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## CLINICAL REPORT

# Characterization of a germline variant MSH6 c.4001G > C in a Lynch syndrome family

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## Abstract

**Background:** Germline variants in the DNA mismatch repair (MMR) genes (*MLH1*, *MSH2*, *MSH6*, and *PMS2*) cause Lynch syndrome, an autosomal dominant hereditary cancer susceptibility syndrome. The risk for endometrial cancer is significantly higher in women with *MSH6* pathogenic/likely pathogenic (P/LP) variants compared with that for *MLH1* or *MSH2* variants.

**Methods:** The proband was tested via a clinical testing, Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT). RT-PCR was performed using patient's blood DNA and cDNA was analyzed by DNA sequencing and a cloning approach.

**Results:** We report a 56-year-old female with endometrial cancer who carries a germline variant, *MSH6* c.4001G > C, located at the last nucleotide of exon 9. While the pathogenicity of this variant was previously unknown, functional studies demonstrated that this variant completely abolished normal splicing and caused exon 9 skipping, which is expected to lead to a prematurely truncated or abnormal protein.

**Conclusion:** Our results indicate that this variant likely contributes to cancer predisposition through disruption of normal splicing, and is classified as likely pathogenic.

## KEYWORDS

c.4001G > C, germline, Lynch syndrome, MSH6, splice site variant

## 1 | INTRODUCTION

Lynch syndrome (LS), also known as hereditary non-polyposis colorectal cancer (HNPCC), is an autosomal dominant cancer predisposition syndrome accounting for approximately 1%–5% of all diagnosed colorectal cancers (CRC) (Hampel et al., 2005; Rubenstein et al., 2015;

Vasen, 2005). LS is characterized by a significantly increased risk of developing endometrial cancer (EC) and CRC, as well as ovarian, small bowel, stomach, hepatobiliary, urinary, brain or central nervous system cancer, and sebaceous tumors (Cohen & Leininger, 2014). LS is caused by a defect in the DNA mismatch repair (MMR) pathway, (Kunkel & Erie, 2005; Tamura et al., 2019), or by a

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deletion in the epithelial cell adhesion molecule (*EPCAM*) (OMIM#: 185535) gene, leading to a transcriptional read-through which silences the downstream mutS homolog 2 (*MSH2*) (OMIM#: 60309) gene (Goel et al., 2011; Kuiper et al., 2011; Niessen et al., 2009).

The MMR proteins MLH1, MSH2, MSH6, and PMS2 are encoded by MutL homolog1 (*MLH1*) (OMIM#: 120436), *MSH2*, mutS homolog 6 (*MSH6*) (OMIM#: 600678) and post-meiotic segregation increased 2 (*PMS2*) (OMIM#: 600259) genes, respectively. MSH2 couples with either MSH6 or MSH3, and MLH1 interacts with PMS2 or MLH3 to form heterodimeric complexes (Jiricny, 2006). These complexes are responsible for surveillance and correction of errors made during DNA replication, repair, and recombination (Jiricny, 2006). Germline P/LP variants in the MMR gene generally lead to tumors with characteristic mutational signature, microsatellite instability (MSI), and loss of expression of one or more MMR proteins detected by immunohistochemistry (IHC) (Boland et al., 2008). Although *EPCAM* is not an MMR gene, germline deletions of 3' end of the *EPCAM* gene leads to epigenetic silencing of the neighboring MMR gene *MSH2* by hypermethylation (Kuiper et al., 2011; Ligtenberg et al., 2009).

