

A Clinical Scoring System for Selection of Patients for *PTEN* Mutation Testing Is Proposed on the Basis of a Prospective Study of 3042 Proband

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Cowden syndrome (CS) and Bannayan-Riley-Ruvalcaba syndrome are allelic, defined by germline *PTEN* mutations, and collectively referred to as *PTEN* hamartoma tumor syndrome. To date, there are no existing criteria based on large prospective patient cohorts to select patients for *PTEN* mutation testing. To address these issues, we conducted a multicenter prospective study in which 3042 probands satisfying relaxed CS clinical criteria were accrued. *PTEN* mutation scanning, including promoter and large deletion analysis, was performed for all subjects. Pathogenic mutations were identified in 290 individuals (9.5%). To evaluate clinical phenotype and *PTEN* genotype against protein expression, we performed immunoblotting (*PTEN*, P-AKT1, P-MAPK1/2) for a patient subset ($n = 423$). In order to obtain an individualized estimation of pretest probability of germline *PTEN* mutation, we developed an optimized clinical practice model to identify adult and pediatric patients. For adults, a semiquantitative score—the Cleveland Clinic (CC) score—resulted in a well-calibrated estimation of pretest probability of *PTEN* status. Overall, decreased *PTEN* protein expression correlated with *PTEN* mutation status; decreasing *PTEN* protein expression correlated with increasing CC score ($p < 0.001$), but not with the National Comprehensive Cancer Network (NCCN) criteria ($p = 0.11$). For pediatric patients, we identified highly sensitive criteria to guide *PTEN* mutation testing, with phenotypic features distinct from the adult setting. Our model improved sensitivity and positive predictive value for germline *PTEN* mutation relative to the NCCN 2010 criteria in both cohorts. We present the first evidence-based clinical practice model to select patients for genetics referral and *PTEN* mutation testing, further supported biologically by protein correlation.

Introduction

Cowden syndrome (CS [MIM 158350]), presenting in adulthood, and Bannayan-Riley-Ruvalcaba syndrome (BRRS [MIM 153480]),¹ a pediatric syndrome, show overlapping clinical features and may present with multi-system disease, including macrocephaly, various cancers, and skin, neurologic, and gastrointestinal manifestations.^{2,3} Because subsets of these two syndromes, together with other seemingly unrelated clinical syndromes,⁴ share a common etiology germline *PTEN* (MIM 601728) mutation,² they are allelic and collectively referred to as *PTEN* hamartoma tumor syndrome (PHTS).⁵ Inheritance of this disorder is autosomal dominant, and penetrance is believed to be high (around 80%).⁵ The *PTEN* tumor suppressor gene, located on 10q23.3, encodes a dual-specificity phosphatase that can dephosphorylate both protein⁶ and phospholipid substrates.⁷

We formulated the International Cowden Consortium (ICC) operational diagnostic criteria⁸ 14 years ago to select families and affected individuals for purposes of identifying the specific mutated gene. Over the last 10 years, we have continually revised this set of criteria for

referral of patients for clinical germline *PTEN* mutation testing,^{9,10} with early expert opinion and clinical data derived from families studied from initial consortium studies suggesting that 85% of patients with CS had an identifiable *PTEN* mutation.^{2,11} The most recent National Comprehensive Cancer Network (NCCN) 2010 criteria,¹² based primarily on these operational criteria, are useful, but they also have several disadvantages. These include the inability to quantitatively evaluate individual patients for their probabilities of testing positive for a *PTEN* mutation. Additionally, with the multisystem involvement of Cowden syndrome and rapid expansion of the clinical spectrum to include phenotypes such as autism¹³ and polyposis syndrome,¹⁴ the complexity of the current NCCN criteria involving multiple possible combinations of major and minor criteria render them challenging for use outside of a specialist community. Further, the current NCCN criteria is not refined for adult and pediatric populations, considering that CS and BRRS are clinically and epidemiologically distinct as a result of age-related penetrance and variable expression, even of the common underlying *PTEN* mutation.³ Finally, we note that there are no existing criteria based on large prospective patient

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cohorts for selection of patients for *PTEN* mutation testing.

Concurrently, advances in the understanding of the biology of *PTEN* in a patient-oriented setting have been inhibited by the lack of a common reference for measuring severity of phenotypes arising from *PTEN* protein deficiency or dysfunction. This is crucial, given insights supported by increasing laboratory evidence on the importance of tumor suppressor gene dosage. Small changes in the expression levels of tumor suppressor genes have been proposed to influence susceptibility to cancer,¹⁵ and there is experimental support from animal experiments to support a view that subtle variations of *Pten* dosage may also result in increased cancer susceptibility.¹⁶ Nonetheless, to date, there has been no evidence in the setting of a human population to support this view. From a molecular angle, as a lipid phosphatase that dephosphorylates phosphatidylinositol-3,4,5-triphosphate (PIP3) to phosphatidylinositol-4,5-phosphate (PIP2), *PTEN* is the key negative regulator of the phosphatidylinositol-3-kinase (PI3K) signal transduction cascade, inhibiting pathways of growth, proliferation, and survival.¹⁷

PTEN inactivation is associated with the activation of the AKT/mTOR signaling pathway when *PTEN*'s lipid phosphatase is most relevant and with the mitogen-activated protein kinase (MAPK) signaling pathways with activated MAPK1/2 when *PTEN*'s protein phosphatase pathway is more relevant.^{18–20}

We thus conducted a prospective multicenter, multinational study, collecting phenotypic data on patients who met relaxed criteria for CS,²¹ in order to quantitatively assess both adult and pediatric patients for a priori risk of *PTEN* germline mutation in two large patient cohorts with a semiquantitative score. Subsequently, we evaluated whether there was correlation between *PTEN* protein dosage and the clinical score.

Subjects and Methods

Research Participants

For the two independent cohorts from the Cleveland Clinic (CC) and The Ohio State University (OSU; C.E., Principal Investigator), a total of 3042 patients was recruited into a protocol approved by the Institutional Review Boards for Protection of Human Subjects from both institutions (CC: 2005–2010; OSU: 2000–2006). Individuals who were probands and who met relaxed International Cowden Consortium operational criteria for CS (pathognomonic criteria, or at least two criteria, either major or minor)^{9,10} (Table 1) were recruited prospectively for these cohorts (Table 2). These patients were recruited from both community and academic medical centers throughout North America, Europe, and Asia. Upon providing informed consent, checklists to document presence or absence of specific features were completed by specialist genetic counselors or physicians concurrently with submission of samples. Specialist genetics staff reviewed all checklists and corresponded with the enrolling center; if necessary, further primary documentation of medical records was obtained for phenotype confirmation with patient consent.

