ARTICLE

Full-length transcript amplification and sequencing as universal method to test mRNA integrity and biallelic expression in mismatch repair genes

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Abstract

In pathogenicity assessment, RNA-based analyses are important for the correct classification of variants, and require gene-specific cut-offs for allelic representation and alternative/aberrant splicing. Beside this, the diagnostic yield of RNA-based techniques capable to detect aberrant splicing or allelic loss due to intronic/regulatory variants has to be elaborated. We established a cDNA analysis for full-length transcripts (FLT) of the four DNA mismatch repair (MMR) genes to investigate the splicing pattern and transcript integrity with active/inhibited nonsense-mediated mRNA-decay (NMD). Validation was based on results from normal controls, samples with premature termination codons (PTC), samples with splice-site defects (SSD), and samples with pathogenic putative missense variants. The method was applied to patients with variants of uncertain significance (VUS) or unexplained immunohistochemical MMR deficiency. We categorized the allelic representation into biallelic ($50 \pm 10\%$) or allelic loss ($\leq 10\%$), and $>10\%$ and $<40\%$ as unclear. We defined isoforms up to 10% and exon-specific exceptions as alternative splicing, set the cut-off for SSD in cDNA + P to 30–50%, and regard $>10\%$ and $<30\%$ as unclear. FLT cDNA analyses designated 16% of all putative missense variants and 12% of VUS as SSD, detected MMR-defects in 19% of the unsolved patients, and re-classified $>30\%$ of VUS. Our method allows a standardized, systematic cDNA analysis of the MMR FLTs to assess the pathogenicity mechanism of VUS on RNA level, which will gain relevance for precision medicine and gene therapy. Diagnostic accuracy will be enhanced by detecting MMR defects in hitherto unsolved patients. The data generated will help to calibrate a high-throughput NGS-based mRNA-analysis and optimize prediction programs.

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Introduction

Heterozygous germline defects in the four MMR genes *MLH1*, *MSH2*, *MSH6*, and *PMS2* represent the genetic cause for Lynch Syndrome (LS), the most common hereditary predisposition for colorectal cancer (CRC) and associated tumors with autosomal dominant inheritance [1] showing a gene-specific difference in penetrance [2]. LS tumors typically display high microsatellite instability (MSI-H) and immunohistochemical (IHC) loss of the respective MMR protein. For 65% of the patients with suspicion of LS documented in the German HNPCC Consortium, the diagnosis is molecularly confirmed by the identification of a (likely) pathogenic MMR germline variant [3], according to the InSiGHT criteria [4]. Thirty-five percent of our patients fulfilling the clinical criteria of LS remain unsolved due to the detection of either a germline VUS (20%), or no relevant variant at all (15%) in the MMR genes [3, 5]. For several

sporadic cases, the accumulation of two somatic hits may serve as an explanation, but for those with familial tumor clustering the suspicion of a hereditary tumor syndrome remains [6]. Among VUS, missense variants can exert a functional defect on protein level, for which in silico prediction programs and in vitro assays are performed to define their pathogenicity [7–10], but they can also confer a splicing defect. Splice prediction programs are very reliable only for variants within the conserved splice sites [11, 12], but not for variants altering an exonic splice enhancer (ESE) or generating an exonic splice silencer as well as intronic motifs. Furthermore, the in vivo effect of SSD variants can be versatile and incomputable in silico. There is also growing evidence that several supposed missense variants in the MMR genes in fact generate a SSD [11, 13]. The same may be true for assumed synonymous variants, small indels [11, 13, 14], and even for truncating variants [15–17]. For a correct pathogenicity assessment of variants, an SSD should be excluded prior to functional analyses on protein level. The identification of SSD as a primary cause of disease implies the possibility of therapeutic approaches that target splicing [18, 19], and thus, a correct assessment of a variants' pathogenicity mechanism is mandatory.

The interpretation of cDNA analyses results, which are necessary to investigate the effect of VUS on mRNA level, is impeded by the presence of a variety of alternatively spliced isoforms of the MMR genes already reported in literature [20–23], which have to be discriminated from aberrant, allele-specific splicing [24, 25]. Alternative splicing (AS) is a common phenomenon occurring in 74% of the human multi-exon genes [26], but hitherto its functional relevance, if any, has only been speculated. For the four MMR genes, AS is regarded to result in no or non-functional proteins [16, 27]. This implies that a variant-induced SSD in the MMR transcripts exceeding a certain threshold has to be considered as pathogenic, even for exons known for AS, and for isoforms with in-frame (*if*) deletions. This is different, e.g., for *BRCA1* and *BRCA2*, where splicing *if* can result in a functional protein, and is not regarded as pathogenic even with abundance of 50% of the transcripts [28, 29]. The detection of aberrant splicing of MMR transcripts due to a VUS helps in pathogenicity assessment of that variant, especially when a complete, allele-specific SSD can be demonstrated. However, transcripts encoding an early PTC by a nucleotide change, deletion, frameshift, or SSD are subjected to degradation by NMD [30], and might barely be detected by RNA-based analysis. For pathogenicity assessment of variants on RNA level, we established a robust and universal protocol for cDNA analysis of the four MMR FLT by leukocyte culture, investigation of active/inhibited NMD by incubation with/without puromycin (cDNA-P/+P) [31], RT-PCR of the FLT and sequencing. We defined the normal AS pattern and

biallelic expression in normal controls, and the cut-off values for SSD and allelic loss (AL) in samples with pathogenic variants generating an early PTC or conferring an SSD. The method was applied to samples with VUS or molecularly unsolved patients suspected of having LS.

