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Gene-Specific Criteria for *PTEN* Variant Curation: Recommendations from the ClinGen PTEN Expert Panel

Jessica L. Mester[1], **Rajarshi Ghosh**[2], **Tina Pesaran**[3], **Robert Huether**[4], **Rachid Karam**[3], **Kathleen S. Hruska**[1], **Helio A. Costa**[5], **Katherine Lachlan**[6,7], **Joanne Ngeow**[8], **Jill Barnholtz-Sloan**[9,10], **Kaitlin Sesock**[11], **Felicia Hernandez**[3], **Liying Zhang**[12], **Laura Milko**[13], **Sharon E. Plon**[2], **Madhuri Hegde**[†,14,15], and **Charis Eng**[†,9,10,16]

[1]GeneDx, Inc, Gaithersburg, MD

[2]Baylor College of Medicine, Houston, TX

[3]Ambry Genetics, Aliso Viejo, CA

[4]Tempus Labs, Chicago, IL

[5]Stanford University School of Medicine, Stanford, CA

[6]Wessex Clinical Genetics Service, University Hospitals Southampton, Southampton, UK

[7]Human Genetics and Genomic Medicine, Faculty of Medicine, University of Southampton, Southampton, UK

[8]National Cancer Centre Singapore, Singapore

[9]Case Comprehensive Cancer Center, Cleveland, OH

[10]Case Western Reserve University School of Medicine, Cleveland, OH

[11]Counsyl, Inc, San Francisco, CA

[12]Memorial Sloan Kettering Cancer Center, New York, NY

[13]University of North Carolina, Chapel Hill, NC

[14]Emory University, Atlanta, GA

[15]PerkinElmer Genetics, Pittsburgh, PA

[16]Cleveland Clinic Genomic Medicine Institute, Cleveland, OH

Abstract

The ClinGen PTEN Expert Panel was organized by the ClinGen Hereditary Cancer Clinical Domain Working Group to assemble clinicians, researchers, and molecular diagnosticians with PTEN expertise to develop specifications to the 2015 ACMG/AMP Sequence Variant Interpretation Guidelines for *PTEN* variant interpretation. We describe finalized *PTEN*-specific variant classification criteria and outcomes from pilot testing of 42 variants with benign/likely

Corresponding Author: Jessica Mester, GeneDx, Inc, 207 Perry Parkway, Gaithersburg, MD USA 20877-2142, jmester@genedx.com, (216) 399-6678.

[†]Madhuri Hegde and Charis Eng should be considered joint senior author.

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benign (BEN/LBEN), pathogenic/likely pathogenic (PATH/LPATH), uncertain significance (VUS), and conflicting (CONF) ClinVar assertions. Utilizing these rules, classifications concordant with ClinVar assertions were achieved for 14/15 (93.3%) BEN/LBEN and 16/16 (100%) PATH/LPATH ClinVar consensus variants for an overall concordance of 96.8% (30/31). The variant where agreement was not reached was a synonymous variant near a splice donor with non-canonical sequence for which *in silico* models cannot predict the native site. Applying these rules to six VUS and five CONF variants, adding shared internal laboratory data enabled one VUS to be classified as LBEN and two CONF variants to be as classified as PATH and LPATH. This study highlights the benefit of gene-specific criteria and the value of sharing internal laboratory data for variant interpretation. Our PTEN-specific criteria and expertly reviewed assertions should prove helpful for laboratories and others curating *PTEN* variants.

Keywords

PTEN; variant; classification; criteria; ClinGen

Introduction

ClinVar is a publicly available resource of genetic variants and classifications submitted by clinical and research laboratories as well as gene or disease-specific expert groups (Landrum et al., 2016). To help users understand the level of evidence behind each assertion, ClinVar and the Clinical Genome Resource (ClinGen) developed a ranked review status system, with ClinGen-designated Expert Panels (EP) at a high level of review supporting the assertion of clinical significance (Rehm et al., 2015). For some genes or disorders an expert variant curation group already existed, but others, such as *PTEN*, were without such a resource. Germline pathogenic variants in *PTEN* (MIM# 601728) occur over an extremely heterogeneous clinical spectrum and may be present in individuals described clinically as having Cowden syndrome (CS), Bannayan-Riley-Ruvalcaba syndrome (BRRS), and other phenotypes such as macrocephaly and autism, collectively described as PTEN Hamartoma Tumor syndrome (PHTS) (J. Mester & Eng, 2013). The ClinGen PTEN EP was the first to form under the guidance of the ClinGen Hereditary Cancer Clinical Domain Working Group and was tasked with making PTEN-specific assessment and modifications where applicable to the 2015 American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) Variant Interpretation Guidelines (Richards et al., 2015). These guidelines have been adopted by ClinGen as a standardized framework for sequence variant interpretation; however, they were designed to be broadly applicable, making expert involvement necessary to define gene-specific guidance for application and level of strength for criteria within each evidence type. Here, we present the PTEN EP gene-specific variant curation guidelines along with the rationale and data supporting the specifications made for each criterion, as well as results from pilot testing the criteria on variants with benign/likely benign (BEN/LBEN), pathogenic/likely pathogenic (PATH/LPATH), uncertain significance (VUS), and conflicting (CONF) ClinVar assertions.

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Methods

The ClinGen PTEN Expert Panel (EP) includes individuals with PTEN-specific expertise in the areas of clinical research (JM, JN, KS, CE), patient care (JM, TP, KL, JN, KS, CE), basic science (RH, RK, HC, LZ, CE), and molecular diagnostics (JM, TP, RH, RK, KH, MH). EP members were drawn from international academic, clinical, and diagnostic laboratory settings and include a balance of geneticists, genetic counselors, research scientists, and variant curation experts. All EP members disclosed potential conflicts of interest as required by ClinGen.