Table 1. Operational Criteria for Cowden Syndrome without Family History of Known *PTEN* Mutation

Pathognomonic Criteria
Adult Lhermitte-Duclos disease (cerebellar tumors)
Mucocutaneous lesions ^a
- Facial trichilemmomas, any number ^a (at least two biopsy-proven trichilemmomas ^b)
- Acral keratoses
- Papillomatous papules
Mucosal lesions
Autism spectrum disorder and macrocephaly ^b
Major Criteria
Breast cancer
Thyroid cancer (nonmedullary)
Macrocephaly (megalcephaly) (i.e., 97 th percentile and above)
Endometrial cancer
Mucocutaneous lesions ^b
- One biopsy-proven trichilemmoma
- Multiple palmoplantar keratoses
- Multifocal cutaneous facial papules
- Macular pigmentation of glans penis
Multiple GI hamartomas or ganglioneuromas ^b
Minor Criteria
Other thyroid lesions (e.g., adenoma, multinodular goiter)
Mental retardation (i.e., IQ of 75 and below)
Gastrointestinal hamartomas ^a (single gastrointestinal hamartoma or ganglioneuroma ^b)
Fibrocystic disease of the breast
Lipomas
Fibromas
Genitourinary tumors (especially renal cell carcinoma)
Genitourinary malformations ^a
Uterine fibroids
Autism spectrum disorder ^b

^a Present in this section as defined by ICC criteria only.

^b Present in this section as defined by NCCN 2010 criteria only.

PTEN Mutation and Deletion Analysis

To understand the relationship between *PTEN* (NM_000314.4) mutations and the clinical phenotype, we had all subjects undergo *PTEN* mutation analysis, as described below. Genomic DNA was extracted from peripheral blood leukocytes via standard methods.²² Scanning of genomic DNA samples for *PTEN* mutations was performed as previously reported with a combination of denaturing gradient gel electrophoresis, high-resolution melting curve analysis (Idaho Technology), and direct Sanger sequencing (ABI 3730xl).²³ Deletion analysis with the multiplex ligation-dependent probe amplification (MLPA) assay²⁴ was performed with the P158 MLPA kit (MRC-Holland) according to

Table 2. Baseline Data for the Study Cohorts

	CC Adult Cohort		CC Adult Mutation Cohort		CC Pediatric Cohort		CC Pediatric Mutation Cohort		OSU Cohort		PTEN Mutation Cohort (OSU)	
		%		%		%		%		%		%
Number	2007		105		92		28		943		157	
Age, median (range)	55.9 (18.0–98.2)		43.9 (3.0–78)		8.2 (1.8–16.9)		8.3 (3.0–16.9)		48.5 (1.2–91.7)		36.1 (1.7–73.1)	
Gender, male	117	5.8	34	32.4	59	64	21	75	164	17.4	70	44.6
Gender, female	1890	94.2	71	67.6	33	36	7	25	779	82.6	87	55.4
Neurological												
Macrocephaly, presence	538	26.8	79	75.2	87	95	28	100	385	40.8	135	86
Extreme macrocephaly (male, ≥ 63 cm)	15	13	11	32	2	3	2	10				
Extreme macrocephaly (female, ≥ 60 cm)	67	4	24	34	4	12	2	29				
Lhermitte Duclos disease	17	0.8	9	8.6	1	1	0	0	24	2.5	9	5.7
Autism or developmental delay	41	2	13	12.4	78	85	23	82	115	12.2	34	21.7
Breast and Gynecological												
Invasive breast cancer												
<30	29	1.5	1	1.4	0	0	0	0	7	0.9	2	2.3
30–39	164	8.7	6	8.5	0	0	0	0	76	9.8	6	6.9
40–49	475	25.1	13	18.3	0	0	0	0	160	20.5	10	11.5
≥ 50	625	33.1	14	19.7	0	0	0	0	179	23	8	9.2
Fibrocystic breast disease	849	44.9	30	42.3	0	0	0	0	269	34.5	23	26.4
Endometrial cancer												
<30	11	0.6	1	1.4	0	0	0	0	2	0.3	1	1.1
30–39	23	1.2	2	2.8	0	0	0	0	7	0.9	3	3.4
40–49	50	2.6	5	7	0	0	0	0	21	2.7	7	8
≥ 50	171	9	5	7	0	0	0	0	44	5.6	1	1.1
Fibroids	794	42	27	38	0	0	0	0	290	37.2	18	20.7
Gastrointestinal												
Colorectal cancer												
<30	2	0.1	0	0	0	0	0	0	0	0	0	0
30–39	3	0.1	1	1	0	0	0	0	1	0.1	0	0
40–49	15	0.7	3	2.9	0	0	0	0	5	0.5	2	1.3
≥ 50	33	1.6	2	1.9	0	0	0	0	10	1.1	0	0
Polyposis syndrome (≥ 5)	74	3.7	36	34.3	2	2	2	7	168	17.8	51	32.5
Intestinal hamartoma, single	23	1.1	16	15.2	5	5	3	11	11	1.2	10	6.4
Intestinal hamartoma, multiple ^a	11	0.5	8	7.6	0	0	0	0				
Intestinal ganglioneuroma, single	14	0.7	9	8.6	2	2	2	7	11	1.2	5	3.2
Intestinal ganglioneuroma, multiple ^a	10	0.5	7	6.7	0	0	0	0				
Glycogenic acanthosis	21	1	16	15.2	1	1	1	4	14	1.5	9	5.7

Table 2. Continued

	CC Adult Cohort		CC Adult Mutation Cohort		CC Pediatric Cohort		CC Pediatric Mutation Cohort		OSU Cohort		PTEN Mutation Cohort (OSU)	
		%		%		%		%		%		%
Skin												
Trichilemmomas, biopsy proven	13	0.6	6	5.7	0	0	0	0	112	11.9	39	24.8
Oral papillomas	160	8	16	15.2	3	3	1	4	210	22.3	72	45.9
Penile freckling	22	18.8	10	29.4	16	27	5	24	61	37.2	38	54.3
Acral keratoses	128	6.4	28	26.7	5	5	2	7	54	5.7	16	10.2
Arteriovenous malformations	19	0.9	12	11.4	4	4	2	7	14	1.5	10	6.4
Skin lipomas	528	26.3	45	42.9	25	27	13	46	336	35.6	89	56.7
Fibromas	121	6	17	16.2	0	0	0	0	2	0.2	1	0.6
Endocrine												
Thyroid cancer												
<20	18	0.9	5	4.8	2	2	0	0	11	1.2	3	1.9
20–29	56	2.8	3	2.9	0	0	0	0	16	1.7	1	0.6
30–39	116	5.8	7	6.7	0	0	0	0	58	6.2	4	2.5
40–49	149	7.4	8	7.6	0	0	0	0	45	4.8	3	1.9
≥ 50	203	10.1	4	3.8	0	0	0	0	74	7.8	1	0.6
Thyroid goiter, nodules, or adenomas	710	35.4	72	68.6	4	4	3	11	182	19.3	48	30.6
Hashimoto's thyroiditis	151	7.5	22	21	0	0	0	0	19	2	5	3.2
Genitourinary												
Renal cell carcinoma	94	4.7	7	6.7	1	1	0	0	43	4.6	5	3.2
Testicular germ cell tumor	4	3.4	2	5.9	0	0	0	0	1	0.6	1	1.4
Ovarian germ cell tumor	5	0.3	2	2.8	0	0	0	0	1	0.1	1	1.1
Congenital genitourinary malformations	58	2.9	6	5.7	15	16	8	29	14	1.5	2	1.3

^a Data on specific head size measurement and exact polyp number are not available in the OSU cohort. For intestinal hamartoma and ganglioneuroma, OSU data are therefore presented without breakdown.

manufacturer's protocol. All patients underwent resequencing of the *PTEN* promoter region as previously described.²⁵ The primers used are available in Table S1 available online. Promoter mutations were defined as previously reported,^{11,25} except for -1084T>C; this variant has been reported in population controls of European descent (2/150),²⁶ and further work is required to characterize it.

