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SHORT REPORT

Combined mismatch repair and *POLE/POLD1* defects explain unresolved suspected Lynch syndrome cancers

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Many suspected Lynch Syndrome (sLS) patients who lack mismatch repair (MMR) germline gene variants and *MLH1* or *MSH2* hypermethylation are currently explained by somatic MMR gene variants or, occasionally, by germline *POLE* variants. To further investigate unexplained sLS patients, we analyzed leukocyte and tumor DNA of 62 sLS patients using gene panel sequencing including the *POLE*, *POLD1* and MMR genes. Forty tumors showed either one, two or more somatic MMR variants predicted to affect function. Nine sLS tumors showed a likely ultramutated phenotype and were found to carry germline ($n = 2$) or somatic variants ($n = 7$) in the *POLE/POLD1* exonuclease domain (EDM). Six of these *POLE/POLD1*-EDM mutated tumors also carried somatic MMR variants. Our findings suggest that faulty proofreading may result in loss of MMR and thereby in microsatellite instability.

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INTRODUCTION

Inactivation of the mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6* and *PMS2* causes Lynch syndrome (LS), an autosomal dominant predisposition for colorectal and endometrial cancer.[1] Inactivation of the mismatch repair pathway can also occur sporadically, through somatic *MLH1* hypermethylation[1] or by acquired biallelic somatic inactivation (variant affecting function or loss of heterozygosity (LOH)) of the MMR genes.[2,3] Inaccurate DNA repair leads to a high frequency of somatic variants, with loss of MMR leading to 'hypermutated' tumors with 10–100 variants/Mb.[4] LS tumors are characterized by microsatellite instability (MSI) and immunohistochemical loss of expression of MMR proteins.[1] However, germline variants affecting function cannot be detected in up to 59% of patients displaying MSI and/or loss of MMR, referred to as 'suspected LS' (sLS).[5]

Recently, germline and somatic variants in the exonuclease domains (EDMs) of DNA polymerase ε (*POLE*) and polymerase ₫ (*POLD1*) were described.[6–14] These *POLE/POLD1* variants affect proofreading function and lead to an ultramutated phenotype with a variant incidence exceeding 100 variants/Mb. Germline *POLE*-EDM variants can result in a LS phenotype and microsatellite instable colorectal cancers (CRCs).[6,15] The exact role of somatic *POLE/POLD1* variants in tumors with high microsatellite instability (MSI-H) remains unclear.

The aim of our study was to identify the underlying genetic cause of disease in a cohort of 64 sLS cases – selected on the basis of MSI, loss of MMR, young onset and often a family history for LS – by screening the MMR, *POLE* and *POLD1* genes in both leukocyte and tumor DNA.

SUBJECTS AND METHODS

This study included 64 patients with Lynch-associated tumors recruited in four academic centers in the Netherlands between 1997 and 2014: Leiden University Medical Centre ($n = 37$), Maastricht University Medical Centre ($n = 11$), Erasmus Medical Centre ($n = 9$) and University Medical Centre Utrecht ($n = 7$). Demographic and clinical data, as well as informed consent, were obtained at the time of diagnosis. This study was approved by the local medical ethical committee of the LUMC (P01-019E). Patients were selected based on loss of MMR (as indicated by immunohistochemical staining) and/or MSI. Unexplained tumors with low MSI or tumors with inconclusive IHC results were also included in this study (see Supplementary Table 1 and Supplementary Methods).

Fifty-eight (91%) patients fulfilled Bethesda criteria,[16] and families of 24 (38%) patients also fulfilled Amsterdam II criteria.[17] Patients were previously screened in a diagnostic setting for germline MMR variants. Whereas 57 patients showed no disease-causing germline variants, 7 patients were found to have a germline variant of unknown significance (VUS). Of the total cohort, 75% of patients presented with CRC ($n = 48$), 14% with endometrial cancer (EC, $n = 9$) and 11% with another LS-associated tumor (see Supplementary Table 1).