Materials and methods

Study cohort

All patients meet at least the revised Bethesda criteria [32] and provided their informed consent for cancer genetics research compliant with ethical standards. We applied the recent InSiGHT guidelines [4] (2018-06_InSiGHT_VIC_v2.4 at <https://www.insight-group.org/criteria/>) to categorize variants as class 5 (pathogenic), class 4 (likely pathogenic), class 3 (VUS), class 2 (likely not pathogenic), or class 1 (not pathogenic).

Control cohort 1 included samples from patients with MMR-proficient tumors and normal germline testing of the four MMR genes. For each MMR transcript, ten sample pairs (cDNA-P/+P) were analyzed, and all had a heterozygous variant in the transcript (informative variant) in *MLH1*, *MSH6*, or *PMS2* of class 1–2 (likely) benign. For *MSH2* coding variants are so rare that only three of the ten samples had an informative variant.

Control cohort 2 comprised 25 samples from patients with a (likely) pathogenic class 4–5 variant (shown in Table 1 above), four of these patients additionally harbored a class 3 VUS.

Patient cohort 1 was composed of 22 patients with a variant of uncertain significance (21 different types) detected in one of the MMR genes (Table 1) including potential SSD variants, missense variants or deletions of one amino acid, synonymous variants, and an exon duplication (“class 3 variant group”) to test the method on VUS.

Patient cohort 2 comprised 26 patients with deficiency for at least one MMR protein in IHC staining, no potentially causative MMR germline sequence alteration (classes 3–5) detectable (“with unsolved MMR-deficiency”), beside an informative variant (class 1) in the target MMR transcript. IHC deficiency was reported for *MLH1*/*PMS2* in 16 patients, for *PMS2* in 5 patients, for *MSH2*/*MSH6* in 2 patients, and for *MSH6* in 3 patients. Somatic *MLH1* promoter methylation was excluded by MS-MLPA (MRC-Holland) [33, 34] in 13 of 16 *MLH1*-deficient patients for which tumor DNA was available.

cDNA preparation

Lymphocytes were separated from human whole blood using LeucoSep tubes (Greiner Bio-One), cultured for

Table 1 List of MMR gene variants analyzed with location given in consecutively numbered exon (e) or intervening sequence (IVS)

Cohort	Variant types	Σ patient samples	MMR variant	Class prior/posterior	Effect on mRNA % splicing
Control cohort 2	Early PTC (nonsense, frameshift, exon deletion <i>oof</i>)	10	<i>MLH1</i> e12 c.1342G>T p.(Glu448*)	5	? 15% (+AL)
			<i>MSH2</i> e2 c.242_243insA p.(Ser81Argfs*2)	5	–(AL)
			<i>MSH2</i> e7 c.1147C>T p.(Arg383*)	5	–(AL)
			<i>MSH2</i> e7 c.1165C>T p.(Arg389*)	5	–(AL)
			<i>MSH2</i> e7 c.1215C>A p.(Tyr405*)	5	–(AL)
			<i>MSH2</i> e8 c.1321dupA p.(Thr441Asnfs*2)	5	–(AL)
			<i>MSH6</i> e4 c.1421_1422dup p.(Gln47SCysfs*7)	5	–(AL)
			<i>PMS2</i> e6 c.697C>T p.(Gln233*)	5	–(AL)
			+VUS e13 c.2249G>A p.(Gly750Asp)	3/ <i>in trans</i>	–
			<i>PMS2</i> e11 c.1666G>T p.(Glu556*)	5	–(AL)
	Late PTC (frameshift, exon deletion <i>oof</i>)	3	<i>PMS2</i> e11 c.(1144+1_1145-1)_(2006+1_2007-1)del	5	–v.g.del.
			+VUS e13 c.2181G>C p.(Gln727His)	3/ <i>in cis</i>	–
			<i>MSH6</i> e9 c.3969_3979del p.(Phe1323Leufs*14)	5	SSD 35%+v.g.del.
			<i>PMS2</i> e14 c.(2275+1_2276-1)_(2445+1_2446-1)del	5	–v.g.del.
			2 cases		
	Splice site variant	6	<i>MLH1</i> IVS11 c.1039-2A>T	4	SSD 30%
			<i>MLH1</i> IVS13 c.1558+1G>A	4/5	SSD 15% (+AL)
			<i>MLH1</i> IVS13 c.1559-1G>C	4/5	SSD 50%
			<i>MSH2</i> e5 c.942+3A>T	5	SSD 50%
			<i>MSH2</i> e16 c.2635-1G>T	4	SSD 50%
			<i>PMS2</i> e8 c.903G>T p.(Lys301Asn)	4/5	SSD 50%
	Missense variant, deletion of one amino acid <i>if</i>	6	<i>MLH1</i> e16 c.1852_1854del p.(Lys618del)	5	–
			<i>MLH1</i> e4 c.350C>T p.(Thr117Met)	5	–
			<i>MLH1</i> e10 c.793C>T p.(Arg265Cys)	5	SSD 45%
			<i>MLH1</i> e11 c.986A>C p.(His329Pro)	5	SSD 40%
			<i>MLH1</i> e11 c.986A>C p.(His329Pro)	5	SSD 30%
			+VUS e2 c.191A>G p.(Asn64Ser)	3/2 <i>in trans</i>	–
			<i>MLH1</i> e17 c.1984A>C p.(Thr662Pro)	4	SSD 30%
			+VUS e14 c.1653C>T p.(Asn551Asn)	3/2 <i>in cis</i>	–
	Variants of uncertain significance	3	<i>MLH1</i> IVS17 c.1989+4_1989+5insC p.?	3/5	SSD 50%
			<i>MSH2</i> e7 c.1275A>G p.(Glu425=)	3	SSD 35%
			<i>MSH6</i> IVS6 c.3556+3_3556+13del p.?	3/5	SSD 50%
	Missense variant or in-frame deletion of an amino acid	13	<i>MLH1</i> e10 c.799_800delGTinsAG p.(Val267Arg)	3	–
			<i>MLH1</i> e11 c.1013A>G p.(Asn338Ser)	3	–
			<i>MLH1</i> e13 c.1418A>G p.(His473Arg)	3	–
			<i>MLH1</i> e17 c.1964T>C p.(Ile655Thr)	3	–
			<i>MLH1</i> e19 c.2135G>C p.(Trp712Ser)	3	–
			<i>MSH2</i> e2 c.319G>C p.(Ala107Pro)	3	–
			<i>MSH2</i> e8 c.1316_1318del p.(Pro439del)	3	–