Analysis of PTEN and Other Downstream Proteins by Immunoblotting

Human immortalized lymphoblast-derived cell lines were obtained from each patient and cultured in RPMI-1640 supplemented with 20% fetal bovine serum. All cell lines were cultured at 37°C and 5% CO₂.²² Whole-cell lysates were prepared with Mammalian Protein Extraction Reagent (Pierce) supplemented with protease inhibitor cocktail (Sigma-Aldrich). Lysates were separated by SDS-PAGE and transferred onto nitrocellulose. Antibodies used included anti-PTEN mouse monoclonal (Cascade Biosciences) at 1:5000, anti-phospho-AKT1 rabbit polyclonal

(Cell Signaling) at 1:1000, anti-phospho-MAPK1/2 rabbit polyclonal (Cell Signaling) at 1:2000, anti-GAPDH rabbit monoclonal (Cell Signaling) at 1:20,000, and anti-actin mouse monoclonal at 1:20,000. The blots were scanned digitally with the Odyssey Imaging System (Li-Cor Biotechnology). Detected fluorescence intensities for protein bands were background adjusted and normalized between gels with the median expression of individual proteins on each blot.

Statistical Methods for Score Derivation and Validation

Risk data for the various phenotypes were obtained from a variety of sources. For breast, endometrial, and thyroid cancer, age-adjusted cumulative risk data were drawn from the Surveillance Epidemiology and End Results database. For other phenotypes, corresponding prevalence data were derived from the literature, with North American reports preferred: oral papillomas,²⁷ arteriovenous malformations,²⁸ autism/developmental delay,²⁹ thyroid

goiter,³⁰ and fibroids.³¹ Where no standard population-based estimates were available, baseline prevalences were estimated based on best available expert opinion; where rarity of a feature precluded reliable estimates, an estimate of 0.01% baseline prevalence was used. Patients were classified into adult and pediatric age groups at a threshold of 18 years of age. Frequency data were generated from the CC data set, with corresponding relative risks (RR) calculated relative to baseline population risk. For the pediatric CC data set, a relatively high prevalence of *PTEN* mutations allowed ready derivation of highly predictive features from the relative frequencies of cases, and validation in the external OSU data set was directly performed. We refer to the criteria developed here in the pediatric setting as the CC criteria, which is distinct from the CC score for adults. For the adult data set, which was considerably larger, derivation of a score was more challenging. This may be due to a referral bias, which increased recruitment for patients with oncologic conditions, preventing the use of conventional regression modeling. Hence, we adopted a clinically driven modeling approach for data reduction and validation for weighing adult criteria with the CC data set, validating our approach in the separate OSU data set.³²

We first selected the most predictive phenotypic features at initial clinical assessment, excluding certain characteristic dermatologic features of CS such as fibromas or skin (but not oral) papillomas due to loss of specificity in a community setting. For each feature, we considered the relative frequency against baseline, potential for referral bias (most evident in the cancers), and the prevalence of each feature. We weighted each feature with a prespecified approach: an RR relative to baseline of 1–5 corresponded to a weight of 1; RR of 5–10 with a weight of 2; RR of 10–25 with a weight of 4; RR of 25–50 with a weight of 6; RR of 50–100 with a weight of 8; RR > 100 with a weight of 10. High referral bias for individual features was recognized clinically, being additionally evident through relatively high RR when comparing prevalences in the study population relative to the community. We adjusted the weights for these features empirically through reduction by one risk tier, except where the weight was already at the lowest tier of 1, whereupon it was maintained. A score for each individual was obtained by the sum of weights of all positive features. We subsequently performed internal validation of this semiquantitative score in the CC and external validation in the OSU adult data set. All corresponding data fields were available in both data sets, with the exception of data on extreme macrocephaly and exact gastrointestinal polyp number in the OSU data set. Consequently, when evaluating the score performance in the OSU data set, we omitted extreme macrocephaly and matched polyp presence status with corresponding multiple polyp status. These changes would be expected to penalize the accuracy of the CC score relative to the NCCN criteria in the OSU data set; thus, our validation represents a conservative assessment of the CC score's performance. Performance of this score in both data sets was evaluated via measurements of sensitivity, specificity, accuracy, positive and negative predictive value, and receiver-operator characteristic (ROC) methodology. Likelihood-ratio analysis of each model nested in a summary model^{32,33} was performed to compare the performance of the CC score against the NCCN criteria. Calibration to obtain bias-corrected estimates of predicted versus observed values was performed with 200 bootstraps. All analyses were performed with R 2.11.1.³⁴ For analysis correlating protein quantitation with *PTEN* variants, four sample groups were defined (wild-type, common variants of unknown significance [or SNPs; >1% prevalence], rare variants of unknown significance [<1%],

and mutations), analysis of variance (ANOVA) testing was used for comparison between multiple groups, and t tests were used for two-group comparisons. Linear regression was used in evaluating the association between the CC score and *PTEN* protein. All tests were two-sided, and $p < 0.05$ was deemed significant.

Results

PTEN Mutation Spectrum in Patients

Of the 3042 individuals tested, 290 (9.5%) were found to have germline pathogenic *PTEN* mutations (Table S2). To be conservative, we excluded variants of unknown significance without proof of functionality from the mutation-positive group. In total, 85 (29%) missense mutations, 92 (32%) nonsense mutations, 42 (14%) small deletions, 24 (8%) small insertions, 3 (1%) indels, 8 (3%) large deletions, 19 (7%) splice-site donor mutations, 9 (3%) splice-site acceptor mutations, and 8 (3%) promoter mutations were found. The mutation spectrum for the 290 probands (Figure 1) reveals mutations that are distributed irregularly across all exons, with hot spots in exon 5 ($n = 93$, 32%), exon 7 ($n = 37$, 13%), and exon 8 ($n = 45$, 16%). A total of 45 mutations (16%) occurred within the exon 5 hot spot (aa 123–130), corresponding to the catalytic motif of the N-terminal phosphatase domain. A total of 101 mutations (35%) occurred within the C2 domain of the *PTEN* protein (aa 186–351).

