RESEARCH ARTICLE

Characterization of an APC Promoter 1B deletion in a Patient Diagnosed with Familial Adenomatous Polyposis via Whole Genome Shotgun Sequencing [version 1; peer review: 2 approved]

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Abstract
Recently, deletions have been identified and published as causal for Familial Adenomatous Polyposis in the 1B promoter region of the APC gene. Those deletions were measured using multiplex ligation-dependent probe amplification. Here, we present and characterize an ~11kb deletion identified by whole genome shotgun sequencing. The deletion occurred in a patient diagnosed with Familial Adenomatous Polyposis, and was located on chr5, between bases 112,034,824 and 112,045,845, fully encompassing the 1B promoter region of the APC gene. Results are presented here that include the sequence evidence supporting the presence of the deletion as well as base level characterization of the deletion site. These results demonstrate the capacity of whole genome sequencing for the detection of large structural variants in single individuals.

Keywords
APC, Familial Adenomatous Polyposis, Clinical Sequencing, Next Generation Sequencing
Familial Adenomatous Polyposis (FAP) is an autosomal dominant condition characterized by the development of hundreds to thousands of polyps in the colon. This condition results in colon cancer in adult individuals in their late 20s to early 30s with nearly 100 percent penetrance. Mutations in two genes, the adenomatous polyposis coli (APC) and mutY homolog (MUTYH) loci, have been identified as causative for this disease. The majority of the mutations occur in the APC locus. The APC mutations often take the form of simple nucleotide substitutions or small insertions or deletions in the coding region of the gene that produce premature stop codons, or frame shifts respectively. These result in a change in function. The exact mechanism by which these mutations affect the disease is unknown. However, deletions of APC promoter 1B are known to cause a significant change in transcription levels of the APC RNA marked by allele specific differences in transcription. Several mutations have been reported in the promoter region of the APC gene, identified either by sequencing, or by multiplex ligation-dependent probe amplification (MLPA).

The patient analyzed in this work is a 50 year-old Caucasian female who has a personal and maternal family history of FAP. She developed colon polyps at 14 years of age and underwent a partial colectomy at 16 years. The patient had a complete colectomy and a Whipple procedure in her 20’s. Her mother and multiple avuncular cousins on the maternal side are affected. One sibling has a clinical diagnosis of FAP and three siblings are unaffected. The patient’s maternal grandfather died of colon cancer later in life, but a diagnosis of FAP was not confirmed.

Previously, DNA testing in family members had failed to identify a causative mutation. Therefore, the patient and her family participated in a linkage analysis project through the Mayo Clinic in Rochester, Minnesota to identify at-risk family members. The FAP in the family showed linkage to the APC locus on chromosome 5. The patient underwent molecular testing of the APC gene (sequence analysis and Southern blot) and MUTYH gene (analysis for 2 common mutations) in 2008. No mutations were detected. A variant of unknown significance (referred to as Glu1317Gln) was found in the APC gene. However, this variant was absent in other affected family members and was present in the patient’s unaffected child. It was later classified as likely benign. The multiplex ligation-dependent probe amplification (MLPA) assays for the APC locus in use at the time did not characterize the APC promoters, and was negative for APC mutations for this patient.

In an effort to comprehensively search for potential mutations, the patient’s genomic DNA was sent to Illumina whole genome sequencing. A deletion of ~11kb encompassing the APC promoter 1B was identified, and is consistent with the deletion identified recently by Snow et al. via an updated MLPA assay for APC that now includes promoter 1B and by Lin et al.

In this work, we present a comprehensive characterization of this deletion using Illumina short reads, including base level resolution of the deletion site. Further, it is demonstrated that this deletion is detectable using the MLPA assay for the APC locus current at the submission of this article, and would be ambiguous if this, or any single patient were analyzed solely via whole exome sequencing.
deleted region was common to both pairs. Full description of the primers is in provided in Table 1.