Table 1 (continued)

Cohort	Variant types	Σ patient samples	MMR variant	Class prior/posterior	Effect on mRNA % splicing
			<i>MSH2</i> e14 c.2399T>C p.(Leu800Pro)	3	–
			<i>MSH6</i> e5 c.3264_3266del p.(Phe1088del)	3	–
			<i>MSH6</i> e5 c.3286C>T p.(His1096Tyr)	3	–
			<i>MSH6</i> e8 c.3758T>A p.(Val1253Glu)	3	–
			<i>PMS2</i> e11 c.1437C>G p.(His479Gln)	3	–
			<i>PMS2</i> e13 c.2240G>C p.(Arg745Thr)	3	–
			<i>PMS2</i> e12 c.(2006+1_2007-1)_(2174+1_2175-1)dup r.? p.?	3/4	–v.g.dup.
			2 cases		
			<i>MLH1</i> e12 c.1401C>T p.(Ser467=)	3/2	–
			<i>MSH2</i> e10 c.1560A>G p.(Gly520=)	3/2	–
	Exon duplication	2	<i>MSH6</i> e1 c.135C>T p.(Gly45=)	3/2	–
			<i>MSH6</i> e4 c.1914T>G p.(Leu638=)	3/2	–
	Synonymous variant	4			

Control cohort 2 was selected upon the presence of a variant classified as 4–5 (likely) pathogenic according to the LOVD database/InSiGHT. Four cases additionally harbor a class 3 VUS indicated as (+), for which the allelic position in cis or in trans was determined by cDNA analyses. The 21 MMR variants of uncertain significance (class 3) found in 22 patients represent patient cohort “class 3 variant group” for which we investigated the variants' effect on splicing and allelic expression in cDNA samples.

Prior/posterior indicates the classification according InSiGHT before and after cDNA analyses for re-classification, * not according to InSiGHT guidelines. The variants' effect on mRNA is summarized (– indicating no effect on splicing, ? effect of uncertain significance, SSD significant splice site defect, and maximum sum of isoform intensities observed in cDNA+P in %, AL allelic loss, v.g.del. verified genomic deletion in cDNA, v.g.dup. verified genomic duplication in cDNA). PTC premature termination codon, *oof* out-of-frame, *if* in-frame. Variants in bold represent supposed missense variants which turned out to be splicing defects

72–96 h at 37 °C, followed by incubation with puromycin for 5 h at 37 °C [25, 31], resulting in cDNA + P. Simultaneously, lymphocytes were cultured without the addition of puromycin and denominated as cDNA-P. Total RNA was extracted according to the QIAamp RNA blood mini kit (QIAGEN) including a DNase digestion. For many patients, RNA was also isolated using the PAXgene tubes and blood RNA extraction kit (PreAnalytics) [25]. For cDNA synthesis, 1 µg of total RNA was subjected to the iScript Select cDNA synthesis kit (BioRad) and Oligo-(dT)₁₈ primer following the manufacturer's instructions, but altered incubation temperature (48 °C) and extended time (75–90 min). A test-PCR for feasibility of cDNA amplification was carried out for *MLH1* exons 7–8, and showed absence of genomic contamination if the cDNA fragment (132 bp) was present in the agarose gel in absence of the genomic fragment (280 bp) [33].

cDNA amplification and sequence analysis

From 1–2 µl cDNA a long-range RT-PCR (LR-PCR) was carried out for the transcript of *MLH1*, *MSH2*, *MSH6*, or *PMS2* with the forward primer located in the 5' untranslated region (UTR) of the first exon, and the reverse primers in the 3'UTR of the last exon of the longest transcript (primers

details in Supplemental Table 1). To enable allelic discrimination for as many samples as possible, we included the *MLH1* promoter variant c.–93G>A with an allelic frequency (AF) of 0.441 in Europeans, which made it necessary to establish the LR-PCR for *MLH1* for the isoform with the longest 5'UTR, as described in Genbank. Further benign variants with considerable AF located in the coding regions of *MLH1*, *MSH2*, *MSH6*, and *PMS2* (listed in Supplemental Table 2) were used to investigate the allelic representation (AR). For annotation we used the mRNA and DNA RefSeq NM_000249.3, NG_007109.2 for *MLH1*, NM_000251.2, NG_007110.2 for *MSH2*, NM_000179.2, NG_007111.1 for *MSH6*, and NM_000535.5, NG_008466.1 for *PMS2*, and exons were numbered according to their appearance.

