International Cancer Conference Journal
https://doi.org/10.1007/s13691-021-00474-2

CASE REPORT

Immunohistochemistry and RNA-sequencing have been useful in evaluating the pathological significance of a non-consensus site intronic variant in suspected cases of Lynch syndrome

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Received: 9 October 2020 / Accepted: 8 February 2021

Abstract
Immunohistochemistry of mismatch repair proteins is a universal strategy for Lynch syndrome screening. In this case, Lynch syndrome was suspected, because MLH1 and PMS2 expression was negative by IHC. However, mismatch repair genetic analysis revealed a variant of unknown significance of c.454-13A > G in *MLH1*. Therefore, we performed reverse transcription-PCR using mRNA extracted from the patient's lymphocytes and detected a heterozygous gene allele indicating splicing abnormalities that complex splicing, with exon 5 followed by only the first codon (ACG) of exon 6 and leading to exon 7 of the *MLH1*. Two years later, this mutation was corrected to "likely pathogenic". For Lynch syndrome in which mismatch repair protein expression is undetectable by immunohistochemistry, reverse transcription-PCR may be useful to identify an intronic variant of unknown significance as the likely pathogenic variant.

Keywords *MLH1* · Variants of unknown significance · Splicing defect · Non-consensus site · Reverse transcription-PCR

Introduction

Lynch syndrome (LS) is mainly caused by heterozygous germline pathogenic variants in mismatch repair (MMR) genes, *MLH1*, *MSH2*, *MSH6*, and *PMS2*, with approximately 80% of these variants identified in *MLH1* (41.1%) and *MSH2* (39.3%) [1].

The universal strategy for LS screening of high-risk individuals is immunohistochemistry analysis (IHC) of MMR protein expression. IHC can reveal damaged molecules and predict genetic mutations causing LS, because MMR proteins form heterodimeric complexes [2, 3].

While pathogenic variants in MMR genes are detected in coding regions, a high percentage of single-nucleotide substitutions identified in *MLH1* and *MSH2* are splicing alterations, including variants directly affecting splice sites or altering potential exon splicing regulatory elements [4]. Moreover, approximately 16% of pathogenic variants are located in introns (intervening sequences: IVS) [5]. mRNA analysis with reverse transcription-PCR (RT-PCR) and cDNA sequencing (RNA-sequencing) is a useful tool to identify splicing loss of the subsequent exon.

We found that a variant at − 13 position distant from the acceptor site of the intron 5 in *MLH1* has obvious pathogenicity and is due to a complex splicing abnormality.

Case presentation

A 28-year-old woman was diagnosed with colorectal carcinoma. Laparoscopic-assisted right hemicolectomy was performed with D3 dissection. Histopathological examination revealed ascending colon carcinoma stage II (tubular

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Published online: 06 March 2021

adenocarcinoma well-differentiated type). The adjuvant chemotherapy after surgery was not performed, because there were no high-risk factors for recurrence such as T4 and perforation.

Her family history matched Amsterdam Criteria II [6]. Her father had suffered from small bowel carcinoma at age 56 years and her paternal grandfather had suffered from colorectal carcinoma at age of 90 years (Fig. 1). IHC showed that MLH1 and PMS2 proteins were not expressed, suspecting the diagnosis of LS (Fig. 2).

MMR genetic testing by a commercial genetic testing company indicated c.454-13A > G (heterozygous) variant in *MLH1* and c.3488A > T (p.Glu1163Val) variant in *MSH*. The former was evaluated as VUS, because it is located at the so-called the non-consensus splice site, and the latter was evaluated as polymorphism. Results of IHC and MMR genetic analyses were inconsistent. Since the former mutation was located in the intron, it was considered that splicing abnormality may have occurred.

Determining whether this variant is pathological was necessary for medical intervention in this patient and her relatives. RT-PCR analysis was performed using mRNA extracted from the patient's lymphocytes to investigate whether the A > T change upstream of *MLH1* exon 6 affected splicing.