### DNA extraction, PCR, and Sanger Sequencing

Whole blood was fractionated by spinning at 5,000 rpm for 10 minutes at room temperature. White cells were transferred to sterile, nuclease free microcentrifuge tubes and stored at -20°C until processing. Genomic DNA was isolated from 250μL buffy coat with Gentra Puregene Genomic DNA purification buffers (Qiagen, Valencia, CA). Separate amplification of the wild type or deletion APC fragments were performed in a 20μL reaction containing 0.4μL Phusion HF DNA Polymerase (Thermo Fisher Scientific, Pittsburg, PA), 1x Phusion Reaction Buffer, 200μM dNTP’s (Promega Corporation, Madison, WI), 200ng gDNA, and 0.5μM each primer. The cycling conditions were as follows: 98°C for 30s followed by 35 cycles of 98°C for 10s, 60°C for 30s, and 72°C for 60s, ending with a final extension of 72°C for 7min. The amplicons were sequenced with BigDye® Terminator v3.1 (Life Technologies Corporation, Carlsbad, CA) utilizing the PCR primers and standard sequencing conditions. The sequence reactions were purified with Performa DTR Ultra 96-well filtration plates (Edge Biosystems, Gaithersburg, MD) and processed on the ABI 3130xl Genetic Analyzer (Life Technologies Corporation, Carlsbad, CA).

The resulting gel for the PCR products is shown in Figure 1, and the sequencing results are shown in Figure 2, rendered in Geospiza’s FinchTV, (http://www.geospiza.com/Products/finchtv.shtml).

*Table 1.* Primers used to genotype samples for the presence or absence of the deletion described in this work.

| Primer Pair 1 | Primer Pair 2 |
|--------------|--------------|
| **Left Primer** | **Right Primer** | **Product Length-No Deletion** | **Product Length-With Deletion** |
| GGGTCTAGTTCATTCGTTGCT | GACACCTACCATTGTGTTACCATT | 1058 Bases | No Product |
| GAGGGGGTTGCTCTTGAAA | GAGGGGGTTGCTCTTGAAA | 11653 Bases | 653 Bases |

*Figure 1.* Gel images for the PCR products produced in the Louisville Index case, as well as the seven kindreds reported by Snow et al., using the two primer pairs described in Table 1. The relationship between the lanes and the nine kindreds defined in Snow et al. are Lane 1: Ladder, Lane 2: Kindred 8, Lane 3: Kindred 43, Lane 4: Kindred 44, Lane 5: Kindred 256, Lane 6: Kindred 509, Lane 7: Kindred 685, Lane 8: Kindred 691, Lane 9: Kindred 353 (APC c.426_427delAT) And Lane 10: Kindred 6699 (APC c.532–941G>A). These images demonstrate heterozygous deletions in eight of the samples analyzed. PCR products corresponding to the bands circled in red were sequenced using Sanger technology. Those results are shown in Figure 2.

Dataset 1. Raw Gel electrophoresis image for Figure 1

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The gel image represented in Figure 1, showing in lane 1 a control human sample that was not part of this work which was cropped from the in-text figure10.
Paired end whole genome sequence data was generated at ~40X coverage for the patient, and mapped to the human reference assembly Build-37.1. Given the clinical phenotype our initial analysis of the data was limited to the APC and MUTYH loci. Variation analysis was performed in the region defined by the 5’ and 3’ most exons of the longest reported transcript for APC and MUTYH, plus and minus 50,000 bases respectively (described in detail in Methods). The resulting counts of single nucleotide variations (SNVs) and small indels are shown in Table 2–Table 4. The corresponding VCF file, along with mapped reads for these regions are available for download or visualization at http://dx.doi.org/10.13013/J6QN64N8. When viewing in IGV, navigate to the APC locus by entering APC in the text box at the top of the frame.

All missense variants identified had corresponding records in dbSNP and are listed in Table 4. None are reported as deleterious. There were no non-sense SNVs or frame shifting small insertions or deletions identified. The search was then turned toward larger structural variants. Visual inspection of the VCF file for the APC locus revealed a region of approximately 10kb with 17 measured SNVs or small insertions relative to the reference. None of their respective genotypes were classified as heterozygous. This loss of heterozygosity suggested a deletion. Upon further inspection, there were other signatures characteristic of a deletion, that included a cluster of paired end reads whose mate mapped ~11kb from their respective start, and several mates that were soft trimmed because they spanned the deletion site. These soft trimmed mates were identified (described in methods), and aligned via BLAT to hsBuild-37.1, revealing the deleted region to be of length 11,020 bases, located on chr5, between bases 112,034,824 and 112,045,845, spanning the annotated APC promoter 1B. This deletion is illustrated in Figure 3, along with the positions of commercial probe sets, and other annotation relevant to this work. Given that this deletion was consistent with the deletion reported by Snow et al., the primers used for verification in this work, were run on the kindreds studied in that work. It was verified that the deletion reported there was identical to the one reported here. Also, this deletion is identical to a deletion published by Lin et al., identified in kindreds from Missouri, Illinois, and Idaho not known to be related to each other.

