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Report

Germline Inactivation of *PTEN* and Dysregulation of the Phosphoinositol-3-Kinase/Akt Pathway Cause Human Lhermitte-Duclos Disease in Adults

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Lhermitte-Duclos disease (LDD), or dysplastic gangliocytoma of the cerebellum, is an unusual hamartomatous overgrowth disorder. LDD can be familial or, more commonly, sporadic. It has been only recently recognized that LDD may be associated with Cowden syndrome (CS). Over 80% of patients with CS carry germline mutations in *PTEN*. It remains unclear whether all cases of LDD, even without features of CS, are caused by germline *PTEN* mutation and whether somatic *PTEN* mutation occurs in sporadic LDD. We obtained paraffin-embedded LDD lesions from 18 unselected, unrelated patients and performed mutational analysis of *PTEN*. Overall, 15 (83%) of 18 samples were found to carry a *PTEN* mutation. All individuals with mutations were adult-onset patients, but the three without mutations were diagnosed at the ages of 1, 3, and 11 years. Germline DNA was available from six adult-onset cases, and all had germline *PTEN* mutations. Of these six, two had CS features, one did not have CS features, and three were of unknown CS status. Immunohistochemistry revealed that 75% of the LDD samples had complete or partial loss of PTEN expression accompanied by elevated phosphorylated Akt, specifically in the dysplastic gangliocytoma cells. These data suggest that the loss of PTEN function is sufficient to cause LDD. The high frequency and spectrum of germline *PTEN* mutations in patients ascertaining by LDD alone confirm that LDD is an important defining feature of CS. Individuals with LDD, even without apparent CS features, should be counseled as in CS.

PTEN/MMAC1/TEP1 (MIM 601728), on 10q23.3, encodes a lipid and protein phosphatase (Li and Sun 1997; Li et al. 1997; Steck et al. 1997). PTEN signals down the phosphoinositol-3-kinase (PI3K)/Akt pathway (Maehama and Dixon 1998; Stambolic et al. 1998; Weng et al. 1999). Proper PTEN signaling, via both PI3K/Akt-dependent and -independent pathways, leads to G1 cell cycle arrest and/or apoptosis (Furnari et al. 1998; Li and Sun 1998; Stambolic et al. 1998; Weng et al. 1999, 2001*a*, 2001*b*, 2001*c*, 2001*d*).

Germline mutations in *PTEN* cause Cowden syndrome (CS [MIM 158350]), an autosomal-dominant condition with age-related penetrance characterized by multiple hamartomas affecting derivatives of all three germ layers and by a high risk of breast, thyroid, and endometrial cancers (Liaw et al. 1997; Marsh et al. 1998; Eng 2000). Germline *PTEN* mutations have been found in 81% of CS probands and in 60% of patients with Bannayan-Riley-Ruvalcaba syndrome (BRRS [MIM 153480]), a related but apparently distinct hamartoma syndrome characterized by neonatal onset, macrocephaly, hemangiomas, lipomatosis, and speckled penis (Liaw et al. 1997; Marsh

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et al. 1997, 1998, 1999). A subset of seemingly unrelated clinical conditions, such as Proteus syndrome (PS [MIM 176920]), Proteus-like syndromes, and VATER association with macrocephaly and ventriculomegaly, have been associated with germline *PTEN* mutations (Zhou et al. 2000*b*, 2001; Reardon et al. 2001; Smith et al. 2002).