The 2015 ACMG/AMP Variant Interpretation Guidelines (Richards et al., 2015) includes eight different evidence types: population data, computational/predictive data, functional data, segregation data, *de novo* data, allelic data, and "other". Given the diverse backgrounds of EP members, work began by familiarizing the membership with the ACMG/AMP criteria as well as current knowledge related to *PTEN* within each evidence type. Workgroups were assembled to research and present information on each evidence type to the entire EP to ensure all members had the comprehensive background knowledge necessary for rule review and to make informed decisions regarding the utility and strength of individual criterion.

The EP decided to develop and test benign criteria on a set of *PTEN* variants defined as benign or likely benign (BEN/LBEN) per multiple ClinVar submitters and agreed upon by EP members, and then repeat the process for pathogenic criteria and a similar pathogenic/likely pathogenic (PATH/LPATH) *PTEN* variant test set. The ClinGen Genomic Variant Working Group, which provided preliminary review and feedback on the criteria during the development phase, recommended a minimum 80% concordance with the consensus ClinVar classifications prior to rule acceptance. Finally, variants with classifications of uncertain significance (VUS) or with conflicting interpretations by multiple submitters (CONF) were curated by two independent biocurators to assess inter-curator concordance and process workflow. Variants are annotated using GenBank reference sequence NM_000314.4 and NC_000010.10 (GRCh37/hg19).

Results

PTEN-Specific Variant Curation Rules

The final PTEN EP specifications to the ACMG/AMP variant curation criteria were approved by the Sequence Variant Interpretation working group (SVI) and are summarized in **Table 1.** Five of the 28 original ACMG/AMP criteria were removed due to lack of relevance to PTEN or, as in the case of PP5/BP6, based on recommendation from the SVI (Biesecker & Harrison, 2018). Four of the remaining 23 criteria were left unchanged, and disease-specific modifications (DS) and/or modification to the criteria strength (SM) were made to the others. The EP made no modifications to the rules outlined by ACMG/AMP to combine criteria to arrive at a classification.

Evidence assessment and modification

A. Population Data (BA1, BS1, PM2, PS4)—PHTS is considered a rare disorder. A study within the Dutch population reported CS as occurring in approximately 1 in 250,000

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individuals (Nelen et al., 1999); however, at the time CS was the only clinical diagnosis associated with PTEN, thus this study did not include individuals with other PHTS phenotypes and is likely an underestimate of true disease prevalence, which is to date unknown. *PTEN* pathogenic variants have been identified in 1% or fewer of unselected individuals with breast, thyroid, or endometrial cancers, and higher rates (up to 17% in one study) among patients with autism and macrocephaly (Butler et al., 2005; Nagy et al., 2011; Ring et al., 2016; Tung et al., 2016; Wong et al., 2016). To consider the maximum possible frequency of PHTS in the population, the group considered the population incidence and percent causation owed to PHTS for these four PHTS-component features (Supp. Table S1). The highest estimates of disease incidence and attribution to PHTS were used to calculate conservative estimates of allele frequency attributable to PHTS. PTEN is the only gene associated with PHTS, and while a few recurrent variants have been reported, pathogenic variants of diverse types (missense, truncating, splicing, large rearrangement) have been reported across the gene (J. Mester & Eng, 2013), making further adjustments for genetic heterogeneity unnecessary. Additionally no founder effect exists and *de novo* variants often occur (J. Mester & Eng, 2012).

Summing the estimates gave a total allele frequency of 0.000961 (0.0961%). Rounding up, the EP accepted an allele frequency ≥0.001 (≥0.1%) as the cutoff for application of BS1. Based on these data, the EP also felt comfortable lowering the allele frequency for BA1 set by ACMG/AMP from ≥0.05 (≥5%) to ≥0.01 (≥1%). These values are purposefully conservative to account for the unknown population prevalence of PHTS and penetrance of several component features, sub-clinical disease presentations, and unknown population prevalence and percent of disease attributable to PHTS for many other component phenotypes. Additionally, per the ExAC database the missense constraint (z=3.71) and probability of loss of function (LOF) intolerance (pLI=0.98) scores for *PTEN* suggest high constraint, making it unlikely that a pathogenic missense or LOF variant would rise to high allele frequency. The EP also adopted the recommendation by the SVI that the variant be present in at least 5 alleles with a minimum number of 2,000 alleles analyzed within the population of interest to minimize the risk of sequencing error or chance inclusion of an affected individual.

With respect to PM2 (variant is absent within population databases), the group initially tested their criteria with this rule as written, requiring that a variant be completely absent in order to apply this criterion. During the rule testing process, it was noted that several loss of function alleles were present at ultra-rare frequency (1/240,000+ alleles) in the gnomAD cohort (Lek et al., 2016), including PTEN c.50_51delAA, listed as pathogenic per multiple ClinVar submitters and selected for the PATH/LPATH test set. While LOF is an established disease mechanism for PHTS, and variants leading to LOF would be expected to be PATH/LPATH, the ACMG/AMP guidelines require additional pieces of evidence in addition to PVS1 in order to achieve a PATH or LPATH classification. Although the gnomAD cohort excludes individuals with severe pediatric-onset disease (Lek et al., 2016), some of the cohorts, such as the Swedish Schizophrenia & Bipolar Studies cohort (dbGaP accession phs000473.vs.p2) contributing to the dataset include individuals as young as 18 years of age. Additionally, some individuals also came from The Cancer Genome Atlas cohort (http://cancergenome.nih.gov/) and are positive for cancer phenotypes. Cumulative cancer risk in

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PHTS is estimated to be less than 50% by age 40 (Bubien et al., 2013; Nieuwenhuis et al., 2014), and the benign features associated with this condition are often under-recognized (Eng, 2003), making it possible that affected individuals could be included in a general population cohort. Thus the group chose to modify PM2 to permit use if present at <0.00001 (0.001%) allele frequency in the gnomAD dataset or another large sequenced population, with allele frequency <0.00002 (0.002%) in an ancestry-specific subpopulation if multiple alleles are present.

