Exome Sequencing Reveals Germline SMAD9 Mutation That Reduces Phosphatase and Tensin Homolog Expression and Is Associated With Hamartomatous Polyposis and Gastrointestinal Ganglioneuromas

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Hamartomatous polyposis syndromes (HPS) account for a small but appreciable proportion of inherited gastrointestinal cancer predisposition syndromes; patients with HPS have an increased risk for colon and extracolonic malignancies. We present a unique case of familial juvenile polyposis syndrome associated with gastrointestinal ganglioneuromas of unknown etiology. The patient was tested to express SMAD9V90M had reduced expression of phosphatase and tensin homolog; this reduction was also observed in a polyp from the patient. We have therefore a new susceptibility locus for HPS. Patients with hamartomatous polyposis in the colon associated with ganglioneuromatosis should be referred for genetic assessments.

Keywords: Hamartomatous Polypos; Ganglioneuromas; Colorectal Cancer; SMAD Signaling.

Six distinct autosomal-dominant inherited hamartomatous polyposis syndromes (HPS) have been described (Supplementary Table 1). It is now known that many of these syndromes carry a substantial risk for developing colonic and extracolonic malignancies, depending on which gene(s) are involved. Clinical management is informed by the specific gene.

It is important to note that a significant number of patients have no family history. Therefore, the diagnosis of these syndromes remains a clinical process primarily. These important, potentially heritable, polyp conditions are a challenge for clinicians because individuals present with features that overlap one or more of the syndromes.

We describe here a novel hamartomatous polyposis susceptibility gene discovered while investigating a 38-year-old white man with HPS, presenting with diarrhea and weight loss, because of the persistence of his symptoms. Colonoscopy revealed diffuse 3- to 5-mm polyps in his ascending, transverse, descending, and sigmoid colon. Thirty of these were removed, and reported as showing hamartomatous polyps admixed with either mature adipose or ganglioneuromatous proliferation. Two ascending colon polyps blocks was re-reviewed by our gastrointestinal pathologist and confirmed as ganglioneuromatous polyps (Figure 1A). Upper endoscopy was unremarkable.

His family history is significant; his father had diffuse polyposis and died in his 40s; the patient did not know the cause of death. His paternal uncle and aunt died from early-onset colorectal cancer in their 40s (Figure 1B). The genetic differential diagnosis for gastrointestinal ganglioneuromas includes Cowden syndrome1,2 (CS), multiple endocrine neoplasia type 2B, and type I neurofibromatosis (Supplementary Table 1). Because physical examination did not reveal the stigma of multiple endocrine neoplasia type 2B or type I neurofibromatosis, CS was considered. He had no other CS-related individual features, such as macrocephaly, mucocutaneous manifestations, or personal history of cancer. Histologically, he did not present with characteristic juvenile polyps or Peutz-Jehgers polyps. This patient therefore did not fulfill clinical testing criteria for HPS and was instead enrolled in a prospective research study for HPS. He underwent sequencing and deletion/duplication analysis for BMRP1A, SMAD4, phosphatase and tensin

Abbreviations used in this paper: BMP, bone morphogenetic protein; CS, Cowden syndrome; HPS, hamartomatous polyposis syndromes; JPS, juvenile polyposis syndrome; miR21, microRNA 21; mRNA, messenger RNA; PTEN, phosphatase and tensin homolog; WT, wild type.
domains of these SMADs recognize DNA through a mutation. The modeled SMAD8 (protein encoded by SMAD9V90M) as a gain-of-function mutation. We saw increased phosphorylation of SMAD9 in Flag-tagged SMAD9V90M transfected cells as compared with Flag-tagged SMAD9 wild-type (WT) SMAD9WT transfected HEK293 cells (Figure 2A). We observed increased inhibitor of differentiation 1 messenger RNA (mRNA) level in SMAD9WT-transfected HEK cells with BMP4 stimulation (Figure 2B) with a greater increase in inhibitor of differentiation 1 expression in SMAD9V90M-transfected HEK cells compared with that in SMAD9WT transfected cells. Interestingly, we observed that both protein (Supplementary Figure 1C) and mRNA levels (Figure 2C) of PTEN in lymphoblastoid cell lines derived from our patient were lower compared with lymphoblastoid cell lines from healthy participants, suggesting that SMAD9V90M mutation affects PTEN expression. Corroborating the lymphoblastoid cell line observations, reduced PTEN protein expression was evident in the patient’s polyp (Figure 1A).

