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A mosaic *PTEN* mutation causing Cowden syndrome identified by deep sequencing

Colin C. Pritchard MD, PhD[1], Christina Smith MT(ASCP)[1], Tatyana Marushchak MT(ASCP)[1], Karen Koehler MT(ASCP)[1], Heidi Holmes MS, CGC[2], Wendy Raskind MD, PhD[2,3], Tom Walsh PhD[2] and Robin L. Bennett MS, CGC[2]

Purpose: Mosaic *PTEN* mutations are not well described in Cowden syndrome. We report a 40-year-old woman with a clinical diagnosis of Cowden syndrome including Lhermitte–Duclos disease, who had a mosaic *PTEN* mutation detected by next-generation deep sequencing.

Methods: Complete *PTEN* gene sequencing by the Sanger method and deletion/duplication analysis performed on DNA extracted from blood leukocytes at a commercial clinical laboratory did not identify a mutation. Because of high suspicion of a *PTEN* mutation, we repeated testing by next-generation sequencing using the ColoSeq assay, which sequences the entire *PTEN* locus at >320-fold average coverage.

Results: ColoSeq identified a frameshift *PTEN* mutation (c.767_768delAG) in 1.7% of sequencing reads from peripheral blood leukocytes (21/1,184 reads), which is below the limit of detection of most Sanger sequencing methods. The mutation was detected at full heterozygous levels in skin fibroblasts and a cerebellar tumor, and at approximately the 25% level in colonic and endocervical mucosa, confirming somatic mosaicism.

Conclusion: Our report highlights the power of deep next-generation sequencing to identify mosaic mutations that can be missed by traditional less sensitive approaches. We speculate that mosaic *PTEN* mutations are more common in Cowden syndrome than previously described.

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Key Words: ColoSeq; Cowden syndrome; massively parallel sequencing; mosaic; mosaicism; next-generation sequencing; *PTEN*

Inherited mutations in *PTEN* cause a spectrum of hereditary cancer syndromes, referred to as *PTEN* hamartoma tumor syndromes, including Cowden syndrome and Bannayan–Riley–Ruvalcaba syndrome.[1] Somatic mosaicism is not thought to be common in autosomal-dominant hereditary cancer syndromes (such as Li–Fraumeni syndrome and hereditary breast ovarian cancer syndrome), although it is well characterized in familial adenomatous polyposis and neurofibromatosis type 2 families.[2,3] We are aware of only two previous reports of *PTEN* somatic mosaicism, with only one of the reports associated with Cowden syndrome in a single proband.[4,5]

Massively parallel "next-generation" sequencing technology has dramatically increased throughput and reduced the cost per nucleotide sequenced as compared with traditional Sanger methods, enabling cost-effective sequencing of multiple genes simultaneously in the clinical laboratory setting. Target enrichment is generally required to achieve adequate read depth for accurate identification of the full spectrum of mutations.[6] We recently reported a validation study demonstrating the accuracy and feasibility of solution-based targeted capture and next-generation sequencing for genes that are implicated in Lynch and polyposis syndromes.[7] Here, we describe the application of this deep sequencing approach to identify a low-level mosaic *PTEN* mutation causing Cowden syndrome. Of note, this mutation was undetectable in DNA extracted from blood using traditional sequencing methods, suggesting the possibility of underrecognition of somatic *PTEN* mosaicism as a cause of Cowden syndrome.

MATERIALS AND METHODS

Patient samples

In the proband, we tested DNA extracted from peripheral blood leukocytes and cultured skin fibroblasts obtained from a left forearm skin biopsy. Moreover, we tested DNA prepared from archived formalin-fixed paraffin-embedded tissue samples obtained from three different anatomical sites: (i) endocervical mucosa with squamous metaplasia, (ii) normal colonic mucosa at the site of suspected rectal polyp, and (iii) tumor tissue from a dysplastic gangliocytoma of the right cerebellum (Lhermitte–Duclos disease). Genomic DNA was prepared with the Gentra Puregene DNA Isolation Kit (Qiagen, Germantown, MD, catalog no. 158489). Hematoxylin–eosin–stained slides were reviewed before DNA extraction for all formalin-fixed paraffin-embedded tissue samples. Clinical specimens were obtained in accordance with the declaration of Helsinki and the ethics guidelines of Human Subjects Division of the University of Washington.

