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Molecular analysis of cancer genomes in children with Lynch syndrome: Exploring causal associations

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Abstract

Lynch syndrome (LS) predisposes to cancer in adulthood and is caused by heterozygous germline variants in a mismatch repair (MMR) gene. Recent studies show an increased prevalence of LS among children with cancer, suggesting a causal relationship. For LS-spectrum (LSS) cancers, including high-grade gliomas and colorectal cancer, causality has been supported by typical MMR-related tumor characteristics, but for non-LSS cancers, causality is unclear. We characterized 20 malignant tumors of 18 children with LS, including 16 non-LSS tumors. We investigated second hits, tumor mutational load, mutational signatures and MMR protein expression. In all LSS tumors and three non-LSS tumors, we detected MMR deficiency caused by second hit somatic alterations. Furthermore, these MMR-deficient tumors carried driver variants that likely originated as a consequence of MMR deficiency. However, in 13 non-LSS tumors (81%), a second hit and MMR deficiency were absent, thus a causal link between LS and cancer development in these children is lacking. These findings demonstrate that causality of LS in children with cancer, which can be determined by molecular tumor characterization, seems to be restricted to specific tumor types. Large molecular and epidemiological studies are needed to further refine the tumor spectrum in children with LS.

KEYWORDS

cancer predisposition, Lynch syndrome, mismatch repair, mutational signatures, pediatric cancer

What's new?

Lynch syndrome, caused by heterozygous germline variants in a mismatch repair (MMR) gene, predisposes to cancer in adulthood. Recent studies have also shown increased prevalence of

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Lynch syndrome among children with cancer. However, a large proportion of the cancers in children with Lynch syndrome do not fall within the established Lynch syndrome tumor spectrum. In this systematic analysis of causality based on tumor genomic characteristics, the majority of children with Lynch syndrome and cancers outside the spectrum had no signs of MMR deficiency in those tumors, indicating a lack of causal relationship between tumor development and the underlying syndrome.

1 | INTRODUCTION

Lynch syndrome (LS, OMIM #120435) is a cancer predisposition syndrome (CPS) associated with an increased risk of developing cancers during adulthood, in particular colorectal carcinoma (CRC) and endometrial cancer (EC). LS is caused by mono-allelic germline variants in one of the mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6* and *PMS2* or by 3′ *EPCAM* deletions causing epigenetic silencing of the downstream located *MSH2* gene. In the majority of malignancies occurring in individuals with LS, a second hit in the affected MMR gene results in a complete MMR defect, driving cancer development.[1] Biallelic pathogenic germline variants in these genes cause constitutional MMR deficiency (CMMRD, OMIM #276300), which is associated with a complete MMR defect in all cells, resulting in a strongly increased risk to develop cancer in a different spectrum of tissues and at a much younger age.[2] While CMMRD is clearly a childhood CPS, the incidence of childhood cancer in families with LS is not increased when compared to families without LS,[3] supporting the notion that LS is primarily an adult CPS. Nevertheless, large genomic studies in cohorts of children with cancer are frequently reporting heterozygous germline pathogenic or likely pathogenic (P/LP) variants in MMR genes.[4–6] This discrepancy highlights our limited understanding of the causal relationship between LS and childhood cancer.

In a recent meta-analysis study, a significantly increased prevalence of P/LP germline variants in MMR genes was observed among children who developed brain and non-brain solid tumors when compared to healthy populations.[7] These findings are partially in line with the established tumor spectrum in adults with LS, which includes high-grade brain tumors, although solid malignancies in the GI-tract, female reproductive system, hepatobiliairy system and renal system are more prevalent.[1] Several case reports confirm that high-grade brain cancers and CRC are also diagnosed occasionally in children with LS, suggesting a possible association.[8–10]

