

Deep sequencing with intronic capture enables identification of an *APC* exon 10 inversion in a patient with polyposis

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Purpose: Single-exon inversions have rarely been described in clinical syndromes and are challenging to detect using Sanger sequencing. We report the case of a 40-year-old woman with adenomatous colon polyps too numerous to count and who had a complex inversion spanning the entire exon 10 in *APC* (the gene encoding for adenomatous polyposis coli), causing exon skipping and resulting in a frameshift and premature protein truncation.

Methods: In this study, we employed complete *APC* gene sequencing using high-coverage next-generation sequencing by ColoSeq, analysis with BreakDancer and SLOPE software, and confirmatory transcript analysis.

Results: ColoSeq identified a complex small genomic rearrangement consisting of an inversion that results in translational

skipping of exon 10 in the *APC* gene. This mutation would not have been detected by traditional sequencing or gene-dosage methods.

Conclusion: We report a case of adenomatous polyposis resulting from a complex single-exon inversion. Our report highlights the benefits of large-scale sequencing methods that capture intronic sequences with high enough depth of coverage—as well as the use of informatics tools—to enable detection of small pathogenic structural rearrangements.

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Inherited deleterious mutations in the *APC* gene cause familial adenomatous polyposis (FAP) and have also been associated with Gardner and Turcot syndromes.¹ Sanger sequencing of all 15 coding exons in the *APC* gene has become the initial standard screening test for *APC* mutations. Sanger sequencing of *APC* exons has about 55% sensitivity for mutations in patients with >100 colorectal adenomas.² Assays for large rearrangements of the *APC* gene detect mutations in an additional 3% of patients with FAP.^{3,4} Beyond this, testing for two common mutations in *MUTYH* will identify 7% of patients with classic polyposis as carriers of biallelic mutations in *MUTYH*, which has an overlapping phenotype.^{2,5} Therefore, current screening for *APC* and *MUTYH* using these three separate tests has a cumulative sensitivity of about 65% for causative mutations in patients with classic polyposis, which is defined as the condition of having >100 polyps.² Of the mutations in *APC* that are detected using current protocols, Sanger sequencing detects frameshift, nonsense, and splice-site mutations, which represent 43, 42, and 9%, respectively, of identified mutations, in addition to detecting missense mutations that have been categorized as pathogenic.^{2,3} The remaining 6% of mutations detected with current protocols are detected by multiplex

ligation-dependent probe amplification or fluorescence in situ hybridization.^{3,4}

Several assays have been designed to rapidly screen for mutations in *APC* that are not detectable with Sanger sequencing or to confirm the pathogenicity of detected mutations. Assays such as conformation-sensitive denaturing gel electrophoresis or denaturing high-performance liquid chromatography can rapidly scan for variants in amplified exons.^{6,7} Some laboratories use the protein truncation test to evaluate the pathogenicity of mutations that may not have obvious effects.⁸ However, many mutations are not detectable with methods that target coding exons. A small proportion of patients with FAP have complex rearrangements or somatic mosaicism; these are also not detected by routine screening.^{4,9,10}

High-throughput “next-generation” sequencing technology has dramatically reduced the per-base cost of sequencing, making sequencing of intronic segments in addition to exons at high depth economically practical. Consequently, next-generation detection strategies allow for more comprehensive detection of disruptive mutations, including point mutations, splice-site mutations, intronic mutations, deletions, duplications, large rearrangements, and complex structural rearrangements.

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ColoSeq is a recently validated next-generation sequencing assay that interrogates both the intronic and exonic sequences of 19 genes associated with colon cancer and polyposis.¹¹ Herein, we describe the identification of a complex genomic inversion spanning exon 10 of the *APC* gene.

MATERIALS AND METHODS

DNA samples of patients

We tested DNA extracted from peripheral blood leukocytes and prepared genomic DNA with the Gentra Puregene DNA Isolation Kit (Qiagen, Germantown, MD; catalog no. 158489). Clinical specimens were obtained in accordance with the Declaration of Helsinki and the ethics guidelines of the 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 been described in detail previously.¹¹ Briefly, genomic DNA was sheared and SureSelect probes were used to capture exonic and intronic sequences of multiple genes associated with Lynch syndrome and polyposis (Agilent Technologies, Santa Clara, CA). Custom-designed targets included exonic and intronic sequences in *MLH1*, *MSH2*, *MSH6*, *PMS2*, *EPCAM*, *APC*, *MUTYH*, *CDH1*, *PTEN*, *STK11*, *TP53*, *SMAD4*, *BMPRIA*, *POLE*, *POLD1*, *GALNT12*, *GREM1*, *AKT1*, and *PIK3CA*. Paired-end sequencing of amplified targets was carried out on an Illumina HiSeq2000 system (Illumina, San Diego, CA) according to standard protocols. Single-nucleotide polymorphisms and insertions/deletions (indels) were called as described previously.¹¹ To evaluate structural variation, reads were mapped to the human reference genome (hg19) using BWA, and variants were identified using BreakDancer¹² and CREST,¹³ as described elsewhere.¹⁴ Split reads at inversion breakpoints were identified using SLOPE.¹⁵ Inversion breakpoints and exact structures were confirmed using Sanger sequencing (primer sequences available from authors).