Pediatric Clinical Criteria Derivation

To derive appropriate clinical criteria to guide selection for *PTEN* testing, we analyzed genotype and phenotype data from pediatric (<18 years) and adult individuals separately, focusing on the CC data set (Table 2) to generate the criteria for validation testing in the OSU data set. For pediatric individuals, we determined that the presence of macrocephaly (occipitofrontal circumference [OFC] > 2 standard deviation [SD] over the population mean, or 97.5th percentile) was a necessary criterion for diagnosis, based on 100% prevalence at the point of diagnosis (Table 3). Neurologic (autism and developmental delay) and dermatologic (lipomas, oral papillomas) features represented extremely common secondary features; either or both systems were involved in 100% of patients with germline *PTEN* mutation. However, given that dermatologic features may often be overlooked, less-prevalent features in patients at first presentation in the pediatric setting are likely to be at least as important, such as vascular (such as arteriovenous) malformations, gastrointestinal polyps, thyroid goiter, and early-onset cancers (thyroid and germ cell).

Adult Clinical Score Derivation

For adults, additional weighing of the criteria was performed, resulting in the derivation of an appropriately weighted semiquantitative scoring system, referred to here as the CC score (Table 4). The generated score is useful for the calculation of point pretest probability of *PTEN*

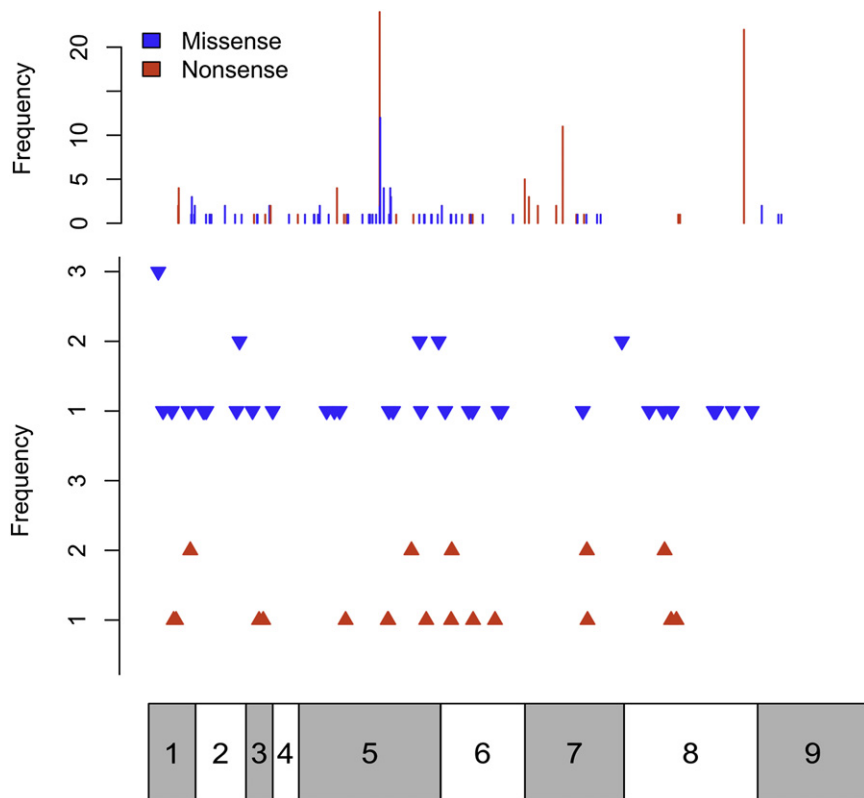


Figure 1. Consolidated *PTEN* Mutation Spectrum

Distribution and number of substitutions (missense and/or nonsense), small insertion mutations, and small deletion mutations across the gene. In the top panel, blue bars represent missense mutations and red bars represent nonsense mutations. In the second panel, the blue arrowheads represent small deletions and the red arrowheads represent small insertions along the gene. Complex mutations (indels, splice-site mutations, and large deletions) and promoter mutations are not depicted. For both panels, frequencies of both the substitution mutations and the insertion/deletion mutations are shown on the left. The bar below corresponds to the multiple exons of the *PTEN* cDNA molecule, with exon 1 on the left to exon 9 on the right, allowing for matching of mutation to exon. As evident, exons 5, 7, and 8 are sites of common mutations.

mutation. It is presented together with examples to illustrate its use (Figure 2). Increasing risk score was strongly associated with mutation-positive status ($p < 0.001$, Mann-Whitney test). ROC-based analysis demonstrates the variation of sensitivity and specificity in both data sets at multiple thresholds (Figures S1 and S2). At a threshold CC score of 10 (corresponding to a point pretest probability of approximately 3%; including and

above the threshold score), sensitivity for diagnosis is 90%. Sensitivity falls accordingly as the threshold for CC score rises, so that when the threshold CC score is 15 (point pretest probability of 10%), the sensitivity is 72%. We report better performance for our overall approach relative to the NCCN 2010 criteria via conventional clinical and epidemiologic measures (Table 5). To evaluate for overfitting in the CC score, we used bootstrap validation on the resulting score within the CC data set ($n = 200$), demonstrating excellent calibration (Figure 3) and yielding an optimism-corrected concordance index of 0.91. Formal likelihood-ratio testing demonstrated considerably higher adequacy and strongly significant statistical benefit for the CC score relative to the NCCN criteria (Figure 4). The performance of the CC scoring system exceeded that of the NCCN criteria in cohorts from both centers, consistently showing superior predictive power, concordance indices, sensitivity, and specificity for the detection of *PTEN* mutations relative to the NCCN 2010 criteria.

***PTEN* Genotype, Clinical Score, and Downstream Pathway Proteins**

We next sought to determine whether there was a correlation between *PTEN* mutation status and expression of proteins considered to be downstream readouts for *PTEN* signaling. For *PTEN* mutant patient-derived lymphoblasts ($n = 24$), *PTEN* protein was decreased and P-AKT1 was relatively increased (Figure 5) compared to wild-type *PTEN*, common variants (SNPs), and rare variants of unknown

Table 3. Pediatric Clinical Criteria for *PTEN* Testing^a

Clinical Features	Percent Prevalence in CC Data Set of Pediatric Probands with <i>PTEN</i> Mutation
1. Macrocephaly (≥ 2 SD)	100%
2. At least one of the following four additional criteria should be present:	
- Autism or developmental delay	82%
- Dermatologic features, including lipomas, trichilemmomas, oral papillomas, penile freckling	60%
- Vascular features, such as arteriovenous malformations or hemangiomas	29%
- Gastrointestinal polyps	14%

^a In addition, pediatric-onset thyroid cancer and germ cell tumors (testicular cancer and dysgerminoma) are recognized associations of Cowden syndrome and should provoke consideration of *PTEN* testing.