RNA derived from the patient's lymphocytes was subjected to RT-PCR of *MLH1* exon 3 to exon 9. The primers used were as follows: MLH1Ex3-9F 5′-CGAGGTGAG GCTTTGGC-3′, MLH1Ex3-9R 5′-CTTCTTCACTGAGTA GTTTGC-3′. Small 376 bp fragments were also observed in addition to the expected 468 bp for patient-derived cDNA (Fig. 3). cDNA was re-extracted from each fragment and performed sequencing. The 468 bp fragments were spliced by each exon in turn, while the smaller fragment is a complex splicing, with exon 5 followed by only the first codon (ACG) of exon 6 but lacking 92 bp, and suddenly leading to exon 7 (Figs. 4, 5). The two nucleotides following the ACG used for transcript in exon 6 were the same GT as the consensus sequence of the intron donor site. Therefore, it was suggested that a new cryptic donor site was used in this allele. The anomalous transcript could be predicted to cause premature stop codon in exon 7.

Two years later, we got the report from genetic testing company that this mutation was "likely pathogenic". DNA test by ClinVar showed "likely pathogenic" in five individuals. Therefore, we did not examine *MLH1* promoter methylation and the *BRAF* V600E mutation.

Discussion

LS is an autosomal dominant genetic disease in which colorectal carcinoma and endometrial cancer, etc. frequently occur from an early age due to disordered DNA mismatch repair. Confirmation of pathological mutations in MMR genes is necessary for the definitive diagnosis.

IHC is a universal strategy of LS screening before genetic analysis. IHC in LS is considered to have high sensitivity and specificity of 80.8–100.0% and 80.5–91.9%, respectively [7]. Therefore, in cases where the loss of mismatch repair proteins of MLH1 and PMS2 by IHC is not consistent with the genetic analysis as VUS, further