MMR deficiency in the tumor can indicate causality of the underlying LS in cancer development. MMR deficiency can be confirmed by demonstrating the presence of a second hit, including loss of heterozygosity (LOH), in the MMR gene, a high tumor mutational load (TML) and contributions of mutational signatures reminiscent of MMR defects. Additionally, negative immunohistochemistry (IHC) for the MMR proteins and presence of microsatellite instability (MSI) are an indication of MMR deficiency. Four recent studies describing individuals with LS and childhood cancer have applied part of these techniques on malignant tumors of 17 children. Because of the small sample size and the variety of analyses applied, their combined results do not provide a definitive answer, but they do indicate that in brain tumors and CRCs there is evidence for MMR deficiency.[6,11–13] Together, these studies suggest involvement of the underlying LS in the development of LS-spectrum (LSS) cancers. For non-LSS cancers, currently too little information is available to determine causality.

In this study, we describe a cohort of 18 individuals with LS and pediatric cancer and investigate the somatic landscape of individual malignancies to explore a causal relation with LS. We selected 14 children that developed malignant tumors other than high-grade brain cancers and CRC, here defined as non-LSS tumors. Four children who did develop LSS cancers were included as a control group. By examining second hits, tumor mutational load (TML), mutational signatures and the presence of the MMR proteins, we determined whether there is molecular evidence for MMR deficiency in the tumors of children with LS. For malignancies in which we found molecular evidence of MMR deficiency, we further explored causality by analyzing whether tumor-specific driver variants could have been the result of loss of MMR activity.

2 | MATERIALS AND METHODS

2.1 | Cohort selection

All children with cancer diagnosed with LS in the Princess Máxima Center between 2018 and 2022 were included in the cohort ($n = 13$), data was collected as part of this center's biobanking initiative (project number PMCLAB2021-269). Data of five additional children with LS and pediatric cancer was included through the INdividualized Therapy FOr Relapsed Malignancies in Childhood (INFORM) registry.[14]

In the Princess Máxima Center, pathogenic germline variants in the MMR genes were discovered through exome sequencing during routine diagnostics, in research settings investigating cancer predisposition (through the individual Therapies [iTHER][15] and Predisposition to Childhood Tumors [PrediCT] studies) or during studies investigating mutational signatures. The Polymerase Epsilon (*POLE*) and Polymerase Delta 1 (*POLD1*) genes were screened separately for possible pathogenic germline or somatic variants. Additional pathogenic germline variants, if present, were discovered in a research setting (iTHER).[15] Within the INFORM study, all children were screened for pathogenic germline variants in cancer predisposing genes using exome and/or

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genome sequencing and virtual gene panels. These panels include all MMR genes, *POLE*, *POLD1*, as well as other genes associated with CPS. In all cases, only LP (class 4) and P (class 5) variants are reported. Variants are reported on the following reference transcripts or assembly: NM_000249.4 (*MLH1*), NM_000251.3 (*MSH2*), NM_000179.3 (*MSH6*), NM_000535.7 (*PMS2*), NM_000314.8 (*PTEN*), NM_006231.4 (*POLE*), NC_000002.12 (chromosome 2).

2.2 | Genome sequencing

Exome and/or genome sequencing was performed on a total of 20 tumor and 18 normal samples, according to different procedures depending on the time of sequencing and the origin of the samples (Table S1). Samples from the Princess Máxima Center were handled as described by van Belzen et al.[16] For exome sequencing, libraries were captured with KAPA MedExome or KAPA HyperExome (Roche). INFORM registry samples were handled as described by Worst et al.[17] For a subset of exome sequencing samples, libraries were captured with Agilent SureSelect version 7. For one tumor-normal pair, high-coverage genome sequencing data was generated as well. Preprocessing of all data was performed using the GATK4 preprocessing for variant discovery workflow, including mapping of raw reads to human reference genome hg38 with the Burrows-Wheeler aligner.[18]

