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Assessing pathogenicity of mismatch repair variants of uncertain significance by molecular tumor analysis

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ABSTRACT

Functional analyses are the main method to classify mismatch repair (MMR) gene variants of uncertain significance (VUSs). However, the pathogenicity remains unclear for many variants because of conflicting results between clinical, molecular, and functional data. In this study, we evaluated whether whole exome sequencing (WES) could add another layer of evidence to elucidate the pathogenicity of MMR variants with conflicting interpretations. WES was performed on formalin-fixed paraffin-embedded tumor tissue of eight patients with a constitutional MMR VUS (seven families), including eight colorectal and two endometrial carcinomas and one ovarian carcinoma. Cell-free CIMRA assays were performed to assign Odds of Pathogenicity to these VUSs. In four families, seven tumors showed MMR deficiency-associated mutational signatures, supporting the pathogenicity of the VUS. Moreover, somatic (second) MMR hits identified in the WES data were found to explain MMR staining patterns when the MMR staining was discordant with the reported germline MMR gene variant. In conclusion, WES did not significantly reclassify VUS in these cases but clarified some phenotypic aspects such as age of onset and explanations in case of discordant MMR stainings.

1. Introduction

Lynch syndrome (LS) is a hereditary condition that increases the risk of colorectal cancer (CRC), endometrial cancer (EC), and other extracolonic cancers (Dominguez-Valentin et al., 2020). Biannual colonoscopies are recommended for LS carriers to reduce CRC risk and improve overall survival (Seppälä et al., 2021). Therefore, it is crucial to identify all patients with LS. Universal tumor screening (UTS), MMR gene, or oncogene panel testing are currently used to detect LS (Feliubadaló et al., 2019). However, these methods not only identify (likely) pathogenic variants but also variants of unknown significance (VUS) in MMR genes. When a VUS is detected, further testing is necessary to determine its potential pathogenicity, which can influence the screening advice given by clinicians in shared decision-making.

Various tools have been established to assess the pathogenicity of gene variants, many of which are included in the universal guidelines of the American College of Medical Genetics and Genomics (ACMG), the Association for Molecular Pathology (AMP), the American Society of

Abbreviations: LS, Lynch syndrome; CRC, Colorectal cancer; EC, Endometrial cancer; UTS, Universal tumor screening; MMR, Mismatch Repair; VUS, Variants of Unknown Significance; ACMG, American College of Medical Genetics and Genomics; AMP, Association for Molecular Pathology; MSI, Microsatellite instability; IHC, Immunohistochemistry; TMS, Tumor mutational signatures; WES, Whole exome sequencing; ID, Indels; SBS, Single base substitutions; COSMIC, Catalogue Of Somatic Mutations in Cancer; NGS, Next-generation sequencing; CIMRA, Cell-Free In Vitro MMR Activity; LOVD, Leiden Open Variation Database; FFPE, Formalin-fixed paraffin-embedded; TMB, Tumor mutational burden; SNVs, Single nucleotide variants; MSH, MutS homolog; PMS2, Postmeiotic segregation increased 2; MLH1, MutL homolog 1; POLE, DNA polymerase epsilon; MMRd, Deficient Mismatch Repair; LOH, Loss of heterozygosity; MSI-H, Microsatellite instability-high; MSS, Microsatellite stability.

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Clinical Oncology, and the College of American Pathologists (Li et al., 2017). Clinical information, such as de novo appearance or segregation data, can aid in reclassifying a VUS as (likely) benign or (likely) pathogenic (Barnetson et al., 2008; Rosenthal et al., 2017). For instance, if only affected relatives inherit the VUS, it supports the pathogenicity of this variant. However, interpreting segregation data can be challenging when penetrance is low. Additionally, it is relevant to determine the occurrence of the variant in unaffected controls/relatives, as most variants are likely inconsequential polymorphisms (Song et al., 2016; Barnetson et al., 2008).

Secondly, if not already done, tumor analysis can contribute to assessing a VUS's effect on protein level and its association with MMR deficiency. Immunohistochemistry for MMR proteins or detection of microsatellite instability (MSI) are commonly used methods to assess MMR deficiency in the tumor itself. Also, DNA analysis in tumor material regularly detects somatic hits that potentially explain the MMR deficiency, thereby shedding light on the causality of the VUS.