The average age of onset was 52.1 years. Two patients were excluded from the analysis because of poor DNA quality. Of the remaining 62 tumors, tumor and leukocyte DNA was sequenced for variants in the exonic regions of *MLH1*, *MSH2*, *MSH6*, *PMS2*, *POLE* and *POLD1* using the Ion PGM System (Life Technologies, Carlsbad, CA, USA). Raw data analysis, alignments and variant calling was carried out using the default parameters in Torrent Suite v4.0 (Thermo Fisher Scientific, Waltham, MA, USA; see Supplementary Methods). Variants were functionally annotated using ANNOVAR.[18] The full data set was filtered and prioritized by variant frequency ($>10\%$) and coverage ($>50\times$). Interesting variants under 10% were manually curated. The *in silico* prediction programs were used to predict effect on function (see Supplementary Methods). All variants (likely) affecting function, including two variants with a 9% variant frequency, were validated with Sanger sequencing. For all *PMS2*

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variants, *PMS2*-specific primers were created to confirm that the variant is present in *PMS2* and not in a *PMS2* pseudogene.

LOH was determined for every heterozygous SNP by comparing the ratio of allele A with allele B in leukocyte and tumor DNA samples. Furthermore, for every heterozygous SNP, the allelic imbalance factor (AIF)[19] was calculated and Fisher's exact test was performed to determine whether the difference between normal and tumor is significant. If all heterozygous SNPs of one gene showed LOH with an AIF > 2 and Fisher's exact *P*-value < 0.05, LOH was called (Supplementary Table 1).

RESULTS

Seven patients with a germline MMR VUS (class 3) were included in this study (see Supplementary Table 1). In all cases, the variant was detected with NGS in leukocyte and tumor DNA. During the course of the study, three of these germline variants were reclassified as class 4 or 5 ((probably) affects function) by the International Society for Gastrointestinal Hereditary Tumors Incorporated (InSiGHT). Four tumors displayed a second somatic MMR variant predicted to affect function. One tumor displayed LOH (Supplementary Table 1). Six cases with somatic *MLH1* hypermethylation fulfilling revised Bethesda criteria (three from families fulfilling Amsterdam II criteria) were sequenced for underlying hereditary defects explaining the family history, but no germline variants were found. One of these *MLH1* hypermethylated tumors carried a somatic *MLH1* variant likely to affect function and one displayed *MLH1* LOH (Supplementary Table 1).

One ($n = 27$, 44%) or two ($n = 13$, 21%) somatic aberrations (variant or LOH) in a MMR gene were found in a total of 62 tumors (see Supplementary Table 1). Biallelic inactivation was concordant with IHC. Of the 13 tumors with two somatic aberrations, 12 had variants in *MLH1* or *MSH2* and were MSI-H. The thirteenth tumor, sLS-07, showed expression loss of MSH6 and was MSS.

Although the majority (83%) of tumors showed < 10 somatic variants in the genomic region analyzed, 10 cases displayed a larger number of somatic variants, ranging from 16 to 375 somatic variants within the sequenced area of 31 kb. Out of 10 tumors, 9 showed a *POLE* or *POLD1* variant that (probably) affects function (Table 1). Of the highly mutated tumors, two carried novel germline heterozygous *POLE/POLD1* variants that are predicted to affect proofreading (Supplementary Table 2). Of these two germline cases, tumor sLS-67 was also found to carry two somatic *MLH1* variants, explaining the tumor phenotype (loss of MLH1 and PMS2 expression and MSI-H). The second tumor, sLS-16, was MSI-L, showed positive MLH1 and MSH2 staining (MSH6 and PMS2 were not tested) and had no somatic MMR variants.

Seven of the highly mutated tumors showed somatic *POLE/POLD1* variants likely to affect function. Six tumors carried a somatic *POLE/POLD1*-EDM hot spot variant (*POLE*: c.857C > G, c.856C > T, c.1231G > T, c.1366G > C, c.1367C > T or c.1376C > T and *POLD1* c.1433G > A) that has previously been described to impair proofreading.[4] In the seventh tumor (sLS-105), a novel *POLE* c.846_847delinsTT variant was detected. This variant lies close to a known *POLE* hot spot site (*POLE* c.857) and is predicted to affect function by two out of three prediction programs (Supplementary Table 2). All *POLE* variants were heterozygous, in agreement with previous research.[7,9,10,13,15] Four *POLE/POLD1*-EDM mutated tumors displayed additional somatic nonsense *POLE* variants outside the exonuclease domain (see Supplementary Table 3). Only one of these was upstream of the exonuclease domain (sLS-16).