The *MLH1* transcript of 2.452 bp is amplified from exon 1 to 19 c.–148_*33 with IProof High-Fidelity DNA Polymerase (BioRad), annealing temperature 61 °C for 30 s, extension time 1 min 20 s in 39 cycles. The *MSH2* transcript of 2.965 bp is amplified from exon 1 to 16 c.–41_*121 with IProof High-Fidelity DNA Polymerase (BioRad), annealing temperature 59 °C for 30 s, extension time 1 min 30 s in 39 cycles. The *MSH6* transcript of 4.163 bp is amplified from exon 1 to 10 c.–37_*43 with PrimeSTAR GXL DNA Polymerase (TAKARA Clontech), annealing temperature 60 °C for 15 s, extension time 4 min 30 s in 30 cycles. The

Fig. 1 Alternative splicing (AS) of a complete exon, a combination of two exons (with bracket), or a partial exon (black triangle) is depicted for the four MMR transcripts, as we detected it by LR-PCR in ten MMR-proficient control-pairs of cDNA-P/+P. The maximum relative intensity of AS in cDNA+P is given in % below the exon number, the

frequency how often AS was found in the 20 samples is indicated by +/light gray for 1–3 samples, ++/medium gray for recurrence in 4–6 samples, and +++/dark gray for frequent AS detected in seven or more samples, and the predicted effect of AS on the reading frame is given: if = in frame, oof = out-of frame

PMS2 transcript of 2.775 bp is amplified from exon 1 to 15 c.-9_*177 with LA Taq DNA Polymerase (TAKARA Clontech) in a two-step PCR program with 68 °C for 2 min 30 s in 30 cycles. In particular samples, additional short PCR amplifications were performed to investigate allele-specificity of a SSD or the *cis/trans* status of variants (primers see Supplemental Table 1). PCR products were visualized on a 1% agarose electrophoresis gel to check for successful FLT amplification, which was the case for 104 of 106 lymphocyte culture cDNAs-P/+P each, and for 26 out of 73 cDNAs from PAXgene indicating an insufficient quality of RNA from PAXgene tubes for FLT amplification. PCR products were treated with Exo-SAP kit (USB) and directly subjected to Sanger sequencing using several internal primers to generate overlapping reads of 600–900 bp length in one direction by using Big Dye v1.1 (Applied Biosystems). Sequences were run on ABI PRISM 3100 Avant.

Visual inspection of the FLT Sanger sequences was performed especially at known variants and exon–exon-junctions to assess the AR and the splicing pattern by calculation of relative intensities % from the respective peak heights using Sequence Scanner v2.0 (Applied Biosystems)/Mutation Surveyor 3.1 (SoftGenetics) and rounded to intervals of 5. First, the AR of known heterozygous variants in the transcript (informative variants) was investigated in cDNA-P and cDNA+P in comparison to a wild-type (wt) sequence (examples in Supplemental Fig. 1A–D). The larger peak allele of a variant was defined as one allele representing 50%, and the intensity of the other allele was calculated accordingly from the peak heights (Supplemental Fig. 1C, D). In a second step, the splicing patterns were assessed and compared between cDNA-P and cDNA+P. Isoforms become visible by presence of an additional sequence usually starting at exon boundaries [25]. In sequences with two or three traces, we defined the wt, assigned the origin of additional sequences, and calculated intensities from their average peak heights (see Supplemental

Fig. 1E) with the assumption that the sum of FLT and AS isoform intensities is 100%. Nomenclature is compliant with HGVS standard recommendations (<http://varnomen.hgvs.org/recommendations>) referring to the genomic position in hg19. For SSD we mention the exon number for complete exon skipping, partial exon skipping of the 5'-end by p, or q for skipping of the 3'-end, and intron/pseudo-exon inclusion as an insertion. We used the software Alamut Visual Version 2.10 (Interactive Biosoftware, France, <http://www.interactive-biosoftware.com>) to predict the variants' effect upon splicing (SpliceSiteFinder-Like, MaxEntScan, NNSPLICE, GeneSplicer, Human Splice Finder) and the variants' effect on protein function (Align GVGD, SIFT, Mutation Taster, MAPP, PolyPhen), evolutionary nucleotide conservation, and AF from the GnomAD browser or 1000genomes. The cDNA results were submitted to the LOVD3 (<https://databases.lovd.nl/shared/references/DOI:10.1038/s41431-019-0472-8>).

Results

All results are based on cDNA from lymphocyte cultures enabling FLT analysis for 98% of the cDNAs, as PAXgene resulted in poor cDNA quality allowing FLT analysis in 36% of the cDNAs. Detailed results are documented in Supplemental Table 2, and summarized in Table 1 and Fig. 2.

cDNA analysis in MMR-proficient samples (control cohort 1)

The benign variants in ten sample pairs for *MLH1*, *MSH6*, and *PMS2*, and three sample pairs for *MSH2* showed an AR with 40–55% intensity (Supplemental Fig. 2A), so that biallelic expression was defined as 50% AR, allowing 10% standard deviation. AS was observed with an intensity of 5–10% in cDNA-P for at least one isoform in

Fig. 2 Schematic presentation of splicing intensities in cDNA+P found for variants in the four MMR transcripts *MLH1*, *MSH2*, *MSH6*, and *PMS2* sorted by exon numbers. Exon skipping in normal controls (gray dots) was defined as alternative splicing, with their frequency correlated to the thickness of the line, the height giving the range of splicing intensity in %. Alternative splicing usually ranged from 0% to 10%, but showed a few exon-specific exceptions. Based on this, the

lower, light gray zone comprises splice-neutral variants. In samples with a designated splice site defect aberrant splicing was expected and found in a range of 30–50%. The gray zone above was defined to designate splicing defects. After definition of the cut-off values, we investigated the splicing of the variant-bearing exon in samples with a PTC, pathogenic missense variant, or VUS including potential SSD, predicted missense or synonymous variant

each MMR gene except for *MSH2*. In cDNA+P, additional isoforms were present, and some exon-specific AS intensities reached up to 15% for *MLH1* or *PMS2*, and up to 25% for *MSH6*. We found AS skipping of a single exon, two neighboring exons, or a part of an exon, for details see Fig. 1.

cDNA analysis of samples with pathogenic variants (control cohort 2)