Lhermitte-Duclos disease (LDD), or dysplastic gangliocytoma of the cerebellum, is an unusual condition usually occurring sporadically but also in a familial form (Albrecht et al. 1992). LDD is believed to be a hamartomatous overgrowth of hypertrophic ganglion cells that replace the granular-cell layer and Purkinje cells of the cerebellum, resulting in global thickening of the cerebellar folia (Ambler et al. 1969; Russell-Jones et al. 1981; Albrecht et al. 1992; Nowak and Trost 2002). Clinically, therefore, patients with LDD may present with ataxia, signs and symptoms of increased intracranial pressure, and seizures (Ambler et al. 1969; Russell-Jones et al. 1981; Albrecht et al. 1992; Nowak and Trost 2002). There are ~134 single cases or selected small series of patients with LDD that have been reported in the literature to date (reviewed by Vinchon et al. 1994; Liaw et al. 1997; Iida et al. 1998; Marsh et al. 1998; Koch et al. 1999; Nelen et al. 1999; Sutphen et al. 1999; Robinson and Cohen 2000). Of these, 67 cases had features suggestive or diagnostic of CS, although a definitive diagnosis of CS in the majority of these cases was not established. Prior to the identification of *PTEN* as the CS-susceptibility gene (Liaw et al. 1997), the co-occurrence of documented CS cases and families with LDD (Padberg et al. 1991; Albrecht et al. 1992; Eng et al. 1994) suggested, but did not show conclusively, that LDD and CS share a common etiology. Subsequently, germline *PTEN* mutations have been identified in three well-documented CS kindreds segregating LDD (Liaw et al. 1997; Nelen et al. 1997; Marsh et al. 1998), thus lending some molecular credence to the previous clinical hypothesis. Conditional *Pten* disruption in the mouse cerebellum resulted in a LDD-like phenotype, further suggesting that loss of function of PTEN might be sufficient to cause human LDD (Backman et al. 2001; Kwon et al. 2001). However, it is not known if all cases of human LDD, even without features of CS, are caused by germline *PTEN* mutation and if somatic *PTEN* mutation can account for sporadic LDD. To this end, we have accrued 18 unselected, unrelated patients with LDD, without regard to the presence or absence of other features and have performed an extensive analysis for the presence of *PTEN* mutations. We also examined the expression levels of PTEN and phosphorylated Akt (P-Akt) in archived affected cerebellar tissues using immunohistochemistry.

Archived paraffin-embedded tissue from 18 unselected, anonymized patients with LDD (with linked demographic, clinical, and pathologic information) was accrued from multiple centers in the United States and Europe, in accordance with protocols approved by the Dana-Farber Cancer Institute (1996–1998) and The Ohio State University (1998–present) institutional review boards. For all patients, the diagnosis of LDD was confirmed by pathology after surgical resection or biopsy. All histopathologic sections were subjected to centralized re-review by C.D.M. and A.R.C. Available clinical information from all 18 patients was reviewed to determine if any of them met the operational diagnostic criteria for CS, as delineated by the International Cowden Consortium or the U.S. National Comprehensive Cancer Network (Eng 2000) (table 1). Of the 18 patients reviewed, 4 individuals (LDD-7, LDD-8a, LDD-8b, and LDD-14), all female, could be clinically classified as having CS. In 3 patients (LDD-1, LDD-5, and LDD-17), the diagnostic features of CS were absent. Insufficient clinical information was available to conclusively determine CS status in the remaining 11 patients.

DNA was extracted from archival paraffin-embedded cerebellar tissue sections with lesions using a QIAamp DNA Mini Kit (Qiagen). The procedure was performed according to the manufacturer's instructions, except that a prolonged (2-d) proteinase-K digestion at 65°C was added. The samples were then subjected to *PTEN* mutation analysis, which was performed without knowledge of other clinical information. The entire coding sequence, the exon-intron boundaries, and the flanking sequences of *PTEN* were analyzed for mutations using PCR-based denaturing gradient gel electrophoresis (DGGE) and sequencing, as described elsewhere (Marsh et al. 1998; Mutter et al. 2000). A mutation-positive result led us to obtain germline DNA from paraffin-embedded adjacent normal tissue, if present, using laser capture microdissection (LCM) (Kurose et al. 2002); LCM-derived germline DNA was then subjected to *PTEN* mutation analysis. A mutation-negative result in tissue denotes the absence of *PTEN* mutation in both the germline and in the tissue. Samples that were mutation negative in the coding and flanking intronic regions were subjected to *PTEN* promoter mutation analysis, as described elsewhere (Zhou et al. 2003).

