Germline Mutations in *BMPR1A/ALK3* Cause a Subset of Cases of Juvenile Polyposis Syndrome and of Cowden and Bannayan-Riley-Ruvalcaba Syndromes*

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Juvenile polyposis syndrome (JPS) is an inherited hamartomatous-polyposis syndrome with a risk for colon cancer. JPS is a clinical diagnosis by exclusion, and, before susceptibility genes were identified, JPS could easily be confused with other inherited hamartoma syndromes, such as Bannayan-Riley-Ruvalcaba syndrome (BRRS) and Cowden syndrome (CS). Germline mutations of *MADH4* (*SMAD4*) have been described in a variable number of probands with JPS. A series of familial and isolated European probands without *MADH4* mutations were analyzed for germline mutations in *BMPR1A*, a member of the transforming growth-factor β–receptor superfamily, upstream from the SMAD pathway. Overall, 10 (38%) probands were found to have germline *BMPR1A* mutations, 8 of which resulted in truncated receptors and 2 of which resulted in missense alterations (C124R and C376Y). Almost all available component tumors from mutation-positive cases showed loss of heterozygosity (LOH) in the *BMPR1A* region, whereas those from mutation-negative cases did not. One proband with CS/CS-like phenotype was also found to have a germline *BMPR1A* missense mutation (A338D). Thus, germline *BMPR1A* mutations cause a significant proportion of cases of JPS and might define a small subset of cases of CS/BRRS with specific colonic phenotype.

Introduction

The major hamartomatous-polyposis syndromes comprise juvenile polyposis syndrome (JPS [MIM 174900]), Peutz-Jeghers syndrome (PJS [MIM 175200]), Cowden syndrome (CS [MIM 158350]), and Bannayan-Riley-Ruvalcaba syndrome (BRRS [MIM 153480]). Whereas CS and a subset of BRRS are allelic (Marsh et al. 1997*a*, 1999), current evidence suggests that CS and BRRS are genetically distinct from JPS and PJS (for reviews, see Eng and Ji 1998; Eng and Parsons 2001). PJS is an autosomal dominant disorder characterized by perioral pigmented spots, hamartomatous polyposis, and a risk for colon and breast cancers (Boardman et al. 1998; for review, see Eng et al. 2001). Germline mutations of the nuclear serine-threonine–kinase gene *LKB1/STK11* cause most cases of PJS (Hemminki et al. 1998; Jenne et al. 1998). CS is a poorly recognized autosomal dominant cancer syndrome characterized by multiple ha-

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Table 1

Clinical Features Present in Probands and Families with JPS

	Cancer		Anomalies		
Proband or Family	Colon	Other	Cardiac	Head/Facial	Other
BMPR1A-mutation positive:					
16[a]	Yes	No	No	No	No
18[a]	Yes	Esophageal	No	No	No
10[a]	Yes	No	No	No	No
19[a]	Yes	Unknown	No	No	No
FT[a]	Yes	No	No	Hypertelorism, macrocephaly	No
SM316	No	No	No	Hypertelorism, macrocephaly	No
VR	Unknown	No	No	No	Porphyria, malrotation of gut
1469	Unknown	Unknown	Unknown	No	No
JP7/19[a]	Yes	No	Ventricular septal defect, unspecified defects	No	No
JP8/1	Yes	Adrenal hamartoma, Wilms tumor	Ebstein anomaly	No	No
BMPR1A-mutation negative:					
MD[a]	Yes	No	No	Hypertelorism, macrocephaly	Telangiectasia
5[a]	Yes	Stomach	No	No	No
6[a]	Yes	No	No	Macrocephaly	No
1[a]	Yes	Small bowel	No	No	No
14[a]	Yes	No	No	No	No
22[a]	No	No	No	Macrocephaly? (diameter 56 cm [female])	Pigment naevi on trunk
CV	Unknown	No	Unknown	No	No
KS[a]	Unknown	No	No	No	No
YC[a]	Yes	No	No	No	No
SM106	No	No	Ventricular septal defect	Hypertelorism	No
12[a]	Yes	Small bowel	Aortic regurgitation	Subarachnoid hemorrhage	No
DM[a]	Yes	Stomach	Unknown	No	No
WN[a]	Yes	No	Unknown	Unknown	Unknown
JP1/1	Yes	Melanoma	No	No	Osler disease, epilepsy, empty sellar syndrome
JP2/13[a]	Yes	No	Unknown	Unknown	Unknown

Note.—By definition, all probands or families have at least one member with juvenile polyps.