Per the ACMG/AMP criteria, PS4 may be applied in one of two ways: 1) significant case-control data, or 2) counting multiple unrelated patients with the same phenotype. Not surprisingly given the rarity of PHTS, the PTEN EP could not identify an existing case-control study for which application of PS4 in the first manner would be appropriate. Should such a study come to light, PS4 may be applied to a case-control study finding an odds ratio >2 with $p<0.05$ and 95% confidence interval with lower limit >1.5. The EP has adapted the second application for use of PS4, counting multiple unrelated probands, to incorporate phenotype specificity (PP4) as described in section F (Phenotype).

B. Computational and Predictive Data (BP1, BP3, BP4, BP7, PP2, PP3, PM1, PM4, PM5, PS1, PVS1)—As a gene with several pathogenic missense variants, BP1 (missense variants in a gene for which primarily truncating variants cause disease) does not apply to *PTEN*. Likewise, PTEN does not contain a repetitive region without known function as would be used to apply the ACMG/AMP definition of BP3 (in-frame deletions/insertions in a repetitive region without known function), leading the EP to remove that criterion as well. However, PTEN is a highly conserved protein, with numerous pathogenic missense variants causing disease (J. Mester & Eng, 2013) and as of March 2018, no missense variants classified as benign or likely benign by any ClinVar submitter. Thus the EP opted to maintain PP2 as written to be applied to PTEN. The EP also decided to adopt PM5 (novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before), adding that the missense variant in question need not be novel, but should contain a BLOSUM62 (Henikoff & Henikoff, 1992) value equal to or less than the known variant. The EP also decided that this rule may be applied when the known variant is likely pathogenic unless applying would lead to a higher (pathogenic) classification for the variant being assessed.

Again given the lack of BEN/LBEN *PTEN* missense variants, the EP was left without a set of established BEN/LBEN missense variants to be used to test the accuracy of *in silico* predictors to be used as evidence to apply BP4 or PP3 to *PTEN* missense variants. While investigating potential *in silico* tools, the EP also came to find that some algorithm predictions were highly sensitive to sequence alignment, further limiting confidence in these tools. Should the EP classify several missense variants as BEN/LBEN, another attempt will be made to validate *in silico* tools to apply PP3/BP4 for missense variants.

The EP did maintain PP3/BP4 use for intronic or synonymous variants which may impact splicing. HSF version 3.1 (http://www.umd.be/HSF3/index.html; (Desmet et al., 2009)), NNSplice 0.9 version (http://www.fruitfly.org/seq_tools/splice.html; (Reese, Eeckman, Kulp, & Haussler, 1997)), and MaxEntScan (http://genes.mit.edu/burgelab/maxent/

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Xmaxentscan_scoreseq_acc.html; (Yeo & Burge, 2004)) scores correlated well with published data, providing concordant predictions for 23/23 (100%) variants functionally proven to impact splicing and 8/11 (72.7%) with no splicing impact or that were putatively benign based on gnomAD population frequency data (Lek et al., 2016) (Supp. Table S2). At least two of these three predictors were capable of detecting the native splice sites for every splice donor/acceptor with the exception of the intron 1 splice donor, and the intron 6 splice acceptor could not be predicted by NNSplice and was weakly predicted by MaxEntScan. The *PTEN* intron 1 splice donor site has a non-canonical sequence (CCTgtatcc – single nucleotide code) that leaves existing *in silico* tools unable to predict its presence or interrogate the impact of potential splicing variants in this region. The intron 6 splice acceptor has nearby potential acceptor sites of comparable strength that may compete with the native site. Additionally, only one variant near the intron 1 splice acceptor, *PTEN* c.80–3C>G, had undergone splicing analysis with no impact observed (Chen, Romigh, Sesock, & Eng, 2017), and *in silico* predictors were discordant, predicting a splicing impact. Therefore, the EP will not consider applying PP3/BP4 for variants which may impact the intron 1 splice donor or acceptor sites, and will approach variants around the intron 6 splice acceptor with caution.

The group agreed to adopt BP7 (synonymous variant for which splicing prediction algorithms predict no impact) as defined by ACMG/AMP, and extended this criteria to apply to intronic variants at or beyond the +7 or −21 positions. For intronic variants, it was further stipulated that the nucleotide position should not be conserved; conserved positions were defined as having a PhastCons score=1 or a PhyloP score >0.1 (Pollard, Hubisz, Rosenbloom, & Siepel, 2010; Siepel et al., 2005).

The EP also maintained PVS1 (null variant in a gene where LOF is a disease mechanism) as defined by ACMG/AMP, applying it to nonsense or frameshift variants predicted to cause nonsense-mediated decay (NMD), canonical splice site variants, and single or multi-exon deletions. Additionally, internal data from EP laboratory members as well as published literature report *de novo* variants in individuals with a strong PHTS-related phenotype as 3' as c.1120_1121dupCC (p.D375fs) (Vanderver et al., 2014). Although this variant occurs within exon 9, *PTEN*'s final exon, and NMD is not predicted to occur, it is predicted to result in the disruption of the C-terminal domain which includes PEST motifs, residues that undergo phosphorylation, and a PDZ domain-binding motif (X. Wang & Jiang, 2008), leading the EP to set this position as the 3' boundary for use of PVS1. For protein truncating variants causing disruption 3' of c.1121 or protein extension, the group agreed to apply PM4 (protein length changes) as currently defined. The group also agreed to apply this criterion to small in-frame deletions or duplications disrupting one of PTEN's catalytic motifs, which include the WPD loop (residues 90–94), P-loop (also described as phosphatase core, residues 123–130), and the TI-loop (residues 166–168) (NP_ 000305.3) (Lee et al., 1999). These three regions also comprise the residues for which PM1 (mutational hot spot and/or critical and well-established functional domain) may be applied for missense variants.

The EP applied PS1 (same amino acid change as a previously established pathogenic variant) as currently defined by ACMG/AMP, and expanded it to include a different nucleotide substitution for an intronic splice site variant if the predicted impact is equal to or

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greater than the known pathogenic variant per *in silico* splicing tools. A caveat that caution should be used when applying this criteria to exonic variants causing aberrant splicing was included.