PCR-based DGGE and subsequent sequencing revealed 16 intragenic *PTEN* mutations in 15 (83%) of the 18 samples (table 1). By using LCM, we were able to microdissect affected and adjacent nonaffected tissues in four samples (LDD-G5, LDD-5, LDD-7, and LDD-19). Nonaffected tissue sections were available for another two patients (LDD-9a and LDD-14) and served as sources of germline DNA. Thus, six had matched germline DNA, and all six were found to have germline mutations. In the sample from patient LDD-19, in addition to the germline mutation Y88H, which was detected in DNA from both nonaffected bone tissue and affected cerebellar tissue, a second mutation, H141R,

Table 1

Summary of Patients with LDD and *PTEN* and P-Akt Status by Immunohistochemistry

	Characteristics of Patients with LDD		Results of Analysis				
Patient	Sex/Age (years)	CS/Features of CS	*PTEN* Mutations	Germline/Somatic	LOH[a]/ROH[b]	PTEN Levels	P-Akt Levels
LDD-G1	F/53	?	16–18 del AA			++	++
LDD-G2	M/38	?	381–4 del AAAG			−	++
LDD-G3	F/1	?	−			++	−
LDD-G4	F/55	?	R130X			+	−
LDD-G5	M/27	?	99 del T	Germline	LOH	−	++
LDD-1	F/51	No	758 ins A			+	++
LDD-2	F/11	No	−				
LDD-5	F/35	No	P246L	Germline	ROH	+	++
LDD-7	F/50	Yes	53 del A	Germline	LOH	+	
LDD-8a	F/43	Yes	Y16X			+	++
LDD-8b	F/34	Yes	Q110X			+	++
LDD-8c	F/?	?	R130X			−, ++	++, −
LDD-9a	?	?	Y88H	Germline			
LDD-11	?	?	G36R			−	
LDD-12	?	?	K80E			++	
LDD-14	F/43	Likely	347–51del ACAAT	Germline		−	−
LDD-17	M/3	Unlikely	−				
LDD-19	M/28	?	Y88H	Germline	NI[c]	+	++
			H141R	Somatic			

Note.—Blank = not done because of lack of sample material.

[a] LOH = loss of heterozygosity.

[b] ROH = retention of heterozygosity.

[c] NI = not informative.

was only detected in DNA from the affected cerebellar tissue, suggesting that the latter is a somatic mutation. Of the 16 mutations found in 15 patients with LDD, 10 were truncating mutations, comprising 4 nonsense mutations and 6 frameshifting microdeletions or -insertions, and 6 were missense mutations. Mutation R130X was detected in two unrelated patients, LDD-G4 and LDD-8c. Similarly, mutation Y88H was detected in the germline of two unrelated patients, LDD-9a and LDD-19 (table 1). The recurrent R130X and Y88H as founder mutations were excluded by genotype analysis (data not shown). No sequence variants in the promoter region were found in the three LDD samples without intragenic *PTEN* mutations (LDD-G3, LDD-2, and LDD-17) (table 1).

Loss-of-heterozygosity (LOH) analysis was performed on four LDD samples using two microsatellite markers flanking *PTEN*, D10S1765 and D10S541, as previously described (Marsh et al. 1999). Of the four samples analyzed, three were informative at a minimum of one marker, and one was not informative for either marker. LOH was scored when at least one of the two polymorphic loci showed evidence of LOH. We found that two LDD samples had LOH at the *PTEN* locus in the affected cerebellar tissue, thus leaving the remaining germline mutant allele (LDD-G5 and LDD-7) (table 1). No LOH was detected in the third informative LDD sample (LDD-5).

We next examined the PTEN expression level and P-Akt level in these LDD samples by immunohistochemistry using antibodies against PTEN (6H2.1) and P-Akt (Ser 473) (Cell Signaling Technology), respectively. Immunohistochemical detection of PTEN and P-Akt was carried out following protocols described elsewhere (Perren et al. 1999; Zhou et al. 2000*a*). As noted in other studies (Perren et al. 1999; Gimm et al. 2000; Zhou et al. 2000*a*), the vascular endothelium serves as an internal positive control for PTEN. Levels of PTEN immunostaining in the vascular endothelium are remarkably constant among various tissues, including breast (Perren et al. 1999), thyroid (Gimm et al. 2000), pancreas (Perren et al. 2000), and colon (Zhou et al. 2002). PTEN immunostaining intensities equal to that of the vascular endothelium in a particular sample were scored as ++ in table 1, weak or decreased staining intensity as +, and no immunostaining as −. Overall, 11 (78%) of 14 of the patients with LDD showed negative (−) or weak (+) PTEN immunostaining in their dysplastic gangliocytoma cells in the cerebellum, and the remaining 3 had PTEN staining intensity in the dysplastic gangliocytoma cells equivalent to that in the neurons of the granular-cell, Purkinje-cell, and molecular layers of the adjacent