[a] Familial.

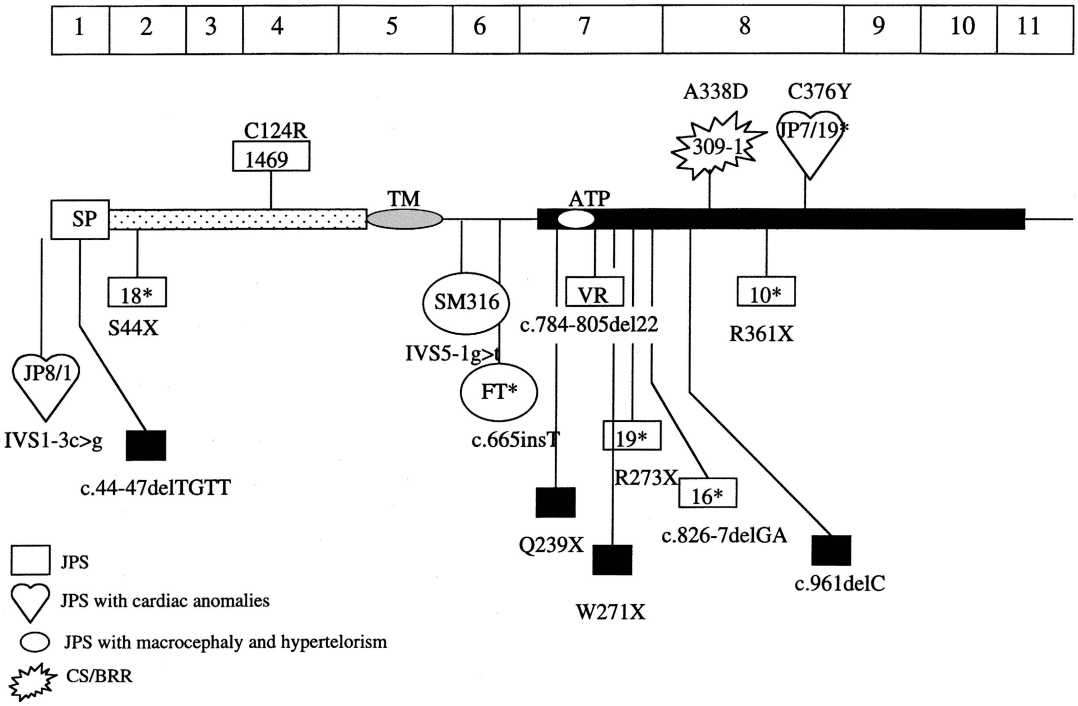

Figure 1 Spectrum of germline *BMPR1A* mutations in 14 probands with JPS and in 1 family with CS/BRRS. The exons are depicted by the numbered boxes at the top, and the domains of the receptor are depicted below. Signal peptide (SP), transmembrane domain (TM), ATP-binding domain (ATP), extracellular domain (*dotted bar*), and kinase domain (*black bar*) are shown. Black squares represent the four families' mutations published by Howe et al. (2001).

martomas and by a high risk for breast, thyroid, and endometrial cancers (Eng 2000). Although gastrointestinal hamartomatous polyposis can be documented if systematically searched for (Weber et al. 1998), the polyps are rarely symptomatic in CS, in contrast to the other three syndromes. BRRS is a congenital disorder characterized by macrocephaly, lipomatosis, thyroid problems, and pigmented macules on the glans penis in males (Gorlin et al. 1992); in BRRS, gastrointestinal hamartomatous polyposis can be quite prominent and symptomatic (Tsuchiya et al. 1998). Germline mutations in the tumor-suppressor gene *PTEN* cause 80% of cases of classic CS and 60% of cases of BRRS (Marsh et al. 1998*b*, 1999). There is little, if any, linkage evidence of genetic heterogeneity in CS (Nelen et al. 1996). The extent of genetic heterogeneity in BRRS is unknown. Clinical diagnosis of JPS is by exclusion, and JPS is characterized by gastrointestinal hamartomatous polyposis and by a risk for gastrointestinal cancers (for review, see Eng et al. 2001). Germline mutations in *MADH4* (*SMAD4*) have been described in a proportion of cases of JPS (Howe et al. 1998). From a nonsystematic survey of North American probands with JPS, it was estimated that ~35–60% of cases of JPS would harbor germline *MADH4* mutations (Howe et al. 1998); however, 3%–28% (weighted average 15%) of cases of JPS originating mainly from Europe have been found to carry *MADH4* mutations (Houlston et al. 1998; Friedl et al. 1999; Roth et al. 1999; Woodford-Richens et al. 2000*a*, and in press). Thus far, genes encoding several other SMADs have not been found to be associated with JPS (Bevan et al. 1999; Roth et al. 1999). Recently, germline truncating mutations in *BMPR1A/ALK3/SKR5* were described in four of four families segregating JPS (Howe et al. 2001). *BMPR1A*, on 10q21-q22, encodes a bone morphogenic-protein–receptor serine-threonine kinase that belongs to the transforming growth-factor β (TGFB)–receptor SMAD superfamily (for reviews, see Massagué 2000; Eng 2001). Members of the TGFB-receptor superfamily can homo-oligomerize or hetero-oligomerize.