Germline variants in MMR genes result in a cumulative risk of up to 60% to develop CRC in men, and up to 50% in women; and a risk of up to 50% to develop EC at 75 years of age (Dominguez-Valentin et al., 2020). *MLH1* and *MSH2* P/LP variants account for more than 50% of all LS colorectal cancer in many studies (Hampel et al., 2008; Moller et al., 2017; Sjursen et al., 2016; Yurgelun et al., 2015). *MSH6* and *PMS2* germline P/LP variants are less common, accounting for about 6%–17% and less than 15% of all MMR gene deleterious variants in LS patients respectively (Bonadona et al., 2011; Hampel et al., 2008; Moller et al., 2017; Sjursen et al., 2016). However, more recent studies indicate *MSH6* and *PMS2* P/LP variants account for 24%–29% and 22%–24%, respectively, of all germline P/LP variants associated with Lynch syndrome which is much more prevalent than the previous studies (Espenschied et al., 2017; Latham et al., 2019). Germline *EPCAM* deletions occur in at least 1%–3% of the LS families (Tutlewska et al., 2013). Individuals with *MSH6* P/LP variants tend to develop CRC at an older age than those who carry *MLH1* or *MSH2* P/LP variants and have reduced penetrance (Baglietto et al., 2010; Berends et al., 2002; Hendriks et al., 2004; Wijnen et al., 1998). In women harboring *MSH6* P/LP variants, the risk for colorectal cancer is significantly lower than that in individuals harboring *MLH1* and *MSH2* P/LP variants, while the risk for endometrial cancer is significantly higher by age 70 (Hendriks et al., 2004). The cumulative risk for diagnosis of endometrial cancer through lifetime is 16%–49% for individuals who contains *MSH6* P/LP variants (Baglietto et al., 2010;

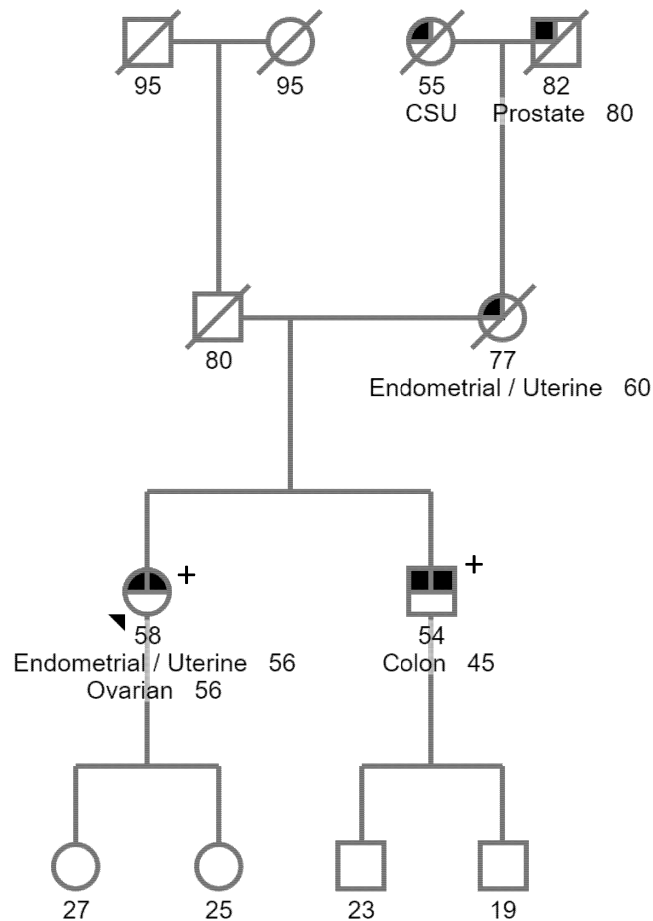
Bonadona et al., 2011; Moller et al., 2018). The incidence of EC is 26-fold higher in women who carry *MSH6* pathogenic variants, compared with incidence for the general population (Baglietto et al., 2010). Therefore, determining *MSH6* variant pathogenicity is of significant clinical relevance, particularly for predicting cancer risks.

The clinical interpretation of variants involving the last nucleotide of an exon is difficult due to uncertain molecular effects of such alterations. Variants at this position may result in missense substitutions (Kanai et al., 1999), and/or disruptions of normal splicing leading to skipping of one or more exons (Barreiros et al., 2018; Vettore et al., 2010; Yamada et al., 2007). Typically, variants in the last nucleotide of an exon are classified as a variant of uncertain significance (VUS) according to the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) guidelines (Richards et al., 2015) in the absence of additional functional, segregation, and splicing studies. A variant in the last nucleotide of exon 9 of *MSH6* gene (c.4001G > A) has been reported to segregate with disease in multiple LS families (Hendriks et al., 2004; Klarskov et al., 2011; Wijnen et al., 1999), and has been classified as pathogenic. However, there are no functional data supporting the pathogenicity of the other variants affecting the same nucleotide, including c.4001G > C. In this report, we demonstrated that the *MSH6* c.4001G > C variant, identified in 56-year-old woman diagnosed with uterine endometrioid carcinoma with microsatellite instability high (MSI-H) and loss of MSH6 protein expression, disrupts normal splicing and results in complete loss of exon 9, which presumably leads to premature protein truncation or abnormal protein. Our results indicate that this variant likely contributes to cancer predisposition through disruption of normal splicing, and can be classified as likely pathogenic based on ACMG/AMP guidelines.