C. Functional Data (BS3, PS3)—As part of its role as a tumor suppressor, PTEN functions as a phosphatase, catalyzing the conversion of phosphatidylinositol triphosphate ($PI(3,4,5)P_3$) to $PI(4,5)P_2$ and leading to inhibition of the AKT pathway. When this phosphatase activity is diminished, over-activation of AKT occurs, driving tumor development (Myers et al., 1998; Stambolic et al., 1998). Numerous *PTEN* missense variants have been identified which alter the structure of the active site pocket such that phosphatase activity is impacted, and several independent researchers have used this assay as a means of measuring PTEN dysfunction (Han et al., 2000; Mighell, Evans-Dutson, & O'Roak, 2018; Rodríguez-Escudero et al., 2011). The EP therefore felt PS3 (well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect) would be suitable for a missense variant found to cause over a 50% reduction in lipid phosphatase activity or any variant found to cause aberrant splicing on functional interrogation. Decreased PTEN or increased pAKT expression, although not as direct a measure as phosphatase activity, have been significantly associated with presence of pathogenic *PTEN* variation (Spinelli, Black, Berg, Eickholt, & Leslie, 2015; Tan et al., 2011). In addition to phosphatase activity, pathogenic *PTEN* variants have been found to disrupt protein cellular localization (Gil et al., 2015; He et al., 2012; Lobo et al., 2009) and lead to aberrant cellular phenotypes, including defective cell migration, proliferation, and invasion (Costa et al., 2015; Malek et al., 2017). Knock-in and knock-out *Pten* mouse models have also been developed, with mice harboring pathogenic variants or haploinsufficient *Pten* demonstrating PHTS-related phenotypes and increased tumor burden (Di Cristofano, Pesce, Cordon-Cardo, & Pandolfi, 1998; J. L. Mester, Tilot, Rybicki, Frazier, & Eng, 2011; Tilot et al., 2014). The EP elected to apply a supporting level of evidence (PS3_supporting) for *in vitro* cellular assays not meeting PS3 and transgenic model organism studies.

Although normal lipid phosphatase activity may suggest retained function, *PTEN* missense variants have been described which retain lipid phosphatase activity, but lead to dysfunction via loss of protein phosphatase activity, protein instability, abnormal cellular localization, or other mechanisms unrelated to lipid phosphatase activity (Davidson et al., 2010; Gil et al., 2015; Yang et al., 2017). The EP would therefore consider applying BS3_supporting to variants with one functional study demonstrating results comparable to wild-type. BS3 (well-established *in vitro* or *in vivo* functional studies show no damaging effect) may be applied at the strong evidence level for variants with both lipid phosphatase activity AND results from a second assay appropriate to the protein domain demonstrating no statistically significant difference from wild type. BS3 may also be applied for intronic or synonymous variants demonstrated to *not* cause a splicing impact via RNA, mini-gene, or other splicing assays. Phosphatase assays for which BS3/PS3 may be applied must include a catalytic dead control, such as p.C124S, and at least three biological replicates would be required to apply criteria to results of any of these or similar readouts of PTEN function (Costa et al., 2015; Malek et al., 2017).

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D. Segregation Data (BS4, PP1)—The EP adopted the approach being taken by other ClinGen EPs (Gelb et al., 2018; Kelly et al., 2018) and supported by the SVI and other work (Jarvik & Browning, 2016) that 3 or 4 meioses be required for application of PP1, with additional meioses meriting higher evidence levels (5 or 6 meioses for moderate, 7 or more meioses for strong) based on estimated LOD scores of 0.9, 1.5, and 2.1, respectively (Kelly et al., 2018). The EP further stipulated that cosegregation with disease must be observed across at least two families for application of the criteria at the strong evidence level, to avoid the possibility that a true undiscovered disease allele is present in linkage disequilibrium with the variant under interrogation.

The EP defined that BS4 can be used when a PHTS phenotype is present in several individuals from one side of a family, and the variant in question is found to be inherited from the opposite side. For application of BS4 at its pre-defined strong evidence level, the EP specified that such lack of segregation with disease must be seen in two or more families. A supporting level of evidence (BS4_supporting) may be used when one such family is identified.

E. *De Novo* Data (PM6, PS2)—The EP agreed to adopt the definitions of PM6 and PS2 as defined by ACMG/AMP, and decided to follow the SVI-approved approach to apply higher levels of evidence with increasing numbers of *de novo* probands or to lower the evidence level when phenotypic specificity is lacking (https://www.clinicalgenome.org/site/assets/files/8490/recommendation_ps2_and_pm6_acmgamp_critiera_version_1_0.pdf) (**Table 1,** Supp. Table S3). In accordance with ACMG/AMP guidance, family history must also be consistent with a *de novo* event, and the patient's phenotype should be a match for PHTS, but they need not meet the strict criteria for PS4/PP4 outlined in the following section.

F. Phenotype (PS4/PP4)—Given the extreme population rarity of any one specific pathogenic *PTEN* variant, it is unlikely that a case-control study will find statistically significant results for a specific variant, leading the EP to adopt the second use of PS4 (multiple unrelated individuals with the same phenotype). PHTS causes increased risk for a diverse combination of phenotypes, several of which are specific to this disorder and uncommon in the general population. Phenotype among affected individuals is also highly variable, even within the same family, and is also age- and gender-dependent (Lachlan, Lucassen, Bunyan, & Temple, 2007), necessitating a careful approach whereby PP4 (phenotype specificity) is wrapped into the PS4 criteria. For adults, a scoring system called the Cleveland Clinic (CC) score predicts *a priori PTEN* mutation risk based on personal history, with findings highly specific to PHTS, such as Lhermitte-Duclos disease or hamartomatous polyps, given higher weight (Tan et al., 2011) (Supp. Table S4). The EP decided that either one adult with phenotypic features specific enough to result in a CC score ≥30 (≥80% mutation risk), or two with score of 25–29 (62–80% mutation risk) would qualify for PS4_supporting. In other words, an adult with a CC score of 35 would merit 1 phenotype specificity point; an adult with a CC score of 28 would merit 0.5 points.