We have examined *BMPR1A* for germline mutations, in a cohort of familial and sporadic cases of JPS, with the hypotheses that this mainly European cohort with a relatively low *MADH4*-mutation frequency would have a high frequency of *BMPR1A* mutations with a distinct mutational spectrum. Furthermore, because of the location of this gene in proximity to *PTEN* (Dahia et al. 2000) and, perhaps, because of its function, it also became a good candidate gene for susceptibility in *PTEN*-mutation–negative cases of CS and of BRRS.

Families, Material, and Methods

Families

Eighteen unrelated families with JPS and seven isolated cases of JPS were ascertained by clinical criteria described elsewhere (Marsh et al. 1997*b*) and were already known not to carry germline *MADH4* mutations. Although all families and individuals met the diagnostic criteria for JPS, some affected individuals had developed other tumors (table 1)—predominantly, colorectal adenomas and/or cancer—as is common in this condition (Woodford-Richens et al. 2000*a*). Twenty-one probands with CS/BRRS or CS/BRRS-like phenotype without germline *PTEN* mutations were ascertained by the revised operational diagnostic criteria of the International Cowden Consortium (Eng 2000) and by criteria described elsewhere (Marsh et al. 1998*a,* 1999). Probands and families with CS/BRRS-like phenotype have component features of CS/BRRS but do not meet the operational diagnostic criteria set forth by the International Cowden Consortium. All specimens were collected and analyzed, after informed consent was obtained, under protocols approved by each institution's Human Subjects Protection Committees.

Mutation Analysis

Genomic DNA was extracted from peripheral leukocytes, by standard protocols (Mathew et al. 1987). As template, 20–100 ng of DNA was used for 35 cycles (94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, after a hot start) of PCR amplification of each of the 11 coding exons (thus, ALK3E3 corresponds to exon 1, etc.) and flanking intronic regions of *BMPR1A,* by use of the following primers: ALKE3F (5′-TCCAAAATTCAGTTGTATTCC-3′), ALKE3R (5′-CACATACATTACTAAAATGAACACTG-3′), ALKE4F (5′-GTCACGAAACAATGAGCTTT-3′), ALKE4R (5′-TTAAGAAGGGCTGCATAAAA-3′), ALKE5F (5′-CATTCAGACTCAAATTTCGTT-3′), ALKE5R (5′-TCTCATGGGTCCCAAATTA-3′), ALKE6F (5′-CCAAACCATTTCTAATTTTATCA-3′), ALKE6R (5′-CATGCTCCGACTTTTCTC-3′), ALKE7F (5′-CCAGGCTACCTAGAATTGAA-3′), ALKE7R (5′-AACAGCGGTTGACATCTAAT-3′), ALKE8F (5′-CCTCAAGGTTTTTCTTAGGG-3′), ALKE8R (5′-TCAACACACCATTCATGTCT-3′), ALKE9F (5′-TCATCAAGAGCTCAAACCTT-3′), ALKE9R (5′-ACCTCACTAGCCTTGTCAAA-3′), ALKE10F (5′-CCCTAGCCTATCTCTGATGA-3′), ALKE10R (5′-AACAGTGGGGCAAAGAAC-3′), ALKE11F (5′-TATTTTATTTTTGGCCCTCA-3′), ALKE11R (5′-TGATGAGTAAATCAACATAATCAG-3′), ALKE12F (5′-ATTTTTGTGCCCATGTTTT-3′), ALKE12R (5′-AATCACTTCTTCAGGGGACT-3′), ALKE13F (5′-ACTCAGTCCCCTGAAGAAGT-3′), and ALKE13R (5′-CTAGAGTTTCTCCTCCGATG-3′). The amplicons were gel- and column-purified and then were subjected to semiautomated PCR-based sequence analysis by an ABI-377a or a Perkin-Elmer 3700, as described elsewhere (Mutter et al. 2000).