Next-generation deep sequencing by ColoSeq

ColoSeq solution-based targeted gene capture, genomic library preparation, and massively parallel sequencing methods have

[1]Department of Laboratory Medicine, University of Washington, Seattle, Washington, USA; [2]Division of Medical Genetics, Department of Medicine, University of Washington, Seattle, Washington, USA; [3]Department of Psychiatry and Behavioral Sciences, University of Washington, Seattle, Washington, USA. Correspondence: Colin C. Pritchard (cpritch@uw.edu)

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been described previously.[7] Briefly, DNA was sonicated on a Covaris S2 instrument (Covaris, Woburn, MA) to a peak of 200 bp, Illumina paired-end adapters (Illumina, San Diego, CA) were ligated, and adapter-ligated library was PCR amplified for five cycles with Illumina primers 1.0 and 2.0 (Illumina). Individual paired-end libraries were hybridized to a custom design of complementary RNA biotinylated oligonucleotides targeting 1.1 Mb of DNA in 30 genomic regions including the entire *PTEN* gene and flanking regions.[7] Sequencing was performed with 2 × 101 bp paired-end reads using SBS v3 chemistry on a HiSeq2000 (Illumina). Sequence alignment and variant calling were performed against the reference human genome (University of California, Santa Cruz hg19). Sequencing reads were aligned using BWA[8] and MAQ;[9] single-nucleotide variants and small insertions and deletions, as well as large deletions and duplications, were detected as previously described,[7,10,11] and using the Genome Analysis Toolkit[12] (version 1.6) and Varscan 2.0.[13]

Allele-specific PCR and Sanger sequencing confirmation

Allele-specific PCR and Sanger sequencing primers were designed to confirm that the mutation was present on the functional *PTEN* gene and not on the pseudogene. The primer sequences (all 5′–3′) were: *PTEN* mutation-specific forward: TGTGTGTGGTGATATCAAAGTAGTT, wild-type forward: TGTGTGTGGTGATATCAAAGTAGAGTT, and common reverse: GCCATAAGGCCTTTTCCTTC. Sanger sequencing was performed using the following primers, which flanked the *PTEN* c.767_768delAG mutation: forward: TTGATTTGCTTGAGATCAAGATT and common reverse: GCCATAAGGCCTTTTCCTTC.

RESULTS

Case presentation

In 2009, the 40-year-old Caucasian female proband was referred for genetic evaluation after resection of a dysplastic gangliocytoma of the cerebellum (Lhermitte–Duclos disease), considered a pathognomonic feature of Cowden syndrome. She had other consistent features of Cowden syndrome, including macrocephaly with occipitofrontal circumference of 59 cm, parietal stroke, mucosal papillomas, multiple keratoses on her hands and feet (most prominent on her feet), four confirmed hamartomatous polyps and a ganglioneuroma on screening colonoscopy in 2011, multiple lipomas (not biopsied), and thyroid goiter. Family history was noncontributory: she had a healthy 11-year-old son with attention-deficit disorder and occipitofrontal circumference in the normal range (55 cm), her younger brother and sister were healthy, and both parents were alive at the age of 61 years. There was no family history of cancers associated with *PTEN* hamartoma tumor syndrome such as breast, thyroid, endometrial, or colon cancers. Consanguinity was denied.