For children, no similar tool existed, and the CC scoring tool could not be applied given the age-dependent penetrance of many PHTS phenotypes. Therefore, creation of a separate

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scoring system for children was required. EP members from two U.S.-based testing laboratories and a clinician in the United Kingdom who used a national referral lab for testing contributed de-identified phenotype data from children who had undergone *PTEN* mutation analysis. Age- and gender-matched pediatric patients were selected with either positive (PATH) or negative (no variants identified) results. The EP phenotype workgroup drafted scores for findings observed in children with PHTS, assigning higher scores to features more specific to PHTS and lower ones to those observed more often in children referred for genetics evaluation and/or testing (Table 2). Using this scoring system, paired *t*-test identified significantly higher scores for children with PATH variants compared to those with negative *PTEN* testing (4.45 vs. 2.95, p=0.009, Supp. Table S5). When a threshold of 5 was applied, 100% specificity was achieved for both boys and girls, leading the EP to adopt this score as the cutoff for application of PS4_supporting (1 phenotype specificity point) for pediatric cases. Using a cutoff of 4, specificity dropped to 50% due to the high prevalence of autism/developmental delay (DD)/intellectual disability (ID) diagnoses across the cohort, but sensitivity increased to 80% (Supp. Table S6). Thus, similar to the approach accepted for adults, a score of 4 was accepted as appropriate for 0.5 phenotype specificity points, with the caveat that autism/DD/ID may not contribute to the score given the common nature of this phenotype for pediatric genetics referral (Woodward, Alves, & Butler, 1993).

Similar to *de novo* evidence, EP members chose to apply PS4_supporting at higher evidence levels when multiple probands meeting these phenotype criteria were identified. Given the strict criteria developed for applying phenotype specificity points, the duplicative approach to reach higher evidence levels for *de novo* criteria was employed, but with 16 or more specificity points required for a Very Strong evidence level to more closely match the exponential increase in odds for pathogenicity between the Strong and Very Strong evidence levels supported by the SVI (Tavtigian et al., 2018). Table 3 provides a summary of the phenotype specificity scores required for increased levels of evidence with examples for application. The EP also specified that this criterion not be applied when BS1 is met to avoid coincidental accumulation of proband specificity points for variants with high allele frequency.

G. Allelic Data (BS2, BP2, BP5)—Murine *Pten* homozygous knock-out or knock-in models demonstrate embryonic lethality (Di Cristofano et al., 1998; H. Wang et al., 2010). To date, we are not aware of a human individual with homozygosity or compound heterozygosity for pathogenic *PTEN* variants. Only one homozygous potentially hypomorphic missense variant, p.L182S, has been reported in siblings with a PHTS phenotype by exome analysis whose heterozygous parents were phenotypically normal (Schwerd et al., 2016). However, this variant has not been reported elsewhere. Individuals have been reported with one germline pathogenic *PTEN* variant and a somatic “second hit”, with affected tissues demonstrating overgrowth, vascular malformations, and lipomatosis reminiscent of Proteus syndrome (Caux et al., 2007). Together these data suggest that germline homozygosity or compound heterozygosity for pathogenic *PTEN* variants in humans may not be consistent with life, or would result in a severe PHTS phenotype. Thus the PTEN EP wished to apply BS2 (observed in a healthy adult individual) when a variant is present in the homozygous state in at least one individual whose homozygous status was

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confirmed via parental testing or in two individuals without such confirmation, so long as the individual(s) are confirmed as healthy/unaffected persons or do not exhibit features that would be expected for PHTS. A supporting level of evidence may be used when at least two homozygous observations exist in the absence of clinical data, or the criteria for BS2 is met but BS1 (allele frequency greater than expected for disorder) has also been applied. The caveat regarding BS1 was added to ensure a variant could not reach benign status (BS1 + BS2) driven mainly by homozygous occurrences due to population frequency lower than the threshold set for application of BA1. BP2 may be applied when a variant is observed *in trans* with a known *PTEN* pathogenic variant, or observed *in cis* or with unknown phase with three or more different pathogenic *PTEN* variants.

The PTEN EP also agreed to adopt BP5 (variant found in a case with an alternate molecular cause for disease) for two or more co-occurrences with pathogenic variants in a different gene that fully explained the patient's phenotype, but specific circumstances would need to be met in order for a case to be considered for inclusion. First, the variant in the other gene must be considered highly penetrant, with both the individual's age and gender taken into consideration. Additionally, the patient's personal and family history (including up to 2nd degree relatives) should not overlap with features seen in PHTS. As an example, an individual with a personal and family history of breast cancer who harbored a *PTEN* variant in addition to a pathogenic *BRCA2* variant would not apply, because breast cancer is a known risk in PHTS and the *PTEN* variant might have contributed to this individual's breast cancer risk. However, an individual with a personal and family history of ovarian and pancreatic cancer who was positive for a *PTEN* variant as well as a pathogenic *BRCA2* variant **would** be considered for BP5 application, as neither ovarian nor pancreatic cancer are associated with PHTS.

Rule Piloting

For the BEN/LBEN test set (Table 4), 12/15 (80%) variants achieved a BEN/LBEN classification based on initial data accumulated from literature review, population databases, and *in silico* models. The three variants initially classified as VUS included c.−1026C>A, c.−1311T>C, and c.75G>A (p.L25=). Internal data from group members were used to identify cases with homozygous occurrences, segregation data, or co-occurrence data that might be applied. In the case of c.−1026C>A, both homozygous observations (BS2_P) as well as co-occurrences with pathogenic variants in other genes explaining the patient's phenotype (BP5) were identified, leading to a final LBEN classification. As of March 2018, PTEN c.−1311T>C was present in gnomAD at an allele frequency of 1.42% (23/1618) within East Asian populations. Despite the denominator being less than 2000, BA1 was applied with approval from the Genomic Variant Working Group given the allele frequency would remain >1% if 2000 alleles were present in the studied population (23/2000 = 1.15%) and 95% confidence interval of the proportion (0.9–2.1%). *PTEN* c.75G>A is near a predicted U12-dependent splice donor for which *in silico* tools are not available. Although BP7 was applied based on the synonymous nature of this variant, no additional criteria apply at present, leaving this variant at VUS. Thus, the BEN/LBEN test set achieved a final concordance of 93.3% (14/15) with consensus ClinVar classifications.