Loss of Heterozygosity (LOH) Analysis

Available component tumors from *BMPR1A*-mutation–positive and *BMPR1A*-mutation–negative cases of JPS were subjected to LOH analysis with markers ALK3ca, ALK3ggaa, and D10S573, by techniques described elsewhere (Marsh et al. 1998*c;* Woodford-Richens et al. 2000*b*). Two component tumors from proband JP8/1 (table 1) was analyzed by sequencing of the amplicon containing the germline mutation, to examine allelic contribution.

Reverse-Transcriptase PCR (RT-PCR) Analysis

To assess the putative splice-site mutation in proband JP8/1, RNA was extracted from her component tumors, a Wilms tumor (table 1), and a colon carcinoma, and cDNA was synthesized. RT-PCR was performed using the primers 5′-GCATAGGTCAAAGCTGTTTGG-3′ and 5′-GCAAGGTATCCTCTGGTGCT-3′, with Ampli*Taq* Gold (Perkin-Elmer) at and annealing temperature of 60°C. Amplicons were fractionated through 2% agarose, were stained with ethidium bromide, and then were visualized with UV trans-illumination. Any aberrant bands noted on the gel were cut out of low-melting-point agarose, were gel- and column-purified, and then were subjected to sequence analysis.

Results

All 11 coding exons, splice junctions, and flanking intronic regions of *BMPR1A* were examined in 18 unrelated *MADH4*-mutation–negative families with JPS and in seven unrelated *MADH4*-mutation–negative individuals with isolated JPS. All available polyps from these cases showed no loss of SMAD4 expression. Overall, of 25 unrelated probands with JPS, 10 (40%) were found to have germline *BMPR1A* mutations (fig. 1): 6 (33%) of the 18 familial cases and 4 (57%) of the 7 isolated cases had germline mutations. In the mutation-positive familial cases in which samples from family members were available, the respective mutations were shown to segregate with affected status (data not shown)

Of the 10 germline *BMPR1A* mutations found in probands with JPS, all except 2 were nonsense, frameshift, or splice-site mutations predicted to result in truncated receptors (fig. 1). The missense mutations found in cases of JPS were examined in cohorts of 50 race-matched normal controls. None of the 100 normal control chromosomes were found to carry these missense mutations; furthermore, in the familial cases of JPS with C376Y, this mutation was found to segregate with dis-

ease. Loss of the wild-type allele in three component tumors—all of which were villous adenomas and two of which also had adenocarcinomatous components—from an affected family member was also demonstrated (fig. 2). The splice-site mutation IVS1-3c→g was shown to result in skipping of exon 1, and the component tumor (a Wilms tumor; table 1) from the proband had loss of the wild-type allele. A colorectal carcinoma from the proband with the IVS1 splice mutation did not show LOH. Thus, of five component tumors from *BMPR1A*-mutation–positive individuals, four were found to have loss of the wild-type allele. In contrast, 24 component tumors from 13 familial and isolated cases without germline *BMPR1A* mutations showed no LOH in that region (data not shown).

Although limited because of small sample size, genotype-phenotype associations were examined, especially those with respect to cardiac anomalies or to head/facial features (table 1 and fig. 1). Both among the 10 *BMPR1A*-mutation–positive families and among the 15 *BMPR1A*-mutation–negative families, 2 had cardiac anomalies; because of the limited size of sample, these were not considered statistically different. Similarly, there appeared to be no difference between the numbers of mutation-positive and mutation-negative families and individuals with macrocephaly or hypertelorism. Although there were only two probands/families with mutations as well as with the clinical features of hypertelorism and macrocephaly, both of these mutations—IVS5-1g→t (SM316) and c.665insT (FT)—occurred in the juxtatransmembrane domain (fig. 1).