Table 4. CC Adult Score Derivation

	Population Risk	Relative Risk (Referral/Community)	Relative Risk (Mutants/Community)	Weight
Neurological				
Macrocephaly, presence	2	10.7	30.1	6
Extreme (male, OFC \geq 63 cm)	0.2	130	160	10
Extreme (female, OFC \geq 60 cm)	0.2	20	170	10
Lhermitte Duclos disease	0.01	80	860	10
Autism or developmental delay	5	0.2	1.2	1
Breast and Gynecological				
Invasive breast cancer				
<30	0.06	23.3	23.3	4
30–39	0.43	20.2	19.8	4
40–49 ^a	1.45	17.3	12.6	2
\geq 50	10.93	3	1.8	1
Fibrocystic breast disease	10	4.5	4.2	1
Endometrial cancer				
20–29	0.01	50	140	10
30–39	0.06	20	46.7	6
40–49	0.19	13.7	36.8	6
\geq 50	2.43	3.7	2.9	1
Fibroids	13	3.2	2.9	1
Gastrointestinal				
Polyposis syndrome (five or more, any type)	1	3.7	34.3	6
Intestinal hamartoma or ganglioneuroma, any number	0.01			10
Glycogenic acanthosis	0.01	100	1520	10
Skin				
Trichilemmomas, biopsy proven	0.01	60	570	10
Oral papillomas	0.46	17.4	33	6
Penile freckling	1	18.8	29.4	6
Acral keratoses	10	0.6	2.7	1
Arteriovenous malformations	0.25	3.6	45.6	6
Skin lipomas	20	1.3	2.1	1
Endocrine				
Thyroid cancer				
<20	0.01	90	480	10
20–29 ^a	0.06	46.7	48.3	4
30–39 ^a	0.13	44.6	51.5 ^b	4
40–49 ^a	0.17	43.5	44.7	4
\geq 50 ^a	0.57	17.7	6.7	1

(Continued on next page)

Table 4. Continued

	Population Risk	Relative Risk (Referral/Community)	Relative Risk (Mutants/Community)	Weight
Thyroid goiter, nodules, adenomas, or Hashimoto's thyroiditis (one or more features)	5	7.1	14.4	4
Genitourinary				
Renal cell carcinoma	1.49	3.2	4.5	1

^a For these specific features, referral bias was clinically recognized and accounted for by a standard downward modification of weight by a risk tier; this bias may also be recognized in a high RR (study population/community, relative to the RR in patients with *PTEN* mutations/community). Individual features for which high referral bias was evident, but where weight was already 1, were not further adjusted downward.

^b For this borderline RR just above the threshold of 50, given the relatively consistent RRs in adjacent groups for all populations, this weight was adjusted to 4 for consistency between the adjacent age groups.

significance, whereas no significant overall difference was seen for P-MAPK1/P-MAPK2, actin, or GAPDH. It was noted on inspection that PTEN and P-AKT1 protein expression for the rare *PTEN* variants was intermediate between that of mutant and wild-type samples, although there was no statistical significance discerned between the protein profiles of rare variant lymphoblasts (n = 15) and the other groups of samples.

Finally, we evaluated whether an association existed between PTEN/P-AKT1 protein expression and CC score, which might serve as a potential index of phenotypic load of syndromic features. When analyzing the full data set, a strong inverse association was apparent between PTEN protein expression and CC score (p < 0.001; Figure 6). This association remained (p = 0.036) after excluding patients with pathogenic mutations (Figure 7). No correlation between P-AKT1 and CC score was noted in any subgroup of individuals.

Discussion

The criteria described here represent the collective experience derived from the largest prospective cohort of

patients with germline pathogenic *PTEN* mutations to date (290 probands) with standard-of-care testing. This represents a core strength of this study. To our knowledge, outside of these two specialist centers, the largest single reported series from a single center includes 21 patients,³⁵ and, to date, a total of 211 patients (both probands and affected family members) have been reported in the entire published medical literature.³⁵ Our study is limited to probands only, this being critical for an accurate evaluation of the relative importance of each feature in initial screening through the reduction of ascertainment bias. In terms of the mutation spectra, our summary study confirms our previously reported profiles from smaller data sets.³⁶

Pediatric Criteria

The classification of our recommendations for *PTEN* mutation testing into distinct adult and pediatric criteria has demonstrated considerable clinical utility in our cohorts. This success is likely multifactorial in nature, with reasons including age-specific phenotypic penetrance and, importantly, the different health services utilized in the distinct age groups: for example,

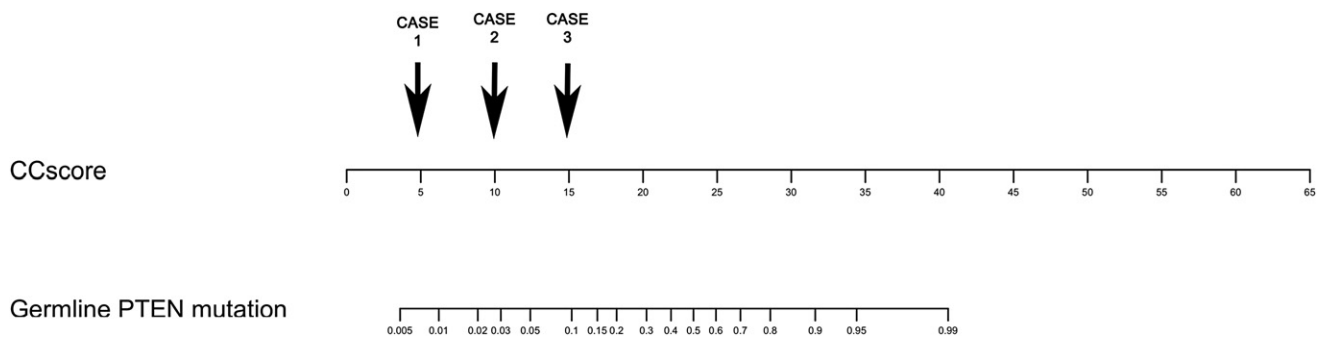


Figure 2. CC Score Nomogram for Obtaining a Corresponding Individual Point Pretest Probability of Germline *PTEN* Mutation
 The CC score is first derived by a sum of the weights of positive features that is provided in Table 4. To illustrate, three hypothetical cases are presented, each corresponding to a CC score of 5 points, 10 points, and 15 points, respectively. Case 1 may present with breast cancer at age 55 (1 point), with background of thyroid cancer at age 44 (4 points), with a final score of 5 and corresponding point probability < 1%. Case 2 may present with breast cancer at age 38 (4 points) and concurrent macrocephaly (6 points), with a final score of 10 and corresponding point probability of 3%. Case 3 may present with a single hamartomatous gastrointestinal polyp (10 points) found on endoscopy, Hashimoto's thyroiditis (4 points), and lipomas (1 point), for a final score of 15 and corresponding point probability of 10%.