The proband was assumed to have Cowden syndrome/*PTEN* hamartoma tumor syndrome, and a blood sample was sent to a commercial clinical laboratory. A mutation was not identified by Sanger DNA sequencing in exons 1–9 or the promoter region of the *PTEN* gene. Concurrent targeted chromosomal microarray analysis with exon-level resolution did not reveal a pathogenic deletion or duplication of *PTEN* including the upstream promoter region.

Still suspecting the patient had *PTEN* hamartoma tumor syndrome, we retested the patient in 2012 with next-generation deep sequencing using ColoSeq, a multi-gene panel that includes *PTEN*.

ColoSeq identifies a mosaic *PTEN* mutation

ColoSeq testing on peripheral blood leukocyte DNA from the proband identified a *PTEN* mutation in 1.7% of sequencing reads (21/1,184 total reads; **Figure 1a**). The mutation, predicted to result in a frameshift and premature protein truncation, is designated NM_000314.4:c.767_768delAG, p.Glu256Valfs*41 in Human Genome Variation Society (HGVS) nomenclature, and occurs at chr10:89,717,742-89,717,743 in Hg19 genomic coordinates. The mutation was confirmed to be on the functional *PTEN* gene by mutation-specific PCR (**Figure 1b**). Sanger sequencing of multiple tissues from the proband revealed that the mutation was present at heterozygous levels in skin fibroblasts and dysplastic gangliocytoma tissue (cerebellar tumor) and at approximately the 25% level in normal colon and normal endocervical mucosa (**Figure 1c**).

Her adolescent son did not have the *PTEN* mutation as measured in DNA from blood lymphocytes (and he was not suspected to be affected on the basis of clinical presentation). Her parents refused testing but would not be expected to carry the *PTEN* mutation.

DISCUSSION

To our knowledge, this is only the third report of a mosaic *PTEN* mutation associated with a *PTEN* hamartoma tumor syndrome. In the first report, a *PTEN* nonsense mutation (p.R130*) was detected as a recurrent "second hit" in multiple different neoplastic tissues from a Proteus-like syndrome patient with a germline *PTEN* p.R335* mutation, suggesting that the p.R130* mutation was mosaic rather than tumor acquired.[5] In a second, very recent report, a 20-year-old woman with colonic ganglioneuromas was diagnosed with a germline *PTEN* frameshift mutation, prompting evaluation of her parents.[4] In that case, the patient's 48-year-old macrocephalic father carried the mutation at approximately the 10% level in peripheral blood lymphocytes, consistent with mosaicism, although additional tissues were not tested. In both earlier studies, Sanger sequencing was used, a method that was not sensitive enough to detect the mosaic mutation in the peripheral blood of our patient.

Our report supports several important insights relevant to clinical genetics. The first is that next-generation deep-sequencing methods are likely to offer improved sensitivity to detect mosaic mutations as compared with traditional Sanger sequencing. Sanger sequencing generally has a sensitivity of only ~10–20% mutant allele. Current next-generation deep-sequencing methods can detect a mutation at as low as a 1% level, and emerging specialized methods are likely to further improve this