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Given that clinical laboratory data provided helpful evidence for the BEN/LBEN variants, these data were incorporated for the initial review of the pathogenic criteria tested on 16 consensus ClinVar PATH/LPATH variants (Table 5). On initial review, 15/16 (93.8%) variants achieved a PATH/LPATH classification. PTEN c.50_51delAA was present in 1/246,272 alleles in gnomAD, and the EP's initial PM2 language permitted use only when a variant was completely absent in sequenced populations. Thus the EP modified PM2 as previously described, permitting use for this variant and other LOF variants present in gnomAD at ultra-rare allele frequency and leading to a final 100% (16/16) concordance with consensus ClinVar classifications for the PATH/LPATH test set. This process also highlighted the need for sharing internal laboratory data, as PTEN c.103A>G (p.M35V) would have been classified as VUS without PS2 applied due to a confirmed *de novo* occurrence.

The final set of variants curated for pilot purposes included five with conflicting interpretations in ClinVar (CONF) and six with assertions of uncertain significance (VUS) by multiple submitters, with curation performed by two independent biocurators (Table 6). Biocurators had complete concordance with respect to criteria and classification for five of the six VUS. For the five CONF variants, initial criteria applied by each biocurator differed slightly, but preliminary classifications did not differ by more than one "step" (VUS vs. LBEN, PATH vs. LPATH, etc.). Following brief discussion between biocurators and addition of internal laboratory data, complete agreement for criteria and preliminary classification was achieved for three of the five CONF variants, for a final inter-biocurator concordance of 72.7% (8/11) for criteria and 81.8% (9/11) for classification within the VUS and CONF variant sets. Notably, shared internal laboratory data provided evidence leading to resolution of classification for two of the five CONF variants (1 PATH, 1 LPATH) and an LBEN classification for one of the six VUS.

Discussion

Moving forward, the PTEN EP plans to meet on a quarterly basis to conduct variant curation, and will publicly share classification assertions and supporting evidence in ClinVar. A minimum 75% group attendance, including at least one co-chair, will be required for quorum to be met. Variants with CONF or VUS assertions per multiple submitters have the greatest need for EP review and will thus be given top priority. The EP will also reserve a portion of each meeting for variants expected to be less-challenging, such as variants with PATH/LPATH or BEN/LBEN assertions, prioritizing those with no assertion criteria or with assertion by only a single submitter. Date of ClinVar submission will also be considered, with review priority also given to variants with earliest submission date.

Variants classified as being of uncertain significance or with conflicting assertions present several clinical challenges and may be distressing for providers and patients alike (Lumish et al., 2017). These challenges are compounded for highly-penetrant cancer susceptibility genes such as *PTEN*, where presence of a pathogenic variant may influence surgical-decision making in addition to other medical management and recurrence risk counseling (Balmaña et al., 2016). The PTEN EP hopes that these gene-specific variant curation rules

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and the assertions provided for variants reviewed by the EP will be helpful to clinicians, clinical laboratories, and others interpreting *PTEN* variants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1:

Summary of Gene-Specific Criteria for *PTEN* Variant Classification

Pathogenic Criteria			
Evidence Level	**Rule Code**	**Specification Type**[†]	**Rule Description**
Very Strong	PVS1	DS	Null variant (nonsense, frameshift, canonical ± 1 or 2 splice sites, initiation codon, single or multi-exon deletion) predicted to result in nonsense-mediated decay or causing truncation/frameshift at or 5' to c.1121 (NM_000314.4).
	PS2_VS/PM6_VS	SM	Two proven OR four assumed OR one proven + two assumed *de novo* observations.
	PS4_VS	SM	Proband specificity score ≥16 (see text).
Strong	PS1	DS	Same amino acid change as a previously established pathogenic variant regardless of nucleotide change OR different variant at same nucleotide position as a pathogenic splicing variant, where *in silico* models predict impact equal to or greater than the known pathogenic variant.
	PS2	No change	*De novo* (both maternity and paternity confirmed) in a patient with the disease and no family history.
	PS3	DS	Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect on the gene or gene product. Defined to include studies showing phosphatase activity <50% of wild-type or RNA, mini-gene, or other assay showing impact on splicing.
	PS4	DS	Use 1: The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls. Use 2: Proband specificity score of 4-15.5 (see text).
	PM6_S	SM	Two probands with presumed *de novo* occurrence (maternity/paternity not confirmed).
	PP1_S	SM	Co-segregation with disease in multiple affected family members, with ≥7 meioses observed across at least two families.
Moderate	PM1	DS	Located in a mutational hot spot and/or critical and well-established functional domain. Defined to include residues in catalytic motifs: 90-94, 123-130, 166-168 (NP_000305.3).
	PM2	DS	Present at <0.00001 (0.001%) allele frequency in gnomAD or another large sequenced population. If multiple alleles are present within any subpopulation, allele frequency in that subpopulation must be <0.00002 (0.002%).
	PM3	Removed	For recessive disorders, detected *in trans* with a pathogenic variant.
	PM4	DS	Protein length changes due to in-frame deletions/insertions in a non-repeat region or stop-loss variants. Applies to in-frame insertions or deletions impacting at least one residue in a catalytic motif (see PM1), protein truncation with disruption starting 3' of c.1121 (NM_000314.4), and variants causing protein extension.
	PM5	DS	Missense change at an amino acid residue where a different missense change determined to be pathogenic or likely pathogenic has been seen before. In addition, variant being interrogated must have a BLOSUM62 score equal to or less than the known variant.
	PM6	No change	Assumed *de novo*, but without confirmation of paternity and maternity.
	PS4_M	SM	Proband specificity score of 2-3.5 (see text).
	PP1_M	SM	Co-segregation with disease in multiple affected family members, with 5 or 6 meioses observed.
Supporting	PP1	DS	Co-segregation with disease in multiple affected family members, with 3 or 4 meioses observed.
	PP2	No change	Missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease.