Table 5. Comparison of the CC Pediatric Criteria and Adult Score Relative to the NCCN 2010 Criteria

		Cleveland Clinic		Ohio State University	
		Nonmutant	Mutant	Nonmutant	Mutant
Adult					
CC score ^a	Fails threshold	1426	10	445	8
	Meets threshold	476	95	267	102
	Sensitivity/specificity		90%/75%		93%/62%
	Concordance index	0.83 ^c	0.83 ^c	0.78	0.78
NCCN criteria ^b	Fails criteria	1310	27	474	17
	Meets criteria	592	78	238	93
	Sensitivity/specificity		74%/69%		85%/67%
	Concordance index	0.72	0.72	0.76	0.76
Pediatric					
CC criteria ^a	Fails threshold	6	0	7	1
	Meets threshold	58	28	68	46
	Sensitivity/specificity		100%/10%		98%/9%
NCCN criteria ^b	Fails criteria	5	3	56	20
	Meets criteria	59	25	19	27
	Sensitivity/specificity		89%/8%		56%/74%
Overall					
CC score ^a	Fails threshold	1432	10	493	11
	Meets threshold	533	124	294	146
	Sensitivity/specificity		93%/73%		93%/63%
	Concordance index	0.83 ^c	0.83 ^c	0.76	0.76
NCCN criteria ^b	Fails criteria	1315	30	530	37
	Meets criteria	650	104	257	120
	Sensitivity/specificity		78%/67%		76%/67%
	Concordance index	0.72	0.72	0.72	0.72

^a For adults, using a threshold Cleveland Clinic (CC) score of 10 points and above for recommendation of *PTEN* testing.

^b National Comprehensive Cancer Network (NCCN) criteria, 2010.

^c Optimism corrected with 200 bootstraps.

macrocephaly and delayed development are well-established clinical problems for pediatricians who use head circumference measurements routinely, whereas cancers and polyps are common presenting problems in adult medicine. Indeed, it is possible that patients who present in childhood are more readily identified than adult patients, given that features such as macrocephaly and autism are likely to result in early consults with pediatricians, who are often familiar with issues of genetics. At the same time, patients presenting and being diagnosed during adulthood with *PTEN* mutations tend to have fewer overt disease manifestations because of many reasons, including a selection bias, in which more severely affected individuals may already have been diagnosed during childhood, true age-related penetrance, and less physician familiarity. In terms of the

phenotypic spectrum, the particularly high prevalence of neurologic features such as autism and developmental delay (>80%) validate early observations by our group,¹³ demonstrating previously unrecognized *PTEN* mutations in patients with autism spectrum disorder and macrocephaly. The inclusion of this feature into our clinical criteria is particularly noteworthy, because autism was not hitherto recognized in pediatric patients with BRRS, and germline *PTEN* mutation is now recognized as one of the most common single gene causes of autism.^{26,37} Thus, the pediatric CC criteria that we have developed de novo here, maximizing sensitivity, represents an important guideline for pediatricians to select children for referral to genetic professionals for *PTEN* mutation testing. Such an approach is aided by the routine use of head

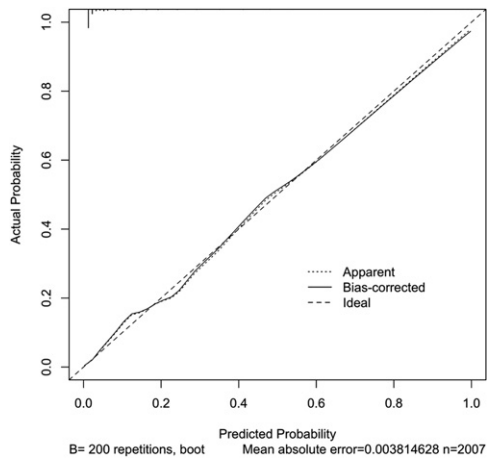


Figure 3. A Calibration Plot for Model-Based Predicted Probabilities of *PTEN* Mutation and Actual Outcomes

The calibration plot shows excellent bias-corrected correlation between observed and predicted values for the developed model within the CC data set, suggesting good internal calibration for the CC score model, that is, individual predicted and actual outcomes are similar. The dashed line at 45° ($y = x$) represents ideal agreement between observed and predicted probabilities of *PTEN* mutation.

circumference measurements, and this macrocephaly is present in all patients diagnosed in childhood. Indeed, because macrocephaly is similarly present in almost all

adult patients, it is likely that active evaluation of pediatric patients with macrocephaly may result in earlier diagnosis.

Adult Clinical Scoring

For adult patients, our study establishes useful clinical thresholds that can be readily translated to community practice for referral of patients for specialist evaluation. We recommend a threshold CC score of 10 (or a point pretest probability of 3%) and above for referral to medical genetics for specialist evaluation, this permitting a sensitivity of at least 90%. We present a range of scenarios that would satisfy this threshold. It is clear that age of presentation for cancer profoundly influences risk: patients presenting with endometrial, thyroid, or breast cancer below the age of 40 must be evaluated for other suggestive features of germline *PTEN* mutation, particularly macrocephaly. For patients presenting with first onset of cancer above 40, combinations of the above cancers, together with other suggestive nonmalignant features, are more common. Similarly, gastroenterologists or surgeons who encounter patients with polyposis syndromes (at least five polyps) should also assess the past medical history of these individuals for the presence of other types of cancer, particularly breast, endometrial, or thyroid cancer, or relevant combinations. These

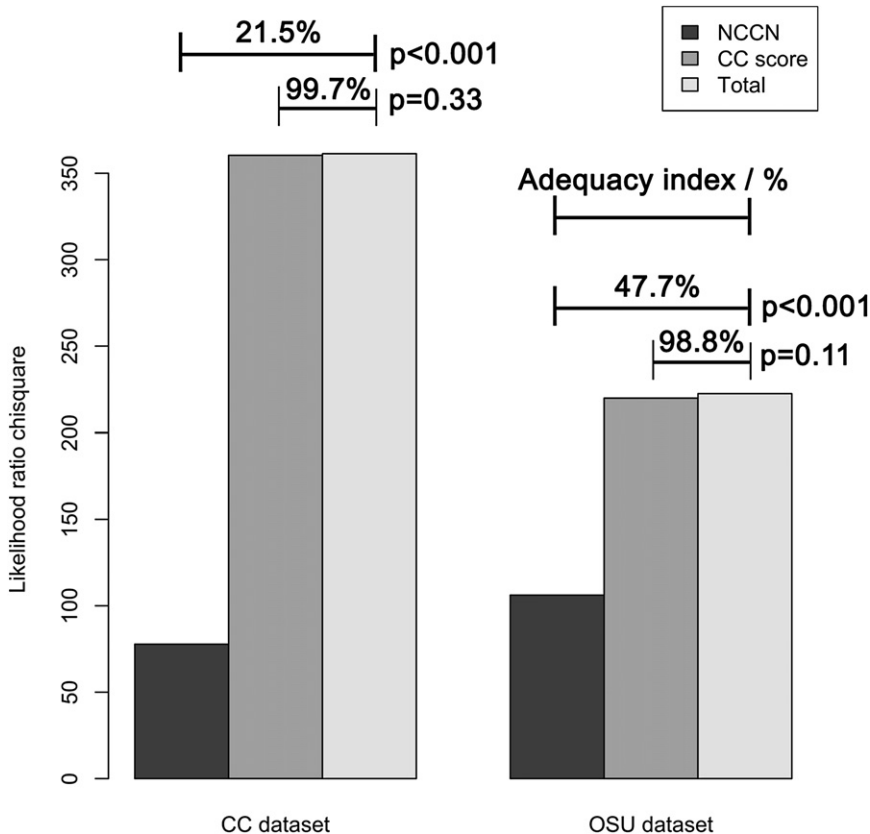


Figure 4. A Graph Showing that the CC Score Yields Superior Predictions Relative to the NCCN Criteria in Both the CC and the OSU Data Sets

Each data set shows corresponding likelihood ratio chi-squares for the NCCN criteria, the CC score, and a full model comprised of both criteria. The CC score confers statistically significant benefit to the NCCN criteria in *PTEN* mutation prediction, but the NCCN criteria do not confer benefit to the CC score. Higher adequacy indices are observed for the CC score relative to the NCCN criteria in both data sets.