## 2 | MATERIAL AND METHODS

### 2.1 | Subject

Our proband is a 58-year-old woman who was diagnosed with uterine endometrioid carcinoma at age 56 with MSI-H and loss of MSH6 protein expression. A four-generation pedigree (Figure 1) indicated that at least three family members were affected with LS-related cancers. The proband's maternal grandfather was affected with prostate cancer. The proband's mother and brothers were diagnosed with LS. The proband was tested via a clinical-grade testing using NYSDOH- and CLIA-approved Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) and was identified to carry the *MSH6* (NM\_000179.2) c.4001G > C.



**FIGURE 1** Patient pedigree. The patient described here is a 58-year-old female who was diagnosed with endometrial and ovarian cancers at age 56. Her brother who also carries the variant *MSH6* NM\_000179.2 c.4001G > C was affected with colon cancer at age 45 and her mother was diagnosed with endometrial cancer at her 60s.

This variant was classified as VUS and likely pathogenic by different laboratories in ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/variation/233214/>). Given the conflicting interpretations of pathogenicity of this variant, the patient was assigned onto an IRB-approved protocol and agreed to provide additional blood samples for further characterization of this variant at Memorial Sloan Kettering Cancer Center (MSKCC). Peripheral blood samples were collected and submitted to the Diagnostics Molecular Genetics Laboratory at MSKCC. Control RNAs were from unrelated cancer patients who do not carry the *MSH6* variant.

## 2.2 | In silico analysis

Sequence data spanning the *MSH6* locus for *Homo sapiens* [Chromosome 2: 47,783,082.0-47,810,101] was obtained from the Ensembl Genome Browser (<http://www.ensembl.org/index.html>). Primers were

designed using the Primer 3 software (<http://bioinfo.ut.ee/primer3-0.4.0/>). In silico evaluation of the variants was performed through Alamut (Interactive Biosoftware), which include SSF, MaxEnt, NNSPLICE, and GeneSplicer tools.

## 2.3 | cDNA analysis

Total RNA from the patient was extracted using the PAXgene BloodRNA Kit (PreAnalytiX, Qiagen, Valencia, CA) and was subsequently used for cDNA synthesis (Superscript III First-Strand Synthesis SuperMix, Invitrogen Life Technologies, Carlsbad, CA). Control RNA was extracted from another unrelated cancer individual who did not carry the *MSH6* variant. RT-PCR was performed through SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen) for RT and then the JumpStart REDTaq Ready Mix (Sigma) for PCR, with control cDNA or the patient's cDNA in the presence of M13-tagged forward and reverse primers (Forward, E7F: 5'-GTA AAA CGA CGG CCA GT TGAAACTGCCAGCATACTCAT-3'; Reverse, E10R: 5'-CAG GAA ACA GCT ATG AC TCAACTCAAAGCTTCCAATG-3'). Each PCR reaction contains 12.5  $\mu$ l 2 $\times$  JumpStart REDTaq Ready Mix, 2  $\mu$ l 10  $\mu$ M primers (1  $\mu$ l for each primer), 2  $\mu$ l cDNA, and water to make a final volume of 25  $\mu$ l. PCR reactions were performed under the following conditions: 96°C for 5 min, 94°C for 30 s (35 $\times$ ), 64°C for 45 s (35 $\times$ ), and 72°C for 60 s (35 $\times$ ) with a final extension at 72°C for 5 min (1 $\times$ ).