The Illumina paired end short read data that provides evidence for the deletion relative to the reference has been isolated from the
Table 2. Single nucleotide variants (SNVs) identified in our patient for the APC and MUTYH loci.

| Gene  | SNVs | Intronic | 5’ UTR | In Coding Region | 3’ UTR |
|-------|------|----------|--------|------------------|--------|
|       |      |          |        | Silent           | Missense | Nonsense |        |
| APC   | 154  | 143      | 2      | 6                | 2       | 0        | 1      |
| MUTYH | 7    | 5        | 0      | 1                | 1       | 0        | 0      |

Table 3. Insertions and Deletions identified in our patient for the APC and MUTYH loci.

| Gene  | In/Dels | Intronic | 5’ UTR | Frame Shifting | 3’ UTR |
|-------|---------|----------|--------|----------------|--------|
| APC   | 26      | 25       | 1      | 0              | 0      |
| MUTYH | 3       | 3        | 0      | 0              | 0      |

Table 4. Positions of the missense variants detected in the MUTYH and APC loci. Single Nucleotide Polymorphism database (dbSNP) accession numbers and Human Genome Variation Society (HGVS) names for the gene, including the amino acid change and position are also listed.

| Gene  | Chr | Coordinate | Reference Allele | Variant Allele | dbSNP     | HGVS Names             |
|-------|-----|------------|------------------|----------------|-----------|------------------------|
| MUTYH | 1   | 45800156   | C                | T              | rs3219484 | NP_001041636.1 Val22Met |
| APC   | 5   | 112175240  | G                | C              | rs1801166 | NP_000029.2 Glu1317Gln |
| APC   | 5   | 112176756  | T                | A              | rs459552  | NP_000029.2 Val1822Asp |

Figure 3. A rendering of human chromosome 5 with features between bases 111,978,904 and 112,052,349 for human genome build 37.1 in the Integrative Genomics Viewer. Records from the VCF file for the patient described here are displayed in the top track indicating a region with a loss of heterozygosity consistent with a deletion. We also render the exon identified as APC promoter 1B, the MLPA probes used commercially to analyze this locus, the region selected for pull-down in the TruSeq exome capture kit, the deletion reported by Rohlin et al. in 2008, and the position of the deletion described here relative to all these features.
larger dataset, and is made available in its own binary alignment map file for inspection at the DOI included above.

In order to confirm the deletion, PCR primers were designed to specifically interrogate it. These primers produce a product of approximately 1 kb for individuals with no deletion, and a second pair of primers was designed that flank the deletion site. This placement produces a product of 0.6 kb from chromosomes with the deletion, and 1.7 kb in chromosomes without. As the NGS data suggests a heterozygous deletion, the expectation was a single band with the first primer pair, and two bands, one strong from the 0.6 kb amplicon, and one weak (if detectable) for the 12 kb amplicon. This was confirmed in the gel represented in Figure 1. The ~1 kb and ~6 kb bands were cut from the gel and sequenced using Sanger technology. The trace images are shown for the two different alleles in Figure 2. One read shows the deletion, and the second allele is consistent with the reference. The deletion is confirmed by the Sanger sequence data, and the primers are provided as a definitive Sanger sequencing assay for it. The second PCR image in Figure 1, and third read included in Figure 2 confirmed that our respective kindred shares the same deletion as the seven families reported by Snow et al. We predict that all families descend from a common founder.

Although this deletion was identified by visual inspection, the binary alignment map file for the region was analyzed by the application BreakDancer to determine if the deletion could be identified algorithmically from whole genome sequence data. BreakDancer identifies putative deletions by identifying read pairs, clustered by genomic coordinate, that have similar inferred insert sizes which are either much larger or smaller than the standard distribution of insert sizes measured for mapped pairs. Using this algorithm, a deletion was identified on chr5 and was approximated to lie between bases 112,034,793 and 112,045,844, corroborating the finding presented here.

