173/B1016[a]) | BRR | familial | P246L | exon 7 |
| B/CS6 | BRR/CS | familial | IVS7–4insT | IVS7 |
| Ban14 | BRR | sporadic | c.866insCT | exon 8 |
| Ban15 | BRR | familial | c.950–953delTACT | exon 8 |
| B/CS7 | BRR/CS | familial | c.987–990delTAAA | exon 8 |
| B/CS8 | BRR/CS | familial | R335X | exon 8 |
| Ban16 | BRR | sporadic | IVS8–8G→A | IVS8 |
| Ban17 | BRR | sporadic | 46XY, del(10)(q23.3–q24.1) | gross deletion |
| Ban18 | BRR | sporadic | 46XY, t(10;13)(q23.2;q33) | balanced translocation |
| B/CS9 | BRR/CS | familial | no mutation | – |
| Ban19 | BRR | familial | no mutation | – |
| Ban20 | BRR | sporadic | no mutation | – |
| Ban21 | BRR | sporadic | no mutation | – |
| Ban22 | BRR | familial | no mutation | – |
| Ban23 | BRR | sporadic | no mutation | – |
| Ban24 | BRR | familial | no mutation | – |
| Ban25 | BRR | familial | no mutation | – |
| Ban26 | BRR | sporadic | no mutation | – |
| Ban27 | BRR | familial | no mutation | – |
| Ban28 | BRR | familial | no mutation | – |
| Ban29 | BRR | sporadic | no mutation | – |
| Ban30 | BRR | sporadic | no mutation | – |
| Ban31 | BRR | sporadic | no mutation | – |
| Ban32 | BRR | sporadic | no mutation | – |
| Ban33 | BRR | familial | no mutation | – |
| Ban34 (B85[a]) | BRR | sporadic | no mutation | – |

[a]Mutation analysis was performed on DNA extracted from cultured skin fibroblasts.

### Fine mapping of 46XY, del(10)(q23.2–q24.1)

The peripheral blood karyotype of a patient with sporadic BRR had previously shown a region of interstitial deletion on 10q encompassing *PTEN*, 46XY, del(10)(q23.2–q24.1) (32). The patient's parents were shown to have normal chromosome complements (32). Fine structure mapping using the proband's and both parents' germline DNA and the above panel of polymorphic markers was performed to define at least one end of this deletion and to assign the parent of origin (Fig. 2B). Retention of heterozygosity was observed at *D10S219*, the most centromeric marker used, while clear hemizygosity was observed for *D10S2491* and *D10S541*. The marker *D10S579* was non-

**Table 2.** Correlations between BRR and BRR/CS phenotypes and *PTEN* mutations

| Phenotype | Mutation | No mutation | *P*-value[a] |
|---|---|---|---|
| Cancer or breast fibroadenoma present | 11 | 1 | |
| Cancer or breast fibroadenoma absent | 15 | 16 ($n$ = 43) | 0.014 |
| Lipoma(s) present | 25 | 12 | |
| Lipoma(s) absent | 1 | 5 ($n$ = 43) | 0.028 |
| Sporadic BRR | 7 | 9 | |
| Familial BRR | 19 | 8 ($n$ = 43) | 0.113 |
| Phenotype | Truncating mutation | Non-truncating mutation | |
| Cancer or breast fibroadenoma present | 10 | 0 | |
| Cancer or breast fibroadenoma absent | 8 | 6 ($n$ = 24) | 0.024 |
| Phenotype | Mutation frequencies (%) | | |
| CS | 81 | | |
| BRR | 60 | | |
| BRR/CS | 91 | | |

[a]Determined by Fisher's exact test (two-tailed).

informative; thus, the centromeric breakpoint of this deletion is distal to *DI0S219* and might even be defined by *D10S2491* (Fig. 2B). Inspection of the alleles inherited from both parents demonstrated a paternal origin for this deletion (Fig. 2B).