Nonsense-mediated decay in class 4–5 early and late PTC variants

For each of the four major MMR transcripts at least one early PTC was investigated (1 × *MLH1*, 5 × *MSH2*, 1 × *MSH6*, 3 × *PMS2*, for details see Table 1). The ten cDNAs-P showed an almost monoallelic expression of the wt, whereas the early PTC was present with 0–10% intensity due to active NMD and defined as AL. In cDNAs+P the early PTC allele was restored, showing intensities of 15–40% for eight of the ten variants (Supplemental Table 2) including aberrant splicing for one sample (Table 1, Fig. 2). Two of the five early PTC in *MSH2* were only visible with maximum 5% or 10% intensity in forward and reverse sequences despite NMD-inhibition, and no additional aberrant splicing was detected. For these two samples with *MSH2* variants in exon 2 (c.242_243insA p. (Ser81Argfs*2)) and exon 7 (c.1215 C > A p. (Tyr405*)) additional short PCRs from cDNA+P spanning exons 1–4, or 4–12 and 5–8 showed the same underrepresentation of

the early PTC allele as in LR-PCR, making an AL due to primer selection unlikely. We tested NMD evasion on three sample pairs with late PTC variants in *MSH6* or *PMS2* which were not subjected to NMD, and detected aberrant splicing for one sample (Supplemental Table 2, Table 1, Fig. 2).

Aberrant splicing in class 4–5 splice site variants

To define cut-off values for aberrant splicing resulting in a SSD, we analyzed cDNAs with six variants designated as class 4/5 due to assumed SSD. Aberrant transcripts out-of-frame (*oof*) generating an early PTC had intensities of 0–10% in cDNA-P due to NMD, whereas aberrant transcripts *if* showed 50% intensity.

MSH2 c.942+3A>T and *PMS2* c.903G>T exerted a complete skipping of the affected exon with 50% intensity in cDNA+P. *MLH1* c.1039-2A>T and *MLH1* c.1558-1G>C created several isoforms in cDNA+P (in total 30–50% intensity) skipping the affected exon and also neighboring exons. *MSH2* c.2635-1G>T induced a deletion of the first four nucleotides of exon 16 (r.2635_2638del p. Gln879Valfs*12) with 50% intensity. *MLH1* c.1558+1G>A inserted a part of intron 13 and enhanced AS of exons 9–10 with 15% intensity each. The informative variant c.655A>G in this sample showed only 15–20% intensity in cDNA+P for the G allele, indicating a reduced AR of the pathogenic allele and explaining the low percentage of aberrant splicing of 15%. In InSiGHT database, another variant *MLH1* c.1558+1G>T was designated as class 5

generating the same SSD with unknown intensity. Detailed information about the different SSD is given in Table 1 and Supplemental Table 2.

Taken together, the AR in a heterozygous variant is found with relative intensities of 40–60% in normal controls. The detection of a reduced AR of an informative variant (<40%) indicates an abnormality so that potential splice site aberrations may not be assessed accordingly and needs further investigation. AS occurs with intensities up to 10% and exceeds this value for only few specific exons. Transcripts with an early PTC were underrepresented with 0–10% in cDNA-P due to NMD and defined as AL, but can be restored up to 15–40% intensity in cDNA+P by NMD-inhibition in most of the cases. Variants in the conserved splice sites can reliably be predicted as SSD, and aberrant splicing was visible with 30–50% intensity at least in cDNA+P, or 15% in combination with a reduced AR (Fig. 2, Supplemental Table 2).

class 4–5 missense variants or deletions of one amino acid

Five missense variants assumed to affect one amino acid and already classified as pathogenic were analyzed in cDNA to test for the mechanism of pathogenicity. *MLH1* c.793C>T p.(Arg265Cys) and *MLH1* c.986A>C p.(His39Pro) both created several isoforms by skipping different exons with aberrant transcript intensities from 30% to 45% in cDNA+P, and the variant conferring the SSD was absent in the transcript (0–5% intensity). The location of the variant *MLH1* c.191A>G p.(Asn64Ser) *in trans* to a pathogenic variant in the absence of constitutional mismatch repair-deficiency (CMMRD) features allows a re-classification of this VUS to class 2. *MLH1* c.1984A>C p.(Thr662Pro) showed 20% intensity (reduced AR), and an *if* skipping of exon 17 with 15–30% intensity. As exon 17 also shows AS, we checked for an aberrant allele-specific splicing by analyzing the VUS c.1653C>T p.(Asn551Asn) in *MLH1* exon 14 additionally present in the sample, which showed biallelic expression in cDNA-P/+P in the FLT, but a reduced AR of 20% intensity when selecting for the presence of exon 17. *MLH1* c.1852_1854del p.Lys618del and c.350C>T p.Thr117Met showed an AR of 50% intensity in cDNA-P/+P in the absence of aberrant splicing, and are designated as splice-neutral. Detailed information about the different aberrant transcripts is given in Table 1 and Supplemental Table 2. In conclusion, three *MLH1* class 4–5 putative missense variants are in fact SSD (Fig. 2, Supplemental Fig. 2) even though not located next to a splice site, and not predicted as SSD by bioinformatics. The FLT analysis furthermore determined four VUS as splice-neutral, and their allelic status towards the pathogenic variant in the transcript (Table 1).

cDNA analysis for class 3 MMR variants (patient cohort 1)

By applying our FLT analysis method, we investigated the effect of further 21 different class 3 VUS from patient cohort 1 (Table 1) upon mRNA splicing (results depicted in Fig. 2, Supplemental Fig. 2, Supplemental Table 2):