To functionally understand this observation, we transfected Flag-tagged SMAD9WT or Flag-tagged SMAD9V90M into HEK293 cells. Consistently, in SMAD9V90M-transfected HEK293 cells, PTEN protein levels were significantly decreased compared with that in SMAD9WT cells (Figure 2A). Additionally, quantitative reverse transcription polymerase chain reaction for PTEN transcript in transfected cells showed lower PTEN mRNA level in SMAD9V90M cells compared with that in SMAD9WT cells (Figure 2D). However, SMAD9 was unable to directly bind the PTEN promoter, suggesting that it is not acting as a transcription factor (Supplementary Figure 1D). We then turned our attention to a direct downstream effector of BMP-SMAD9 signaling pathway, microRNA 21 (miR21), which has been shown to target PTEN directly and suppress PTEN expression.3 We showed that miR21 expression level was increased significantly in BMP4-treated SMAD9V90M-transfected cells compared with SMAD9WT-transfected cells (Figure 2E) due to increased binding of SMAD9V90M to pri-miR21 (Supplementary Figure 2C) with consequent increased processing of pri-miR21 into mature miR21. We surmise, therefore, that SMAD9V90M is a gain-of-function mutation leading to increased miR21 expression, with consequent decreased PTEN stability at both mRNA and protein levels.

We subsequently screened 40 juvenile polyposis syndrome (JPS) patients and 40 CS patients with HPS who were mutation negative for BMPR1A, STK11, SMAD4, and PTEN for SMAD9 mutations but did not detect any with SMAD9 mutations. BMPR1A and SMAD4 germline mutations account for 40%–45% of patients who meet criteria for JPS; in contrast, this SMAD9 mutation is likely to be a private mutation, as we did not find a mutation in SMAD9 outside of

Figure 1. (A) Histological features (at 100× magnification). Clockwise from top left: (i) H&E stains showing spindle and polygonal nerve and ganglion cells; (ii) S100 stain highlights the lesional neural fibers; (iii) decreased PTEN expression; (iv) strongly positive p-ERK expression; and (v) decreased p-AKT expression. Bar = 100 μm. (B) Pedigree of proband-3684TE family.

homolog (PTEN), and STK11 but was mutation negative for these known HPS genes.

Because this was an unique case of familial HPS associated with gastrointestinal ganglioneuromas and colorectal cancer of unknown etiology, we hypothesized that functionally important sequence variants in either the PTEN or transforming growth factor–β/bone morphogenetic protein (BMP) signaling pathways might be affected and proceeded with whole exome sequencing (see Supplementary Materials and Methods).

We analyzed all sequence data for the presence of nonsense, frameshift, consensus splice site, and missense mutations after whole exome sequencing. Given the very unique histologic phenotype of the polyps seen in our patient, we focused our attention on mutations that were likely to be of biological importance, namely those in the canonical HPS signaling pathways (Supplementary Table 1). In this manner, we identified in our patient a novel germline heterozygous SMAD9V90M (SMAD9V90M) missense mutation (Supplementary Figure 1A).

Structural modeling predicts SMAD9V90M as an activating mutation. The modeled SMAD8 (protein encoded by SMAD9) MH1 shows a globular fold containing 5 α-helices and 6 β-strands, where Val90 lies in the α4-helix. The MH1 domains of these SMADs recognize DNA through a β-hairpin made up of β2 and β3. Val90 is located in close proximity with the DNA binding interface and contacts residues Leu87 and His104 through hydrophobic interactions. Mutation of Val90 to a methionine would therefore cause the long side chain of methionine to clash with the neighboring His104. To relieve this steric clash, His104 would move toward the DNA phosphate backbone, therefore, enhancing the DNA binding of SMAD8 (Supplementary Figure 1B).

