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ARTICLE

The complexity of screening PMS2 in DNA isolated from formalin-fixed paraffin-embedded material

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Abstract

Germline variants in the DNA mismatch repair (MMR) gene *PMS2* cause 1–14% of all Lynch Syndrome cancers. Correct variant analysis of *PMS2* is complex due to the presence of multiple pseudogenes and the occurrence of gene conversion. The analysis complexity increases in highly fragmented DNA from formalin-fixed paraffin-embedded (FFPE) tissue. Here we describe a reliable approach to detect true *PMS2* variants in fragmented DNA. A custom NGS panel designed for FFPE tissue was used targeting four MMR genes, *POLE* and *POLD1*. Amplicon design for *PMS2* was based on the position of paralogous sequence variants (PSVs) that distinguish *PMS2* from its pseudogenes. *PMS2* variants in exons 1–11 can be correctly curated based on this information. For exons 12–15 this is less reliable as these undergo gene conversion. Using this method, we screened *PMS2* variants in 125 MMR-deficient tumors. Of the 125 tumors tested, six were unexplained MMR-deficient tumors with solitary PMS2 protein expression loss. In these six tumors two unclassified variants (class 3) and five variants likely affecting function (class 4/5) were detected in *PMS2*. One microsatellite unstable tumor with positive staining for all MMR proteins was found to carry a frameshift *PMS2* variant (class 5). No class 4 or class 5 *PMS2* variants were detected in tumors with other patterns of MMR protein expression loss.

Introduction

Heterozygous germline variants in the MMR genes cause Lynch Syndrome (LS), an autosomal dominant predisposition for mainly colorectal- and endometrial cancer [1]. Most of the reported variants up to now are found in the *MLH1* and *MSH2* gene [2, 3]. However, recent studies show that *PMS2* (and *MSH6*) variants affecting protein function in unselected, population based cohorts are actually much more prevalent [4].

An explanation for this discrepancy is the fact that the colorectal cancer (CRC) risk of *PMS2* variant (that affects function) carriers has shown to be much lower compared with *MLH1* and *MSH2* with risk of CRC around 11–19% by the age of 70 years, and many *PMS2* variant (that affects function) carriers remain undetected [5]. The introduction of population based staining for MMR deficiency in colon and endometrial cancers under age 70 in many countries will very likely result in a higher detection of *PMS2* variants. Supporting a higher prevalence of *PMS2* variants is the fact that homozygous or compound heterozygous variants in the *PMS2* gene are seen more often in patients with constitutional mismatch repair deficiency (CMMRD), a recessive disorder characterized by CRC and childhood hematological- and brain malignancies [6].

The previous underestimation of *PMS2* variant (that affects function) carriers may have also been caused by the presence of multiple *PMS2* pseudogenes, which hamper the analysis of *PMS2* [3, 7, 8]. Fourteen *PMS2*-pseudogenes share a high homology with the 5' end of *PMS2* (exons 1–5), while a fifteenth pseudogene (*PMS2CL*) shares high

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homology with *PMS2* exon 9 and exon 11–15 [2, 8–11]. Additional complexity is added through ongoing gene conversion events between *PMS2* and *PMS2CL* [11]. Germline variant screening strategies propose long-range PCR with a reverse primer in *PMS2* exon 6 or propose designing multiplex ligation-dependent amplification (MLPA) probes, and PCR primers, based on paralogous sequence variants (PSVs) to distinguish *PMS2* exons 1–5 from the fourteen homologous pseudogenes [2, 10, 12]. These PSVs are specific nucleotides that differ between *PMS2* and the pseudogenes, and enable differentiation between two almost complete homologues sequences [3, 9, 10]. This strategy is not reliable in detecting variants in exons 12–15 due to gene conversion events between *PMS2* and *PMS2CL* [11, 13]. Through crossover the sequence corresponding to *PMS2* or *PMS2CL* could be present as the exons 12–15 sequence of *PMS2*, and subsequently expressed [10–12]. To determine which sequence is present, and expressed, long-range PCR on genomic DNA (gDNA) or cDNA is proposed using primers in the unique exon 10 and a nonspecific reverse primer in the 3′ UTR [10, 11, 13–15].