## DISCUSSION

Twenty-six of 43 (60%) unrelated individuals with BRR were found to have a germline *PTEN* mutation. These mutations were found scattered along the length of *PTEN*, with the exception of exons 1, 4 and 9, and included point missense and nonsense mutations, deletions, insertions, deletion–insertions, splice site mutations, a balanced translocation and a gross deletion (Table 1 and Fig. 1A). Of the 17 BRR cases where mutations were not detected, hemizygosity analysis encompassing *PTEN* excluded whole-gene and whole-region deletion. These results would suggest that gross gene deletion was an unlikely mechanism of PTEN inactivation in these cases. However, other possible mechanisms of PTEN inactivation, including mutations in the promoter region, mutations deep within an intron or methylation of promoter sequences, cannot be excluded, although the latter has yet to be described as a germline mechanism of gene regulation in syndromes where imprinting is not involved.

While two anecdotal cases of gross deletion of the 10q23 region encompassing *PTEN* have been reported (32,33), no CS cases have been found to carry a germline hemizygous *PTEN* deletion or chromosomal rearrangement. In this series, only two of 43 (5%) unrelated BRR cases have a gross chromosomal abnormality. In the case with 46XY, del(10)(q23.2–q24.1) (32), we found that this deletion originated on the paternal allele. Interestingly, this patient does not seem to have a more 'severe' phenotype, from a neoplastic point of view, presenting with lipomas, hemangiomas and intestinal polyps without known malignancy (32). However, this patient suffered severe morbidity from his polyposis, which necessitated a total colectomy for control of anaemia and malnutrition (M.T. Geraghty and C. Eng, unpublished data). Together with the fact that germline intragenic *PTEN* mutations are aetiologic for a proportion of BRR, the observation that gross alteration of *PTEN* probably results in haploinsufficiency argues for gross *PTEN* deletion causing BRR as well. These three chromosomal aberration cases (two reported here), especially the deletions, provide an approximate human equivalent of the *Pten*$^{+/-}$ mouse models (22,34,35). Interestingly, neither of these mouse models developed the characteristic tumours of CS, including breast, brain or thyroid cancer. The major tumour of the first model (22,34) was T cell lymphoma. Lymphoma is only rarely known to be associated with CS (36) and it is difficult to determine whether this is a true association or chance occurrence. However, more reminiscent of both BRR and CS, this murine model had microscopic hamartomatous polyps of the colon (34). The other phenotypic features found in the second model (35), such as prostate abnormalities, colon adenocarcinomas and papillary thyroid carcinoma, are atypical of the human syndromes. These cancers are not component to BRR, while papillary thyroid carcinoma is very rarely seen in CS, except in germline *PTEN* mutation-negative and *PTEN* deletion-negative cases (16; C. Eng, unpublished data).

It is of considerable interest that loss of the wild-type *PTEN* allele was reported in thymic lymphomas from the first mouse model (34), while the wild-type allele was retained in various tumours from the second mouse model (35). Our finding of germline deletion in the two BRR patients in the absence of an additional identifiable somatic *PTEN* mutation in their tumours (data not shown) would appear to be in concordance with the second model suggesting that PTEN haploinsufficiency alone may cause developmental defects and tumour formation. However, whilst point mutations could not be identified in the remaining allele in these patients, disruption of the function of wild-type PTEN by alternative mechanisms such as transcriptional inactivation was not excluded. From a phenotypic standpoint based on very small numbers, it would appear that the two gross deletion cases are associated with much more severe