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Pathogenic Criteria			
Evidence Level	**Rule Code**	**Specification Type**[†]	**Rule Description**
	PP3	DS	Multiple lines of computational evidence support a deleterious effect on the gene or gene product. To be applied only to synonymous or intronic variants where at least 2 out of 3 *in silico* models predict a splicing impact.
	PP5	Removed	Reputable source recently reports variant as pathogenic, but the evidence is not available to perform an independent evaluation.
	PS3_P	DS; SM	Abnormal *in vitro* cellular assay or transgenic model with phenotype different from wild type that does not meet PS3.
	PS4_P	DS	Phenotype specific for disease with single genetic etiology. Proband specificity score of 1-1.5 (see text).
Benign Criteria			
Evidence Level	**Rule Code**	**Specification Type**[†]	**Rule Description**
Stand-Alone	BA1	DS	Allele frequency ≥0.01 (1%) in a studied population with ≥2,000 alleles tested and variant present in ≥5 alleles.
Strong	BS1	DS	Allele frequency from 0.001 (0.1%) up to 0.01 (1%) in a studied population with ≥2,000 alleles tested and variant present in ≥5 alleles.
	BS2	DS	Observed in the homozygous state in a healthy or PHTS-unaffected individual. One observation if homozygous status confirmed, two if not confirmed. To be applied at supporting evidence level if BS1 is also applied.
	BS3	DS	Well-established *in vitro* or *in vivo* functional studies show no damaging effect on protein function or splicing. To be applied for missense variants with both lipid phosphatase activity AND results from a second assay appropriate to the protein domain demonstrating no statistically significant difference from wild type. For intronic or synonymous variants, RNA, mini-gene, or other splicing assay demonstrates no splicing impact.
	BS4	DS	Lack of segregation in affected members of two or more families.
Supporting	BP1	Removed	Missense variant in a gene for which primarily truncating variants are known to cause disease.
	BP2	DS	Observed *in trans* with a pathogenic or likely pathogenic *PTEN* variant OR at least three observations *in cis* and/or phase unknown with different pathogenic/likely pathogenic *PTEN* variants.
	BP3	Removed	In-frame deletions/insertions in a repetitive region without a known function.
	BP4	DS	Multiple lines of computational evidence suggest no impact on gene or gene product (conservation, evolutionary, splicing impact, etc.). To be applied only to synonymous or intronic variants where at least 2 out of 3 *in silico* models predict no splicing impact.
	BP5	DS	Variant found in at least two cases with an alternate molecular basis for disease. Other gene/disorder must be considered highly penetrant AND patient's personal/family history should not overlap with PHTS.
	BP6	Removed	Reputable source recently reports variant as benign, but the evidence is not available to perform an independent evaluation.
	BP7	DS	A synonymous (silent) or intronic variant at or beyond +7/−21 (5'/3' exonic) for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not conserved.
	BS2_P	DS; SM	Two homozygous observations with no clinical data provided, or meets criteria for BS2 but BS1 is also applied.
	BS3_P	DS; SM	*In vitro* or *in vivo* functional study or studies show no damaging effect on protein function but BS3 not met.
	BS4_P	DS; SM	Lack of segregation in affected members of one family.

[†]DS = Disease-Specific; SM = Strength Modified

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Table 2:

PTEN Phenotype Scoring for Pediatric Patients

Feature	Score (points)
Macrocephaly of >2 SD to <4 SD	2
Extreme macrocephaly (≥4 SD)	3
PTEN-specific MRI characteristics (dilated Virchow-Robin, prominent perivascular spaces)	2
Autism/developmental delay (DD)/intellectual disability (ID)	2
Penile freckling	3
Lipoma	1
Oral papilloma	3
Hamartomatous polyp(s)	3
Arteriovenous malformation/hemangioma	2
Thyroid cancer	3
Thyroid nodule/Hashimoto's thyroiditis	2

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Table 3:

Phenotype Specificity Score and Examples of Application for Increasing Levels of Evidence

Supporting (PS4_P): 1-1.5 points	**Moderate (PS4_M): 2-3.5 points**	**Strong (PS4): 4-15.5 points**	**Very strong (PS4_VS): ≥16 points**
1 proband with phenotype specificity = 1 (child with pediatric score ≥5 or adult with CC score ≥30) OR	2 probands with phenotype specificity = 1 (total of 2 points)	4 probands with phenotype specificity = 1 (total of 4 points)	16 probands with phenotype specificity = 1 (total of 16 points)
2 probands with phenotype specificity = 0.5 (adult CC score = 25-29, child = 4 omitting autism/DD/ID phenotypes) (total of 1 point) OR	1 proband with phenotype specificity = 1 plus 2 probands with phenotype specificity = 0.5 (total of 2 points)	3 probands with phenotype specificity = 1 plus 2 probands with phenotype specificity = 0.5 (total of 4 points)	14 probands with phenotype specificity = 1 plus 4 probands with phenotype specificity = 0.5 (total of 16 points)
1 proband with phenotype specificity = 1 plus 1 proband with phenotype specificity = 0.5 (total of 1.5 points)	2 probands with phenotype specificity = 1 plus 2 probands with phenotype specificity = 0.5 (total of 3 points)	7 probands with phenotype specificity = 1 plus 5 probands with phenotype specificity = 0.5 (total of 9.5 points)	10 probands with phenotype specificity = 1 plus 15 probands with phenotype specificity = 0.5 (total of 17.5 points)
Etc.	Etc.	Etc.	Etc.