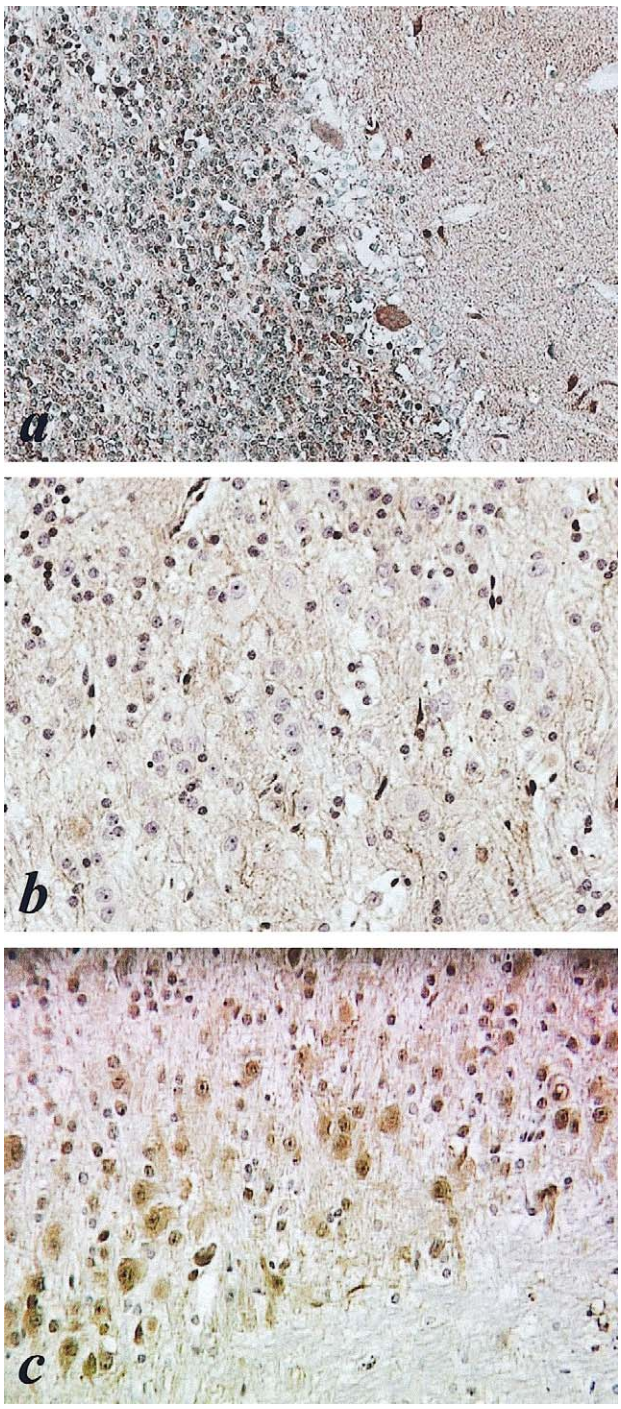

Figure 1 Loss of PTEN expression leads to elevated P-Akt levels detected by immunohistochemistry in LDD. *a*, Moderate to strong PTEN immunostaining intensity (*brown*) in the neurons of the granular-cell, Purkinje-cell, and molecular layers of adjacent normal cerebellar section. *b*, Loss of PTEN immunostaining in dysplastic ganglion cells of LDD. *c*, Markedly elevated P-Akt immunoexpression (*brown*) in the dysplastic neurons, predominantly in the cytoplasm. Sections were counterstained with methyl green (*light blue*) or hematoxylin (*blue*). Original magnification, 20 × .