2.3 | Variant calling

Somatic variant calling was performed on tumor-normal pairs using Mutect2 (GATK v4.2.0.0).[18] Soft clipped bases were excluded from somatic calling and multinucleotide substitution (MNS) calling was omitted. A bed file of the intersecting regions of the different captures, extended by 200 basepairs flanking each region, was used to restrict somatic variant calling. The somatic calls were scored using Mutect filters and after applying the PASS filter, MNS were selected based on chromosomal location and moved to a separate list. Both single base substitution (SBS) and insertion and deletion (indel) somatic calls were filtered to have at least five reads supporting the alternative allele, a variant allele frequency (VAF) of 0.1 or higher in the tumor and a VAF of 0 in the normal sample. Finally, the calls were filtered to have a population frequency of 1% or lower, based on the GnomAD and GoNL databases.[19,20]

For high-coverage genome sequencing data and exome sequencing data captured with MedExome of HyperExome, copy number variants (CNVs) were called according to GATK best practices, using the GATK4 CNV somatic pair workflow.[18] In-house panels of normals were used for normalization. In case sequencing methods of tumor and normal samples did not match, an adapted single sample workflow was used. For tumors with exome sequencing data captured with Agilent SureSelect version 5 or 7, CNV calling was performed on low-coverage genome sequencing data through generation of read-depth plots.[17]

2.4 | Molecular analyses of sequencing data

To calculate the TML, the number of somatic variants remaining after filtering was divided by the combined size of the regions in the bed file used to restrict somatic calling (101.6 Mb). The TML was calculated for the combined number of SBS and indels and is presented as the number of variants per Mb, referred to as mut/Mb.

For each tumor, a mutational profile was constructed for somatic SBSs and indels by determining the proportions of different variant classes in their sequence context.[21] SBS mutational profiles of individual tumors were used for refitting if they contained at least 200 somatic SBS, the number required for a representative mutational profile.[22] For indel mutational profiles, we used a threshold of 150 indels per tumor. Mutational profiles of tumors that met these conditions were subjected to a strict refit approach with a maxdelta value of 0.05 using Mutational Patterns,[23] to determine contributions of reference signatures archived in the Catalogue of Somatic Mutations in Cancer (COSMIC) (http://cancer.sanger.ac.uk/cosmic/signatures). The cosine similarity between the original and reconstructed mutational profiles of all tumors was 0.75 or higher for SBS and 0.8 or higher for indels (Table S2).

2.5 | Immunohistochemistry

IHC was performed at the histopathological section of the laboratory of pediatric oncology in the Princess Maxima Center. For IHC of the MMR proteins, formalin-fixed and paraffine-embedded tissue was sliced into 4 µm sections. IHC was performed using the BOND-RX automated IHC system using the Polymere Refine kit (DS9800, Leica, Wetzlar, Germany). Sections were pretreated with Tris-EDTA for 20 min and incubated for 15 min with monoclonal mouse primary antibodies for in vitro diagnostic use against *MLH1* (clone ES05, Leica, Wetzlar, Germany), *MSH2* (clone 79H11, Leica, Wetzlar, Germany), *MSH6* (clone PU29, Leica, Wetzlar, Germany) and *PMS2* (clone M0R4G, Leica, Wetzlar, Germany).

2.6 | Driver variant analyses

To determine the timing of second hit MMR aberrations, we calculated their clonality based on tumor purity. The tumor purity was estimated based on the peak frequency of somatic variants located on diploid chromosomes. The allele frequency of (partial) chromosomal deletions (in tumors of individuals L04, L07, L14, L15, L17) or near-haploidization (in the tumor of L16) was calculated based on the B-allele frequencies of common heterozygous SNPs in that region.

For tumors with contributions of MMR-associated mutational signatures, a list with driver variants was compiled out of non-synonymous coding and splice site variants in driver genes reported in previous studies.[24–28] Only SBSs and indels with a PHRED-scaled CADD score ≥15 and a VAF indicative of a clonal event were considered as driver variants. For each of these variants, the a posteriori

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probability was calculated as described by Morganella et al.[29] The a posteriori probability reflects the likelihood that the specific driver variants in their sequence context were caused as a direct consequence of the MMR deficiency. Instead of signatures resulting from a de novo extraction, we used signatures resulting from the strict refit approach.