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Table 4:

BEN/LBEN Variant Test Set and Criteria

PTEN Variant	Round 1 review –criteria applied	Initial classification	Round 2 review – criteria added following GVWG review, addition of clinical laboratory data	Final EP classification
c.−9C>G (NC_000010.10:g.89624218C>G)	BA1	BEN		BEN
c.−903G>A (NC_000010.10:g.89623323G>A)	BA1	BEN		BEN
c.−1026C>A (NC_000010.10:g.89623200C>A)	**BS1**	**VUS**	**BS2_P, BP5**	**LBEN**
c.−1059C>G (NC_000010.10:g.89623167C>G)	BA1	BEN		BEN
c.−1311T>C (NC_000010.10:g.89622915T>C)	**None**	**VUS**	**BA1**	**BEN**
c.18A>G (p.L6=)	BP4, BP7	LBEN		LBEN
c.75G>A (p.L25=)	BP7	VUS		VUS
c.132C>T (p.G44=)	BS1, BP7	LBEN	BS2_P, BP5	LBEN
c.360A>C (p.A120=)	BP4, BP7	LBEN		LBEN
c.1104T>C (p.D368=)	BS1, BP4, BP7	LBEN		LBEN
c.79+35C>T (IVS1+35C>T)	BS1, BS3	BEN		BEN
c.165-13_165-10delGTTT (IVS2-13_IVS2-10delGTTT)	BS1, BP4	LBEN		LBEN
c.254-39G-T (IVS4-39G>T)	BP4, BP7	LBEN		LBEN
c.801+23G>A (IVS7+23G>A)	BS1, BP4	LBEN		LBEN
c.1026+32T>G (IVS8+32T>G)	BA1	BEN		BEN

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Table 5:

PATH/LPATH Variant Test Set and Criteria

PTEN Variant	Round 1 review – criteria applied	Initial Classification	Round 2 review - criteria added following PM2 adjustment, additional *de novo*/proband phenotype specificity levels of evidence	Final EP classification
c.50_51delAA (p.Q17RfsX26)	**PVS1, PS4_P**	**VUS**	**PM2**	**PATH**
c.511C>T (p.Q171X)	PVS1, PM2, PM6, PS4_P	PATH	PS4_P→PS4_M	PATH
c.892C>T (p.Q298X)	PVS1, PM2, PS4_P	PATH		PATH
c.964A>T (p.K322X)	PVS1, PM2	LPATH		LPATH
c.987_990delTAAA (p.N329KfsX14)	PVS1, PM2, PS4_P	PATH		PATH
c.80-1G>C (IVS1-1G>C)	PVS1, PM2	LPATH		LPATH
c.165-1G>A (IVS2-1G>A)	PVS1, PM2	LPATH		LPATH
c.493-2A>G (IVS5-2A>G)	PVS1, PM6_S, PM2	PATH		PATH
c.801+1delG (IVS7+1delG)	PVS1, PM2	LPATH		LPATH
c.802-2A>T (IVS7-2A>T)	PVS1, PM2, PS4_P	PATH		PATH
c.1026+1G>A (IVS8+1G>A)	PVS1, PM2, PS4_P	PATH	PS4_P→PS4_M	PATH
c.103A>G (p.M35V)	PS2, PM2, PP2, PS4_P	LPATH	PS4_P→PS4_M	LPATH
c.389G>A (p.R130Q)	PS3, PM6_S, PM1, PM2, PP2, PS4_P	PATH	PS4_P→PS4, PM6_S→PM6_VS	PATH
c.407G>A (p.C136Y)	PS2, PS3, PM2, PP2, PS4_P	PATH		PATH
c.517C>T (p.R173C)	PS3, PM6_S, PM2, PP1, PP2, PS4_P	PATH	PS4_P→PS4_M, PM6_S→PM6_VS	PATH
c.737C>T (p.P246L)	**PS2, PM2, PP2, PS4_P**	**LPATH**	**PS4_P→PS4, PS2→PS2_VS**	**PATH**

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Table 6:

Test Set of Variants with Uncertain Significance (VUS) or Conflicting (CONF) ClinVar Assertions

PTEN Variant	ClinVar Status (as of 10.29.17)	Curator 1 Criteria	Curator 1 Classification	Curator 2 Criteria	Curator 2 Classification	Final EP Criteria	PTEN EP Classification
c.−1170C>T (NC_000010.10: g.89623056C>T))	VUS (3 submitters)	--	VUS	--	VUS	BP5	VUS
c.209+3A>T (IVS3+3A>T)	VUS (2 submitters)	PM2, PP3	VUS	PM2, PP3	VUS	PM2, PP3	VUS
c.235G>A (p.A79T)	**VUS (5 submitters)**	**PP2**	**VUS**	**BS1, PP2**	**VUS**	**BS1, BS2_P, PP2**	**LBEN**
c.304_306dupAAA (p.K102_P103insK)	VUS (2 submitters)	PM2	VUS	PM2	VUS	PM2	VUS
c.1052_1054delTAG (p.V351del)	VUS (2 submitters)	PM2	VUS	PM2	VUS	PM2	VUS
c.1171C>T (p.P391S)	VUS (3 submitters)	PM2, PP2	VUS	PM2, PP2	VUS	PM2, PP2	VUS
c.−764G>A (NC_000010.10:g.89623462G>A)	CONF (1 submitter PATH, 2 VUS)	PM2	VUS	PM2, PM6, PS3_P	VUS	PM2	VUS
c.44G>A (p.R15K)	CONF (1 submitter LPATH, 1 VUS)	PM2, PP2	VUS	PS3, PM2, PP2	LPATH	PM2, PS3_P, PP2	VUS
c.78C>T (p.T26=)	CONF (1 submitter VUS, 2 LBEN)	PM2, BP7	VUS	PM2, BP7	VUS	PM2, BP7	VUS
c.209+4_209+7delAGTA (IVS3+4_IVS3+7delAGTA)	**CONF (2 submitters PATH, 1 VUS)**	**PS3, PM2, PS4_P**	**LPATH**	**PS3, PM2, PM6_S, PP1, PS4_P**	**PATH**	**PS3, PM6_S, PP1_M, PM2, PS4_P**	**PATH**
c.521A>G (p.Y174C)	**CONF (1 submitter PATH, 2 VUS)**	**PS2, PM2, PP2, PS4_P**	**LPATH**	**PS2, PM2, PP2, PS4_P**	**LPATH**	**PS2, PM2, PP2, PS4_P**	**LPATH**

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