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Molecular Case Studies

VARIANT DISCREPANCY RESOLUTION

Classification of the canonical splice alteration *MUTYH* c.934-2A > G is likely benign based on RNA and clinical data

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Abstract *MUTYH*-associated polyposis (MAP) is an autosomal recessive disorder characterized by the development of multiple adenomatous colonic polyps and an increased lifetime risk of colorectal cancer. Germline biallelic pathogenic variants in *MUTYH* are responsible for MAP. The *MUTYH* c.934-2A > G (NM_001128425.1) variant, which is also known as c.850-2A > G for NM_001048174.2, has been identified in our laboratory in more than 800 patients, including homozygous and compound heterozygote carriers. The variant was initially classified as a variant of uncertain significance (VUS) because of lack of a MAP phenotype in biallelic carriers. In two unrelated female patients who were heterozygous carriers of this variant, further testing by RNA sequencing identified an aberrant transcript with a deletion of 9 nt at the start of exon 11 (*MUTYH* r.934_942del9). This event is predicted to lead to an in-frame loss of three amino acids in a noncritical domain of the protein. This was the only splice defect identified in these patients that was not present in the controls, and the aberrant transcript is derived exclusively from the variant allele, strongly supporting the cause of this splice defect as being the intronic variant, *MUTYH* c.934-2A > G. The splicing analysis demonstrating a small in-frame skipping of three amino acids in a noncritical domain, along with the absence of a MAP phenotype in our internal cohort of biallelic carriers, provides evidence that the variant is likely benign and not of clinical significance.

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CASE PRESENTATION

The *MUTYH* gene (NM_001128425.1) encodes an adenine DNA glycosylase that is involved in base excision repair necessary for the repair of oxidative damage and prevention of tumorigenesis (Pezzi et al. 2009). Biallelic pathogenic mutations in *MUTYH* cause the autosomal recessive disorder *MUTYH*-associated polyposis (MAP; OMIM #604933), which is typically characterized by the presence of 20–99 colonic adenomatous polyps (although other types of polyps may occur) and an 80% risk of colorectal cancer by age 70 (Syngal et al. 2015; Kantor et al. 2017). Molecular genetic testing is recommended for patients suspected of having MAP, in which early diagnosis can reduce morbidity and mortality through available surveillance and prophylaxis. National Comprehensive Cancer Network (NCCN) Guidelines recommend colonoscopy and polypectomy every 1–2 yr starting as early as age 25 for biallelic *MUTYH* carriers. The NCCN further recommends prophylactic colectomy and ileorectal anastomosis when adenomas cannot be treated endoscopically (NCCN 2019).

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Table 1. Clinical characteristics and results of RNA sequencing analysis for the two cases presented in this study

Case	Sex	Age at diagnosis	Cancer history	Colorectal polyps	Family history	*MUTYH* NM_001128425.1 variants identified	Zygosity	Results of RNA analysis	Other variants identified
1	F	Early 30s	Colon cancer	No report of polyps	Pancreatic, head and neck, gastric, and breast cancers	c.934-2A > G	Heterozygous	r.934_942del9 (in-frame deletion of 9 nt in exon 11)	None
2	F	No data	No personal history of cancer	7–8 polyps	Breast, pancreatic, prostate cancers, and lymphoma; possible colon and stomach cancers	c.934-2A > G	Heterozygous	r.934_942del9 (in-frame deletion of 9 nt in exon 11)	VUS in *RAD50*

(VUS) Variant of uncertain significance.

Rare, canonical splice site alterations are often classified as likely pathogenic by clinical diagnostic laboratories based on the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) guidelines (Richards et al. 2015; Pesaran et al. 2016). However, *MUTYH* c.934-2A > G occurs at high frequency (~1.5%–2.6%) in East Asian general populations and is reported as homozygous in some of these individuals (Lek et al. 2016). Taken together, these conflicting data led to an initial classification *MUTYH* c.934-2A > G as a variant of uncertain significance (VUS). Furthermore, this variant has not been identified in a patient with MAP in a homozygous state or in conjunction with a second pathogenic mutation in *MUTYH*. In two cases in which this alteration was identified in unrelated Asian females by multigene panel testing (MGPT), further investigation was performed by RNA sequencing (Table 1).