Of 21 unrelated probands with CS/BRRS, without germline *PTEN* mutations, 1 was found to have a germline missense mutation, A338D, in exon 8 of *BMPR1A*. This missense alteration was not observed among 172 race-matched, geographically matched control chromosomes. Interestingly, the proband had only colonic polyposis, which comprised hamartomatous and adenomatous polyps and began at the age of 16 years, and lipomas. Her family history, however, comprised individuals with breast cancer, with renal-cell carcinoma, with brain tumor(s), and with melanoma. Taken together, these features constitute the minimum criteria (i.e., one major and three minor) for the diagnosis of CS (Eng 2000). It is acknowledged that the diagnosis of CS in this family barely met the minimum International Cowden Consortium diagnostic criteria, and some clinicians might consider this family to have a CS-like phenotype. None of the other probands with CS/BRRS or CS/BRRS-like phenotype were found to have *BMPR1A* mutations.

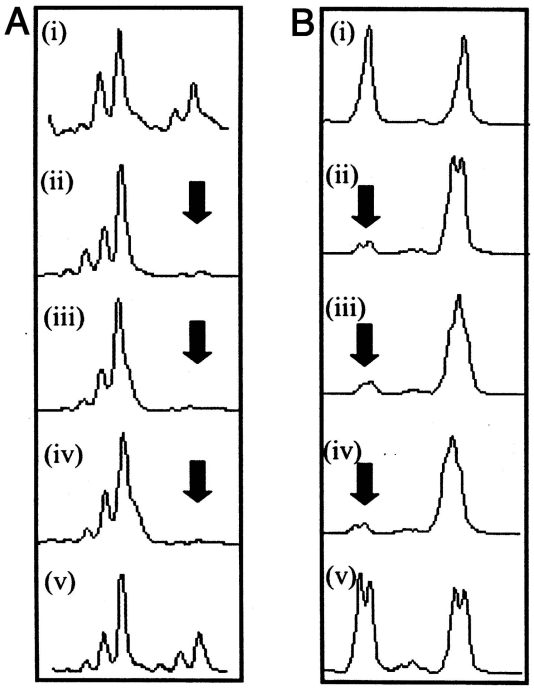

Figure 2 LOH analysis with microsatellite markers alk3ca (*A*) and alk3ggaa (*B*), which lie in proximity to *BMPR1A* (see text), and genomic DNA templates from family JP7/19, whose members harbor a germline missense mutation, generated from peripheral blood leukocytes (*i*), from villous adenoma (*ii*), from two villous adenomas with adenocarcinomatous components (*iii* and *iv*), and from normal tissue originating from the same archival section as one of the villous adenomas with adenocarcinoma (*v*).

Discussion

In this cohort of familial and isolated cases of JPS who are *MADH4*-mutation–negative and who originate mainly from Europe, 40% have been found to harbor germline *BMPR1A/ALK3* mutations. Thus, among European cases of JPS, *MADH4* mutations account for ≤28% of cases and *BMPR1A* mutations account for 40%. No systematic survey of cases of JPS originating in the United States has been performed yet, and it thus is unknown what proportion cases of JPS is due to *BMPR1A* mutations. Nonetheless, at least one other JPS-susceptibility gene should exist.

Overall, to date, 14 different germline *BMPR1A* mutations have been described in probands with JPS—10 in patients from this study and the 4 in U.S. kindreds described elsewhere (Howe et al. 2001). Of these germline *BMPR1A* mutations, 9 (64%) are located within exons 6–8 (exon 1 is the first coding exon; there are two other noncoding exons 5′ of exon 1), encoding part of the intracellular domain of the receptor (fig. 1), and, of these 9 mutations, 8 have occurred in the N-terminal 142 amino acids of the kinase domain, half of which are in close proximity to the ATP-binding site. There are no mutations located in or beyond the C-terminal half of the kinase domain. Interestingly, the two pro-

bands with mutations occurring in exons 5 and 6 both have macrocephaly and hypertelorism.