Thirdly, in silico prediction models and functional analyses contribute significantly to the reclassification of VUSs and provide insights into their potential pathogenicity at protein level. For example, altered splicing can be viewed in SpliceAI, and the protein structure can be explored in SIFT, PolyPhen-2, or MAP-MMR (You and Vilar, 2013; Barnetson et al., 2008). More recently, another comprehensive artificial intelligence-based approach to predict the pathogenicity of missense variants was introduced (Cheng et al., 2023). Also, analysis of MMR protein levels and DNA damage can provide more insights into the remaining MMR activity associated with specific MMR VUSs (You and Vilar, 2013; Drost et al., 2020). Moreover, RNA function measured by RT-PCR can classify up to 25 % of the VUSs (Borràs et al., 2012). Then, functional assays are valuable tools for the classification of VUSs in MMR genes and one of these assays, the Cell-Free In Vitro MMR Activity (CIMRA) assay (Drost et al., 2019; Drost et al., 2020; Rayner et al., 2022) does not require patient material, is fully calibrated and validated and, therefore, will be incorporated as strong evidence for or against pathogenicity of a VUS in the upcoming ACMG/AMP-based diagnostic criteria. However, despite the variety of methods available, the assessment of pathogenicity of a subset of variants might be further enhanced, particularly when clinical data for a VUS are scarce.

To enhance the classification of VUSs, exploring novel methods for variant interpretation is crucial. One potentially promising approach is the utilization of tumor mutational signatures (TMS) through whole exome sequencing (WES) or whole genome sequencing. TMS are distinct patterns of somatic mutation types found within tumors, including single base substitutions (SBS), indels (ID), and doublets (Koh et al., 2021). Different TMS are identified based on the underlying mutational processes. In the context of MMR deficiency (MMRd), several TMS have been associated, such as SBS6, SBS14, SBS15, SBS20, SBS21, SBS26, SBS44, ID1, ID2 and ID7 ('Catalogue of Somatic Mutations in Cancer (COSMIC)', 2022.

A recent study has demonstrated that TMS can effectively discriminate pathogenic MMR variant carriers from non-carriers diagnosed with CRC (Georgeson et al., 2021). Furthermore, we recently showed that MSH6 Lynch tumors show a somewhat distinct molecular phenotype compared to other Lynch tumors (Helderman et al., 2023). In our current study, we conducted WES on tumors (CRC, EC, and ovarian cancer) from patients with an MMR gene VUS to explore whether TMS can serve as an additional tool to elucidate the impact of VUS in cases where functional analyses yield ambiguous or inconclusive results. By integrating TMS analysis, we aim to provide further insights into the pathogenicity of VUSs and improve the interpretation of these variants.

2. Methods

2.1. Cohort description and prior probability

This study was approved by the Medical Ethical Committee of Leiden, The Hague, Delft (protocol P17.098). Patients with an MMR VUS were eligible to participate in this study if they provided written informed consent for using tissue and clinical data. Patient samples were managed in accordance with medical ethical guidelines outlined in the Code of Conduct for the responsible use of human tissue in the context of health research, as established by the Federation of Dutch Medical Scientific Societies. The prior probability of the pathogenicity of the variants was checked in the LOVD database ('Leiden Open Variation Database (LOVD)')Leiden Open Variation Database (LOVD). n.d..

2.2. WES analysis

WES analysis was performed in available tumor samples. Sample preparation, molecular evaluation, and mutational signature analysis were performed as described by Helderman et al.(Helderman et al., 2023). Briefly, tumor and normal tissue were obtained from formalin-fixed paraffin-embedded (FFPE) tissue blocks. DNA was extracted using NucleoSpin®. Samples were prepared for sequencing following the Hybridization Capture procedure using the Agilent SureSelectXT Human All Exon V7 kit. The prepared libraries were then sequenced using the NovaSeq™ 6000 Sequencing System (Illumina Inc., San Diego, US), with each sample yielding a minimum of 12 Gb of sequence data. Sequencing reads were trimmed using Trimomatic v.036 (Bolger et al., 2014) and aligned to GRCh38 using BWA-MEM (Li and Durbin, 2009). Data was analyzed using a self-developed pipeline within ANNOVAR and data parser (source code available upon reasonable request). See also Supplementary S1.

We used MSIsensor2 to enhance MSI detection by calculating the MSI score (number of MSI sites/all valid sites). With a 20 % cutoff and above, tumors are classified as MSI-high (Niu et al., 2014).

2.3. TMS

Mutational signatures were extracted from the WES data using SigProfiler and the SigProfiler reference mutational signatures to score the overall contribution of MMRd to the tumor mutational burden (TMB) in each tumor (Alexandrov et al., 2020). The Catalogue Of Somatic Mutations in Cancer (COSMIC) database was consulted to interpret the signatures ('Catalogue of Somatic Mutations in Cancer (COSMIC)', 2022).

The SNV mutational signatures SBS6, SBS15, SBS20, SBS21, SBS26, and SBS44, along with the INDEL mutational signatures ID1, ID2, and ID7, were identified as being MMRd-signature associated. SBS1, SBS5, and SBS40 were considered to be related to aging (Alexandrov et al., 2013; Consortium, 2020; Tate et al., 2019; Alexandrov et al., 2020; Diaz-Gay and Alexandrov, 2021). The total number of MMRd-signature-associated mutations observed in the CRCs with an *MSH6* VUS was compared to similar data from CRCs with (likely) pathogenic *MSH6* variants obtained Helderman et al. (Helderman et al., 2023), to contextualize our findings within established categories of MMRd signature-associated mutational patterns.