## 2.4 | Cloning

To test whether the mutant allele is able to generate any normal transcript, RT-PCR products were cloned into pCR4 TOPO vectors (Invitrogen, Carlsbad, CA), following procedures of the pCR4 TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). DNA from colonies was amplified using the forward E7F and the reverse primer E10R, and subjected to direct DNA sequencing analysis using the forward PCR primer (BigDye Terminator v3.1 Cycle Sequencing kit and 3730 DNA Analyzer, Applied Biosystems, Foster City, CA).

## 3 | RESULTS

### 3.1 | The *MSH6* c.4001G > C variant disrupts normal splicing and presumably leads to premature protein truncation

This variant c.4001G > C affects the last nucleotide of exon 9 of the *MSH6* coding sequence, which is part of the

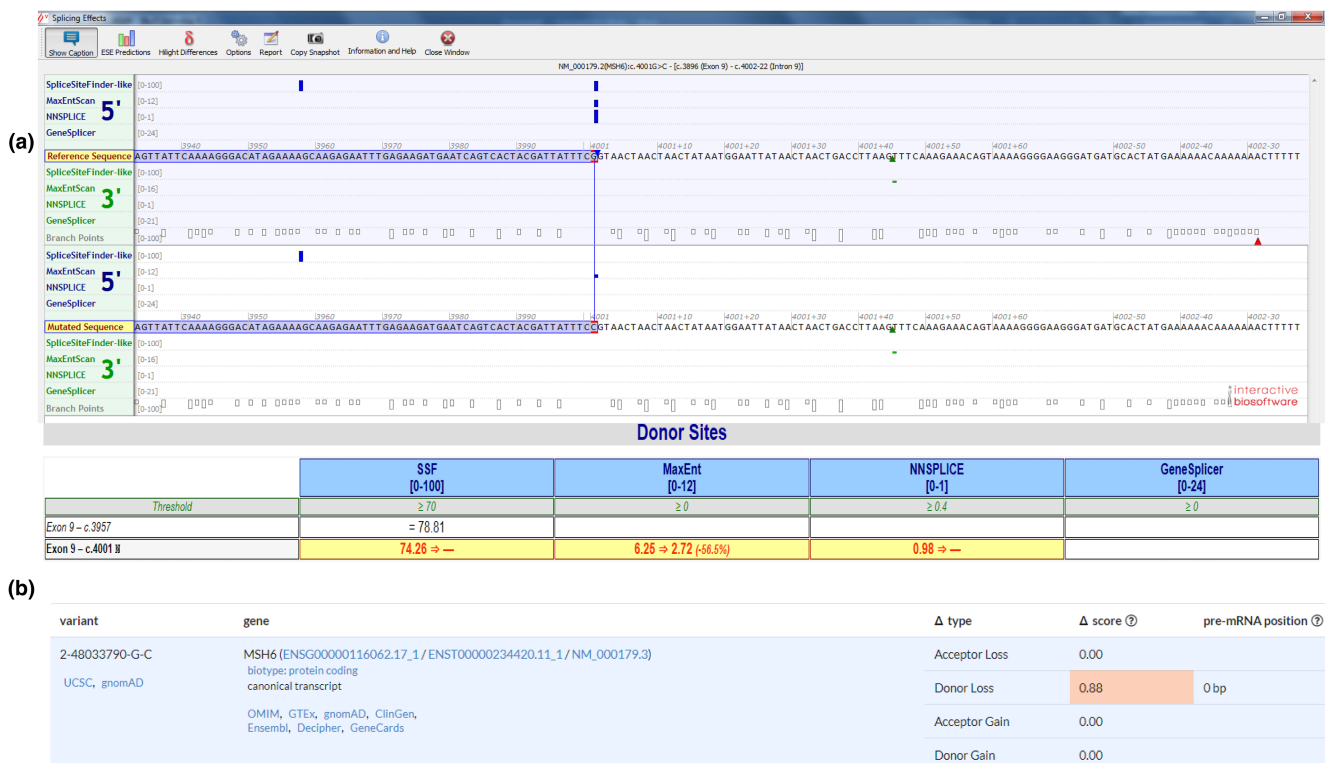
consensus splice site for this exon. To evaluate the potential effects of the variant on splicing, we used Alamut software, which incorporates four tools to predict the potential effect of *MSH6* c.4001G > C on mRNA splicing. Three out of the four tools predicted that the variant significantly weakens the 3' splice acceptor site with two of them predicting a complete loss of the canonical acceptor site and another one predicting a score reduction of 57%. The last tool (GeneSplicer) predicted that this variant may not affect splicing (Figure 2a). Another learning-based splicing tool, SpliceAI (<https://spliceailookup.broadinstitute.org/>), predicts a loss of the canonical donor site at c.4001, with the high prediction score (0.88) (Figure 2b).