While this strategy is very suitable for reliable detection of *PMS2* variants in leukocyte DNA, it is not applicable when using DNA isolated from formalin-fixed paraffin-embedded (FFPE) tissue blocks, which is highly fragmented [16]. There is a high need for reliable detection of somatic *PMS2* variants in DNA isolated from FFPE tissue as it has been recently shown that a large proportion of MMR-deficient tumors without germline MMR variants and without *MLH1* promoter hypermethylation can be explained by two somatic MMR variants [17–19]. Moreover, testing DNA isolated from FFPE will enable screening of deceased index patients of which only FFPE material is available. Lastly, to implement reliable *PMS2* variant screening in molecular tumor diagnostics, a high-throughput strategy should be developed.

Most studies only focus on screening for variants in *MLH1, MSH2* and *MSH6*, possibly because of the complexity of screening for true *PMS2* variants [17]. We now describe possible pitfalls in *PMS2* variant detection and propose a next generation sequencing (NGS) based approach for reliable testing of *PMS2* in FFPE DNA.

Material and methods

Study cohort

Two patient cohorts were included in this study. In the first cohort, 40 patients with LS associated cancer were screened for somatic DNA variants in the MMR genes in a diagnostic setting. Patients presented with colorectal cancer (CRC, $n = 23$), endometrial or ovarian cancer (EC/OC, $n = 12$), sebaceous gland cancer ($n = 2$), breast cancer ($n = 2$) or colorectal adenomas ($n = 1$). The average age of onset was 55.8 years (range 31–87), and 26 patients were female. The patients presenting with breast cancer both had a history of CRC or EC. The majority of tumors screened showed loss of expression of one or more mismatch repair (MMR) proteins with immunohistochemical staining (IHC) and/or microsatellite instability (MSI) ($n = 35$), but five patients with a family history of CRC were also screened, while having a MMR-proficient phenotype. All experiments were performed in the ISO-15189 certified pathology laboratory of the LUMC. For IHC the laboratory routinely participates in NordiQC quality assessment evaluations. All MLH1/PMS2 negative tumors tested negative for *MLH1* promoter hypermethylation. Four tumors had solitary immunohistochemical expression loss of PMS2. In a second retrospective research patient cohort, DNA isolated from FFPE tissue blocks of 85 unexplained suspected LS patients (without germline MMR variants and without *MLH1* promoter hypermethylation) were screened for variants in the MMR genes in a research setting. Two of the MMR-deficient tumors showed isolated PMS2 expression loss with IHC. Average age of onset of the first Lynch-associated tumor was 51.9 years (range 30–81). IHC and MSI had previously been performed at request of board certified Clinical Genetics medical specialists.

NGS panel

Using the Ion Ampliseq™ tool, two custom NGS panels were designed covering *MLH1, MSH2, MSH6, PMS2, POLE* and *POLD1*. Libraries were prepared with Ion AmpliSeq™ Library Kit 2.0 according to the manufacturer's protocol. Both panels had comparable coverage, although the diagnostic panel covered 76.5% of *PMS2* (exons 1–12), while the research panel covered 79.1% of *PMS2* (exons 1–11 and exon 14). Next-generation sequencing data was generated using the Ion Proton™ System (Life Technologies, Carlsbad, CA, USA).

NGS annotation

The unaligned BAM files, generated by the Proton sequencer, were mapped against the human reference genome (GRCh37/hg19) using the TMAP 5.0.7 software with default parameters (https://github.com/iontorrent/TS). A mapping score is calculated for each read, where the read receives a positive score for each base that matches the reference sequence, and a negative score for each mismatch and/or each deletion. A read will receive multiple mapping scores for different genomic locations where it could possibly be mapped. The read is then assigned to the genomic

location with the highest mapping score. In case that a particular read gets the same alignment score at multiple locations, it will be randomly assigned to one of the loci. Subsequently, variant calling was done using the Ion Torrent specific caller, Torrent Variant Caller (TVC)-5.0.2.

All identified *PMS2* variants (likely) affecting function were visually inspected using the Integrative Genomics Viewer (IGV) [20, 21]. The following Genbank reference sequences were used: NM_000249.3 for *MLH1*, NM_000251.2 for *MSH2*, NM_000179.2 for *MSH6*, NM_000535.5 for *PMS2*, NM_006231.2 for *POLE* and NM_001256849.1 for *POLD1*. *PMS2* exons are numbered as for transcript ENST00000265849.11. Classification of the functional effects of the variants was done according to the five-tiered InSiGHT scheme [22]. As per Human Genome Variation Society guidelines the term "affects function" was used instead of "pathogenic". All *PMS2* variants were added to the gene variant database at www.LOVD.nl/PMS2 (individual IDs: 00208595–00208632).