2.4. Targeted next generation sequencing

Supplementary to WES, targeted next-generation sequencing (NGS) was performed to determine the TMB in tumors with failed/uninterpretable WES analysis. In short, the Oncomine Comprehensive Assay Plus™ (Thermo Fisher Scientific) was used on FFPE tissue according to the manufacturer's protocol.

2.5. CIMRA assay

The cell-free In Vitro MMR Activity (CIMRA) assay only requires variant sequence data to assess missense and small indel variants and is fully calibrated and validated, yielding the Odds of Pathogenicity (OddsP) of full penetrance of a VUS (Drost et al., 2019; Drost et al., 2020;

Rayner et al., 2022) and was used to predict the pathogenicity of the VUSs. Available CIMRA results of the included variants were extracted from the INVUSE (Investigation of variants of uncertain clinical significance for use in clinical practice, n.d) database. The INVUSE project is dedicated to investigating the pathogenicity of VUSs in the Mismatch Repair (MMR) genes among Dutch patients. The project employs functional assays to offer patients and their families greater clarity regarding the significance of the gene variants identified.

2.6. Statistical analysis

To further investigate differences in TMS profiles, the subgroup of tumors from patients with an *MSH6* VUS was compared to tumors from patients with a confirmed pathogenic *MSH6* variant. We also examined variations in the number of SNVs and INDELs between these groups. Therefore, statistical analyses were conducted using IBM SPSS Statistics for Windows 2017 (version 25.0) and RStudio (Team R, Integrated Development for R, Boston, MA, 2020) as also described by Helderman et al.(Helderman et al., 2023). Continuous outcome variables were summarized as medians with interquartile ranges and were compared using the Mann-Whitney *U* test for two-group comparisons. Statistical significance was defined as a *p*-value of less than 0.05.

3. Results

3.1. Cohort description

Twenty patients carrying an MMR gene VUS were identified at the LUMC between 2012 and 2020. Among these patients, eight of them, representing seven families, provided written informed consent (one patient with an *MSH2* VUS, six patients with an *MSH6* VUS, and one with an *PMS2* VUS) for the utilization of tissue and clinical data. All patients had a diagnosis of CRC and/or EC. A detailed clinical description and family pedigrees are included in **Supplementary S2 and S3**.

3.2. Description of the CIMRA functional assay and molecular analysis per family

Family 1 – MSH6, c.3014G > A, p.(Arg1005Gln) concerns a woman diagnosed with an endometrioid adenocarcinoma of the endometrium (T1) and an endometrioid type ovarian cancer (T2), both at the age of 48. The endometrial tumor displayed MSI-H status, accompanied by weak staining of MSH2 and the absence of MSH6 staining. Similarly, while MSI analysis was not conducted on ovarian cancer, staining results also revealed weak staining of MSH2 and the absence of MSH6 staining. No somatic mutations or LOH of MSH2 or MSH6 were identified to explain the observed tumor characteristics. A germline VUS was detected in *MSH6*, c.3014G > A, p.(Arg1005Gln). The bioinformatics-derived prior probability (MAPP/PP2 prior $P = 0.3512$) and CIMRA assay results (OddsP: 16.3, moderate evidence of pathogenicity) yielded consistent findings, indicating likely pathogenicity of this VUS, following the AMCG criteria.

WES revealed that multiple signatures were present in both the endometrial and ovarian cancers. In the EC (T1), SBS signatures were found, with 21.2 % MMR-related and 78.8 % related to age. The contribution of the INDEL signature in this tumor was 89.9 % associated with MMR and 10.1 % related to unknown factors. Signatures found in the ovarian cancer (T2) are 71.8 % MMR related and 58.2 % age related. INDEL signatures observed in this tumor are 95.5 % MMR-related and 4.5 % other than MMR or age-related.

A number of MMR-related IDs fit an underlying MSH6 defect (T1: $n = 124$; T2: $n = 126$) and MMR SBSs (T1: $n = 128$; T2: $n = 234$), making the observed variant to some extent more suspect for pathogenicity.

The proband of *Family 2 – PMS2, c.197C > T, p.(Ile66Thr)* was diagnosed with CRC at the age of 47 years. The tumor (T3) showed MSI-low status and loss of staining of PMS2. In the tumor, a class 3 variant was detected in *PMS2* (c.197 T > C, p.(Ile66Thr)). Additionally, LOH of PMS2 was observed in the tumor. Notably, a variant of unknown significance in *POLD1* was also identified in both tumor tissue and normal tissue.