Potential SSD variants

Three VUS potentially exert an SSD, as they are located close to a splice site, show an evolutionary sequence conservation of 62–84%, and two to five algorithms predict a reduction/loss of the splice donor site. *MLH1* c.1989+4_1989+5insC generated an exon 17 *if* skipping (r.1897_1989del p.Glu633_Glu663del) with 50% intensity in cDNA-P/+P, and biallelic expression of the benign variant c.1217G>A p.Ser406Asn in exon 12. An allele-specific 100% SSD was shown by amplification of exons 11–17 selecting for transcripts including exon 17, where the allele c.1217A was monoallelically present. *MSH2* c.1275A>G p.(Glu425=) induced an *if* skipping of the rear part of exon 7 (r.1229_1276del p.Ile411_Gly426del) with 30–35% intensity in cDNA-P/+P. The c.1275G allele was still present with 15% intensity, suggesting an allele-specific but incomplete SSD. The cDNA-P sample of *MSH6* c.3556+3_3556+13del showed an AL in a benign variant in exon 1 due to NMD. In cDNA+P a 50% skipping of exon 6 *oof* (r.3439_3556del p.Ala114Valfs*9) was detected, and the benign variant c.116G>A p.Gly39Glu was biallelically represented with 50%. To sum up, we found aberrant splicing with intensities of 30–50% for all three potential SSD VUS. Two variants were designated as complete SSD, which allowed a re-classification to class 5 (pathogenic), whereas the third variant showed a significant but partial SSD and remained class 3.

Missense variants or small in-frame deletions

Based on a biallelic AR (45–60%) and absence of aberrant splicing in cDNA-P/+P for the remaining 11 missense variants and two variants deleting one amino acid (Table 1), we designated these VUS as splice-neutral. However, for re-classification of these variants the effects on protein level have to be clarified in a consented manner.

Exon duplication

The FLT analysis of two samples with a genomic duplication in *PMS2* exon 12 of unknown localization showed an *if* insertion r.2007_2174dup p.Lys670_Ala725dup with 50% intensity in cDNA-P/+P verifying the aberration of one allele in the *PMS2* transcript. Even though InSiGHT rules

would conclude class 3 for this *if* insertion, we regard it as class 4 because it occurs in the protein domain of functional importance for MLH1 interaction. Furthermore, their offspring with homozygous exon duplication shows CMMRD features and IHC loss of the PMS2 in normal and tumor tissue.

Synonymous variants

Four synonymous variants showed a biallelic expression with intensities of 50–60% in the absence of aberrant splicing. The AS was maximum 5% for the exon harboring the variant or any exon in cDNA-P/+P, even for the variant located in *MSH6* exon 4, for which elevated levels of AS was observed in controls. Our designation of these four synonymous variants *MLH1* c.1401C>T p.Ser467=, *MSH2* c.1560A>G p.Gly520=, *MSH6* c.135C>T p.Gly45=, c.1914T>G p.Leu638= as splice-neutral enabled a reclassification from class 3 to class 2 (Table 1).

cDNA analysis in patients with unsolved MMR-deficiency (patient cohort 2)

By investigating the AR of a benign, heterozygous variant (class 1) in the relevant FLT of *MLH1*, *MSH2*, *MSH6*, or *PMS2* we performed a screening for an AL or SSD, which revealed an AR ranging between 45% and 55% and a normal splicing pattern in cDNA-P/+P for 21 of the 26 patients with unresolved MMR-deficient tumors. In cDNA-P of five patients, an AL was detected with intensities of one allele below 10%, four indicating a germline defect in *MLH1*, and one in *PMS2*. In cDNA+P of three of these patients the NMD-inhibition restored the missing allele with an AR of 30–50% in benign variants. Furthermore, the explanatory pathogenic cDNA changes were visible in cDNA+P sequencing: an early PTC variant in *MLH1* exon 13 (r.1415_1416delGA p.Arg472Thrfs*6), an *oof* skipping of *MLH1* exon 6 (r.454_545del p.Thr152Leufs*3) with 45% intensity, and a *PMS2* insertion of 71 nucleotides prior to exon 8 (r.803_804insAATGTGCCATGTGAACCAACCCCGTCTGAAAAGTGAGAGCCCCCTCTGCCCCG GCAGCCGCCCGTCTGGGAG p.Tyr268*) with 35% intensity (Table 2, Fig. 2). Genomic re-sequencing of these patients revealed two pathogenic germline variants, which were initially overlooked in earlier routine diagnostics screening: the early PTC *MLH1* c.1415_1416delGA p.Arg472Thrfs*6 has not been detected by DHPLC mutation screening performed formerly [35], and the splice site variant *MLH1* c.545+3A>G explanatory for exon 6 skipping was only visible in the forward, but not in the reverse Sanger sequence and was therefore not noticed even by the mutation detection program. A large genomic SVA-insertion of 2.2 kb in *PMS2* c.804-60_804-59insJN866832.1

Table 2 Patient cohort 2 of 26 patients with immunohistochemical (IHC) loss of MMR protein staining but no detectable MMR germline defect (classes 3–5)

IHC loss	Σ patient sample	cDNA-P/+P analysis results	Final (genomic) result
MLH1/ PMS2	1	AL/PTC variant <i>MLH1</i> e13 c.1415_1416delGA 50%	PTC <i>MLH1</i> e13 c.1415_1416delGA p. Arg472Thrfs*6 class 5
	2	AL/aberrant splicing 45% of e6 r.454_545del	SSD <i>MLH1</i> IVS6 c.545 +3A>G p.Glu153Phefs*8 class 5
	3	AL/AL in benign variant	?
	4	AL/AL in benign variant	?
	5–16	biallelic AR in benign variant/biallelic AR in benign variant, absence of aberrant splicing	No <i>MLH1</i> germline defect expected
PMS2	1	AL/aberrant splicing 35% r.803_804insAATGTGCCATGTGAACCAACCCCGTCTGAAAAGTGAGAGCCCCCTCTGCCCCG GCAGCCGCCCGTCTGGGAG	SSD <i>PMS2</i> IVS7 c.804- 60_804-59insJN866832.1 p. Tyr268*
	2–5	Biallelic AR in benign variant/biallelic AR in benign variant, absence of aberrant splicing	No <i>PMS2</i> germline defect expected
MSH2/ MSH6	1–2	Biallelic AR in benign variant/biallelic AR in benign variant, absence of aberrant splicing	No <i>MSH2</i> germline defect expected
	1–3	Biallelic AR in benign variant/biallelic AR in benign variant, absence of aberrant splicing	No <i>MSH6</i> germline defect expected

