Letter to the Editor
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Identification of a Novel Splice Variant (c.423-8A>G) of *APC* by RNA Sequencing

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Dear Editor,

Familial adenomatous polyposis (FAP) is an autosomal dominant disorder characterized by the presence of hundreds to thousands of adenomatous polyps throughout the colon and rectum that evolve into colorectal cancer when left untreated [1]. Variants in the adenomatous polyposis coli (*APC*) gene, a well-known tumor-suppressor gene located on chromosome 5q21, are responsible for the majority of FAP cases. To date, more than 1,600 pathogenic *APC* variants have been reported [2]. We identified a novel splice variant of *APC* in a patient with FAP by next-generation sequencing (NGS) and confirmed its impact on splicing by RNA sequencing. The genetic testing for this patient was performed as a clinical laboratory testing, and RNA sequencing was performed to elucidate the significance of the detected variant. As it is not a part of research subjects, approval from the Institutional Review Board was exempt. Informed consent was obtained from the patient before genetic testing.

In October 2018, a 36 year-old man presented to the Department of Gastroenterology, Asan Medical Center, Seoul, Korea, for further evaluation and management of FAP with adenoma of the ampulla of Vater. His mother, maternal aunt, and maternal cousin had undergone colectomy for FAP in her 50s, her 60s, and his 30s, respectively. Colonoscopy revealed more than 100 small (mostly <3 mm) adenomatous polyps in the colon and rectum, and polypectomy was performed for several large polyps (≥4 mm). Esophagogastroduodenoscopy revealed numerous polyps on the stomach and a mass close to the ampulla of Vater. Biopsy of the mass identified villotubular adenoma with focal high-grade dysplasia, and endoscopic submucosal dissection was performed.

To identify genetic variants causing FAP, we extracted DNA from peripheral blood leukocytes using QIAamp DSP DNA Blood Mini kit (Qiagen, Hilden, Germany) and prepared the library for NGS with a Customized Target Enrichment Kit (Celemics, Seoul, Korea). We conducted NGS with a multigene panel of 128 hereditary tumor-related genes, including colorectal cancer-related genes such as *APC, KRAS, MLH1, MSH2, MSH6, MUTYH, PMS2, SMAD4, STK11,* and *TP53*, using a MiSeqDx V2 sequencing kit (Illumina, San Diego, CA, USA) on a Miseq Dx instrument (Illumina). The mean coverage depth was 463.8x, and 204 variants were identified. Except one variant in the intron of *APC*, all variants were predicted to be benign or likely benign. The identified suspected variant was NC_000005.9 (NM_000038.5):c.423-8A>G, which was heterozygous and found in 105 out of 185 reads (56.76%) (Fig. 1). This variant has never been reported previously. *In-silico* splice-site prediction algorithms used were

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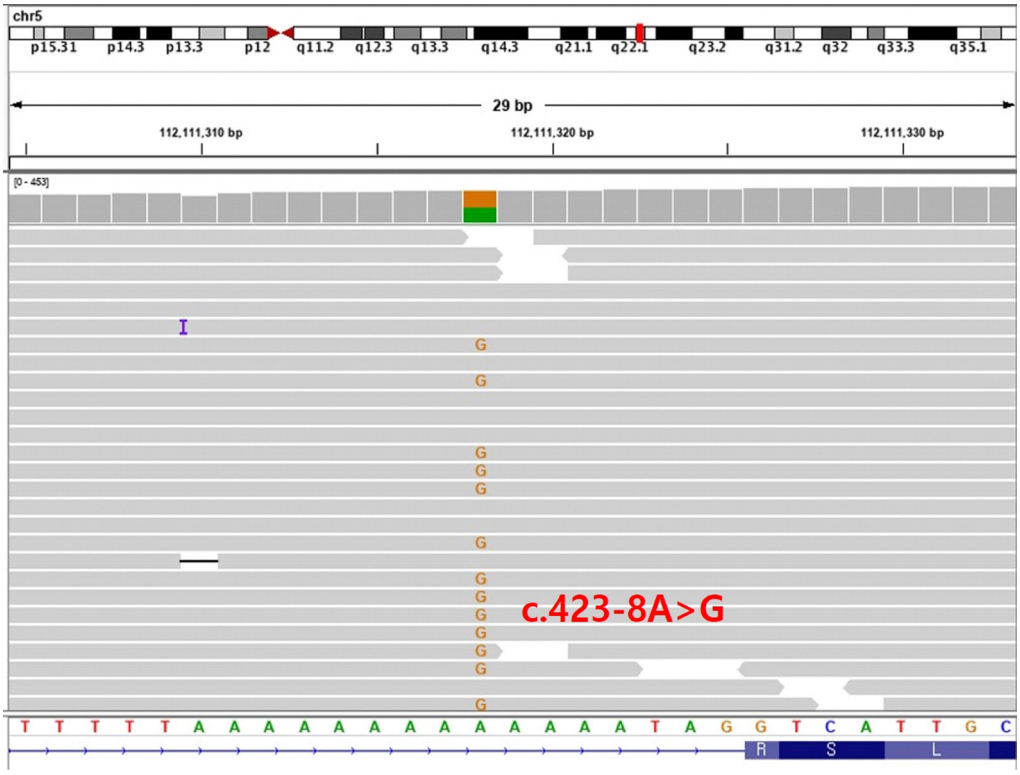

Fig. 1. Next-generation sequencing revealed a variant of *APC*, involving a substitution of guanine (G) for adenine (A) at the intron located –8 from the acceptor site (c.423-8A>G).