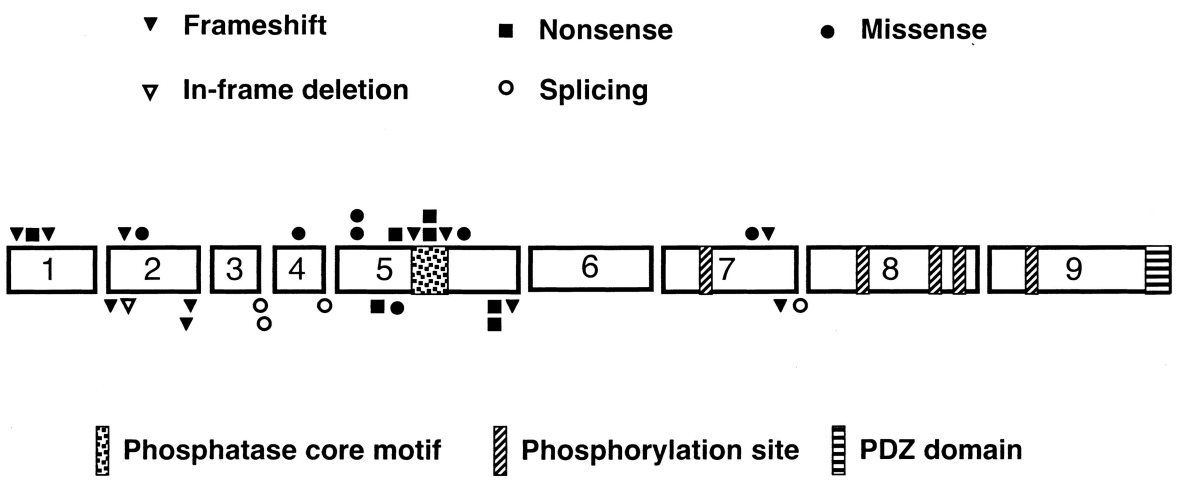

Figure 2 Spectrum of germline *PTEN* mutations detected in patients with LDD. A schematic of the nine-exon gene is displayed. Mutations ($n = 16$) denoted above the bar were detected in this study. Mutations ($n = 14$) underneath the bar were reported elsewhere.

cerebellum and to that in the vascular endothelium. About three-fourths (73%, 8/11) of the LDD samples exhibited strong immunoreactivity against P-Akt in the dysplastic gangliocytoma cells, and one-fourth showed weak or no P-Akt immunoreactivity. One sample, from patient LDD-8c, had heterogeneous staining intensities in different parts of the same tissue section. In general, the P-Akt immunoreactivity was inversely correlated with that of PTEN (table 1 and fig. 1).

In this unique series of 18 unrelated patients with LDD, we found the majority (83%, 15/18) of patients harbored a mutation in *PTEN,* irrespective of whether they had features of CS or not. In the six instances with corresponding germline and LDD tissue available, all six had an adult onset and all had germline *PTEN* mutations. It is interesting to note that the three youngest patients with LDD, diagnosed at ages 1, 3, and 11 years, did not display germline and somatic *PTEN* mutations. This observation is corroborated by a previous report that showed that features of CS did not develop in three children diagnosed with LDD, even after long-term follow-up (Capone Mori et al. 2003). Similar to our cases of childhood-onset LDD, none of these children were found to carry germline *PTEN* mutations either. Therefore, most—possibly all—cases of adult-onset LDD, irrespective of family history or other syndromic features, are likely due to germline *PTEN* mutations. We further demonstrated that these *PTEN* structural alterations result in loss of PTEN protein expression in the dysplastic gangliocytomas, accompanied by elevated levels of P-Akt, reflecting a pro-proliferative state.

The high frequency and spectrum of germline *PTEN* mutations ascertaining only by LDD confirm that LDD is an important defining feature of CS. Germline *PTEN* mutations are associated with >80% of classic cases of CS. The mutation frequency is even higher when mutations in the promoter region are taken into account (Zhou et al. 2003). An overview of all germline *PTEN* mutations occurring in CS, to date, suggests that two-thirds of the mutations occur in exons 1–5, whereas two-thirds of mutations in BRRS occur in exons encoding the C-terminal half (Eng 2003). As in CS, the mutation frequency in LDD is 83%. When this series of 15 mutation-positive individuals with LDD is combined with 14 previously reported mutation-positive individuals with LDD (with obvious CS features) (Iida et al. 1998; Marsh et al. 1998; Koch et al. 1999; Nelen et al. 1999; Sutphen et al. 1999; Robinson and Cohen 2000), there are a total of 29 such cases (fig. 2). Of the 30 mutations found in 29 patients with LDD, 26 are within the first five exons, reminiscent of the spectrum in CS (Eng 2003). Furthermore, of these 30 LDD-associated mutations, ~23% are missense, compared to ~24% of all CS-related mutations reported to date (Eng 2003) ($P > 1$, Fisher's Exact Test). Taken together, these observations suggest that LDD shares a common genetic etiology with CS.