The prior probability is unknown. The CIMRA assay (64.1 % activity of wildtype control; OddsP: 0.8, uncertain significance) provided suggestive but not conclusive evidence regarding pathogenicity of this variant. Analysis of SBS signatures showed that 43.7 % were MMR-related, 53.3 % were age-related, and 3.0 % were attributed to other causes. The contribution of the INDEL signatures was 100 % MMR-related. The WES results further support MMR deficiency, as did the presence of a second hit (LOH). The observed MMR related signatures (ID $n = 93$, SBS $n = 845$) support this finding. Thus, this *PMS2* variant may be moderately pathogenic, resulting in reduced penetrance.

The next patient (*Family 3*, *MSH6* c.3652G > C, p.(Gly1218Arg) was diagnosed with CRC at age 71 at three locations, one proximal (right-sided, T4 proximal, MSI high, weak staining of both MSH2 and MSH6) and two in the rectum (T5–1 MSS, positive MMR staining and T5–2, MSI high, weak staining of both MSH2 and MSH6*)*. Both the prior probability (0.9618) of the found *MSH6* variant (c.3652G > C, p.(Gly1218Arg)) and the results of the CIMRA analysis (OddsP: 70.8) strongly suggest that this variant is pathogenic, and it was, therefore, classified accordingly during the writing of this manuscript.

The SBS signature profile of the proximal colorectal carcinoma (T4) is 42.2 % MMR related, 63.6 % age-related, and 4.7 % had other signatures. The INDEL signature profile of this tumor is 100 % MMR-related. The patient had one MMR-proficient tumor (T5–1); this tumor did not display signatures related to MMR deficiency as expected; instead, it was entirely attributed to age-related factors. Also, the INDEL signature contribution is not MMR-associated. So, in this case, the MMR signature follows the outcome of the IHC, possibly fitting the MSH6 more than the MSH2 deficiency in T4.

In *Family 4,* a variant in *MSH6* (c.3473_3475delGTT, p.(Cys1158del)) was identified in two relatives. Two siblings from this family were included in this study. The brother was diagnosed with three primary CRCs at 67 years: 1) ascending colon (T6), IHC MSH2 weak/MSH6 weak staining2) proximal colon, IHC MSH2 weak/MSH6 weak staining; 3) distal rectum IHC positive MSH6 and PMS2 staining), while his sister was diagnosed with both EC at 49 years and CRC at 60 years (T7, MSI-H, absent staining of MSH2 and MSH6). The EC was classified as a well-differentiated adenocarcinoma, but no data was available regarding MSI or staining results. No germline variant in *MSH2* was found in the sister and no somatic *MSH2* variants were present that could explain the absent or weak staining found in this family. The CIMRA assay of the *MSH6* variant suggested that the variant is pathogenic (OddsP: 59.7). The prior probability of pathogenicity is unknown as the bioinformatics analyses only perform predictions on missense variants.

The SBS signature profile of the CRC diagnosed at 60 years (T7) in the sister showed a high MMR-related contribution (91.1 %), and a smaller age-related contribution (8.9 %). The INDEL signature contribution was 100 % MMR-related. In the case of the CRC diagnosed at 67 years in the brother (T6), the SBS signature profile was predominantly age-related (90 %) with a minor contribution from other causes. The ID signature contribution was 16.7 % MMR-related and 83.3 % related to other causes. The ID and SBS signatures of T6 do not support any MMR-related cause. However, the observed number of signatures in T7 (ID $n = 163$, SBS $n = 1001$) supports the pathogenicity of this variant.

In the following highly affected family (*Family 5*, *MSH6 c.3218C > G, p.(Pro1073Arg)*, one sibling provided informed consent for further tumor analyses. She was diagnosed with CRC at 57 years (T8, IHC MSH2-/MSH6-) and at 71 years (T9, MSI-H, MLH1-/PMS2- due to hypermethylation). The CIMRA assay of the variant suggests that this variant is likely benign (OddsP: 0.08). In agreement, its prior probability (AlphaMissense) was 0.1827, the MAPP, PP2 Prior $P = 0.002$ (=ACMG MMR benign_supportive (BP4)).

For T8, the SBS signature contributions were 49.3 % MMR-related (*n*

= 394) and 50.7 % age-related. The INDEL signatures were 100 % MMR-related (MSI; $n = 14$). The number of MMR-related signatures may be consistent with a pathogenic variant.(Helderman et al., 2023) Although absent staining of MSH2 was seen, no *MSH2* germline variant was found. A possible LOH of *MSH2* and a deletion of *MSH6* were seen but these aberrations were not certain due to low coverage. For T9, the SBS signature contribution was 72.3 % MMR-related, 19.5 % age-related, and 8.2 % related to other causes. The INDEL signatures were 97.5 % MMR-related and 2.5 % related to other causes. In T9, a *POLD1* variant was identified through NGS. The identification of the *POLD1* variant in T9 strongly suggests a secondary somatic MMR deficiency in this tumor, aligning with the presumed benign nature of the *MSH6* germline VUS.