Functionally, we saw evidence to support SMAD9V90M as a gain-of-function mutation. We saw increased phosphorylation of SMAD9 in Flag-tagged SMAD9V90M transfected cells as compared with Flag-tagged SMAD9 wild-type (WT) SMAD9WT transfected HEK293 cells (Figure 2A). We observed increased inhibitor of differentiation 1 messenger RNA (mRNA) level in SMAD9WT-transfected HEK cells with BMP4 stimulation (Figure 2B) with a greater increase in inhibitor of differentiation 1 expression in SMAD9V90M-transfected HEK cells compared with that in SMAD9WT transfected cells. Interestingly, we observed that both protein (Supplementary Figure 1C) and mRNA levels (Figure 2C) of PTEN in lymphoblastoid cell lines derived from our patient were lower compared with lymphoblastoid cell lines from healthy participants, suggesting that SMAD9V90M mutation affects PTEN expression. Corroborating the lymphoblastoid cell line observations, reduced PTEN protein expression was evident in the patient’s polyp (Figure 1A).

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We subsequently screened 40 juvenile polyposis syndrome (JPS) patients and 40 CS patients with HPS who were mutation negative for BMPR1A, STK11, SMAD4, and PTEN for SMAD9 mutations but did not detect any with SMAD9 mutations. BMPR1A and SMAD4 germline mutations account for 40%–45% of patients who meet criteria for JPS; in contrast, this SMAD9 mutation is likely to be a private mutation, as we did not find a mutation in SMAD9 outside of
this family in limited subsets of patients with JPS or CS. Our patient, in addition to JPS, had significant ganglioneuromatosis, which is rare but overrepresented in CS patients with germline PTEN mutations.\(^1,2\) We believe that our patient’s SMAD9 mutation results in gain-of-function SMAD9\(^{V90M}\) leading to reduced PTEN mRNA and protein stability, together, yielding a phenotype (ganglioneuromatous polyps) seen more commonly in patients with germline PTEN mutations. Our study suggests that this might be indirectly regulated by increased miR21 expression (Figure 2F) secondary to increased binding of mutant-SMAD8 to pri-miR21, resulting in increased miR21 biogenesis, the latter consistent with Hata’s demonstration that SMAD proteins controlling DROSHA-mediated maturation of miRs.\(^3\) Our findings will need to be confirmed by future studies to fully understand the complex interactions between SMAD and PTEN signaling. Our study is limited by the unavailability of family members (either deceased or not

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**Figure 2.** Gain-of-function SMAD9\(^{V90M}\) results in increased miR21 expression and reduced PTEN expression. (A) SMAD9\(^{V90M}\)-transfected cells show more phosphorylated-SMAD9 (P-SMAD9 row) and decreased PTEN protein expression (PTEN row) than cells transfected with SMAD9\(^{WT}\). Note equal expression of WT SMAD9 and mutant SMAD9 (FLAG row). (B) SMAD9\(^{V90M}\) induces inhibitor of differentiation 1 (ID1) mRNA expression in BMP4-treated compared with sham-treated (No treat.) HEK293 cells. (C) PTEN mRNA levels (bottom panel) are significantly lower in our patient (3684TE)-derived lymphoblastoid cell lines compared with healthy controls. (D) PTEN mRNA expression in SMAD9\(^{V90M}\)-transfected cells was significantly lower than SMAD9\(^{WT}\)-transfected cells with (BMP4 +) or without BMP4 (No treat.) stimulation. (E) SMAD9\(^{V90M}\)-transfected cells showed higher miR21 expression compared with SMAD9\(^{WT}\)-transfected cells in the presence (BMP4 +) or absence (No treat.) of BMP4. (F) Schematic diagram depicting the effect of SMAD9\(^{V90M}\) on ID1, miR-21, and PTEN expression. Also see text and Supplementary Figure 2C.
contactable) for segregation analysis despite multiple and prolonged attempts to do so.

We do, however, recommend that all patients with a clinical presentation of hamartomatous polyposis, especially those with gastrointestinal ganglioneuromas, be referred for genetic evaluation. It is worth highlighting that use of custom-gene panels relies on known susceptibility genes and will miss novel genes such as this. For select patients, subsequent clinical exome sequencing can prove fruitful.

Supplementary Material
Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2015.06.027.