Results

Two custom MMR panels were designed for detecting variants in DNA isolated from formalin-fixed paraffin-embedded (FFPE) tissue. As FFPE material is known to result in fragmented DNA, the designed amplicons have sizes ranging from 100 to 175 bp. *PMS2* exons 1–11 can be screened due to the *PMS2*-specific PSVs. To be able to distinguish a NGS-read as *PMS2*, every amplicon should at least have one PSV. The two panels (diagnostic and research) covered 96 and 94% of exons 1–11, respectively. A complete overview of PSVs and amplicons is shown in the Supplemental Information, while one of the amplicons (exon 9) is shown in Fig. 1.

By exploiting the presence of PSVs in *PMS2* plus by mapping reads to the full genome and not only to target regions, 125 MMR-deficient tumors (including six tumors with solitary PMS2 expression loss) were screened for variants in *PMS2*. Matching normal colonic mucosa was sequenced when available. Five *PMS2* variants (likely) affecting protein function (class 4/5) and two variants of uncertain significance (VUS, class 3) were detected in the tumors with solitary loss of PMS2 expression (Table 1). The *PMS2* c.(308 C > T/ 308=) (p.(T103I), class 3) and c.1687C > T (p.(R563*), class 5) were found in tumors with a variant in the exonuclease domain of *POLE*, where the *PMS2* variant is expected to be secondary to the *POLE* variant [23]. All patients previously tested negative for germline variants in *MLH1, MSH2, MSH6* and *PMS2*. In addition, one tumor with positive staining for all MMR proteins and a MSI-H phenotype was found to carry a frameshift *PMS2* c.325dupG (p.(E109fs)) variant (Table 1). Interestingly, this patient was previously only tested for germline variants in *MLH1, MSH2* and *MSH6*, but sequencing of leukocyte DNA after detection of the *PMS2* c.325dupG variant detected in the tumor showed that this variant was also present in the germline. In remaining cases with MLH1/PMS2, MSH2/MSH6 or solitary MSH6 expression loss, no *PMS2* variant likely affecting function was detected.

Even though *PMS2* primers amplified more than one locus, due to the presence of PSVs in *PMS2* exons 1–11, the amplified loci are not completely homologous. By aligning the reads to the full genome and by assigning them to the locus with the higher mapping score, variants could be properly called. In addition, IGV was used to visually inspect that reads were mapped to the right locus (Fig. 2). This was performed for all eight *PMS2* variants shown in Table 1, and all variants were found to be present in *PMS2* and not one of the pseudogenes.

Discussion

Using targeted NGS, we now describe how to reliably call *PMS2* variants present in DNA isolated from formalin-fixed paraffin-embedded (FFPE) tissue and how to mitigate the

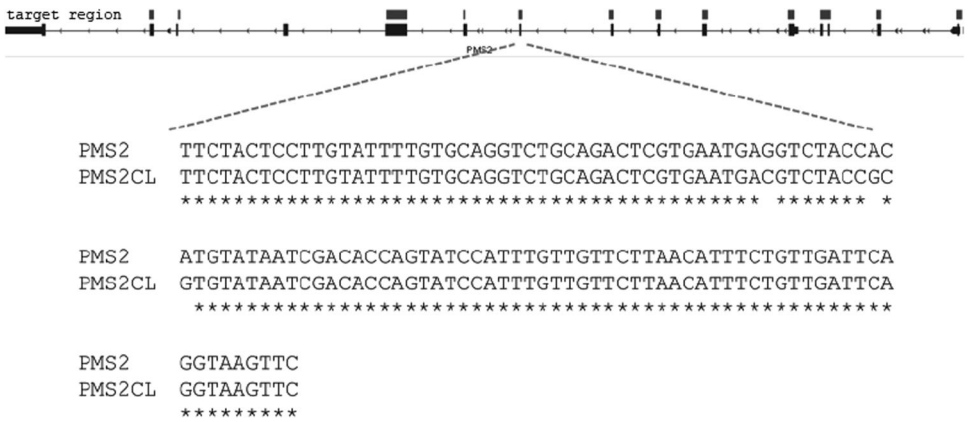

Fig. 1 *PMS2* exon 9 with PSVs. Target regions in *PMS2* (top line), with *PMS2* exon 9 enlarged, showing the paralogues sequence variants (PSVs) between this exon and *PMS2CL*