Case 1: This proband had a history of colon cancer diagnosed in her late 30s and the tumor was microsatellite stable with normal expression of the MLH1, MSH2, and MSH6 mismatch repair proteins by immunohistochemical (IHC) staining. No history of polyps was noted. The IHC staining result for PMS2 was not provided. There was a family history of pancreatic cancer, head and neck cancer, gastric cancer, and breast cancer in several first- and second-degree relatives. The results of MGPT in this individual did not identify any other alterations of potential clinical relevance in *MUTYH* nor in any other gene on the panel.

Case 2: This proband was reported to have seven to eight colon polyps of unspecified histology, but no personal history of cancer. There was a family history of breast cancer, pancreatic cancer, prostate cancer, lymphoma, and possibly colon and stomach cancer in several first- and second-degree relatives. The results of MGPT in this individual did not identify any other alterations of potential clinical relevance in *MUTYH*; however, there was a missense VUS in *RAD50*, c.3902A > G (p.K1301R).

VARIANT INTERPRETATION

MUTYH c.934-2A > G is reported in the ClinVar public database (access date 7/19/2021: VCV000041766.23) with conflicting interpretations of pathogenicity: pathogenic, likely pathogenic, variant of uncertain significance, and likely benign. In addition, there is conflicting evidence of pathogenicity in the literature (Tao et al. 2004; Taki et al. 2016; Thibodeau

et al. 2019). Within the framework of the ACMG/AMP guidelines, canonical splice site alterations are considered as inherently having very strong prior probability of being pathogenic (Richards et al. 2015; Pesaran et al. 2016). However, this weight may be reduced when the predicted splice impact results in an in-frame transcript not subject to nonsense-mediated decay (NMD) and/or when there is a lack of supporting clinical phenotype. In support of this variant being interpreted carefully, thorough review of the literature identified no homozygous or compound heterozygous patients with polyposis despite a high general population frequency of >1% in several Asian populations (Tao et al. 2004, 2008; Miyaki et al. 2005; Kim et al. 2007; Johnston et al. 2012; Bodian et al. 2014; Kurian et al. 2014; Jang et al. 2015; Olfson et al. 2015; Jamuar et al. 2016; Kline et al. 2016; Lin et al. 2016; Taki et al. 2016; Cheng et al. 2017; DeRycke et al. 2017; Hansen et al. 2017; Kobayashi et al. 2017; Zhang et al. 2017; Reuter et al. 2018; Takao et al. 2018). Furthermore, in the general population database, gnomAD, this variant has been observed in the Asian general population five times in a homozygous state (gnomAD, dbSNP: rs77542170). Although the phenotypes of these individuals are unknown, they represent the general population as opposed to a cancer cohort. The frequency of this variant combined with the lack of homozygotes and compound heterozygotes in MAP patients is evidence that this variant is benign.

A retrospective review of our laboratory's clinical testing database was performed to identify all probands with this variant in a homozygous state or a compound heterozygous state with another pathogenic *MUTYH* alteration. Of 418 probands with this variant, one was a confirmed homozygous case, whereas the other two had co-occurrence of this variant with the pathogenic founder mutation *MUTYH* c.1187G > A (p.G396D), but phase was not determined (Fig. 1A). Only one of these reported a history of colon polyps (one to two colon polyps in her 30s). The other two probands reported having colonoscopies, but no polyp history was reported.

For this study, two probands were available and willing to provide an additional blood sample for quantitative RNA sequencing (RNA-seq) analysis of *MUTYH* c.934-2A > G, as described previously (Schafer et al. 2015; Farber-Katz et al. 2018; Karam et al. 2019; Landrith et al. 2020). RNA-seq reflected that the majority of abnormal transcript present in both probands was r.934_942del9 (del9; Fig. 1C,D). Of note, one patient carries the polymorphism *MUTYH* c.1014G > C. Analysis of this polymorphism revealed that all the del9 transcripts were derived exclusively from the allele harboring *MUTYH* c.934-2A > G. There was no allele bias in the amplification of this transcript as the polymorphism was present in equal amounts in the pool of transcripts (data not shown).