Tumor 10 (*Family 6, MSH2 c.796G > A, p.(Ala266Thr)*) involves a male diagnosed with CRC at the age of 44 years (MSS and normal staining of all four MMR proteins). The prior probability (0.9456) and CIMRA assay result (OddsP: 244) strongly suggest, though, that this variant is pathogenic. Unfortunately, the results of WES analysis in this tumor were deemed unreliable, due to the limited number of reported variants. WES analysis did identify two SBS signatures, neither of which were MMR-related, and no INDEL signatures were detected. To validate these findings, the TMB was reassessed using targeted NGS. The TMB was determined to be 5.74 mutations/Mb, which was considered too low to generate a signature profile. The low TMB makes an MMR deficiency in this tumor unlikely. However, this may be a false negative sequencing result, especially if the variant has reduced penetrance or if *MSH6* loss occurs later during tumor development. This challenges the support for the pathogenicity of this variant.

In *Family 7* (*MSH6 c.2342C > A, p.(Pro781Gln)*, a woman was diagnosed with endometrial cancer at 59 years of age. The AlphaMissense-derived Prior probability of pathogenicity was 0.9968 while de CIMRA assay-derived OddsP was 52.2, both strongly indicating pathogenicity. Also, the MAPP, PP2 Prior $P = 0.8291$ (=ACMG MMR (BP3)) suggests pathogenicity.

The tumor (T11) was MSI-H and showed absent staining of MSH6. Notable, the MSIsensor outcome was below 20 % (9,9 %), which is considered MSI low; possibly this is because, in MSH6 tumors, there is a lower rate of insertion/ deletions and more variant changes, which could theoretically result in other outcomes depending on the emphasis laid on the specific type of variant change. No somatic *MSH6* variants or LOH were found though. WES analysis of the tumor revealed a low number of variants, including SBS signatures that are age-related (SBS1: $n = 44$, 53.7 %), MMR-related signatures (SBS40: $n = 24$, 29.3 %); SBS20: $n = 14$, 24.4 %), and INDEL signature ID7 (n = 2, 100 %, MMR-related). To validate these findings, additional targeted NGS analysis was performed, which showed only the presence of the MMR deficiency-related signature SBS6. Despite the limited number of variants called, the results of the functional analysis, along with the clinical data, support MMR deficiency and the likely pathogenicity of the observed variant in *MSH6*. The number of observed signatures cannot strongly support the pathogenicity of this VUS.

Table 1, Fig. 1 (SBS signature contribution per tumor), and Fig. 2 (INDEL Signature contribution per tumor) summarize the above-mentioned results. The signature contribution per tumor is described in detail in Supplementary Figs. **S5.1–5.11**.

3.3. Comparison of SNV's and INDELS in MSH6 VUS tumors vs. MSH6 pathogenic variant tumors

The total number of variants, which includes SNVs and INDELs, in the pathogenic (PV) MSH6 group ($n = 25$) compared to the MSH6 VUS group ($n = 9$) did not exhibit a significant difference ($p = 0.1399$). However, it's worth noting that there were fewer total variants in the latter group. When focusing solely on MMRd signature-associated SNVs, there was also no significant difference between the two groups ($p = 0.2677$). Similarly, for MMRd signature-associated INDELs, no significant difference was observed ($p = 0.5968$). Interestingly, there were more INDELs in the VUS group in this specific category (Fig. 3).

4. Discussion

Classifying a VUS in one of the MMR genes can be a challenging endeavour due to the often limited evidence, which can hinder accurate LS diagnoses. In this pilot study, we explored the potential of using TMS derived from WES data as an additional tool for classifying MMR VUS.

Our findings revealed distinct TMS patterns associated with MMRd in several families (e.g. families 1–3), adding additional proof of possible pathogenicity of MMR VUS. Still, in no cases, the WES led to the definitive reclassification of the VUS to benign or (likely) pathogenic. The TMS patterns concord with the MMR staining results in most cases except for T5, T6, and T10-T11. However, the utility of WES extended beyond this in some cases, allowing us to identify variants or somatic variants that could explain the tumor's phenotype.

For instance, in cases where MSH2 or MSH6 staining was absent (e.g. T1 and T8), without a germline *MSH2* variant present using WES somatic hits were detected in *MSH6*. Furthermore, in certain cases (T3 and T9), we identified likely pathogenic variants in *POLD1*, which make a somatic MMR loss more likely.