Table 1 Overview *PMS2* variants

Tumor characteristics	Variant	(Predicted) Protein effect	Exon	VAF	Class	Location PSVs in amplicon	Panel
CRC45, PMS2−, MSI-H	*PMS2* c.(308 C > T/308=)[†]	p.(T103I)	4	11%	3	c.299, c.298	2
CRC38, MMR+, MSI-H	*PMS2* c.325dupG	p.(E109fs)	4	77%	5	c.299, c.298	2
CRC31, PMS2−, MSI-H	*PMS2* c.486delA	p.(L162fs)	5	34%	5	c.406, c.418, c.429, c.452, c.478, c.492	1
CRC48, PMS2−, MSI unknown	*PMS2* c.619 G > T	p.(G207*)	6	48%	5	NA	1
CRC67, PMS2−, MSI-H	*PMS2* c.903 G > T	p.(Y268*)^	8	52%	4	NA	1
	PMS2 c.1261 C > T	p.(R421*)	11	26%	5	c.1238_1239, c.1360_1361[±]	
EC58, PMS2−, MSI unknown	*PMS2* c.955 C > A	p.(P319T)	9	41%	3	c.924, c.932, c.934	1
EC55, PMS2−, MSI-H	*PMS2* c.1687C > T[†]	p.(R563*)	11	30%	5	c.1556, c.1559, c.1567, c.1688_1689	2

Tumor is shown as type of tumor, followed by age of onset. Tumors were screened with the diagnostics panel [1] or research panel [2], panel-information is shown in the Materials and Methods section

CRC colorectal cancer, *EC* endometrial cancer, *MSI* microsatellite instability, high (H) or unknown, *PMS2*− PMS2 negative staining, *MMR*+ positive IHC MMR staining, *fs* frameshift, *VAF* variant allele frequency, *NA* not applicable, this exon is unique, *PSV* paralogues sequence variant

*Stopcodon

[±]PSV is present in primer sequence

[†]*POLE* mutated tumor

^c.903 G > T was previously described to lead to an exon 8 skip (protein: p.(Y268*)) [29]

presence of pseudogenes by using PSVs. Six out of eight *PMS2* variants detected were located in exons with high homology with one or more of the *PMS2* pseudogenes. By exploring the presence of PSVs in the amplicon and aligning the reads to the complete genome, and not only the target regions, it could be concluded that all variants were truly present in *PMS2* and not in the pseudogenes. This approach was additionally used in a recent study investigating somatic variants in 20 tumors of PMS2-associated LS patients [24]. In this study, the second somatic hit was identified in 16 out of 20 analysed tumors (in nine tumors loss of heterozygosity and in eight tumors a somatic class 4 or class 5 variant) [24].

Although a reliable distinction between *PMS2* and its pseudogenes could be made for *PMS2* exons 1–11, exons 12–15 variants cannot be reliably detected due to the existence of continuous gene conversion targeting these exons. A solution to this challenge is long range PCR of fragments covering *PMS2* exons 12–15 [10, 11]. However, because of the fragmented nature of the DNA this is not possible in FFPE tissue.

Studies that aim to detect *PMS2* variants in DNA from FFPE tissues are very limited. Only six studies describe somatic analysis of *PMS2* [18, 19, 23–26]. We and others achieve a total *PMS2* coverage of 75–80% (100% of *PMS2* exons 1–11) and do not sequence *PMS2* exons 12–15 completely. Haraldsdottir et al. did claim full coverage of *PMS2* in tumor tissue [26]. However, they did not fully explain how they coped with gene conversion of exons 12–15 [26]. For example, one *PMS2* splice site variant in intron 12 was shown without confirmation of its presence in *PMS2* and not in *PMS2CL* through gene conversion, while gene conversion is a frequent event (previously shown to occur in 69% of tested individuals) [11]. This example typically highlights the existing problem with sequencing of *PMS2* exons 12–15. Consensus should be reached whether *PMS2* exons 12–15 should be sequenced in FFPE-tissue, when it cannot be confirmed that these variants are truly present in *PMS2* (and subsequently expressed). Although the current study included *PMS2* exons 12 and 14 in our research panel, caution is needed when analysing these variants. However, it could be considered that a *PMS2* exons 12–15 variant likely affecting protein function detected in a tumor with solitary PMS2 loss of expression with no other *PMS2* variants, is likely present in *PMS2* (and not *PMS2CL)*, and is the cause of the immunohistochemical loss of PMS2 expression. In addition, since expressed genes have elevated mutation rates, if a somatic variant is detected in *PMS2* exons 12–15 it is likely that *PMS2* is expressed [27]. However, only RNA sequencing can confirm whether a variant is expressed.