Protein profiles of patient-derived immortalized lymphoblasts

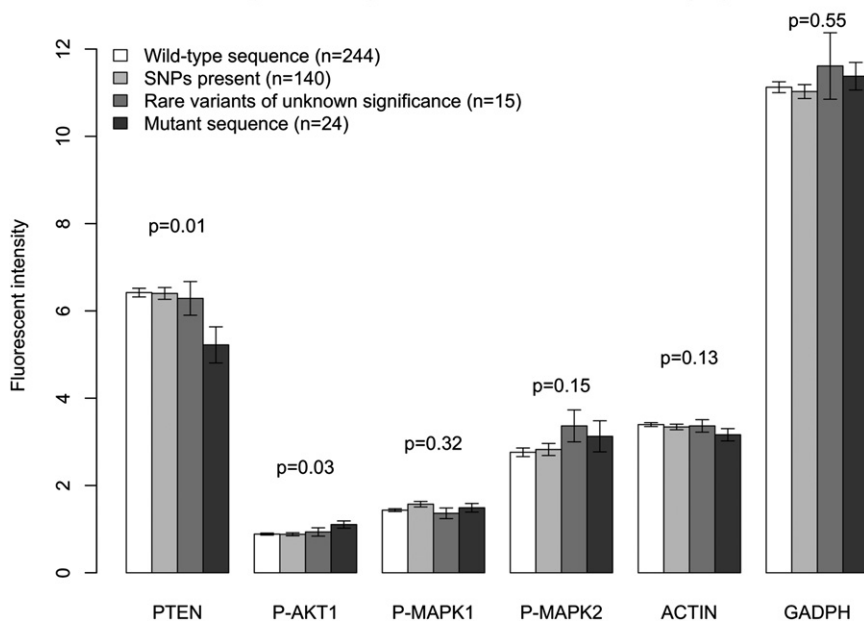


Figure 5. Bar Plot Showing Expression by Immunoblot of Downstream Readout Molecules of PTEN Function for Immortalized Lymphoblasts from 423 Patients

A statistically significant decrease in PTEN protein expression and increase in phospho-AKT1 expression are noted for samples with pathogenic mutations. Standard errors are shown in the error bar. P values are derived from ANOVA testing.

here underline our recent report that *PTEN* mutation may also underpin a distinct gastrointestinal polyposis phenotype in adults,¹⁴ to be considered alongside other polyposis syndromes such as familial adenomatous polyposis and Peutz-Jeghers syndrome. We have demonstrated that the measurement of head circumference is a useful clinical maneuver to facilitate diagnosis in these patients.¹⁴ Although colorectal

scenarios are useful, given that *PTEN* mutation will be considered in the majority of adult patients only after the diagnosis of cancer or polyposis syndrome. Similarly, based on our results, we would recommend consideration of *PTEN* mutation analysis in patients with documented vascular malformations as defined by ultrasound evaluation³⁸ as an important feature of adult PHTS. This feature is recognized in BRRS as an important component of the syndrome, but it is not part of the NCCN diagnostic criteria, even though vascular malformations have been reported in association with CS since the 1970s.³⁹

For adult patients seen by front-line clinicians, genetic etiologies are most often considered if a patient presents with a serious disorder under the age of 40. This common clinical approach for referral of such patients for genetics evaluation is supported by our results, which show that *PTEN* mutation should be considered particularly in patients with breast or endometrial cancer with onset below the age of 40, as well as thyroid cancer with onset below age of 50. However, a not-insignificant proportion of our patients did present with cancer with first onset above age 40, as seen in Table 2. For such patients with late onset of cancer, a majority has distinct phenotypic features or cancer combinations such as breast and thyroid cancers or thyroid and endometrial cancers, which certainly should mandate clinical evaluation. For such individuals, we have demonstrated previously that head circumference measurement alone in the setting of a high-risk breast cancer clinic population was useful in identifying patients with germline *PTEN* mutation.⁴⁰ For these patients who are often first recognized by gastroenterologists or surgeons, we also recommend evaluation by genetics professionals, which may also be useful for consideration of other polyposis syndromes. Indeed, our criteria

cancer is recognized in association with *PTEN* mutation,¹⁴ it does not confer sufficient additional diagnostic value in the community setting.

Advantages of Clinical Scoring over Existing Criteria

The semiquantitative CC score has several advantages over the NCCN criteria. First, the CC score is more accurate and can provide individualized estimates of probability. In particular, the ability to quantify these probabilities is critical for an educated discussion between patients and healthcare providers. Further, adoption of quantitative risk assessment by health management organizations in genetic screening policies underlines their importance. The CC score permits a corresponding estimate for the use of specialist genetics staff and represents a tool for individualized counseling and testing of patients with the wide variety of phenotypes that CS represents. Second, because of the complex and multisystem nature of CS, the current NCCN criteria involve complex rules involving multiplicity of combinations between major and minor criteria inaccessible to most clinicians. A clinical score, represented by a single number as a sum of weights, can be calculated relatively simply. Third, we have found that the age of onset for cancer in patients is crucial for clarifying the diagnosis, and this has been readily incorporated. Finally, accrued clinical experience, together with our active recruitment of patients from the community, demonstrated that certain dermatologic features of CS patients, although characteristic to the eye of the trained specialist, were much more challenging to apply to the community setting.⁴¹

Performance

Although it was clear that the CC score outperformed the NCCN criteria, it is of interest that the fraction of adult

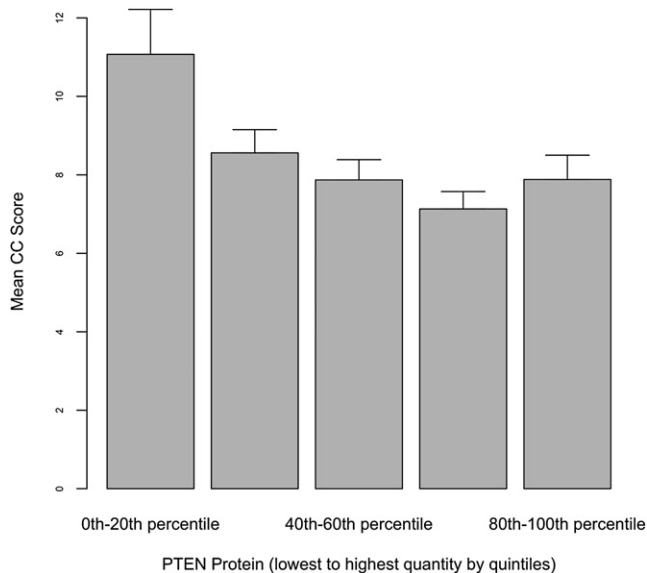


Figure 6. The Relationship between the CC Score and PTEN Protein Quantitation

This relationship between CC score and PTEN protein levels is classified by quintiles, lowest to highest from left to right of each graph, with an inverse association that is demonstrated between rising PTEN protein expression and decreasing mean CC score in the full data set on linear regression ($p < 0.001$). Standard errors are shown by error bar.