Among the 14 different *PTEN* mutations in these 15 subjects with LDD, 3 mutations, Q110X, R130X, and P246L, have been previously described in many probands with CS or BRRS (Eng 2003). Apart from P246L, the other four missense mutations have not been described before. All of the affected amino acids are highly conserved among the vertebrates, and each amino acid change is significant. More importantly, the missense mutations P246L and Y88H have been shown to upregulate Akt (table 1), thus functionally proving pathogenicity. Although K80E results in fully expressed PTEN protein, we do not have information on its P-Akt status. A lysine-to–glutamic acid alteration in a highly conserved amino acid lying in the phosphatase domain,

moreover, would be predicted to be pathogenic. This alteration has never been described in >1,000 normal control subjects in the communal experience.

Although germline *PTEN* mutations account for the majority, perhaps even all, of cases of adult-onset LDD to date (Iida et al. 1998; Marsh et al. 1998; Koch et al. 1999; Nelen et al. 1999; Sutphen et al. 1999; Robinson and Cohen 2000; this report), it is unclear whether the loss of the second *PTEN* allele is necessary for the development of LDD. Only a single report pertinent to the human situation exists: a family with CS segregating a germline *PTEN* mutation also had one individual with LDD. The dysplastic gangliocytoma expressed the mutant allele, suggesting either genetic or epigenetic loss of the wild-type allele, at least in this case (Iida et al. 1998). We have shown that two of the three informative LDD samples with germline mutations had LOH at the *PTEN* locus, and a fourth LDD sample with a germline mutation had a somatic "second hit" mutation in the remaining allele. Furthermore, we have demonstrated that the majority of LDD samples had complete loss or decreased expression of PTEN accompanying elevated P-Akt levels by immunohistochemistry (table 1). These data would suggest that loss of the remaining wild-type *PTEN* allele, either by genetic or epigenetic means, together with the germline mutation, is necessary for the development of LDD. That the *PTEN* genomic alterations, reflected in lost or decreased PTEN protein expression, result in functional consequences is reflected by increased phosphorylation of Akt in all but two mutation-positive LDD samples. Thus, loss of PTEN and subsequent activation of PI3K/Akt pathway is likely the key molecular mechanism in the pathogenesis of LDD.

Two exceptions (LDD-G4 and LDD-14) (table 1) are worthy of some discussion. LDD-G4 harbors a R130X mutation, commonly seen in patients with CS and BRRS (reviewed in Eng 2003), and consequently has decreased PTEN expression in the LDD cells. Similarly, LDD-14, with a germline frameshift mutation in exon 5, has no PTEN expression, likely because of truncation of the product of the mutant allele and either genetic or epigenetic inactivation of the remaining wild-type allele. Yet, in both of these instances, phosphorylation of Akt is not increased. It is possible that, in these two samples, the nonlipid phosphatase, PI3K/Akt-independent pathway (Weng et al. 2001*b*, 2002) downstream of PTEN is dysfunctional, nonetheless resulting in LDD but via a different downstream mechanism.

To date, it would appear that germline *PTEN* mutations have been associated with the majority of CS cases, over half of BRRS cases, up to 20% of PS cases, and, as suggested by our data presented here, even in a subset of isolated LDD cases. Although each of the syndromes can be associated with distinct *PTEN* mutations, it is also clear that identical mutations (e.g., R130X) have been described in all these syndromes and yet result in different phenotypes (reviewed by Eng 2003). Whether the phenotypic differences are due to interaction with other modifying genes and/or the environment is currently unknown.

In summary, our data demonstrate a high PTEN-mutation frequency in adult-onset LDD. Virtually all mutations detected are germline mutations. The majority of these result in decreased or no PTEN protein expression and increased phosphorylation of Akt, which accounts for the pro-proliferative context leading to LDD. The three children with LDD in our study, together with three other children with LDD reported elsewhere (Capone Mori et al. 2003), all exhibited neither *PTEN* mutations nor CS features, despite long follow-up. This may preliminarily suggest that childhood LDD is a distinct entity from adult-onset LDD, a hypothesis that requires further investigation. Thus, consideration should be given to adding adult-onset LDD as a new pathognomonic feature of CS/PTEN hamartoma tumor syndrome (PHTS).

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Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for CS, BRRS, PS, and *PTEN/MMAC1/TEP1*)

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