Eight of the nine ultramutated tumors with a *POLE/POLD1*-EDM variant found in our study showed MSI (3 MSI-H and 5 MSI-L). In six of these ultramutated tumors, IHC detected loss of at least one of the MMR proteins and all six tumors displayed somatic variants in the affected MMR gene likely to affect function. Tumor sLS-19 with two *POLE* variants was found to have two somatic *MLH1* aberrations, as well as two somatic *PMS2* aberrations, and IHC showed solitary loss of PMS2 expression. Reanalysis of staining also showed ambiguous MLH1 staining (cytoplasmic enhancement and vague, focal nuclear staining). Tumor sLS-09 displayed a nonsense *MSH6* variant and missense *MSH2* variant predicted to affect function, and IHC showed loss of MSH6 expression and weak positive MSH2 expression. In three *POLE/POLD1*-EDM mutated tumors with positive or inconclusive MMR expression and MSI-L phenotype, no somatic MMR variants (likely) to affect function were found. However, in one of those three tumors (sLS-05), solitary *MLH1* LOH and *PMS2* LOH without variants was found (see Table 1).

In three non-ultramutated tumors, a *POLE/POLD1*-EDM variant was found (sLS-80, sLS-87 and sLS-101, see Table 1). These variants have not been described before, but are predicted to affect function (see Supplementary Table 2). Two variants co-occur with a germline *MSH6* VUS and a somatic *MSH6* variant (sLS-80 and sLS-87), whereas one (sLS-101) co-occurs with a somatic *PMS2* variant. Four additional non-ultramutated tumors showed *POLE* (sLS-18, sLS-21) or *POLD1* LOH (sLS-12, sLS-49) in all heterozygous SNPs (Supplementary Table 1), without germline or somatic *POLE* variants.

DISCUSSION

POLE-EDM variants are reported to be the mutagenic factor driving ultramutation in tumors.[20] The same report also noted that the increased mutational load seen in *POLE* mutated tumors exceeds that expected because of loss of exonuclease activity.[20] The number of variants detected in the sequenced area in the present study implicates an ultramutated phenotype, with > 100 variants/Mb in all *POLE/POLD1*-EDM mutated tumors in this cohort. As only a limited region (31 kb) was sequenced, we can only extrapolate the total number of variants per Mb. In our cohort, the *POLE/POLD1* mutated MMR-deficient tumors display two deficient pathways increasing the mutational load. Comparing frequencies of the different variants found in these tumors, it might be concluded that faulty proofreading may be the initiating event in some of these tumors, possibly resulting in loss of MMR and thereby in MSI. Interestingly, four tumors show *POLE/POLD1* LOH without germline or somatic *POLE/POLD1* variants. These tumors however do not show the typical ultramutated phenotype, whereas single variants without LOH do show that. This phenomenon of LOH without variants affecting the exonuclease domain has not yet been described. Possibly, the remaining allele is enough to maintain proofreading. Furthermore, three tumors show somatic *POLE/POLD1* variants, without the ultramutated phenotype. All three variants are missense, but are predicted to affect function (Supplementary Table 2). As these variants are not found in ultramutated tumors, evidence of deleterious functional effect is lacking.

In conclusion, targeted next-generation sequencing of 62 sLS cases led to the detection of 9 highly mutated tumors with a germline ($n = 2$) or somatic ($n = 7$) *POLE/POLD1*-EDM variant. Even though *POLE* germline variants have previously been shown to co-occur with somatic MMR variants,[11] in this study we found germline and somatic *POLE/POLD1* variants in a cohort selected for sLS characteristics. Importantly, although current literature mainly addresses *POLE/POLD1* variants in MSS tumors, somatic *POLE/POLD1* variants in sLS patients are likely to be overlooked. However, a very recent recommendation for genetic testing and surveillance states that MMR deficiency should not be an exclusion criterion for genetic testing of