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Conflicts of interest
The authors disclose no conflicts.
Supplementary Materials and Methods

Whole Exome Sequencing

Genomic DNA was extracted from blood. Exome enrichment was performed using the TruSeq SBS version 3 chemistry kit (Illumina Inc, San Diego, CA), followed by 100-bp paired-end sequencing using an Illumina HiSeq 2000 platform. Sequencing was performed at an Illumina Sequencing Service Center (Illumina Inc) in 8 lanes with 34 GB of data generated per lane. The mean sequence coverage was 80×, with >90% of the targeted regions covered by at least 10 reads for each exome. Sequencing data were analyzed and annotated by an in-house optimized pipeline. Raw sequencing reads were mapped to the human reference haploid genome sequence (Genome Reference Consortium human genome build 37, hg19) using the Burrows-Wheeler Aligner (BWA version 0.6.1).1 Indel realignment, base and quality score recalibrations, and removal of polymerase chain reaction (PCR) duplicates from the resultant Binary Alignment Map files were done using the Genome Analysis Toolkit,2-4 Sequence Alignment/Map (SAMtools), and Picard.5 Variant discovery and genotype calling of single nucleotide variations and short insertions and deletions (indels, <50 bp) was performed using the Haplotype Caller of the Genome Analysis Toolkit.

Variant Filtration and Annotation

To prioritize causal variants, we applied the Annovar Variants Reduction pipeline.6 First, we discarded synonymous variants and intronic variants >2 bp from exon boundaries. We retained variants in conserved genomic regions based on a 46-species alignment and removed variants existing in segmental duplication regions. We excluded variants that were observed in the 1000 Genomes Project (April 2012 release) or the NHLBI-ESP6500 exomes with a minor allele frequency >0.0005 and variants reported in dbSNP137 nonflagged database (excluding clinically associated SNPs). To predict the potential impact of missense variants on protein function, we used SIFT6 with a cut-off score of 0.05 and PolyPhen27 with cut-off scores of 0.447 (HumanVar database) and 0.453 (HumanDiv database) to retain predicted deleterious variants. We also used CONsensus DELeteriousness (Condel),8 which combines multiple prediction software algorithms (SIFT, PolyPhen2, and MutationAssessor9) into a single weighted score. All resultant variants were manually inspected through the Integrated Genomics Viewer (IGV, Broad Institute). We also used an in-house database containing variants from 20 exomes from pediatric subjects and 37 CS probands with unrelated phenotypes, sequenced with our patient cohort as an additional internal control filter. We excluded variants that appeared more than once in this in-house exome database. Mutations in candidate genes of interest were validated using PCR-based region-specific mutation analysis through Sanger sequencing.

Structural Biology

To predict the potential structural change caused by the SMAD9V900M missense mutation, homology modeling was performed using SWISS-MODEL.10,11 SMAD9 has 2 isoforms and is confusingly referred to as SMAD8, and so, we refer to it here as SMAD8/9. To verify the role of this residue in DNA binding, the SMAD8/9 MH1 model was superimposed with the structures of SMADs 1, 3, and 4 in complex with their respective cognate SMAD-binding element DNA via Dalilite server.12

RNA Extraction and Phosphatase and Tensin Homolog Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted from peripheral blood derived lymphoblastoid cell lines from controls and patients using GeneJET RNA Purification Kit (Thermo Scientific, Waltham, MA) according to the manufacturer’s protocol, and subsequently treated with DNase 1 (Invitrogen, Carlsbad, CA). DNase treated total RNA was reverse-transcribed into complementary DNA using Superscript III first-strand synthesis system for reverse transcription PCR (Invitrogen), as specified by the manufacturer. Quantitative PCR was performed on 7500 Real time PCR System (AB Applied Biosystems, 850 Lincoln Center drive, Foster City, CA) using the PTEN mRNA specific TaqMan primer-probe, PTEN exon 6—specific TaqMan primer-probe and Actin-β mRNA-specific TaqMan primer probe as endogenous loading control. PTEN mRNA specific primers and probe sequences: forward: 5’-CTGTTAGGTATGTC TTCAAAGGA-3’, reverse: 6’-TATAATTGCAAGTCTGCA CAAT-MGBNFQ. PTEN exon6 specific primers and probe sequences: forward: 5’-CAGTCAGAGGGCTATGTTTGTCT TTCAAAAGGA-3’, probe: 6FAM-TTAATTGCAGAGTTGCA CACACGGAGTACTTG-3’. PTEN exon6 specific primers and probe sequences: forward: 5’-CTGGCACCCAGCACAATG-3’, reverse: 5’-GTGGGTTATGTC TTCAAAAGGA-3’, probe: 6FAM-TTAATTGCAGAGTTGCA CAAT-MGBNFQ. PTEN exon6 specific primers and probe sequences: forward: 5’-CTGGCACCCAGCACAATG-3’, reverse: 5’- CACACGGAGTACTTG-3’, probe: VIC-AGATCAAGATCATTGCT CCT-MGBNFQ.