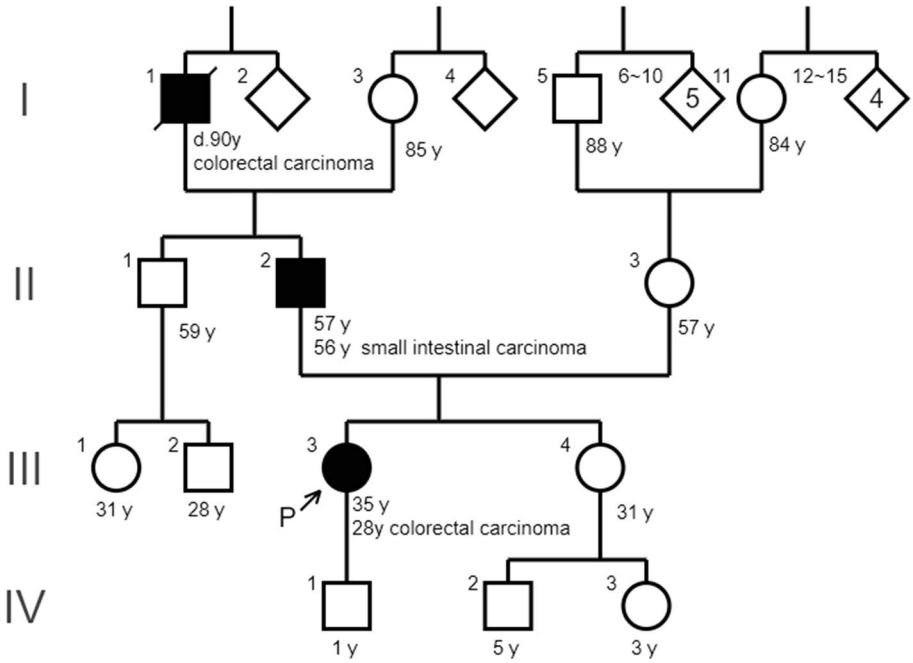

Fig. 1 Family pedigree

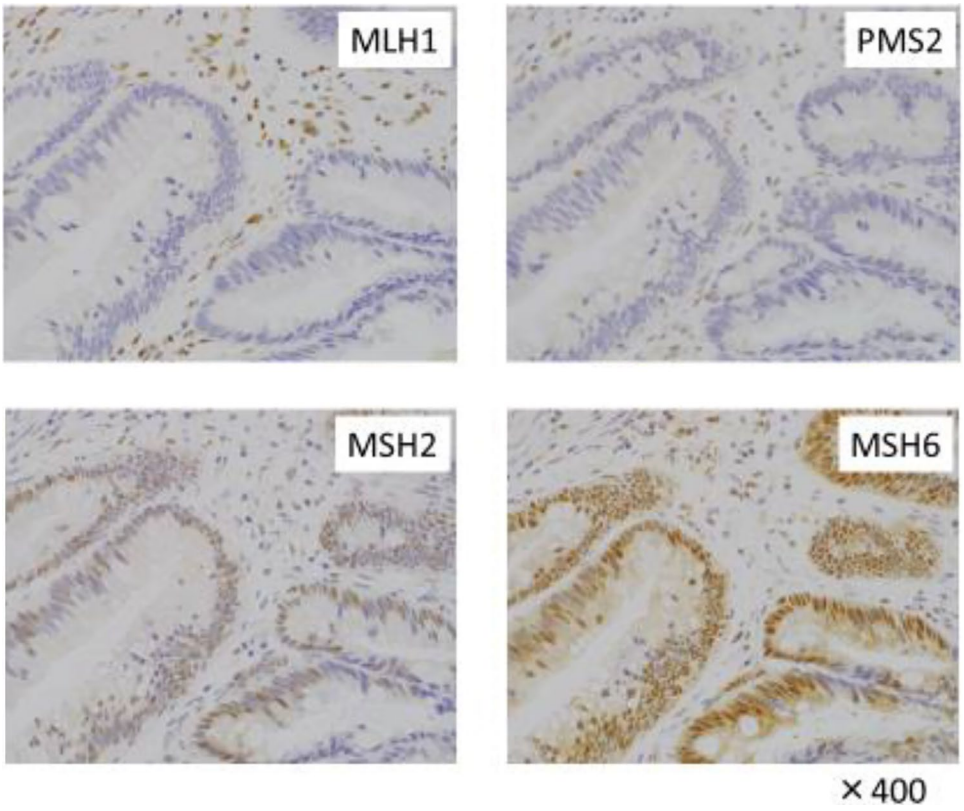

Fig. 2 Immunohistochemistry findings of colorectal carcinoma. Carcinoma epithelium was negative for MLH1 and PMS2 expression and positive for MSH2 and MSH6 expression (hematoxylin staining). All antibodies were obtained from Leica Microsystems, Germany

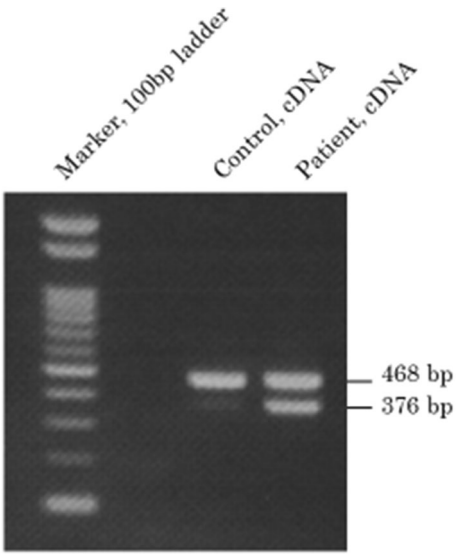

Fig. 3 Results of reverse transcription-PCR analysis. Variant of A > G at position − 13 of *MLH1* intron 6 was analyzed by reverse transcription-PCR using mRNA extracted from the patient's lymphocytes and two fragments were identified; one fragment was the expected size of 468 bp. On the other hand, the other fragment observed in the patient sample was 92 bp shorter (376 bp)

examinations including the MLH1 promoter methylation and the BRAF V600E mutation are necessary [8]. However, we analyzed of RNA extracted from leucocytes of the affected patient, because the mutation was present in intron [9]. Our case also underlined the importance of performing RNA splicing assays to reconcile the disagreement between positive IHC analysis and gene result as intronic variant. In fact, this variant was corrected likely pathogenic later.

Gene-specific distributions by mutation type in MMR genes in LS include pathogenic variants by splicing change in 11% of 1104 patients of *MLH1* and 8% of 883 patients in *MSH2*, respectively [10]. However, many of the variants mapping outside of invariant splice site positions (IVS ± 1, IVS ± 2) tend to classify as VUS [4]. In fact, VUS account for 31% and 28% of all deposited variants in *MLH1* and *MSH2*, respectively [10], though splice site alterations are the most common pathogenic variants in *MLH1* [4]. Therefore, it was clinically very important to confirm whether the variant observed in the non-consensus splice site was pathogenic, that is, the cause of abnormal splicing [11].

In conclusion, although the frequency of *MLH1* abnormality is most common among MMR genes, genetic mutations can be present in introns, which cause splicing error in *MLH1*. LS is strongly suspected due to family history, IHC, and negative *BRAF* V600E mutation, and intronic VUS through MMR gene analysis is necessary to perform exact examination by RT-PCR/sequencing on RNA. In addition, the clinical features of the c.454-13A > G variant of the *MLH1* gene need to be further accumulated.