The c.934-2A > G alteration results in the in-frame deletion of 9 nt from the 5′ end of *MUTYH* exon 11, which is predicted by in silico splice site analysis (Jaganathan et al. 2019). This transcript was absent in normal blood and tissue controls, further confirming the observed splice defect is caused by the variant (Fig. 1C,D). This small deletion is predicted to lead to an in-frame loss of three amino acids (p.V312_Q314del). Another transcript (r.933 + 1_934-1ins79), which results in out-of-frame inclusion of intron 10, was identified in both the probands and control samples. This transcript is known to be a low-level, naturally occurring, alternative splicing event (Fig. 1C; Tao et al. 2004; Taki et al. 2016). RNA data from other published studies of *MUTYH* c.934-2A > G are derived from reverse transcription polymerase chain reaction (RT-PCR) followed by gel electrophoresis, which would be unable to resolve a small-nucleotide change such as del9. In Tao et al. (2004), the authors followed up with quantitative RT-PCR of patient material, but primers designed to detect the variant-type and wild-type transcripts would not detect the del9 transcript. The forward primer for the variant-type transcript was designed to anneal to intron 10, which is absent in the del9 transcript. In addition, the reverse primer for the wild-type transcript spans exons 10/11 and loss of 9 nt from the 5′ end of exon 11 in the del9 transcript would prevent annealing of the primer (Taki et al. 2016). The del9 transcript was detected in the Taki et al. (2016) study after sequencing of the wild-type RT-PCR product from patient samples. A third study