Notably, some families exhibited unexplained discrepancies, with tumors showing presence or absence of MMR deficiency, which was reflected in the TMS results. (e.g. families 4 and 5). Several factors might explain this observation, including chance or tumor heterogeneity, an individual's genetic background, exposure to carcinogens, co-occurrence of genetic alterations, or environmental factors (Kantor and Giovannucci, 2015). Even individuals with the same germline variant could exhibit differences in MMR deficiency and, therefore, differences in TMS due to other genetic or epigenetic factors in their tumors or variations in environmental exposures. Possibly, some of the VUSs that exhibit only moderate functional loss in the functional test may result in a less pronounced mutation overload, as typically observed in MMR deficiency.

While there are some differences in the total number of mutations and MMRd signature associated SNVs between the MSH6 PVs and MSH6 VUS groups, these variations were not statistically significant. The overall mutational landscape between these groups appears relatively similar, with no major distinctions observed.

Future studies are crucial to optimize the implementation of WES analysis in the current VUS classification algorithm, as several obstacles still exist. Firstly, TMS that are associated with MMRd may result from somatic MMRd, such as *MLH1* promotor hypermethylation or double somatic MMR variants, instead of MMRd caused by a constitutional MMR variant (Berger et al., 2018). This means that an MMR VUS may be incorrectly considered pathogenic based on TMS, even though the MMR VUS did not contribute to the TMS. SBS and ID TMS are not (yet) reliable enough to distinguish LS-related CRC from sporadic MMR-deficient CRC (Georgeson et al., 2021). On the other hand SBS and ID TMS can be of help in distinguishing which MMR gene is underlying the deficiency, which might be of help in cases in which IHC MMR is discrepant with the underlying MMR VUS found, for example, the *MSH6* deficiency mutation signature is different than that in *MSH2* deficient tumors (Helderman et al., 2023). Of note, TMB can be inadequately sensitive for MMRd detection in tumors occurring in tissues with a lower proliferative rate than CRC or EC (Davies et al., 2017). Furthermore, pathogenic MMR variants may not always result in significant MSI and/or absent MMR protein staining (Helderman et al., 2023) but can still have an effect on MMR that may be better detectable with TMS although we have no examples of this in our study.

Also WES signatures can have other outcomes than NGS (restricted panel analysis) signatures, as we saw in case T10-T11. This targeted approach can improve the sensitivity and specificity of mutation detection, as the number of reads is usually more constant and higher than in WES analysis. These differences highlight the importance of utilizing complementary sequencing techniques to obtain a

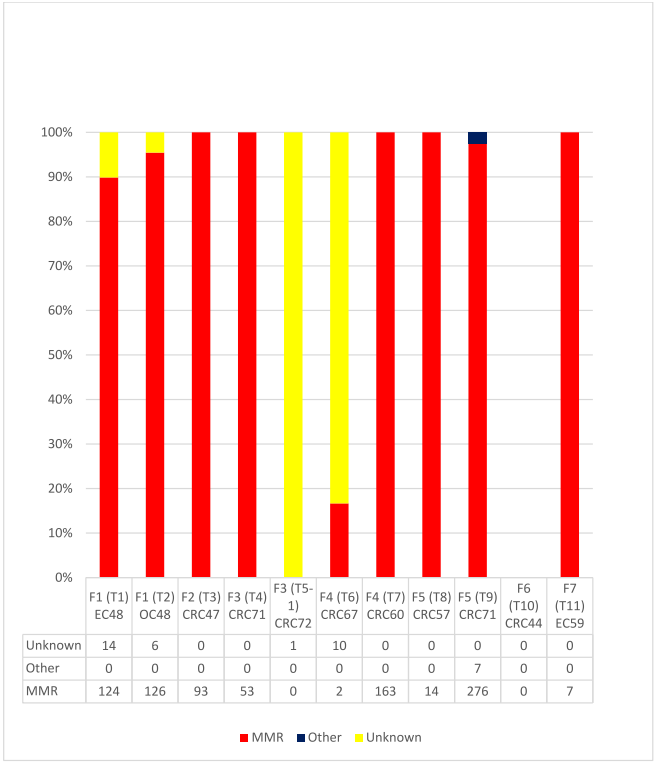
Table 1
MMR = mismatch repair; MAPP/PP2 prior P = Prior probability of pathogenicity; MSI = microsatellite instability; MSS = microsatellite stable; MSI-H = MSI-high; OddsP = posterior odds; NA/NT = not available/not tested IHC = immunohistochemistry of the MMR proteins; LOH = loss of heterozygosity; TMB = tumor mutational burden; FFPE = formalin-fixed paraffin-embedded; VUS = variant of unknown significance;