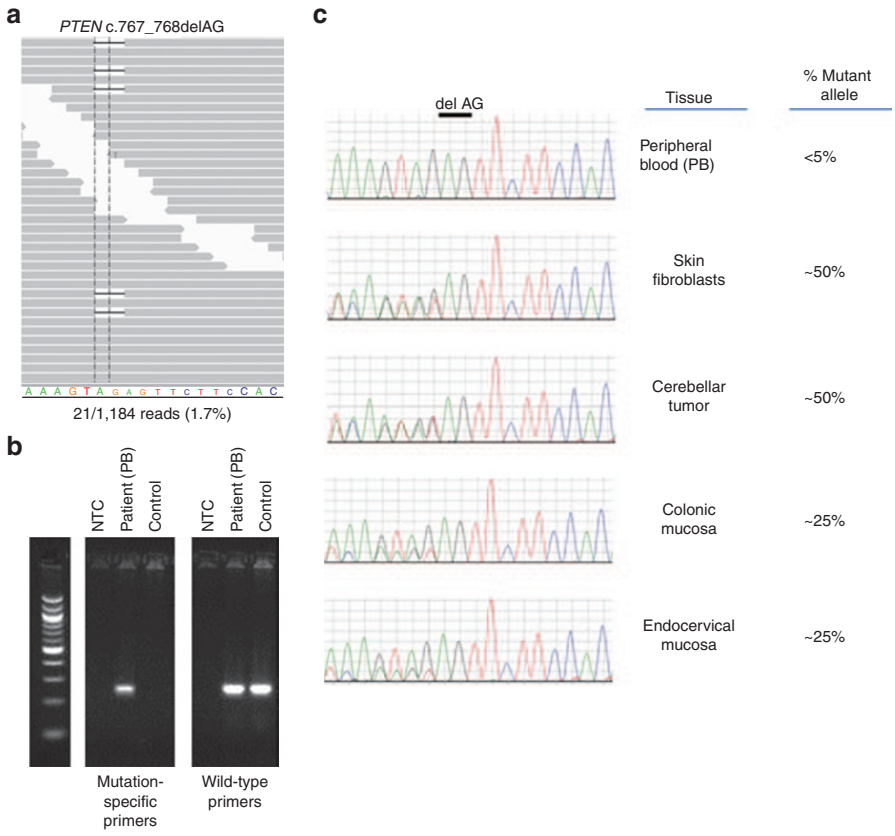

Figure 1 A mosaic *PTEN* mutation causing Cowden syndrome. (**a**) Close-up of next-generation sequencing data from peripheral blood of the proband visualized in the integrated genomics viewer (Broad Institute). Each gray bar represents a unique sequencing read. The five reads with "—" have the *PTEN* c.767_768delAG mutation. A total of 21 of 1,184 reads (1.7%) had the mutation. (**b**) Mutation-specific PCR using primers specific to the functional *PTEN* gene confirms the presence of the mutation in peripheral blood (PB). NTC is no template control. (**c**) Sanger sequencing demonstrates the mutation is present at heterozygous level (~50%) in skin fibroblasts and dysplastic gangliocytoma (cerebellar tumor), at approximately the 25% mutant allele level in colonic mucosa and endocervical mucosa, and below 5% in peripheral blood. Opposite-strand sequencing results are shown in **Supplementary Figure S1** online.

sensitivity.[14] Second, somatic mosaicism in hereditary cancer risk syndromes is probably more common than previously suspected, and germline *de novo* mutations may be less common. For example, *PPM1D* was recently identified as a candidate breast and ovarian cancer risk gene using next-generation sequencing, with all 25 mutations identified being mosaic in peripheral blood lymphocytes.[15] Finally, somatic mosaicism may not predict a milder clinical phenotype, as evidenced by our proband, who had multiple major features of Cowden syndrome/*PTEN* hamartoma tumor syndrome.

Recently, it was demonstrated that Cowden and Cowden-like syndromes can also be caused by germline mutations in *PIK3CA* and *AKT1*.[16] Of note, mosaic mutations of these genes cause CLOVES[17] and Proteus syndrome,[18] respectively.

Because Sanger sequencing is still the mainstay of clinical genetic testing, it is likely that many patients with low-level mosaicism in the peripheral blood remain undiagnosed. The same logic applies to parents of mutation carriers who test mutation-negative, suggesting the mutation is *de novo* in the proband. It was recently reported that up to 47% of *PTEN* mutations might be *de novo*.[19] It is intriguing to speculate that a subset of these presumed *de novo PTEN* mutations were inherited from a parent with somatic mosaicism.

We suggest consideration of next-generation deep sequencing whenever there is a high clinical suspicion of a *PTEN* mutation, particularly with a negative family history.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/gim

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DISCLOSURE

The authors declare no conflict of interest.

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