Confirmatory experimental analysis of splicing errors due to a genomic inversion

Splicing errors due to gene rearrangements (i.e., deletions, duplications, and inversions, involving one or more exons) lead to transcripts of abnormal length. To detect these events, we isolated total RNA from the patient's whole blood within 24 hours of collection using TRIzol LS Reagent (Invitrogen, Life Technologies, Grand Island, NY) and generated complementary DNA (cDNA) by oligo(dT) priming using the SuperScript First-Strand Synthesis System (Invitrogen; Life Technologies). The cDNA was amplified with a primer pair spanning exons 7 and 13 of *APC* (primer sequences available from authors). Products obtained from the reverse transcriptase-polymerase chain reaction on the cDNA were electrophoresed on 2% agarose gels. Aberrant-sized products of polymerase chain reaction were extracted from the gels using QIAquick (Qiagen) and were sequenced in both directions.

RESULTS

Case presentation

The proband is a 40-year-old woman of self-reported Irish and Scottish ancestry who presented to the medical genetics clinic following a history of polyposis of the colon. A colonoscopy performed at 35 years of age was remarkable for the detection of five tubular adenomas. A repeat colonoscopy at 39 years of age noted multiple subcentimeter-sized polyps in the terminal ileum, cecum, and transverse colon. There were too many polyps to be able to ascertain an accurate number. At the hepatic flexure, there were at least 15 subcentimeter-sized polyps. Biopsies obtained from the ileum, cecum, and transverse colon confirmed tubular adenomas. An esophagogastroduodenoscopy at 40 years of age was unremarkable. The proband is an otherwise-healthy individual with a negative review of systems. Her mother was diagnosed with an invasive colorectal cancer at 54 years of age, and the proband's maternal grandmother had a niece (first cousin once removed of the proband) with colorectal cancer at 50 years of age. Consanguinity was denied. Relatives were unavailable for testing.

ColoSeq identifies an inversion in exon 10 of the APC gene

A multigene panel screen of 13 genes associated with colon cancer and polyposis was performed. Average read depth across all genes was 335×, with average read depth of 324× across the *APC* gene. BreakDancer software identified 17 discordant paired-end reads, consistent with an estimated 445-base pair inversion between chr5:112154543 and chr5:112155245, as well as 5 reads with an estimated 676-base pair inversion between chr5:11215434 and chr5:112155245. BreakDancer estimates feature size by comparing differences between expected and actual mapping locations of paired-end reads, highlighting candidate changes without giving accurate breakpoint locations or precise size estimates.¹² Therefore, we used other methods to characterize actual inversion breakpoints. Orthologous analysis using SLOPE revealed a total of 19 split reads consistent with an inversion between chr5:112154359 and chr5:11215008, with an additional 9 split reads between chr5:112154359 and sequence near chr5:112155232,^{15,16} supporting the presence of a complex disruptive rearrangement.

The breakpoint of the genomic inversion was confirmed, and the exact complex rearrangement was defined using Sanger sequencing. The inversion is complex enough that determining the correct Human Genome Variation Society (HGVS) nomenclature is challenging (Figure 1a). In genome build hg19, a large sequence from chr5:112154359-112155228 was inverted, with chr5:112154360-112154371 and chr5:112155008-112155228 duplicated before the inversion event. Near the inversion insertion point, at chr5:112154356-112154360, five base pairs (CTTAT) were deleted, and at the other inversion insertion point, chr5:112155008, eight base pairs (GAACCAGG) were inserted or duplicated from chr5:112155011-112155018 (Figure 1a).