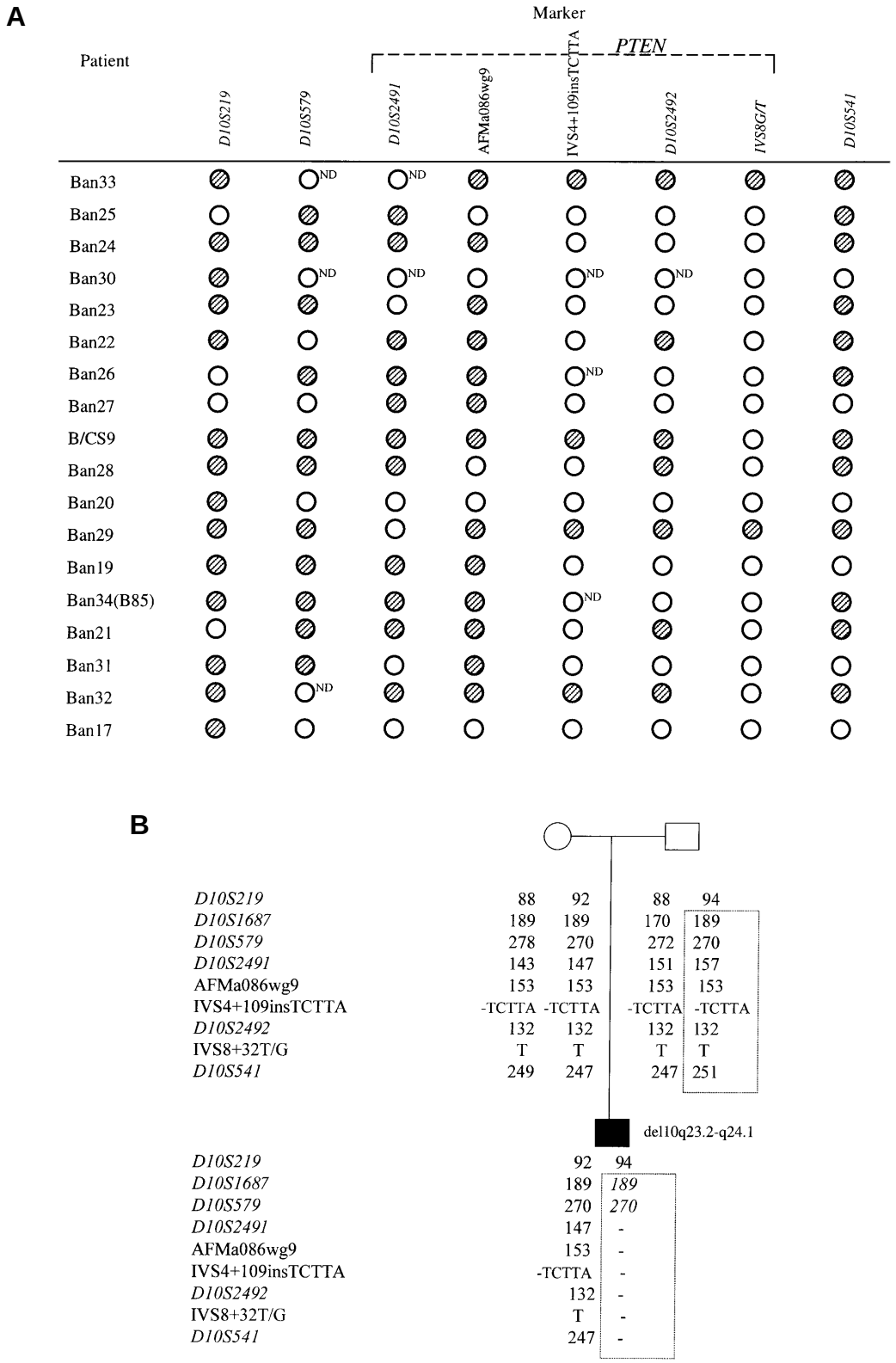


**Figure 2.** (**A**) Hemizygosity analysis of eight polymorphic markers within 10q22–24 in *PTEN* mutation-negative BRR cases. Hatched circles, heterozygosity, i.e. confirmation of the presence of two alleles, at a particular marker site; open circles, homozygosity; ND, marker has not been analysed due to technical difficulties. (**B**) Fine structure mapping of the interstitial deletion 46XY, del(10)(q23.2–q24.1). Retention of heterozygosity is observed at *D10S219* in the proband. The markers *D10S1687* and *D10S579* immediately telomeric are non-informative. Thus, the centromeric breakpoint of this deletion is telomeric of *D10S219.* The deleted paternal chromosome is indicated by a dotted line box.

**Table 3.** Correlations between *PTEN* mutation and the alternative family groupings of CS-only versus BRR/CS versus BRR-only