In conclusion, with a custom NGS panel and by using the presence of PSVs, we were able to reliably detect eight

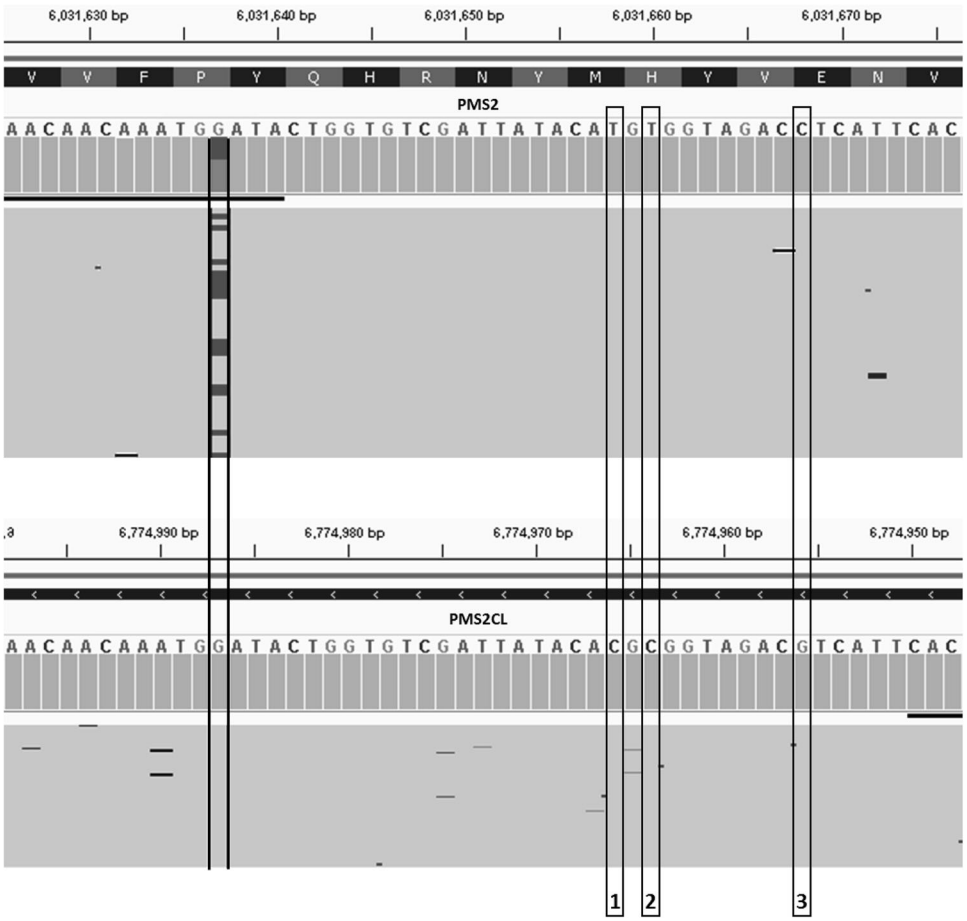

Fig. 2 *PMS2* variants detected with NGS. IGV printout of the *PMS2* c.955 C > A, p.(P319T) shown (left) and the corresponding reads aligned to the *PMS2CL* gene (variant absent). Arrows show the location of three PSVs present in the amplicon (1. c.934 A > G, 2. c.932 A > G and 3. c.924 G > C). All three are absent in the *PMS2* reads, while present in the *PMS2CL* reads. *PMS2* is shown in reverse complement, because *PMS2* is translated on the reverse strand

somatic variants in *PMS2* exons 1–11 in six tumors. Previous studies describe comprehensive strategies for accurate variant detection in *PMS2*, but mainly focus on testing genomic DNA extracted from blood [10, 28]. Since recent studies have shown biallelic somatic inactivation of the MMR genes, there is a growing need for accurate detection of somatic variants in *PMS2* [17–19, 23]. With this guide we show a reliable method to detect *PMS2* variants in DNA from FFPE tissue for exons 1–11 (73–74% of the gene).

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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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