Letter to the Editor
Diagnostic Genetics

Ann Lab Med 2021;41:123-125
https://doi.org/10.3343/alm.2021.41.1.123
ISSN 2234-3806 • eISSN 2234-3814

ANNALS OF LABORATORY MEDICINE

A Novel Splice Variant (c.438T>A) of *APC*, Suspected by Family History and Confirmed by RNA Sequencing

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Dear Editor,

Constitutional variants of the tumor suppressor gene adenomatous polyposis coli (*APC*) cause familial adenomatous polyposis (FAP), an autosomal dominant disorder characterized by numerous adenomatous colorectal polyps [1]. We detected a novel synonymous splice variant of *APC* in a family with FAP by next generation sequencing (NGS) and confirmed its impact on splicing by RNA sequencing.

In August 2019, a 66-year-old man presented to the Department of Gastroenterology, Asan Medical Center, Seoul, Korea, due to increasing polyps since his first FAP diagnosis eight years ago. He reported FAP only in himself and his daughter, who was diagnosed as having an attenuated form of the disease. The daughter's *APC* Sanger sequencing results for leukocytes showed one variant of uncertain significance (VUS), NC_000005.9 (NM_000038.5):c.438T>A (p.Ala146=), and seven benign variants. To identify the FAP-causing genetic variant in the patient, we performed a multi-gene panel NGS for 171 hereditary tumor-related genes, including *APC,* BRCA1 Interacting Protein 1 (*BRIP1*), and Kirsten rat sarcoma viral oncogene homolog (*KRAS*), using a customized Target Enrichment Kit (Dxome, Seoul, Korea) and a MiSeqDx V2 sequencing kit (Illumina, San Diego, CA, USA) with a MiSeqDx instrument (Illumina), after obtaining informed consent for genetic testing. The mean coverage depth was 417.9×, and 300 variants were identified, including all eight *APC* variants found in the daughter plus one additional variant, c.5257G>C (p.Ala1753Pro) (Table 1). This missense variant is registered in ClinVar as a VUS by three submitters, owing to insufficient evidence; it has rarely been found in the healthy population, but was predicted to deleteriously affect protein function by *in silico* tools. Apart from one pathogenic variant of *BRIP1*, NM_032043.2:c.484C>T (p.Arg162*), which has been reported in Lynch syndrome patients [2], and two *APC* VUSs, all other variants were determined to be (likely) benign based on the 2015 ACMG/AMP guideline [3]. As this analysis was performed solely for clinical diagnostic purposes, Institutional Review Board approval was exempted.

The seemingly silent *APC* variant, c.438T>A, has been classified as a VUS on two separate occasions. However, the following characteristics suggested the need for further investigation on its pathogenicity: (1) the variant clustered with symptomatic family members, (2) it is not detected in the healthy population (PM2), (3) it is predicted to have a deleterious effect on splicing by Netgene2 and Human Splicing Finder (PP3), (4) it has not been previously reported, and (5) it affects a moderately conserved nucleotide (PhyloP: 0.347).

To assess the feasibility of PS3 (functional assay) application, total RNA was extracted from the leukocytes of a healthy control

Received: February 27, 2020
Revision received: April 21, 2020
Accepted: July 17, 2020

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Table 1. Characteristics of APC variants identified in the patient and his daughter

Variant*	Proband's zygosity	Daughter's zygosity	gnomAD frequency† East Asian (%)	gnomAD frequency Korean (%)	ACMG/AMP [3] criteria applied	ACMG/AMP classification
c.438T>A	**Hetero**	**Hetero**	**0.00**	**0.00**	**PP3, PM2, PS3‡**	**LP**
c.1458T>C	Hetero	Homo	67.15	75.38	BA1	B
c.1635G>A	Homo	Homo	82.04	85.60	BA1	B
c.4479G>A	Homo	Homo	82.04	85.71	BA1	B
c.5034G>A	Homo	Homo	82.04	85.71	BA1	B
c.5257G>C	**Hetero**	**WT**	**0.03**	**0.10**	**PP3, PM2**	**VUS**
c.5268T>G	Homo	Homo	81.99	85.72	BA1	B
c.5465T>A	Homo	Homo	90.20	92.75	BA1	B
c.5880G>A	Homo	Homo	82.06	85.71	BA1	B

*NC_000005.9 (NM_000038.5) was identified by multi-gene panel NGS or Sanger sequencing; †Allele frequencies are based on gnomAD version 2.1.1.; ‡Based on the 2015 ACMG/AMP applied criteria & classification (variants initially classified as VUS are marked in bold).
Abbreviations: *APC*, adenomatous polyposis coli; NGS, next generation sequencing; ACMG/AMP, American College of Medical Genetics and Genomics/Association for Molecular Pathology; Hetero, heterozygote; Homo, homozygote; WT, wild type; LP, likely pathogenic; B, benign; VUS, variant of uncertain significance.