| Test 1. Mutation present/absent | | | |
|---|---|---|---|
| Phenotype grouping | Present | Absent | *P*-value[a] |
| Test 1a | | | |
| CS | 30 | 7 | |
| BRR/CS | 10 | 1 | |
| BRR (familial only) | 9 | 7 (*n* = 64) | 0.086 |
| BRR (familial + sporadic) | 16 | 16 (*n* = 80) | 0.005 |
| Test 1b | | | |
| CS and CS/BRR | 40 | 8 | |
| BRR (familial only) | 9 | 7 (*n* = 64) | 0.041 |
| BRR (familial + sporadic) | 18 | 16 (*n* = 80) | 0.002 |
| Test 1c | | | |
| CS | 30 | 7 | |
| BRR/CS + BRR (familial) | 19 | 8 (*n* = 64) | 0.378 |
| BRR/CS + BRR (familial + sporadic) | 26 | 17 (*n* = 80) | 0.053 |
| Test 2. Mutation type (truncating/non-truncating) | | | |
| Phenotype grouping | Truncating | Non-truncating | *P*-value[a] |
| Test 2a | | | |
| CS | 24 | 6 | |
| BRR/CS | 10 | 0 | |
| BRR (familial only) | 6 | 3 (*n* = 49) | 0.142 |
| BRR (familial + sporadic) | 8 | 6 (*n* = 54) | 0.050 |
| Test 2b | | | |
| CS + CS/BRR | 34 | 6 | |
| BRR (familial only) | 6 | 3 (*n* = 49) | 0.336 |
| BRR (familial + sporadic) | 8 | 6 (*n* = 54) | 0.057 |
| Test 2c | | | |
| CS | 24 | 6 | |
| BRR/CS + BRR (familial only) | 16 | 3 (*n* = 49) | 1.000 |
| BRR/CS + BRR (familial + sporadic) | 18 | 6 (*n* = 54) | 0.748 |
| Test 3. Mutation position relative to core (5' and within core/3' of core) | | | |
| Phenotype grouping | 5' and within core | 3' of core | *P*-value[a] |
| Test 3a | | | |
| CS | 16 | 14 | |
| BRR/CS | 3 | 7 | |
| BRR (familial only) | 2 | 7 (*n* = 49) | 0.182 |
| BRR (familial + sporadic) | 7 | 8 (*n* = 55) | 0.477 |
| Test 3b | | | |
| CS + CS/BRR | 19 | 21 | |
| BRR (familial only) | 2 | 7 (*n* = 49) | 0.267 |
| BRR (familial + sporadic) | 7 | 8 (*n* = 55) | 1.000 |
| Test 3c | | | |
| CS | 16 | 14 | |
| BRR/CS + BRR (familial only) | 5 | 14 (*n* = 49) | 0.081 |
| BRR/CS + BRR (familial + sporadic) | 10 | 15 (*n* = 55) | 0.419 |
| Test 4. Mutations within the motif | | | |
| Phenotype grouping | Point nonsense | Point missense | *P*-value[a] |
| CS | CS | 2 | |
| All BRR | 0 | 3 (*n* = 10) | 0.167 |

[a]Determined by Fisher's exact test (two-tailed).

gastrointestinal symptoms and mental retardation than those with intragenic mutation. Whether the severity of symptoms in *PTEN* deletion-related BRR is a result of PTEN haploinsufficiency or loss of sequence flanking *PTEN* is not known.

Of the 43 BRR cases studied, 16 were sporadic and 27 were familial. Germline *PTEN* mutations were identified in both groups. In contrast to an earlier report that was unable to detect *PTEN* mutations or deletions in sporadic cases of BRR (37), our data suggest that it is equally likely to be able to identify germline *PTEN* mutations in both sporadic and familial BRR. The small sample size, $n = 3$, of this earlier report may explain this apparent discrepancy. Thus, our results would suggest that the genetic aetiologies of sporadic and familial BRR are similar.

Genotype–phenotype correlation analyses revealed three putative associations. First, in the BRR cohort as a whole, the presence of a germline *PTEN* mutation was correlated with the presence of lipomatosis ($P = 0.028$). Lipomas occur only rarely in CS, unlike in BRR where they are common, thus the association of lipoma and *PTEN* mutation in CS could not be assessed (16). It is possible that BRR may also be caused by additional, as yet unidentified, genes. Our data suggest that, in fact, this may be true in 40% of cases. Unfortunately, the *PTEN* mutation-negative BRR families in the current study are too small effectively to exclude linkage to 10q23 and thus provide further evidence of genetic heterogeneity in BRR. However, it is possible that if BRR in some cases does develop along alternative pathways to those influenced by a *PTEN* mutation, then these pathways do not favour the growth of lipomas.