The effect of *MSH6* c.4001G > C variant on RNA splicing was evaluated by amplifying relevant regions of *MSH6* from cDNA derived from the patient. PCR was designed to generate a fragment that spanned exons likely to be affected by this variant, including a part of exon 7, and the entire coding regions of exons 8, 9, and 10. Identification of an additional PCR product suggested the presence of an aberrantly spliced *MSH6* transcript in the patient sample (Figure 3a). Further sequencing analysis revealed that the entire coding region of *MSH6* exon 9 is deleted from the aberrantly spliced *MSH6* transcript (Figure 3b). The

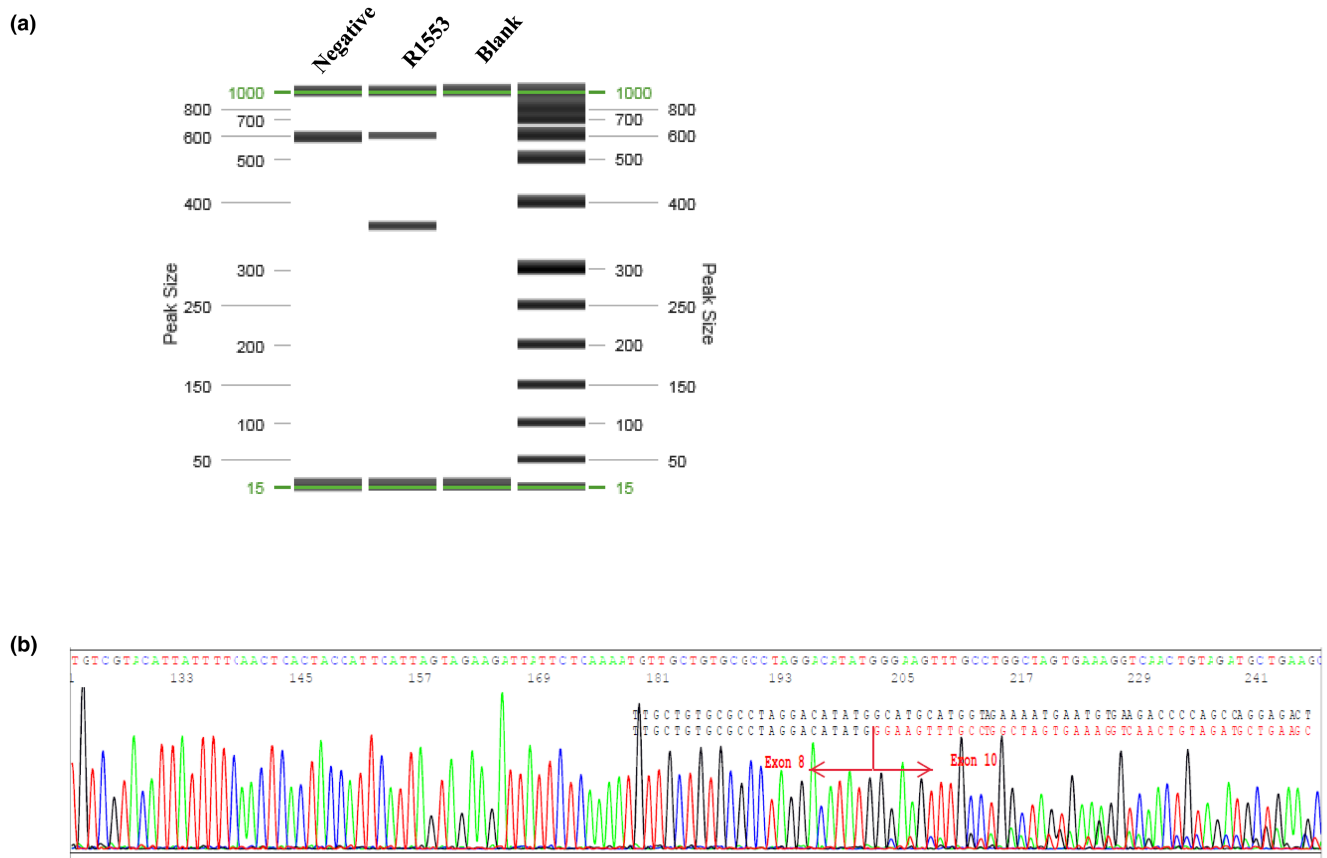
deletion of *MSH6* exon 9 is predicted to result in an absent or truncated protein product NP\_000170.1: (p.?). The truncation disrupts a significant C-terminal portion of the MSH2 interaction domain of the *MSH6* protein (residues Ala1302-Leu1360) (Guerrette et al., 1998; Kariola et al., 2002).