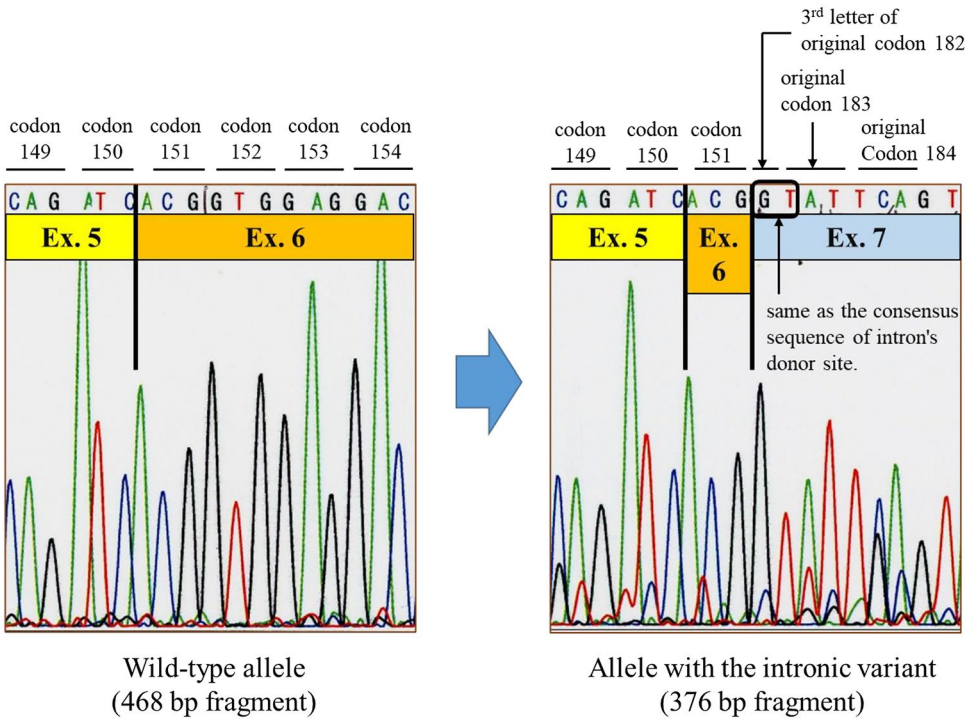

Fig. 4 Results of RNA-sequencing. The 468 bp fragments derived from the wild-type allele were spliced by each exon in turn, while the smaller fragment is a complex splicing, with exon 5 followed by only the first codon (ACG) of exon 6 but lacking 92 bp, and suddenly leading to exon 7. GT were the same as the consensus sequence of intron's donor site. Ex: exon

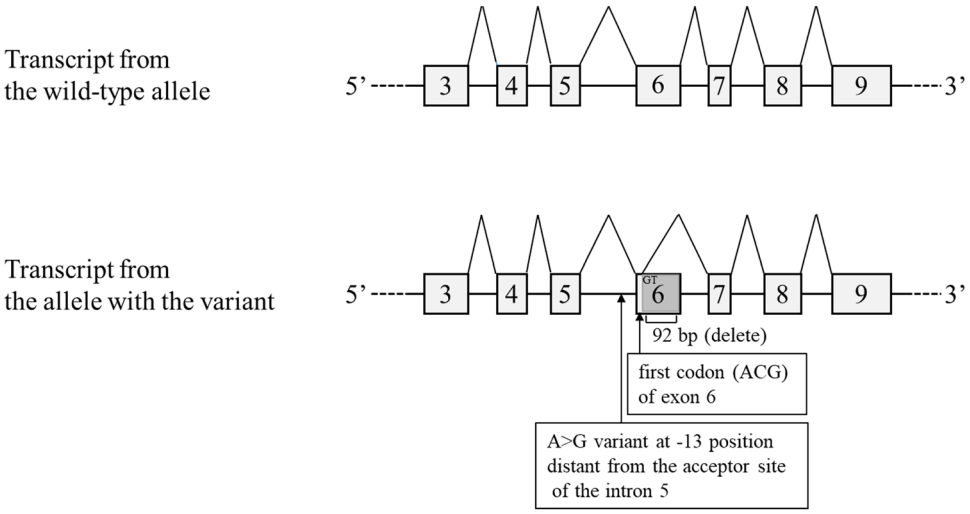

Fig. 5 Schema of the splicing from exon 3 to exon 9 of the *MLH1* gene in patient

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