The methods of Snow et al. used multiplex ligation-dependent probe amplification (MLPA) assays. These are described in a document from MRC-Holland, available at the time of publication at (http://www.mlpa.com/WebForms/WebFormDBData.aspx?FileOID=MCLO2Mc0V%5Cc%7C). Information for those probes, including the partial sequence adjacent to the ligation site, as well as the genomic coordinate derived from a BLAT search using the partial sequence information is reproduced in Table 5, and rendered in Figure 3 relative to the deletion identified in this work. These coordinates are contained within the region deleted for this patient, and as such result in a deletion of the signals corresponding to these probes. The next probe in the set, APC 142, which is outside the deleted region, did not indicate a deletion.

### Table 5. Probe sequence and genomic coordinate information for the MLPA probes that interrogate APC Promoter 1B.

| APC Probe | Partial sequence adjacent to the ligation site | Genomic Coordinate |
|-----------|---------------------------------------------|--------------------|
| 260       | GCACTGATGCT-TCCTACCTCCCA                    | chr5:112,043,195–112,043,218 |
| 274       | TACTTCTGGCCA-CTGGCGAGCGT                   | chr5:112,043,549–112,043,572 |

**Discussion/Conclusion**

Several years ago, a female patient of the University of Louisville Weissskopf Child Evaluation Center presented with Familial Adenomatous Polyposis (FAP). Whole genome shotgun sequencing on the Illumina platform revealed a deletion on chromosome 5 between bases 112,034,824 and 112,045,845, fully encompassing promoter 1B of the APC locus. Deletions that include this promoter have been demonstrated to affect the expression of the full length APC transcript.

In other work by Snow et al., a deletion was identified via MLPA that is consistent with the deletion characterized here. An investigation via PCR of their seven kindreds with the primers used in this work establishes that the deletion is identical to the deletion reported here. Furthermore, this deletion is also reported by Lin et al., in three kindreds not known to be related to each other, or these families. It is likely that this mutation descends from an ancestor common to each of these reported families.

Exome capture has become a popular tool for mutation screening in clinical genetics. The deletion reported here extends several kilobases beyond the region captured by one of the more popular exome capture products (Figure 3). This deletion would have been very difficult to identify by exome capture since the only practical measurements that could have been employed would have been read density and loss of heterozygosity in the captured region.

The whole genome sequencing approach taken here produces an information rich dataset capable of resolving large deletions in individuals. These structural variants result in a number hallmarks that are easily detected. Specifically, the loss of heterozygosity over a large region, a collection of read pairs whose mates consistently map much further apart than the majority of the read pairs, and soft trimmed reads all pinpoint the deletion site unequivocally. We have demonstrated that whole genome sequencing is both a sensitive and accurate approach for the detection and characterization of deletions of this size.

**Data availability**

*F1000Research: Dataset 1. Raw Gel electrophoresis image for Figure 1, 10.5256/f1000research.6636.d50276*  

**Author contributions**

TK and JK conceived of and led the project. TK performed primary and secondary data analyses, and wrote the manuscript. PB and GG served as clinical liaisons. AS and DN performed PCR and background on their sample sets. All provided input during the preparation of the manuscript. All authors have seen and agreed to the final content of the manuscript.

**Competing interests**

T.K. serves as the CEO of Intrepid Bioinformatics.

**Grant information**

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I confirm that the funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Kalbfleisch et al. present an interesting article on identification and characterization of an 11kb APC promoter deletion from whole genome sequencing (WGS) data. The design of the study, the methods used and the presentation of the results are presented in a conclusive and way suitable to the investigation. They have analyzed the WGS data only for the APC and MUTYH locus and identified the deletion by visual inspection of the data. The identified deletion is confirmed and breakpoints identified with sanger sequencing. The deletion is also found to be identical to the previously identified deletions by Snow et al. and Lin et al., and they all descent from a common founder.

Minor points for revision and comments:

1. When a coding DNA reference sequence is used the following recommendations can be followed according to HGVS; the coding DNA reference sequence should be complete and preferably derived from the RefSeq database (format NM_033337.2). In general the longest transcripts are used, and for the MUTYH gene this is NM_001128425.1 (16 exon 549 aa). It would be more appropriate to at least name the variants found according to the NM reference sequence that has been used in table 4. For example the variant rs3219484 if the NM_001128425.1 would have been used the variant would preferable be named NM_001128425.1:c.64A>G, p.Val22Met.