### 3.2 | The variant *MSH6* c.4001G > C completely disrupts normal splicing in the mutant allele

We used a cloning approach to determine whether the c.4001G > C variant completely abolishes normal splicing. The RT-PCR products were cloned into the TOPO sequencing vector, and 135 colonies were sequenced to assess the effect of this variant on splicing. One hundred and nine out of 135 colonies (109/135; 80.7%) contained the aberrantly spliced transcript lacking exon 9 (Figure 4b). The remaining 26 clones (26/135; 19.3%) contained the full-length transcript and all had the wild-type nucleotide at the c.4001 position (i.e., G), indicating that the mutant allele was unable to generate any full-length transcript (Figure 4a).



**FIGURE 2** In silico predictions of the c.4001G > C variant. (a) The Alamut software was used to evaluate the potential effects of the variant on splicing. Three out of the four tools predicted that the variant significantly weakens the 5' donor splice site with two of them predicted complete loss of the canonical donor site and another one predicted a score reduction of 57%. The other tool predicted that this variant does not significantly affect splicing. (b) SpliceAI predicts a loss of the donor site with a high prediction score.



**FIGURE 3** RT-PCR analysis demonstrates c.4001G>C leads to exon 9 skipping. (a) RT-PCR products run on QIAxcel. Two extra bands were observed in the patient, but not in the control. (b) Electropherogram showing that the variant causes exon skipping. The boundary of exons is marked by red arrow.

## 4 | DISCUSSION

Missense changes at the last nucleotide of an exon have been reported to cause missense substitutions, as well as aberrant splicing leading to exon skipping (Kanai et al., 1999; Vettore et al., 2010; Yamada et al., 2007). Therefore, the clinical significance of such variants remains uncertain without further functional, and/or segregation analysis. In this study, we report that a missense substitution c.4001G>C located in the last nucleotide of exon 9 of *MSH6* gene completely abolishes normal splicing of the *MSH6* transcript, and is predicted to lead to a prematurely truncated or absent protein.

The c.4001G>C variant has not, to our knowledge, been previously reported in the literature, and is absent from large reference population databases (e.g., The Genome Aggregation Database) (PM2\_supporting). Although the arginine residue is only moderately conserved, this substitution was classified as likely pathogenic in 2019 by Ambry and as a VUS by Invitae in 2019 (<https://www.ncbi.nlm.nih.gov/clinvar/variation/233214/>). Of note, another variant affecting the same nucleotide (c.4001G>A;

p.Arg1334Gln) is a well-known pathogenic change based on segregation data from two LS families (Hendriks et al., 2004; Klarskov et al., 2011; Wijnen et al., 1999) and observations in other unrelated individuals affected with LS-associated cancers (Overbeek et al., 2007; Susswein et al., 2016; You et al., 2010). The pathogenicity of the c.4001G>C variant was uncertain based on the current version of ACMG/AMP variant interpretation guidelines due to the lack of more definitive functional or segregation data.