All but one of the nine mutations in the cytoplasmic domain are predicted to result in truncated receptors (fig. 1). The truncations all leave an intact transmembrane domain, such that the mutant receptors could be processed, to reach the plasma membrane, but are lacking all or part of the kinase domain. If the mutations in the cytoplasmic domain do result in truncated receptors, then these truncated receptors might be expected to bind ligand, but no signaling could occur. Thus, these intracellular-domain mutations might be predicted to act via dominant negative mechanisms. Family JP7/19 has a missense mutation in the middle of the kinase domain, C376Y. Residue 376 lies within the kinase domain, in close proximity to the active site, and is highly conserved among species—from *Caenorhabditis elegans* to mouse and rat. Four of the five mutations in the extracellular domain are predicted to result in truncated receptors. However, unlike the truncations in the cytoplasmic domain, two of the truncations would result in the lack of all or part of the signal peptide. The third truncation, S44X, results in a very short peptide without a transmembrane domain. Cysteine 124 lies in the cysteine-rich domain, which characterizes receptor kinases and is highly conserved across the TGFB family of type I and type II receptors, as well as across species (Kirsch et al. 2000). The ectodomain of BMPR1A has six intramolecular disulfide bridges between pairs of cysteines, which conformationally allows for BMP2 binding (Kirsch et al. 2000). Cysteine 124 is part of disulfide bond 4, and between the two cysteines forming this disulfide bridge lie nine key residues, which form part of the ligand-binding epitope. Loss of the sulfhydryl group at residue 124, as would be the case for this mutation, would therefore result in severe conformational alterations and in loss of the ability to bind ligand. The splice mutation IVS5-1g→t would be predicted to result in a receptor without a transmembrane domain. Thus, in general, extracellular-domain germline mutations—whether truncating or missense—together with the somatic second hit—as evidenced by LOH in the *BMPR1A* region in the majority of component tumors, both benign and malignant—might result in physical or functional lack of receptor. These observations contrast with those of Howe et al. (2001), who failed to detect LOH in component tumors from mutation-positive families. Our data demonstrating that *BMPR1A* behaves in accordance with the Knudson two-hit theory strongly suggest that *BMPR1A* encodes a tumor suppressor and likely also plays a gatekeeping function (Kinzler and Vogelstein 1998), much like SMAD4 itself (Woodford-Richens et al. 2000*b*).

Although the sample size is small, it would appear that, among the nine cytoplasmic-domain mutations, seven have occurred in familial cases of JPS whereas only two have occurred in isolated cases of JPS. In contrast, of the four extracellular-domain mutations, two occur in familial cases and three occur in isolated cases. Because of the small sample sizes of each subset, no statistical significance can be inferred. However, an interesting hypothesis to test in the future is that *BMPR1A* mutations that occur in the cytoplasmic domain and that are predicted to be dominant negative are associated with higher penetrance and with familial transmission. Because we have demonstrated LOH in component tumors from mutation-positive individuals—and if this hypothesis is correct—then the dominant-negative effect must act against other TGFB-receptor–family partners with which BMPR1A normally hetero-oligomerizes. Extracellular mutations that mainly result in haploinsufficiency, on the other hand, are associated equally with isolated and familial cases.

Because CS and BRRS lie within a single spectrum (Marsh et al. 1999), we chose to examine probands with CS/BRRS and CS/BRRS-like phenotype as one group. Only one such proband with CS/CS-like phenotype was found to harbor a *BMPR1A* mutation—specifically, A338D. This missense mutation occurs in the kinase domain—more specifically, immediately downstream of the kinase catalytic core—and in a residue that is highly conserved across species, from *C. elegans* to mouse and rat. Thus, if an acidic hydrophilic residue (aspartate) were substituted for a neutral nonpolar residue (alanine), the kinase catalytic core would be predicted to be disrupted. Although ligand binding might still be possible, this mutation could be predicted to result either in a loss of substrate specificity or in a receptor that might not be able to bind substrate.

Despite some initial confusion that germline *PTEN* mutations might be associated with rare cases of JPS (Olschwang et al. 1998), over the course of the past 4 years of clinical and molecular-epidemiologic analyses, it has become obvious that the presence of germline *PTEN* mutations defines CS and BRRS, regardless of the manner of clinical presentation (Eng and Ji 1998; Kurose et al. 1999; Marsh et al. 1999). This is germane for clinical cancer genetic practice, because the presence of *PTEN* mutations implies organ-specific surveillance of the patient and of his or her family. On the other hand, detection of a *MADH4* or a *BMPR1A* mutation should be considered diagnostic of JPS. In our opinion, families with CS/BRRS or CS/BRRS-like phenotype with *BMPR1A* mutations must therefore, on the basis of molecular data, be reclassified as having JPS.

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Electronic-Database Information

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Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for JPS [MIM 174900], PJS [MIM 175200], CS [MIM 158350], and BRRS [MIM 153480])

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