2. When discussing MLPA, it would be appropriate to mentioned the versions of the MLPA kit, like P043 (version C1), this is a common way to present which kit has been used, a link to the document describing the assay is not necessary. Probes for the APC promoter 1B were included in the MLPA kit in 2011.

3. In the result section findings of missense and nonsense variants in coding region and small indels are mentioned, what about variants in splice acceptor or donor-sites are these included in the analyses done? It would be nice to mention this in the text and also in the
tables (2 and 4) if anyone’s are found as these variants are important and often constitute disease-causing mutations.

4. Regarding the deleteriousness of the variants found how was this interpreted? It would be nice to mentioned which databases and/or prediction tools has been used since all variants in dbSNP are not benign. I would be recommended to use several prediction tools like for example SIFT, Polyphen-2, Mutation taster, Condel and Combined Annotation-Dependent Depletion (CADD) among others for missense interpretation and also looking at conservation between species. Other important tools used for interpretation of variants found are databases were variants are reported and sometimes also classified often in a 1-5 scale (1 is benign and 5 pathogenic). The InSiGHT database (http://insight-group.org/variants/database/) is commonly used for variants in coloncancer genes, where the APC and MUTYH gene can be found among others, clinvar and HGMD professional (Human Genome Mutation Database) are also databases too use. Information about the minor allele frequency of the variant found can also be found in ExAc (Exome Aggregation Consortium) and ESP (Exome Sequencing Project) and can be included when reporting variants.

5. This deletion was found by manual inspection of the region were the reads maps further apart than expect and by looking at the soft-clipped bases as well as identifying the region having LOH. It would be interesting to discuss the limitation with this visual method regarding sizes and type of rearrangement that can be detected. Some discussion comparing (pros and cons) regarding this method with different algorithms methods like BreakDancer (read-pair methods) and others including read-depth methods and split reads methods for example would also be valuable for an interesting discussion useful for readers trying to find methods to analyze for these types of mutations.

**Competing Interests:** No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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Mutations in the APC gene have been established as a cause of classical FAP. However, in a small
subset (~20%) of affected families, mutations in the coding regions of APC or other polyposis-associated genes such as MUTYH cannot be identified. In this study, the authors perform whole-genome sequencing on one such subject and identify a heterozygous 11kb deletion in the exon 1B / promoter region of APC. The three non-synonymous mutations identified in APC and MUTYH were not felt to be causal mutations. The authors confirmed the presence of the heterozygous deletion through differential PCR and Sanger sequencing.

The authors then went on to investigate nine APC mutation-negative kindreds which were previously found to have exon 1B / promoter deletions using multiplex ligation-dependent probe amplification assays. In all nine kindreds, the same coordinates of the 11kb deletion were inferred.

The study design, presentation of results and conclusions are straightforward. The authors find an APC promoter deletion through whole-genome sequencing and establish the deletion coordinates through Sanger sequencing. Ideally, further investigation of a number of affected and unaffected members in the kindred of this study would provide convincing evidence that this 11kb deletion is associated with the affected state. However, considered in conjunction with the results from Lin et al. and Snow et al., this study shows that whole-genome sequencing is a suitable method for the detection of non-coding mutations in APC-mutation negative FAP individuals. This builds upon mounting evidence that associates APC promoter deletions with FAP. Interestingly, these studies all show that affected members of eleven FAP kindreds in the United States share the promoter deletion with identical coordinates. It might be worth noting in the discussion that the original Snow et al paper using MPLA identified a deletion that was thought to be much larger (>33kb) but that approach did not map the exact coordinates.

Two minor points: (1) In the Results section (p 6 left column second paragraph), it is stated: “these primers produce a product of approximately 1kb for individuals with no deletion.” More accurately, the 1kb product is produced from chromosomes without the deletion (as the authors later state); the 1kb product is also produced in all individuals with the deletion (Fig. 1).

(2) In the discussion, the authors discuss potential difficulties in using targeted capture techniques to discover or assess larger deletions such as the one described. However, we successfully used such a targeted capture assay to discover the promoter deletion described in our study. The analysis methods for detecting individual reads and read pairs that straddle the deletion are applicable and to targeted sequencing strategies.

**Competing Interests:** No competing interests were disclosed.

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.
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