	Summary of results												
Family (patient)	MMR germline variant	MAPP/PP2 prior P (Thompson et al. 2013; 'Leiden Open Variation Database (LOVD)')	CIMRA results		Tumor ID	Tumor (age in years)	MSI	MSI score % (MSIsensor2)	SNVs total	INDELs total	IHC	2nd hit*	Remarks
			% repair	OddsP									
F1 (1)	*MSH6* c.3014G > A, p. (Arg1005Gln)	0.3512	20.9 %	16.3	T1	EC (48)	MSI-H	64.97	604	138	MSH2 weak +, MSH6-	*MSH6* c.3216del	INVUSE: pathogenic
					T2	OC (48)	NA/NT	55.16	560	132	MSH2 weak +, MSH6-	*MSH6* c.3216del	
F2 (2)	*PMS2* c.197 T > C, p. (Ile66Thr)	UNK	64.10 %	0.8	T3	CRC (47)	MSI-low	1.24	1935	93	PMS2-	LOH of PMS2	POLD1 VUS (also in normal tissue)
F3 (3)	*MSH6* c.3652G > C, p. (Gly1218Arg)	0.9618	0 %	70.8	T4	CRC (71)	NA/NT	2.22	1223	53	MSH2 weak +, MSH6 weak +	*MSH6* c.C1444T:p. R482X	INVUSE: pathogenic
					T5–1	CRC (72)	NA/NT	60.32	69	1	MSH2 weak +, MSH6 weak +	None detected	INVUSE: pathogenic POLE VUS: potential artefact
F4 (4)	*MSH6* c.3473_3475delGTT, p. (Cys1158del)	UNK	2.3 %	59.7	T6	CRC (60)	MSI-H	27.33	170	12	MSH2-, MSH6-	None detected	No *MSH2* germline variant
F4 (5)	*MSH6* c.3473_3475delGTT, p. (Cys1158del)	UNK	2.3 %	59.7	T7	CRC (67)	NA/NT	2.53	1099	163	MSH2 weak +, MSH6 weak +	None detected	
F5 (6)	*MSH6* c.3218C > G, p. (Pro1037Arg)	0.002	96.2 %	0.08	T8	CRC (57)	NA/NT	11.37	799	14	MSH2-, MSH6-	Possibly LOH, possible deletion of MSH6 (because of low coverage not certain)	Possible LOH, possible deletion of MSH6.
					T9	CRC (71)	MSI-H	70.44	974	283	MLH1-, PMS2-	None detected	POLD1 variant class 5, MLH1 hypermethylation
F6 (7)	*MSH2* c.796G > A, p. (Ala266Thr)	0.9456	2.0 %	244	T10	CRC (44)	MSS	0.74	2	0	MLH1+, MSH2+ MSH6+, PMS2+	None detected, also no LOH of MSH2	Tumor cell percentage > 30 % INVUSE: likely pathogenic
F7 (8)	*MSH6* c.2342C > A, p. (Pro781Gln)	0.8291	4.2 %	52	T11	EC (59)	MSI-H	9.9	82	7	MSH6-	None detected	INVUSE: pathogenic

[a] Supplementary S4 includes the full details of observed SNVs and INDELs.

	F1 (T1) EC48	F1 (T2) OC48	F2 (T3) CRC47	F3 (T4) CRC71	F3 (T5-1) CRC72	F4 (T6) CRC67	F4 (T7) CRC60	F5 (T8) CRC57	F5 (T9) CRC71	F6 (T10) CRC44	F7 (T11) EC59
Other	0	0	59	57	0	17	0	0	80	2	0
Aging	476	326	1031	388	69	153	98	405	190	0	68
MMR	128	234	845	778	0	0	1001	394	704	0	14

Fig. 1. Percentage of SBS (single base substitutions) signature contribution per tumor – F1 = Family 1, F2 = Family 2, etc.; T1 = tumor 1, T2 = tumor 2, etc.; EC = endometrium carcinoma; OC = ovarian carcinoma; CRC = colorectal carcinoma; MMR = mismatch repair deficiency.



	F1 (T1) EC48	F1 (T2) OC48	F2 (T3) CRC47	F3 (T4) CRC71	F3 (T5-1) CRC72	F4 (T6) CRC67	F4 (T7) CRC60	F5 (T8) CRC57	F5 (T9) CRC71	F6 (T10) CRC44	F7 (T11) EC59
Unknown	14	6	0	0	1	10	0	0	0	0	0
Other	0	0	0	0	0	0	0	0	7	0	0
MMR	124	126	93	53	0	2	163	14	276	0	7

Fig. 2. Percentage of ID (insertion deletion) signature contribution per tumor – F1 = Family 1, F2 = Family 2, etc.; T1 = tumor 1, T2 = tumor 2, etc.; EC = endometrium carcinoma; OC = ovarian carcinoma; CRC = colorectal carcinoma; MMR = mismatch repair deficiency.

comprehensive view of the mutational landscape and accurately assess the signature profiles in tumor samples.