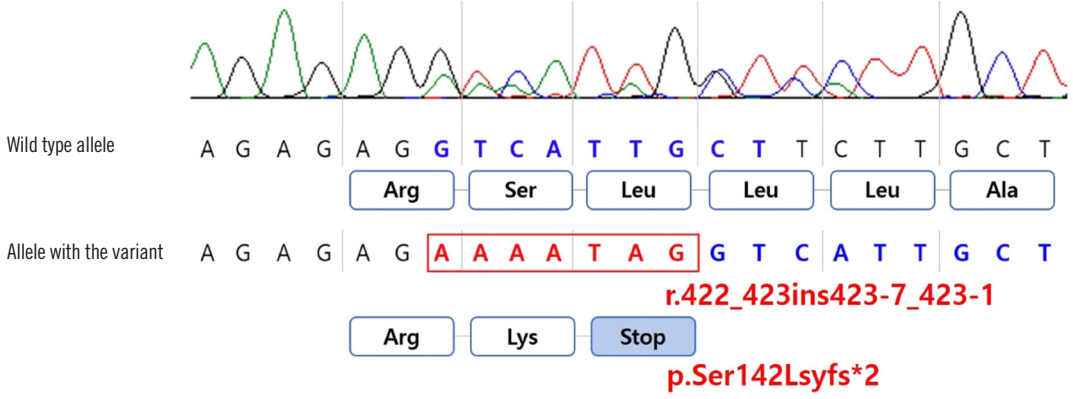

Fig. 2. RNA sequencing revealed an insertion of seven base pairs between r.422 and r.423 (r.422_423ins423-7_423-1). This variant was predicted to cause a premature stop codon (p.Ser142Lysfs*2).

dbscSNV's AdaBoost (adaptive boosting; score: 0.999) [3], Human Splicing Finder [4], and MaxEntScan [5], and Alamut Visual 2 (Interactive Biosoftware, Rouen, France). These algorithms indicated a high chance of splice-site alteration.

As there are numerous adenines close to the acceptor site preceding codon 423 (Fig. 1), presumably, a switch from adenine to guanine at any position in this poly-A site can change the acceptor splice site. The impact of variants on mRNA expression can be highly informative when evaluating their effects at splice junctions and within coding sequences, untranslated regions, and deeper intronic regions [6]. To confirm the splice-site change caused by this variant at the RNA level, we conducted RNA sequencing using primers developed in-house that target the area. RNA was extracted from the patient's leukocytes using High Pure RNA isolation kit (Roche, Indianapolis, IN, USA); RNA was reverse transcribed with RevertAid First Strand cDNA

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Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). Sanger sequencing of the cDNA revealed a change of splice acceptor site (r.422_423ins423-7_423-1; Fig. 2). This variant was predicted to cause a premature stop codon (p.Ser142Lysfs*2). Several variants in this poly-A site preceding codon 423 are registered in gene variant databases such as the Human Gene Mutation Database, but they had not been revealed to affect gene or protein function, except the variant reported by Jarry, *et al*. [7, 8]. These authors reported an *APC* variant identified as c.423-12A>G, which shifted the splice acceptor site up by 11 nucleotides, causing a frameshift and a stop signal 32 codons downstream (p.Ser142Lysfs*32) [8]. So far, studies have not clearly shown an impact of variants between c.423-4 and c.423-16 on gene or protein function [4, 5].

Based on the NGS result in our case, only PM2 (absent in population databases) and PP3 (multiple lines of computational evidence support a deleterious effect) evidence could be assigned based on American College of Medical Genetics and Genomics standards and guidelines, classifying it as "uncertain significance" [3, 6]. However, with the RNA sequencing results added, we could apply PS3 (functional study results) evidence to this variant, classifying it as "likely pathogenic" [6].

Variants in this poly-A site have not been thoroughly validated by RNA sequencing; thus, several variants in this region remain variants of uncertain significance (VUSs), and further evaluation and management of patients with VUSs in this poly-A site and their families may be difficult. With the advancements in sequencing technologies, an increasing number of sequence variants are being detected in the clinical specimens, and the number of VUSs is increasing accordingly. As our study suggests pathogenicity of c.423-8A>G variant via RNA sequencing, we need to make an effort to reduce the number of VUSs by elucidating the impact of sequence variants at the mRNA level when splice-site change is suspected.

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Nothing to disclose.

AUTHOR CONTRIBUTIONS

AK wrote the manuscript. YSH, SBL, and JSB treated the patient and collected samples. WL, SC, WKM, HKK, and SA contributed to the interpretation of the results, and the writing and revision of the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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