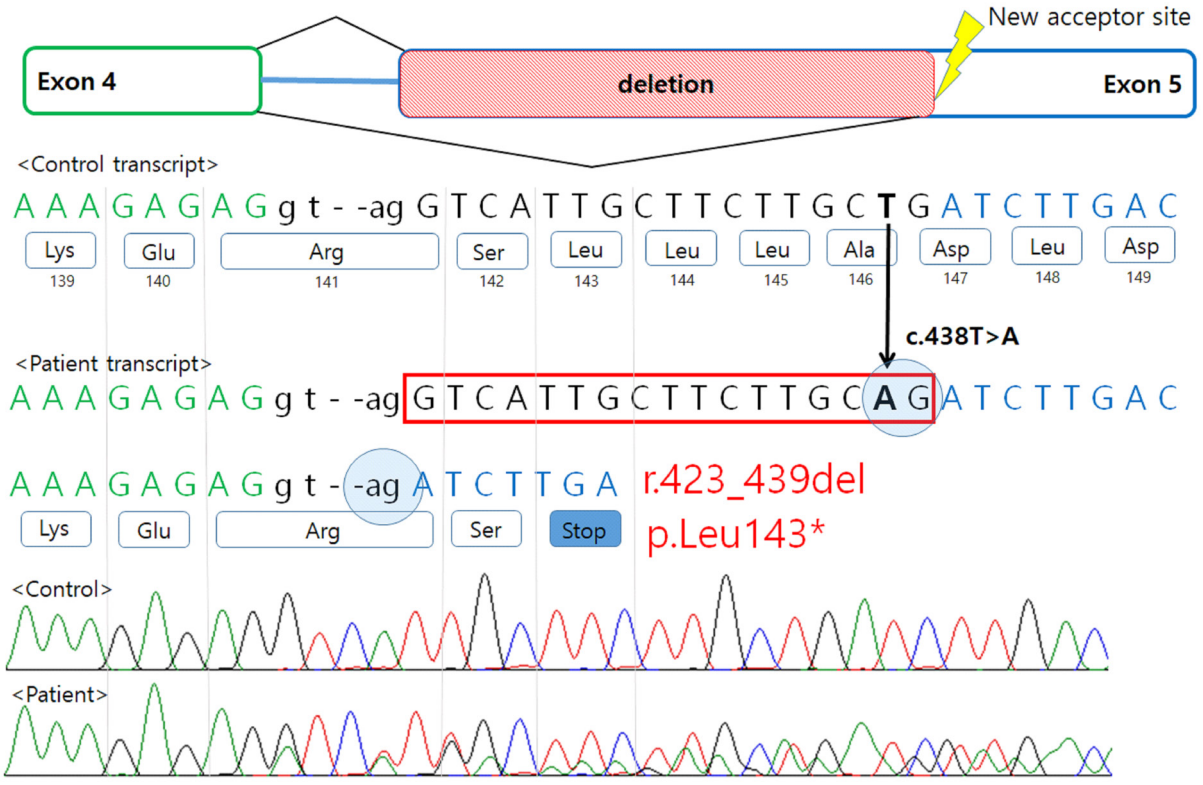

Fig. 1. Schematic diagram of the transcript analysis and sequencing pattern of the control and patient PCR products. The *APC* c.438T>A allele leads to the formation of a new splice acceptor site, which results in the partial deletion of exon 5 (r.423_439del), causing the formation of a premature termination codon (TGA) (p.Leu143*).
Abbreviation: *APC*, adenomatous polyposis coli.

(after obtaining informed consent) and the patient using the High Pure RNA isolation kit (Roche, Indianapolis, IN, USA) and then reverse transcribed using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). cDNA sequencing was performed using target-specific in-house primers: 5′-AGCTATGGCTTCTTCTGGACA-3′ and 5′-ACGACA-

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GCTTTTACAGTCCCA-3′. The reverse transcription-PCR results of the healthy control showed no alternative splicing, whereas those of the patient revealed an open reading frame shift that led to the formation of a new splice acceptor site owing to a 17-bp deletion in the first part of exon 5 (r.423_439del) that caused the formation of a premature termination codon (PTC) (p.Leu143*; Fig. 1). Based on these results, PS3 could be applied, which led to reclassification of the variant as "likely pathogenic." Thus, we defined the genetic cause of FAP in this family.

Approximately 2,000 different *APC* variants have been reported, which can be identified in 60–80% of families with FAP [4]. However, only a few silent/missense *APC* variants have been reported to cause FAP [5]. Although only a small proportion of exonic variants cause splicing alterations, they should not be overlooked and misclassified as synonymous/missense variants. The number of disease-causing missense/splicing variants is underestimated because most clinical sequencing is performed at the DNA level; this leaves functional consequences uncertain [6]. *In silico* analysis of potential splicing variants is not sufficient [5]. RNA sequencing can be highly informative for evaluating the effect of a coding sequence variant (as in this case) or a consensus splice site variant that is deemed pathogenic. The increasing number of VUSs presents a major challenge in providing definitive answers to healthcare providers regarding disease treatment and outcome. The importance of family history and pedigree analysis should not be underestimated. VUSs require periodic re-evaluation as new evidence becomes available, so as not to miss opportunities to diagnose patients [7]. New clinical evidence, such as symptom/sign evolution in patients with a given variant and knowledge of related variants in affected family members, provides a deeper understanding of a variant [3]. Additionally, actively obtained laboratory evidence, such as RNA sequencing results, can lead to the reclassification of a VUS as "(likely) pathogenic" and thus aid in genetic diagnosis.

ACKNOWLEDGEMENTS

None.

AUTHOR CONTRIBUTIONS

HL wrote the manuscript. DY, YSH, SL, and JB managed the patient and provided the clinical information. HK, WL, SC, and WM contributed to the interpretation of the results and revision of the manuscript.

CONFLICTS OF INTEREST

No potential conflicts of interest relevant to this paper are reported.

RESEARCH FUNDING

None declared.

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