Phosphatase and Tensin Homolog Protein Analysis

HEK293 cells were plated 70% confluent in 6-well plates the day before transfection. Flag tagged SMAD9WT, SMAD9V900M plasmids or empty vector were transfected in HEK293 cells with transfection reagent Lipofectamine 3000 (Invitrogen, Grand Island, NY) according to manufacturer’s provided protocol. Forty-eight hours post transfection, cells were treated with MG132 for 8 hours, or cycloheximide for 24 hours, respectively. Cells were then harvested and lysed with M-PER mammalian protein extraction reagent (Thermo Fisher Scientific Inc, Rockford, IL). Western blots were run with the lyses and blotted for anti-PTEN, anti–glyceraldehyde-3-phosphate dehydrogenase and anti-Flag antibodies.
**Inhibitor of Differentiation 1 Quantitative Reverse Transcription Polymerase Chain Reaction**

Total RNA were extracted from Flag tagged SMAD9<sup>WT</sup>, SMAD9<sup>V90M</sup> plasmids or empty vector transfected HECK293 cells 48 hours post transfection. Reverse transcription was processed using Superscript III first-strand synthesis system for reverse transcription PCR (Invitrogen). Inhibitor of differentiation 1 mRNA expression level was measured using quantitative PCR with inhibitor of differentiation 1 specific primers kindly provided by Dr Aldred’s laboratory (Genomic Medicine Institute, Cleveland Clinic).

**Chromatin Immunoprecipitation**

To investigate if V90M mutation in SMAD9 influences its function, firstly, we examined phosphorylation of SMAD9. We transfected Flag-tagged SMAD9<sup>WT</sup> or Flag-tagged SMAD9<sup>V90M</sup> mutant into HEK293 cells. HEK293 cells were plated 70% confluent in 6-well plates the day before transfection. Flag-tagged SMAD9<sup>WT</sup>, SMAD9<sup>V90M</sup> plasmid or empty vector, were transfected in in the cells with Lipofectamine 3000 (Invitrogen) according to manufacturer’s provided protocol. Forty-eight hours post transfection, half of the transfected cells were treated with 8 nM BMP4 for 2 hours. Cells were processed for chromatin immunoprecipitation using Flag antibody (Sigma-Aldrich) and analyzed for PTEN promoter binding ability according to manual (EZ-ChIP™ Chromatin Immunoprecipitation Kit, EMD Millipore Corporation, Billerica, MA).

**MicroRNA 21 Expression**

Using SMAD9<sup>WT</sup> and SMAD9<sup>V90M</sup> plasmid transfected HEK293 cells, total RNA, including miRNA, were extracted from miRNAeasy Mini Kit (Qiagen, Hilden, Germany). Complementary DNA synthesis was performed with TaqMan MicroRNA Reverse Transcription Kit with RT primer specific to miR21 or miRNA66 (TaqMan Small RNA Assay for miR21, TaqMan Small RNA Assay for miRNA66 and TaqMan Universal Master Mix II). miR21 and miRNA66 were amplified for 40 cycles in a 20-μL reaction. miR21 expression levels were analyzed using miRNA66 as internal control.