Table 1 Overview of patients with a _POLE/POLD1-EDM_ mutated tumor

Patient	IHC-negative staining	MSI	Age of onset	Family history	Number of variants	Gene	Variant	Amino acid alteration	%
Somatic POLE/POLD1-EDM variant									
sLS-05	None	L	62	FDR	330	POLE	c.1367C>T	p.(A456V)	28%
						MLH1	LOH		
						PMS2	LOH		
sLS-07	MSH6 (1)	S	39	TDR	37	POLE	c.1231G>T	p.(V411L)	44%
						MSH6	c.2735G>A	p.(W912*)	10%
						MSH6	c.2876 G>A	p.(R959H)	14%
sLS-09	MSH6 (2)	L	42	FDR	16	POLE	c.857C>G	p.(P286R)	38%
						MSH6	c.2539G>T	p.(E847*)	36%
						MSH2	c.1166G>A	P.(R389Q)	38%
sLS-19	PMS2	H	45	FDR	221	POLE	c.1376C>T	p.(S459F)	21%
						POLE	c.856C>T	p.(P286S)	9%
						MLH1	c.199G>A	p.(G67R)	19%
						MLH1	LOH		
						PMS2	c.308C>T	p.(T103I)	11%
						PMS2	LOH		
sLS-24	None	L	34	FDR	115	POLE	c.1366G>C	p.(A456P)	28%
sLS-66	MSH6	L	66	SDR	25	POLD1	c.1433G>A	p.(S478N)	32%
						MSH6	c.3600_3601del	p.(L1201Hfs*13)	28%
sLS-80	MSH2/MSH6	H	52	FDR	5	POLD1	c.1429G>A	p.(V477M)	26%
						MSH6	c.3961A>G**	p.(R1321G)	52%
						MSH6	c.3186C>A	p.(C1062*)	25%
sLS-87	MSH2/MSH6	H	49	FDR	9	POLE	c.1218C>G	p.(N406E)	16%
						MSH6	c.3473_3475del**	p.(C1158del)	53%
						MSH6	c.3311_3312delTT	p.(F1104Trpfs*3)	21%
sLS-101	PMS2	H	55	FDR	5	POLD1	c.1003A>G	p.(I335V)	22%
						PMS2	c.1687C>T	p.(R563*)	30%
sLS-105	MLH1/ PMS2	H	49	No	184	POLE	c.846_847delinsTT	p.(L283F)	13%
						MLH1	c.1614G>A	p.(W538*)	13%
Germline POLE/POLD1-EDM variant									
sLS-16	None (3)	L	41	FDR	185	POLD1	c.961G>A	p.(G321S)	55%
sLS-67	MLH1/ PMS2	H	53	SDR	91	POLE	c.861T>A	p.(D287E)	50%
						MLH1	c.208-1G>A	p.?	14%
						MLH1	c.440_447del	p.(G147Dfs*22)	19%

Abbreviation: EDM, exonuclease domain.
IHC was performed for _MLH1, MSH2, MSH6_ and _PMS2_. Numbers behind staining results indicate: (1) at least MSH6 negative, (2) PMS2 not tested and (3) inconclusive testing. MSI status is defined as MSI-H (H), MSI-L (L) or MSS (S). Age of onset is the age at which the first Lynch-associated tumor occurred. All patients presented with colorectal cancer, except patients sLS-87 and sLS-101 who presented with endometrial cancer. Number of variants depicts the number of somatic variants with a frequency >10% identified in the sequenced region of 31 kb. Stop codons are indicated with an asterisk (*). Germline variants of unknown significance (VUS) are indicated with a double asterisk (**). % shows the percentage of variant reads. Family history is defined as a first-degree relative with LS-associated tumors (FDR), second-degree relative with LS-associated tumors (SDR), third-degree relative with LS-associated tumors (TDR) or no LS in the family (No).

germline _POLE/POLD1_ variants.[21] Our results further emphasize the importance of _POLE/POLD1_ germline and somatic screening in unexplained MSI-H and MMR-deficient tumors.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
AMLJ: experimental work, data analysis and writing; TvW: study concept and design, supervision data analysis and variant calling, critical revision of the manuscript; BEWMvdA and MVG: experimental work; DR: bioinformatic support; CMJT and PD: patient selection; CMJT, AW, TGWL and EBGG: material collection; JTW, FJH and HM: supervision, study concept and design, critical revision of the manuscript and obtained funding.

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