Secondly, the presence of germline *PTEN* mutation in the BRR cohort, including BRR/CS overlap families, showed an association with any cancer or breast fibroadenoma (i.e. a CS phenotype) ($P = 0.014$). Thirdly, an association was observed in the entire BRR cohort between the presence of truncating *PTEN* mutations and cancer or breast fibroadenoma ($P = 0.024$). Perhaps, a parallel correlation observed in CS alone is the presence of *PTEN* mutation and the development of breast carcinoma in a given CS family (16). Continuing this parallel, it may be possible to conclude that the presence of a *PTEN* mutation, whether it be in CS, BRR or BRR/CS families, predisposes to the presence of tumours. However, it would appear that mutation-positive status in BRR predisposes to benign tumours, i.e. lipomas, compared with malignant tumours, i.e. breast or uterine carcinoma, in CS or BRR/CS overlap families. Nonetheless, the associative trend of mutation positivity and tumour in BRR should alert the clinician that cancer might be a formal component of BRR, especially in mutation-positive cases, and specifically in BRR/CS overlap families. As the presence of neoplasms in CS does not appear to occur much earlier than in non-CS cancer-affected individuals, it is possible that a similar phenomenon occurs with BRR. Thus, perhaps longer follow-up periods are required for the diagnosis of neoplasia to be made in this group.

The mutation-positive–any tumour association, together with our observation of 11 BRR/CS overlap families, 10 of which are *PTEN* mutation positive, the similar but not identical mutational spectra of CS and BRR and the four mutations common to both syndromes lead us to suggest that CS and BRR constitute a single syndrome with broad clinical expression. There is little doubt that, at least in the instances of the four identical mutations, Q110X, R130X, R233X and R335X, found in CS-only, BRR-only and BRR/CS overlap families, other genetic and/or epigenetic factors, such as modifier loci, are involved. In the case of BRR, these unknown factors would seem to favour the development of benign tumours; whereas in the case of CS, malignancy, especially breast carcinoma, is favoured. However, for some of the other mutations that are, to date, unique to each group, it is plausible that differences might occur by differentially triggering various signalling pathways. Evidence supporting this theory already exists. While some mutants have been shown to have loss of phosphatase activity against *in vitro* protein substrates (18) and Ptd-Ins(3,4,5)$P_3$, an endogenous substrate, the CS mutation G129E had normal phosphatase activity against non-phospholipid substrates both *in vitro* (23) and in cell lines (38) but had no phosphatase activity against Ptd-Ins(3,4,5)$P_3$. Thus, it would seem likely that PTEN will have multiple substrates including both phospholipids and proteins. It is possible that CS- and BRR-specific mutations may differentially affect PTEN activity towards phospholipid versus proteic substrates and that this may occur in a cell- or tissue-specific manner.

Finally, the overlap of a number of clinical features, the sharing of identical *PTEN* mutations, in addition to the presence of BRR/CS overlap families, are all highly suggestive that BRR and CS are different presentations of a single syndrome and suggest that anticipation may pertain in this syndrome as well. The clinical implications of such a conclusion would be that known affected BRR and CS patients, as well as their at-risk relatives, should receive equal attention with respect to cancer surveillance. This is especially true in the case of *PTEN* mutation-positive BRR and CS. Thus, we believe that *PTEN* mutation-positive CS and BRR can now be grouped together for clinical purposes and classified as the '*PTEN* hamartoma-tumour syndrome' (PHTS).

## MATERIALS AND METHODS

### Patients

Forty-three unrelated BRR patients and, where possible, their affected and unaffected family members, were identified and blood samples collected for *PTEN* mutation screening. The diagnosis of BRR was made with the minimum standard criteria that included at least three of the following four features: macrocephaly, lipomatosis, haemangiomas and pigmented macules of the glans penis (speckled penis) in males. With the exception of one BRR/CS overlap family, B/CSBORD, all BRR cases in this study had macrocephaly. In seven cases, BSN, B85, B172, B173, B1016, B383 and B322, *PTEN* mutation screening was performed on fibroblast lines generated from skin punch biopsies from BRR patients. Of the 43 BRR cases collected, 16 represented sporadic cases of BRR based on clinical history and 27 were familial. Of the 27 familial cases of BRR, 11 were classified as BRR/CS overlap families. Each of these BRR/CS families comprised a minimum of one individual with BRR and one other related member with at least one major feature of CS (4), including breast fibroadenoma, trichilemmomas, papillomatous papules, uterine cancer or breast carcinoma. The comparison cohort of CS-only families had been collected and analysed previously for germline *PTEN* mutation (16). The results of *PTEN* mutation screening in this CS cohort were used for comparison with those of the BRR group in an attempt to identify mutations specific for either CS or BRR.