Additionally, comparing WES to WGS for determining the pathogenicity of VUSs would be helpful. WGS offers a broader genomic context, allowing for the detection of larger structural variations and more comprehensive coverage of non-coding regions (Lelieveld et al., 2015), which might provide additional insights into variant pathogenicity. Including WGS data could enhance our understanding of VUSs and their impact on MMR gene function, as well as improve the resolution of mutational signatures and their associated TMS.

This study has some limitations, including a small sample size and the absence of patients with an *MLH1* VUS. Future research with larger cohorts is required to assess the added value and cost-effectiveness of WES analysis in classifying VUSs on a larger scale. Additionally, it should be investigated whether WES analysis can replace other testing methods such as IHC MMR, MSI analysis, or small MMR panel analysis in the future. Furthermore, future studies should evaluate the discriminative value of TMS in distinguishing LS from sporadic MSI CRCs and the different MMR genes involved.

In conclusion, at this moment the use of WES/TMS to evaluate the potential pathogenicity of a VUS is still limited, and other sources of evidence are necessary, including functional assays, population frequency data, and clinical information, to arrive at a comprehensive interpretation of the variant that considers the specific context of the variant. However, future studies might overcome current pitfalls and evaluate the (cost-) effectiveness of WES/TMS analysis on a larger scale.

A relevant advantage of WES analysis is its potential to uncover alternative explanations when clinical and functional data are challenging to interpret. For example, it can identify variants in POLE/D or other genetic alterations that may clarify the tumor's phenotype, such as age of onset, secondary MMR deficiency, and high mutational burden.

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CRediT authorship contribution statement

Anne-Sophie van der Werf't Lam: Writing – review & editing, Writing – original draft, Visualization, Project administration, Investigation, Formal analysis, Data curation. **Noah C. Helderman:** Writing – review & editing, Formal analysis, Data curation. **Arnoud Boot:** Writing – review & editing, Formal analysis, Data curation. **Diantha Terlouw:** Writing – review & editing, Formal analysis. **Hans Morreau:** Writing – review & editing. **Hailian Mei:** Writing – review & editing, Software, Methodology. **Rebecca E.E. Esveldt-van Lange:** Writing – review & editing. **Inge M.M. Lakeman:** Writing – review & editing. **Christi J. van Asperen:** Writing – review & editing. **Emmelien Aten:** Writing – review & editing. **Nandy Hofland:** Writing – review & editing. **Pia A.M. de Koning Gans:** Writing – review & editing. **Emily Rayner:** Writing – review & editing. **Carli Tops:** Writing – review & editing, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Niels de Wind:** Writing – review & editing, Visualization, Methodology, Formal analysis, Conceptualization. **Tom van Wezel:** Writing – review & editing, Visualization, Methodology. **Maartje Nielsen:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Methodology, Funding acquisition.

Declaration of competing interest

On behalf of myself and the other authors, I declare that we have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

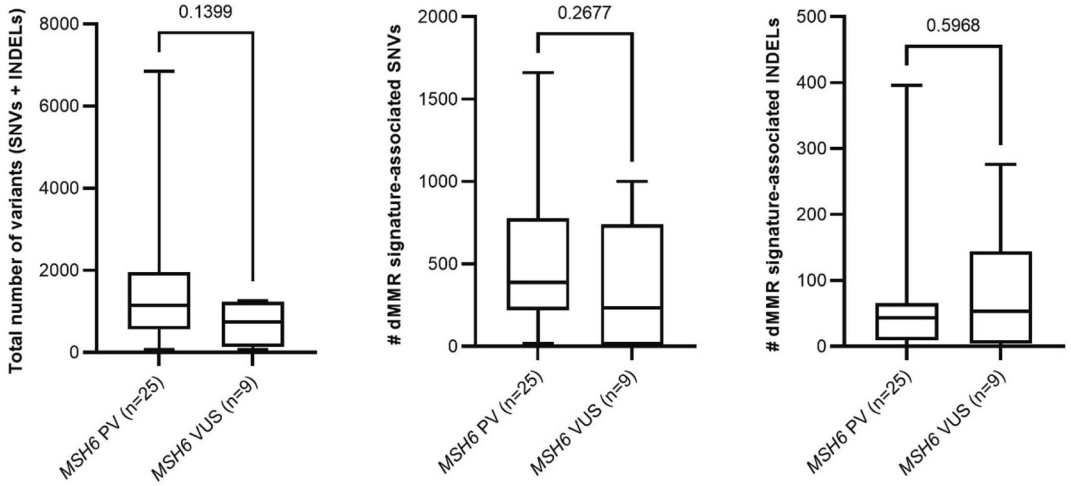

Fig. 3. Comparison of total number of SNVs and INDELS (insertions and deletions) (left), solely mismatch repair deficient (MMRd) associated SNVs (single nucleotide variant) (middle), and solely mismatch repair associated INDELS (right) of tumors of patients with an MSH6 pathogenic variant (PV) versus MSH6 VUS (Variant of Unknown Significance).

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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