In cDNA-P/+P the allelic representation (AR) of an informative variant (class 1) in the target MMR transcript was investigated, and allelic loss (AL) indicates abnormal results, which can be based on a splice site defect (SSD), a premature termination codon (PTC), or so far unknown genomic cause (?)

described previously [36] was found as the underlying genetic cause for the insertion *oof* on cDNA level. This insertion is located in the intronic region of exon 7 which was not analyzed in routine diagnostics at that time. In cDNA+P of two patients the AL of *MLH1* remained unsolved, and was confirmed in new, independent blood samples, but the reasons for this still need to be discovered.

Discussion

Method standardization

We comprehensively describe a robust protocol for the FLT analysis of the four major MMR genes *MLH1*, *MSH2*, *MSH6*, and *PMS2* from patients' heparin blood samples in short term lymphocyte culture with/without puromycin incubation prior to RNA isolation, cDNA synthesis, LR-PCR amplification of the FLT, and Sanger sequencing. We chose direct sequencing to avoid loss of low-abundance isoforms from gel-extraction, as previously described [37]. Lymphocyte culture at short term did not alter expression/splicing in the MMR genes and has proven to be well suitable for FLT cDNA analysis. Even though the relative intensity of alleles and isoforms from Sanger sequences is semi-quantitative, we show that this method reliably allows the designation of a variants' expression to assess its effect on splicing and RNA-stability/integrity.

This is the first report of a FLT PCR protocol at least for *MSH6* and *PMS2*. Previously, FLT amplification and analysis was published for *MLH1* and *MSH2* [13, 37–40], but did not cover the frequent variant *MLH1* c.-93G>A, tested for an AL without sequencing the FLT [40], performed short PCRs and subcloning in a second step before sequencing [39], or performed sequencing with primers of variable orientation [37, 39, 41]. Most cDNA analyses were performed by RT-PCR amplifying only the patients' cDNA in a selected region of interest [7, 11, 15, 42]. Thereby, splicing might be overestimated because shorter isoforms can be preferentially amplified, effects only visible in the FLT such as intergenic inversions might be lost, and different results may be obtained regarding the extent of an SSD due to primer design. Minigene assays examine the inclusion of one specific exon containing a variant in an artificial sequence context in comparison to the wt in a monoallelic ex vivo environment using transfected cell lines. This technique has shown a high concordance to patients' cDNA analysis, but may in some cases differ in the extent and completeness of an SSD, which is depending on the usage of different vectors and types of cell line [12, 13, 43]. Minigene assays are very useful to assess the effect of variants including intronic variants, when patient RNA is not available, and to confirm an unexpected SSD.

We suggest the FLT cDNA analysis as the preferred method to investigate the effect of MMR variants upon splicing in vivo. Within the European Mismatch Repair Working Group we agreed on this FLT protocol to be able to compare cDNA results from different laboratories, and aim to achieve a greater analytical sensitivity and data consistency, as it was shown for cDNA analyses of *BRCA1* and *BRCA2* [44].

Assessment of biallelic expression and normal AS in controls

Our first step in cDNA analyses was to investigate the AR in heterozygous variants in cDNA-P/+P, which ranged 40–60% relative intensity. The AS for all MMR transcripts was set at an intensity of 5–10%, and some exon-specific exceptions were defined with intensities up to 15% in *MLH1* exon 1q, or *PMS2* exon 6p and exon 11, and up to 25% for *MSH6* exon 4 especially in cDNA+P, where transcript degradation was blocked. These higher values of AS for specific exons may be due to a weaker splice site strength, influence of *cis*-regulatory elements, presence of alternative splice sites at other sites than exon boundaries, and for the large exons *PMS2* exon 11 and *MSH6* exon 4 a PCR bias preferentially amplifying shorter fragments may be possible explanations. Other values of splicing above 15% necessitate further examination including allele-specific underrepresentation. This AS reflects the profile in cultured blood lymphocytes and may also be specific for this method (Fig. 1) [25]. Even though many MMR isoforms are reported as AS [27], we reported only those with ≥5% intensity, and selected against isoforms with an alternative start or alternative end with our method by locating primers in the first and last exon in the wt FLT. In each MMR transcript, we detected at least one AS isoform with 5–10% intensity. For *MLH1* several known isoforms were found, and one new isoform lacking exon 1q (r.-62_116del p.), which may be due to the investigation of the transcript with the longest 5'UTR. In previous *MLH1* FLT analyses, no relevant AS was described [38], or specific sequencing primers were used to select against AS [37]. For *MSH2* we recorded AS of exon 12 and 13 also described in partial cDNA analyses [45, 46]. In *MSH6* none of the few alternative isoforms reported [27] showed significant intensity, but we frequently observed skipping of the entire exon 4, which to our knowledge was not described before [38]. For *PMS2* three isoforms were novel to our knowledge (skipping exons 8, 11, and 14), and partial splicing of exon 4q and 6p already reported as AS was confirmed [38]. In previous RT-PCR cDNA studies mostly analyzing only parts of the MMR transcripts, the intensity of AS was rarely quantified, but described as significantly lower compared to the wt [22]. For *MLH1* and *MSH2* AS was measured with

intensities up to 28% by subcloning [39]. Higher intensities of AS in partial cDNA analyses of MMR genes [20–22, 27], e.g., ranging from 20% to 60% for *MLH1* exons 15 and 16 [47] may potentially be overestimated due to a PCR bias for shorter fragments [25]. On the other hand, AS is reported to vary among individuals and tissues [22, 38, 47].