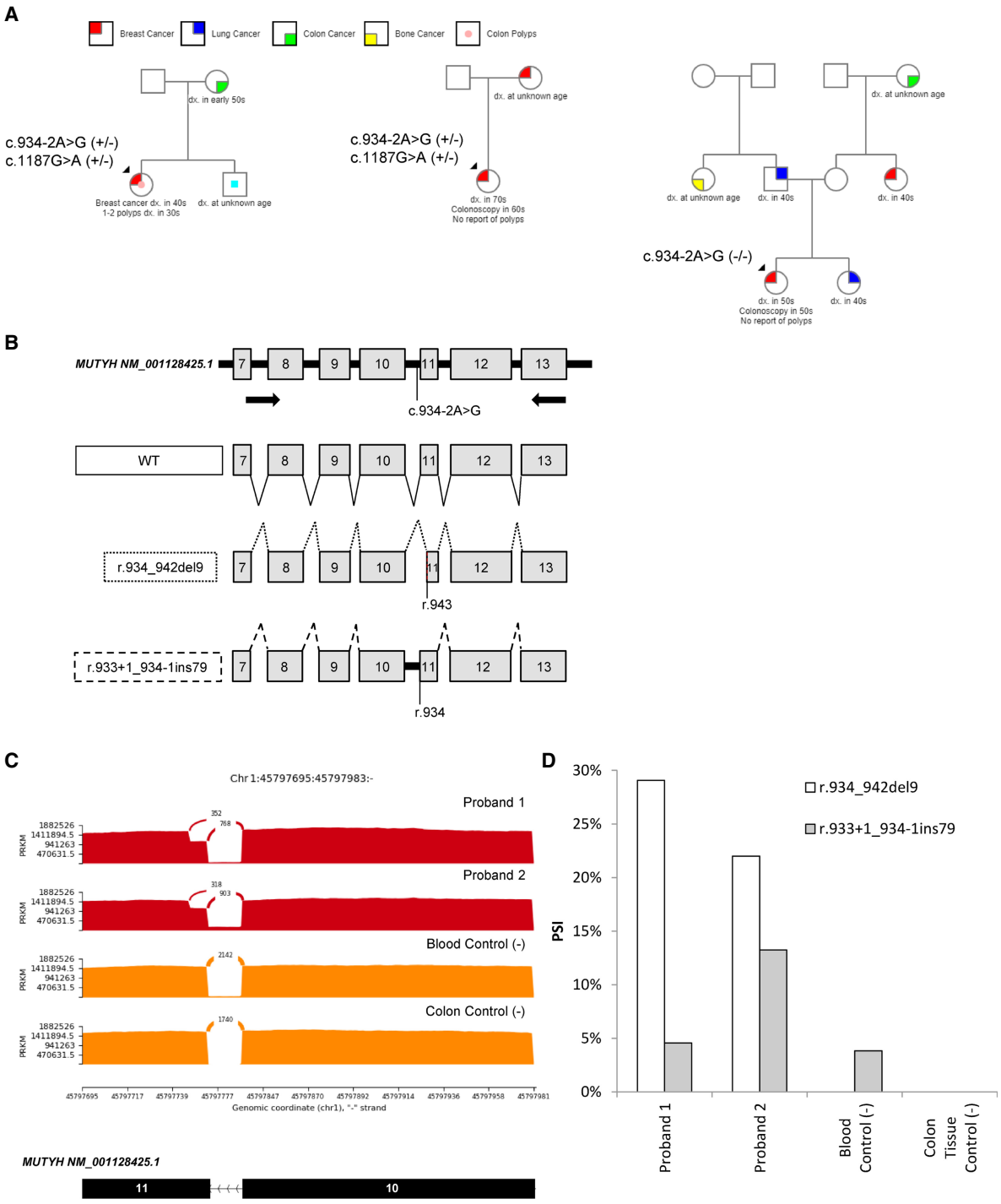

Figure 1. Family histories and massively parallel RNA sequencing (RNA-seq) provide evidence that *MUTYH* NM_001128425.1 c.934-2A > G is likely benign. (*A*) Pedigrees from retrospective analysis of cohort representing a homozygous proband (*right*) and two compound heterozygote probands (*center* and *left*). (*B*) Schematic representation of reverse transcription polymerase chain reaction (RT-PCR) primer design (*top*), the wild type (WT; solid lines) transcript, and the two splicing events observed: r.934_942del9 (dotted lines) and r.933 + 1_934-1ins79 (dashed lines). (*C*) Sashimi plot of the two heterozygous probands (red) and two controls (orange) showing RNA-seq reads supporting the partial exon deletion (*top* arch), wild type (*middle* arch), and intron inclusion (*bottom* line). (*D*) Quantitation of percent spliced in (PSI) for r.933 + 1_934-1ins79 (gray) and r.934_942del9 (white) observed in massively parallel RNA-seq.

identified both the wild-type and del9 transcripts using RNA-seq, but results were not quantitative and a comparison with control samples was not performed (Thibodeau et al. 2019).

The significance of the amino acids predicted to be lost by the del9 transcript (p.V312_Q314del) was investigated by searching for evidence of pathogenicity of missense changes at these three amino acids in public databases (ClinVar, HGMD) and in the literature, although there was limited evidence available. A computational-based structural analysis of this portion of the protein was also limited by the fact that the residues constitute part of a partially flexible linker between the domains and do not engage in any apparent specific interactions (Wang et al. 2015). Flexible linkers are often more tolerant of sequence variation, with any effects often being too subtle to estimate by computational methods.

The cumulative evidence described in this work led to a reclassification of this variant from a variant of uncertain significance to a likely benign variant. The following ACMG criteria were applied: BS1 (subpopulation frequency of 1.539% in East Asian population in gnomAD), BS2 (greater than four to nine homozygotes, presumably without MAP features in gnomAD), and BP2 (co-occurrence with the founder mutation, *MUTYH* c.1187G>A, p.G396D, in two internal cases without MAP features). These data are now available in ClinVar (SCV000183748.8).

SUMMARY

Quantitative RNA sequencing analysis demonstrated that *MUTYH* c.934-2A>G leads to a single aberrant transcript with deletion of 9 nt from the 5′ end of exon 11. This transcript is predicted to lead to an in-frame loss of three amino acids (p.V312_Q314del), which is unlikely to have a significant effect on protein function. Previous RNA studies reported in the literature either failed to detect the del9 transcript in which gel electrophoresis was applied because of its small size difference from wild type, or it was detected as a very minor proportion of aberrant transcripts because of limitations in the study design and methods (Tao et al. 2004; Taki et al. 2016; Thibodeau et al. 2019). This is also the first time the intron 10 retention event was reported in both *MUTYH* c.934-2A>G carriers and wild-type controls; it is apparently the result of routine alternative splicing. *MUTYH* c.934-2A>G was classified as likely benign based on these data combined with high population frequency and homozygous observation of this variant in Eastern Asian general populations, which was further supported by the observation in our laboratory in a homozygous and compound heterozygous state in patients without MAP. Last, it is important to reiterate that caution should be used when applying a very strong prior probability of being pathogenic for canonical splice site alterations if the predicted or demonstrated splice impact results in an in-frame transcript not subject to NMD, especially if there is a lack of clinical phenotype in highly penetrant conditions.

ADDITIONAL INFORMATION

Database Deposition and Access

These data are available in ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) under accession number SCV000183748.8.

Ethics Statement

This study was approved by the Western Institutional Review Board. All patients provided written consent to genetic testing and research-based RNA studies at Ambry Genetics.

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Author Contributions

F.H., B.R.C., M.E.R., H.L., E.C., T.P., and R.K. participated in manuscript preparation and editing. B.R.C. and F.H. participated in data acquisition and analysis. E.C., T.P., and R.K. were responsible for study design.

Competing Interest Statement

All authors were employees of Ambry Genetics for the duration of this study.

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