### Cultured skin fibroblasts

Cultured skin fibroblasts were available from seven BRR patients. B172, B173 and B1016 are affected individuals from the same family, as are B322 and B383. All cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 1× minimum essential medium sodium pyruvate solution, penicillin/streptomycin, 10 mM HEPES buffer solution and 4.4 mM L-glutamine (all from Gibco-BRL Life Technologies, Gaithersburg, MD).

### DNA preparation

Constitutional DNA was extracted from blood leucocytes using standard techniques (39). DNA was extracted from fibroblast cell lines using the QIAam Tissue kit (Qiagen, Chatsworth, CA)

### *PTEN* mutation scanning

A combination of DGGE and temporal temperature gel electrophoresis was used to screen the nine *PTEN* exons and flanking intronic sequences in germline genomic DNA extracted from blood leucocytes or cultured skin fibroblasts. Primer sequences, PCR and gel conditions have been described previously (40–42).

### Intragenic and flanking microsatellite marker analyses

To exclude the possibility of gross deletion in *PTEN* mutation-negative BRR and BRR/CS patients, eight polymorphic markers spanning a physical distance of ~9 cM flanking and within *PTEN* were analysed for hemizygosity. The markers are ordered from centromeric to telomeric and reflect the integrated genetic and physical map of chromosome arm 10q (http://www-genome.wi.mit.edu ): *D10S219–D10S579–D10S2491*–AFMa086wg9–IVS4+109insTCTTA–*D10S2492*/IVS8+32T/G–*D10S541*. These markers were also used for analysis of a BRR patient and his parents, the proband known to have the karyotype 46XY, del(10)(q23.2–q24.1). The five markers *D10S2491*–IVS8+32T/G lie within *PTEN* itself. The IVS8+32G/T polymorphism was screened for by digestion with *Hinc*II under the manufacturer's guidelines (New England Biolabs, Beverly, MA) as described previously (16,28). The IVS4+109insTCTTA polymorphism was detected by sequence analysis. PCR conditions for the microsatellite markers have been described previously (31,40).

### Genotype–phenotype analyses

Genotype–phenotype analyses were carried out on a family-as-a-unit basis (16,43,44). Associations were tested for both within the BRR and BRR/CS overlap group as well as between the BRR-only, BRR/CS overlap group and the CS-only group previously analysed (16). Initially, associations were sought between the presence or absence of *PTEN* mutation and the phenotypic classification of BRR alone, BRR/CS overlap or CS alone. Possible differences between the presence or absence of *PTEN* mutation in sporadic and familial BRR and BRR/CS overlap were also assessed. Mutation type (truncating, non-truncating, gross deletion or translocation) and mutation location (5' of ,and within, the PTPase core motif versus 3' of the PTPase core motif) was also considered, both within the BRR and BRR/CS overlap group and between this group and the CS cohort.

The genotypic information generated by DGGE screening was then cross-tabulated with known phenotype. Firstly, correlations were sought within the entire BRR and BRR/CS overlap group between the presence of a *PTEN* mutation and that of any cancer, skin manifestations or breast fibroadenomas that would be diagnostic of CS, lipomas, intestinal polyps, pigmented macules of the glans penis or café-au-lait spots, thyroid involvement (Hashimoto's thyroiditis, multinodular goitre, follicular thyroid carcinoma or hypothyroidism), or haemangiomas. Secondly, the mutation type (truncating or non-truncating) was cross-tabulated with the BRR phenotypes listed. Thirdly, the mutation position (5' of, or within, the PTPase core motif or 3' of the core motif) was assessed in relation to BRR phenotype.

Comparisons were then made between the CS-only, BRR/CS and BRR-only groups in an attempt to identify trends that may be associated specifically with one or other of these groups. The correlations sought when comparing these three groups included presence or absence of *PTEN* mutation, the mutation type (truncating or non-truncating) and the mutation position (5' of, and within, the PTPase core motif versus 3' of the PTPase core motif). Based on observed differences of the mutation type in the PTPase core motif between the BRR-only and BRR/CS overlap groups and CS-only (Fig. 1), a statistically significant correlation was sought.

For all analyses, the statistical significance of differences between genotype groups was calculated using the two-tailed Fisher's exact test (45,46).

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