NMD inhibition

Late PTC variants had a biallelic AR. All MMR transcripts harboring an early PTC had an AR of only 0–10% in cDNA-P due to NMD, which we categorized an AL. Early PTC transcripts could be restored in cDNA+P with 15–40% intensity for 80% of the samples, whereas caution is advised for *MSH2* where only 5% or 10% intensity may be obtained. In literature, some early PTC alleles in *MSH2* could also be restored to only 12% by NMD inhibition [39]. The usage of an alternative poly-adenylation signal abrogating amplification with the primers used, mRNA instability, or a mechanistic incomplete NMD inhibition for *MSH2* might be an explanation. An elevated AS was observed for some pathogenic variants, but its clinical relevance remains uncertain [11, 27]. Some PTC variants induce a partial SSD, which may be relevant for pathogenicity assessment, if the allele is rescued by an *if* deletion [15–17].

Assessment of aberrant splicing

Splice site variants generated isoforms with 30–50% intensity in at least cDNA+P, indicating a fairly complete SSD. As an exception, one sample showed an intron retention in *MLH1* with only 15% intensity combined with a reduced AR of 15–20%. Here, either amplification of the larger isoform was less efficient, additional larger isoforms may not be amplified, or NMD-inhibition was insufficient. In literature, aberrant splicing was defined for partial cDNA analyses by the detection of an isoform intensity within the range of 35–60% [39], or three-fold higher than AS [48]. However, we found that NMD-inhibition may restore only 15% of the PTC alleles. Therefore, also slightly elevated levels of isoforms may need further investigation, especially when combined with an allelic reduction below 25% intensity [49–51].

Analysis of class 4–5 variants

We designated *MLH1* c.793C>T p.(Arg265Cys) as a complete SSD in cDNA affecting exon 10 and neighboring exons, which is in concordance with a report in (LOVD3 microattribution ID 200068 by Leung), and is supported by the proof of an ESE disruption [43], whereas only a partial splicing defect skipping exon 10 was described in minigene

assays [13, 43]. Previously, for this variant a decreased protein stability [52] and reduced MMR efficiency [10] were reported. For variant *MLH1* c.986A>C p.(His329Pro) classified as pathogenic by segregation in LOVD, we reported a complete SSD, whereas functional assays for the predicted protein indicated pathogenicity on protein level [9, 53]. For variant *MLH1* c.1984A>C p.(Thr662Pro) we found a clear but partial SSD with 30% aberrant splicing, described previously [42], and no evidence for an ESE disruption [43]. Two *MLH1* variants c.1852_1854del p.Lys618del and c.350C>T p.Thr117Met were splice-neutral, and protein instability and MMR impairment have been shown in functional protein assays as the underlying mechanism [54, 55]. Protein predictions revealed a disease-causing effect by three or five of five algorithms for *MLH1* e17 c.1984A>C p.(Thr662Pro) or *MLH1* c.793C>T p.(Arg265Cys) without any hint towards a possible SSD pointing out that missense variants generating an SSD cannot be reliably predicted. Therefore, transcript analysis of variants should ideally precede functional protein tests. Beside this functional misclassification, machine learning systems like in silico prediction tools for splicing and protein function are based on wrong information.

Method application for VUS and patients with unsolved MMR deficiency

Within this small number of 25 different class 3 VUS, we designated 12% of them (3/25) as SSD, which is in concordance with one report [12], while a percentage of 25% SSD amongst missense variants was reported elsewhere [11]. By FLT analysis, we were able to re-classify nine VUS, six as class 2, and three as class 4/5 allowing a clinical relevant interpretation. One variant was an incomplete SSD, the other 15 variants alter an amino acid and remain uncertain on protein level. The analysis of FLT in 26 patients with unsolved MMR deficiency showed abnormal results for five cases, presenting an AL for *PMS2* in one, and for *MLH1* in four cases in cDNA-P. NMD inhibition restored the missing allele in three patients, which were then solved by the detection of genomic pathogenic variants overlooked/not analyzed previously. The underlying cause of the AL of *MLH1* in two patients could not be defined in the scope of this work, but was regarded as sufficient proof for a gene defect (LS) potentially based on a regulatory or structural defect.

Conclusion

By using the FLT MMR analysis in cDNA-P/+P we found 3/19 putative missense variants (16%) and 1/12 PTC variants (8%) to unexpectedly exert an SSD, even

though pathogenicity on protein level was assumed. Of all 25 class 3 VUS of different types, 3 were SSD (12%) and 9 could be re-classified (36%). Furthermore, the method detected a germline defect in five of the 26 patients with unsolved MMR-deficiency (19%). In regard of the need for precision medicine, this method will help to elevate diagnostic accuracy in several regards: first, in finding regulatory or exonic variants missed by routine sequencing or previous screening. Second, the RNA-based pathogenicity assessment of class 3 missense or synonymous variants helps to identify SSD. Third, the correct definition of the mutation mechanism of pathogenic variants, which may constitute SSD, will become important in the context of gene therapy. And fourth, the data will help the education of prediction programs by enabling bioinformaticians to work with correct information. Being aware of the existence of highly homologous pseudogenes, our cDNA analysis for the *PMS2* FLT rules out a co-amplification of pseudogenes, allowing the verification of pathogenic *PMS2* variants, exon deletions/duplications, and the detection of gene conversions between *PMS2* and *PMS2CL* [56]. As a limitation, our method may select against transcripts with large intron retention, duplication of several exons, alternative start or end and fusion transcripts due to our PCR design, which may show up as AL. As this method is tedious, time-consuming and needs a very skilled laboratory, a great achievement of this work is the generation of prerequisite data that will be used in a next step to calibrate high-throughput NGS-based mRNA assays also necessitating cut-offs for biallelic/monoallelic expression and alternative/aberrant splicing.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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