**Pri-Micro RNA21 Reverse Transcription Polymerase Chain Reaction Followed by FLAG Immunoprecipitation in SMAD9-transfected HEK293 Cells**

HEK293 cells were plated 70% confluent in 6-well plates the day before transfection. Flag-tagged SMAD9<sup>WT</sup>, SMAD9<sup>V90M</sup> plasmid or empty vector, were transfected in in the cells with Lipofectamine 3000 (Invitrogen) according to manufacturer’s provided protocol. Forty-eight hours post transfection, cell lysates were extracted and processed for FLAG immunoprecipitation using Flag antibody (Sigma-Aldrich). After centrifuging down Protein A/G plus agarose beads (Santa Cruz Biotechnology, Inc., Heidelberg, Germany), total RNA was extracted with miRNAeasy Mini kit (Qiagen) for reverse transcription with SuperScript III First-Strand kit (Invitrogen). Probe for Pri-miR21 quantitative PCR was provided from TaqMan Pri-miRNA (has-mir-21) Assay kit (Applied Biosystems by Life Technologies, Carlsbad, CA). Pri-miR21 quantitative PCR was amplified for 40 cycles in a 20-μL reaction with triplicates.

**References**

Supplementary Figure 1. (A) SMAD9 V90M mutation. The top panel shows the Integrated Genome Viewer and Sanger sequencing results for the patient showing the heterozygous V90M mutation. The lower panel is the results from one of the internal controls used in this study showing the corresponding wild-type sequence. (B) Possible functional impact of V90M. The modeled SMAD8/9 MH1 shows a globular fold containing 5 α-helices and 6 β-strands, where Val90 lies in the α4-helix. Three residues in SMAD4 (equivalent to R78, Q80, and K85 in SMAD8) are the key residues that are involved in DNA interaction. Those residues are conserved in SMAD4 and SMAD8. Mutation of Val90 to a methionine would cause the long side chain of methionine to clash with the neighboring His104. To relieve this steric clash, His104 would move toward the DNA phosphate backbone, therefore, enhancing the DNA binding of SMAD 8.
Supplementary Figure 2. (A) Immunoblot analysis of PTEN expression in patient-derived lymphoblastoid cells. Immunoblot analysis from lymphoblastoid cell lines was performed using anti-PTEN mouse monoclonal (Cascade Biosciences, Portland, OR) at 1:5000 and anti-ACTIN mouse monoclonal (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:20 000. C1–4 are control research participants, samples 1–4 are from CS patients and samples 5–8 are from HPS patients. PTEN expression was significantly lower in sample 4 (CS patient with germline PTEN deletion) and sample 8, which is our patient 3864TE with the SMAD9 V90M mutation. (B) No direct binding of SMAD9 with PTEN promoter. Chromatin immunoprecipitation (ChIP) analysis was performed on HEK293 cells transfected with empty vector, SMAD9 WT, or SMAD9 V90M plasmids, respectively, using Flag antibody. ChIP elute was then utilized for quantitative PCR using PTEN promoter primers. No significant binding was observed between Flag-SMAD9 and PTEN promoter. (C) Increased binding between SMAD9 V90M and Pri-miR21 in HEK293 cells. Pri-miR21 reverse transcription PCR was performed from total RNAs extracted from FLAG Immunoprecipitation in HEK293 cells transfected with empty vector, SMAD9 WT or SMAD9 V90M vector, respectively. White bar represents Pri-miR21 level being pulled down in HEK293 cell transfected with empty vector. Gray bar represents Pri-miR21 level being pulled down in HEK293 cell transfected with FLAG tagged SMAD9 WT vector. Black bar represent Pri-miR21 level being pulled down in HEK293 cells transfected with FLAG tagged SMAD9 V90M vector.
**Supplementary Table 1.** Characteristics of Heritable Hamartomatous Polyposis Syndromes and Known Involved Signaling Pathways

<table>
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<td><em>PTEN, KLLN, SDHx,</em></td>
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<td>Ganglioneuromas</td>
<td>MEN 2B</td>
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<td>NF1 (diffuse)</td>
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<td>RAS signaling</td>
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AMP, adenosine monophosphate; GNDF, glial cell line–derived neurotrophic factor; MEN 2B, multiple 10 endocrine neoplasia type 2B; MTOR, mammalian target of rapamycin; NF1, type I neurofibromatosis; PI3K, phosphoinositide 3-kinase; PJ, Peutz-Jehgers; TGF, transforming growth factor.