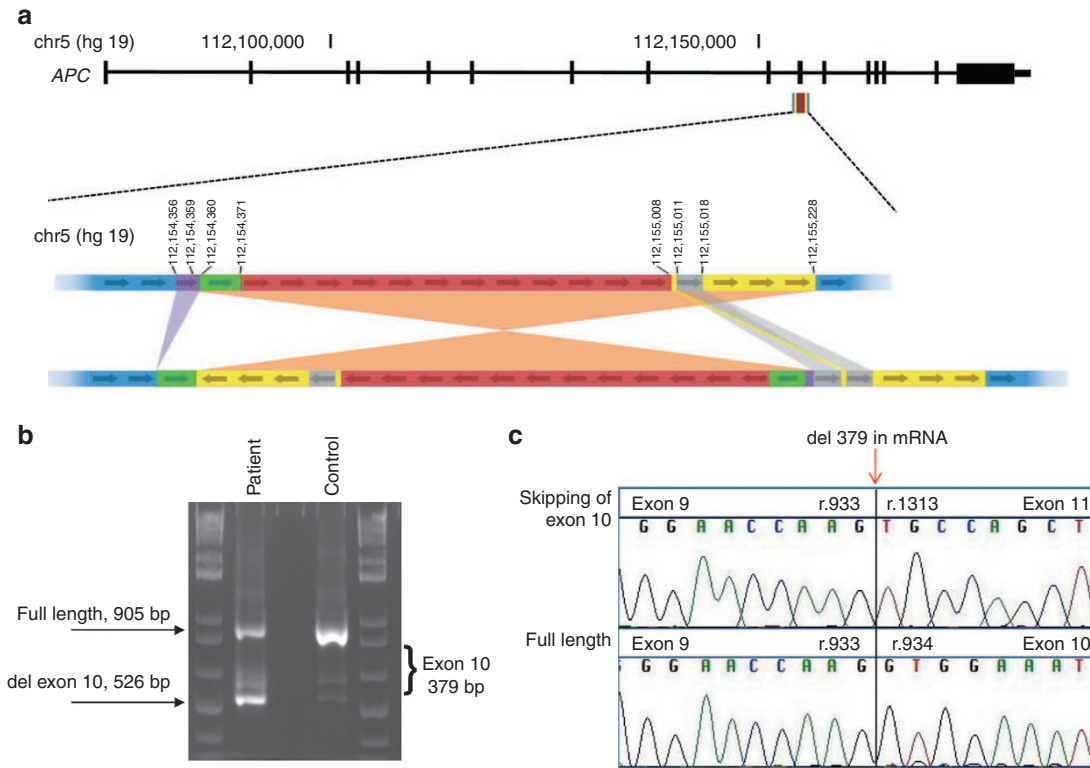


Figure 1 Chromosome 5 inversion spanning exon 10 of the APC gene, causing skipping of exon 10 of APC in the patient's mRNA. (a) Schematic representation of the APC gene with the locations and details of the complex genomic rearrangements; all positions are on hg19 chromosome 5. (b) Gel electrophoresis of cDNA products consistent with skipping of exon 10. (c) cDNA sequence of the resulting APC protein product illustrating the cDNA sequence of exon 9 spliced to that of exon 11. cDNA, complementary DNA.

Confirmatory cDNA analysis of splicing errors due to a genomic inversion

Analysis of cDNA successfully identified a mutant message in APC containing a premature stop codon due to the genomic inversion. The detection of a message of abnormal length suggested that the inversion did not lead to complete transcript degradation due to nonsense-mediated decay. The cDNA product was consistent with the skipping of APC exon 10 in the patient's mRNA: r. 934_1312 del 379, with predicted stop at position 327 of the 2,844-bases-long chain (Figure 1b,c).

DISCUSSION

We are not aware of previous reports of any single-exon inversions in APC causing FAP, and this is the first report of an isolated inversion at exon 10 of the APC gene. However, there are several reports of different small APC rearrangements. One study that examined cDNA transcripts and identified small rearrangements in 8% of FAP families screened¹⁷ and another study that used multiple methods to screen for APC mutations reached the conclusion that tests for detecting splicing defects and larger genomic changes should be included in all diagnostic screening protocols.⁴ The important distinction between these studies and our report is that previous work identified altered transcripts using processed nucleic acid and then followed this with additional studies to identify the underlying genomic alteration. By contrast, our next-generation sequencing assay

detected the small rearrangement at the genomic level in the course of primary clinical testing, and we confirmed the findings in the altered transcript. Had the rearrangement not been so complex, with small deletions and insertions at the breakpoints, we may have been able to identify inversion breakpoints using split reads.

Sanger sequencing that interrogates exonic sequences and intron-exon boundaries, followed by deletion/duplication analysis in cases in which sequencing is negative, has become the standard of care for FAP. Rearrangements, such as the one that we report here, would not normally be detected by either of these methods. The breakpoints of this inversion are such that published primers for exonic sequencing would provide reliable data from the normal copy of the affected exon but would fail to detect the inversion,¹⁸ and it is unlikely that the duplicated exonic sequence of less than 50 base pairs in this complex rearrangement would be detected by multiplex ligation-dependent probe amplification probes. Only a few investigators routinely perform the transcript-based APC analyses that would be expected to detect this complex rearrangement. A next-generation sequencing approach offers significant advantages in allowing identification of sequence variants, deletion/duplication, and structural rearrangements through the use of a single test. Our report demonstrates how the technique of deep next-generation sequencing may obviate the need for multiple screening tests, by enabling detection of small

rearrangements at the genomic level, and illustrates several analytic tools that can be used to identify these variants.

DISCLOSURE

The authors declare no conflict of interest.

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