A variety of software tools have been developed to predict the effect of an alteration on creation of novel, or changes to existing splice sites. Although these tools cannot be used to definitively classify variants in a clinical laboratory setting, they can help prioritize variants of uncertain significance for further investigation, including in vitro splicing studies. Splicing prediction tools, including SplinceSiteFinder-like, MaxEntScan, NNSPLICE, and SpliceAI, suggested that the *MSH6* c.4001G>C variant may affect RNA splicing, which inspired us to pursue further analysis of patient-derived RNA. The c.4001G>C variant completely disrupted normal splicing leading



**FIGURE 4** cDNA cloning demonstrates that the mutant allele does not produce any wild-type transcript. (a) All clones ( $n = 26$ ) with the full-length transcript containing the normal G at the c.4001 position; (b) All clones ( $n = 109$ ) with the exon 9 deletion are generated from the mutant C allele.

to skipping of exon 9 according to in vitro RT-PCR results. Since exon 9 is the penultimate exon of MSH6, the c.4001G > C variant is likely to escape nonsense-mediated decay and result in a truncated protein (p.Ala1268Glyfs\*6) (Karousis & Muhlemann, 2019; Kurosaki et al., 2019).

The MSH6 protein contains two interaction regions which help in forming a heterodimer with the MSH2 protein (Guerrette et al., 1998; Kariola et al., 2002): the amino-terminal interaction region (residues 326 to 575), and the carboxy-terminal interaction region (residues 1302 to 1360) (Guerrette et al., 1998; Kariola et al., 2002). Our RT-PCR and cDNA sequencing analysis demonstrated that the MSH6 c.4001G > C variant, which may lead to a truncated protein (p.Ala1268Glyfs\*6), is expected to disrupt a significant portion of the C-terminal MSH2 interaction domain (PS3). Notably, multiple truncating variants downstream of Ala1268 have been reported in individuals with LS (Baglietto et al., 2010; Barnetson et al., 2006; Devlin et al., 2008; Raskin et al., 2011). Furthermore, MSH6 c.3984\_3987dup (p.Leu1330Valfs\*12), a well-characterized LS founder mutation

in the Ashkenazi Jewish population (Goldberg et al., 2010; Raskin et al., 2011), has been shown to segregate with disease in a family affected with colorectal cancer (Peterlongo et al., 2003), and result in lack of MSH6 staining and tumor microsatellite instability (Goldberg et al., 2010). Taken together, these reports strongly indicate that the last 31 amino acid residues are critical for MSH6 protein function (PM1), and the c.4001G > C variant likely results in a loss-of-function. It is worth noting that tumor from the proband exhibits MSI-H and loss of MSH2 and MSH6 protein expression, and harbors a second hit with the variant c.3646+1G > A located in intron 7 of MSH6, but not in *MSH2* as tested by MSK-IMPACT (Data not shown), indicating that the loss of MSH2 is likely due to loss of MSH6. The proband's brother who was diagnosed with colon cancer at age 45 also had the same germline variant (PP1). Taken together, the strong LS family history (PP4) and our data indicate that the *MSH6* c.4001G > C variant results in a likely loss-of-function, and should be classified as likely pathogenic according to ACMG/AMP guidelines.

The improved understanding of this variant has significant impact on the patient's medical management and for counseling of the patient and family members regarding disease risk and reproductive planning.

## AUTHOR CONTRIBUTIONS

Ciyu Yang and Liying Zhang designed this study. Ciyu Yang performed the experiments and drafted the manuscript. Maksym Misyura and Vikas Rai assisted with generating laboratory data. Sarah Kane and Alicia Latham provided clinical data. All authors reviewed the manuscript.

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## CONFLICT OF INTEREST

Dr. Zhang reports honoraria (Future Technology Research LLC, BGI, Illumina); honoraria and Travel and accommodation expenses (Roche Diagnostics Asia Pacific). Family members hold leadership position and ownership interests of Decipher Medicine.

## DATA AVAILABILITY STATEMENT

The data was submitted in Clinvar. The submission ID is SCV002060005.

## ETHICAL STANDARDS

The patient signed the IRB-approved protocol (#12–245) approved by Memorial Sloan Kettering Cancer Center.

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