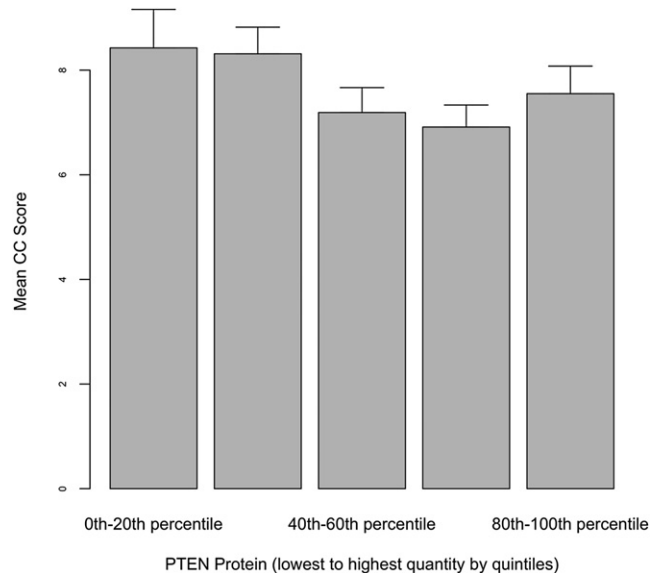


Figure 7. The Relationship between the CC Score and PTEN Protein Quantitation after Exclusion of Patients with PTEN Mutations

The relationship between CC score and PTEN expression levels is classified by quintiles, lowest to highest from left to right of each graph. An inverse association between rising PTEN protein expression and decreasing CC score is seen in samples derived from patients without pathogenic germline *PTEN* mutations ($p = 0.036$). Standard errors are shown by error bar.

patients with *PTEN* mutations in the CC and OSU cohorts is relatively low (7.6%). Certainly the relaxed recruitment criteria (any two features) for recruitment with the goal of detecting more subtle phenotypes were likely to be the main reason. The strictly applied ICC/NCCN criteria, which we first developed on early consortium data based on a retrospective and cross-sectional series, were previously reported to result in a high *PTEN* mutation frequency (85%). Based on this study, it is now clear that the ICC/NCCN criteria have a lower positive predictive value of 15%–30% in a prospective setting, where patients are accrued in a setting closer to the community. That germline alterations of other genes may contribute to this phenotype is of considerable interest, and we have previously identified germline *SDHB* and *SDHD* mutations in 10% of research participants with a similar clinical phenotype and without germline *PTEN* mutations.²² Furthermore, we have recently identified ~35% of individuals, who have similar clinical phenotypes but without *PTEN* mutations, with germline hypermethylation of the *KILLIN-PTEN* bidirectional promoter resulting in downregulation of *KILLIN*.⁴²

Molecular Correlates of Clinical Score

In addition to the clinical dimension of a disease, useful clinical scoring should also reflect biological and molecular aspects of the disease. Experimental data from nonhuman models has accrued, supporting the molecular concept of subtle variations of Pten protein dosage deficiency as a key influence on carcinogenesis, with a Pten dose reduc-

tion of 20% associated with manifestation of murine breast cancer and altered steady-state biology of mammary tissue.¹⁶ Our study provides direct support from human clinical data for the idea that *PTEN* protein dosage may influence a multisystem phenotype, showing that the molecular phenotype of *PTEN* protein deficiency is correlated with increased CC score and consequently increased CS phenotypic load. Thus, even though the average decrease of *PTEN* protein is relatively subtle, its correlation with *PTEN* mutation and elevated CC score is highly provocative in light of these insights. Although the mechanisms of this dose reduction remain unclear, possible explanations include hereditary variants in other genes that regulate *PTEN* expression or its localization.^{43–45} It is also possible that extended upstream *PTEN* promoter variants may affect this expression. Either way, our results imply that phenotypes comprising a complex multisystem syndrome may be underpinned by more genetic variation than would be expected, manifesting in a common *PTEN* protein deficiency pathway with a genetic basis. The provocative idea that *PTEN* protein deficiency may underpin a pathogenic clinical phenotype, however, requires additional validation. When evaluating other proteins that are downstream readouts of the *PTEN* signaling pathway, the increased expression of phospho-AKT1 in *PTEN*-mutated samples is expected.⁴⁶ The absence of association between phospho-MAPK1/2 protein expression and *PTEN* protein expression or mutant status, as would be expected,¹⁹ requires additional investigation

of the relative impairments of the lipid and protein phosphatase activities of the mutations. Overall, our results highlight that the CC score, in correlation with protein quantitation, represents a novel resource for the interrogation of PTEN function in the clinical setting, consistent with the “phenomic” approach we have previously advocated.²¹

In terms of limitations, our data are derived from two referral cohorts representing patients recruited at two major cancer genetics centers, with consequent possible referral bias. It is also likely that referral bias for certain clinical features (particularly adult cancers) may result in overrepresentation of certain patient groups in our patient cohort. We have described our method to adjust for this referral bias through the reduction of score weightage for these features. The most important test of this model and adjustment approach for referral bias is whether it may be validated externally. We were able to demonstrate excellent performance through both calibration and external validation, demonstrating that the approach we have undertaken performs well in a prospective real-world setting. Until a population-based sampling approach to screen community patients for CS is performed, our approach represents the best available evidence-based clinical approach to identifying patients with germline *PTEN* mutations, over the current NCCN criteria. Most importantly, its excellent performance in the real-world setting that we have validated here implies that it is of practice-changing importance.

Overall, we have developed a useful semiquantitative scoring system to evaluate patients for the prior probability of *PTEN* germline mutations, validated in two large separate prospective cohorts, representing an evidence-based advance on existing NCCN criteria. We make practice recommendations with regard to the evaluation of patients for germline *PTEN* mutations, guided by the data accrued above. Additionally, in demonstrating correlation between this score and *PTEN* protein expression in immortalized lymphocytes, we provide direct support from clinical studies for the concept of gene dosage for *PTEN*, highlighting the contribution of *PTEN* protein deficiency to the complex phenotypic features recognized in CS.

Supplemental Data

Supplemental Data include two figures and two tables and can be found with this article online at <http://www.cell.com/AJHG/>.

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Web Resources

The URLs for data presented herein are as follows:

Cleveland Clinic Genomic Medicine Institute: adult and pediatric criteria for individualized risk estimation, <http://www.lerner.ccf.org/gmi/ccscore/>

National Comprehensive Cancer Network (NCCN), <http://www.nccn.org/>

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/>

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