3 | RESULTS

3.1 | Cohort description

In this study, we have systematically investigated causality of germline P/LP MMR variants for tumor formation in 18 individuals with LS who developed pediatric cancer. We included three children who developed a high-grade brain tumor and one child with CRC, malignant tumor types associated with LS in children, as well as 14 children who developed malignant tumors for which this association is uncertain, including three non-high-grade brain tumors, eight non-brain solid tumors and five hematological malignancies (Table 1). Among these children, germline heterozygous P/LP variants in *PMS2* were most prevalent ($n = 8$), followed by *MSH6* ($n = 5$), *MLH1* ($n = 3$) and *MSH2* ($n = 2$) (Table 1). The variants were detected during routine diagnostic testing ($n = 8$), in research settings investigating cancer predisposition ($n = 8$) or through research exploring mutational signatures ($n = 1$). In one child, L04, germline genetic testing was directed by family history and cancer type indicative of predisposition. For 10 children, the germline MMR variant was not yet known to occur in the family. CMMRD was considered unlikely in all individuals based on the lack of a second germline P/LP variant in the MMR genes and the absence of typical abnormal pigmentation of the skin. All children were checked for other germline alterations leading to a CPS. Polymerase proofreading-associated polyposis (PPAP) and combined PPAP and LS, a digenic condition known as POL-LYNCH, which both have a tumor spectrum similar to LS,[30–32] were excluded, as no germline P/LP variants in the exonuclease domain of *POLE* and *POLD1* were found. In L05, who developed an ovarian dysgerminoma, an additional germline variant in *PTEN* was found, which is known to cause PTEN Hamartoma-Tumor Syndrome (PHTS) (Table 1). Ovarian dysgerminoma is not a tumor type that typically develops in individuals with PHTS, but it has been described previously.[33] Primary tumors in children in this cohort were diagnosed between the ages of 1 and 17 (median 10), with five children developing relapses and one child developing a second primary tumor (Table 1).

3.2 | Second hits resulting in MMR deficiency

Because tumor development in individuals with LS typically initiates after a somatic second hit causing local MMR deficiency, all 14 primary tumors and six relapses were examined for somatic second hits, including variants or loss of heterozygosity in the respective MMR gene. As expected, a second hit was detected in all three diffuse pediatric high-grade gliomas (DPHGG) and in the CRC, in the form of a loss of the wildtype allele (L15, L16 and L17) or an additional somatic pathogenic variant (L18) (Table 2). Among the non-LSS tumors, we found a somatic loss of the wildtype allele of *PMS2* in three malignancies of three patients: the therapy-related acute myeloid leukemia (t-AML) of L04, the precursor B-cell acute lymphoblastic leukemia (BCP-ALL) of L07 and the relapsed osteosarcoma (OS) of L14 (Table 2).

3.3 | Tumor mutational load and mutational signatures

High TML is an indicator of MMR deficiency because, under these circumstances, mismatches acquired during replication are no longer repaired and will accumulate in the tumor genome. Typically, a TML above the hypermutation threshold (10 mut/Mb) is considered an indication for MMR deficiency.[34] The TML in the malignancies of patients in our cohort was found to range between 0.2 and 52.5 mut/Mb (median 1.2 mut/Mb) (Table 2). Six patients had tumors with a TML above the hypermutation threshold. These included all four LSS tumors, as well as the BCP-ALL and the relapsed OS (L07 and L14) (Table 2). The DPHGG of individual L17 had a remarkably high TML of 52.5 mut/Mb, likely caused by the somatic missense variant c.1370C>T, leading to p.(Thr457Met) in the exonuclease domain of *POLE*. This variant is classified as a variant of uncertain significance, but surrounding variants are known to be pathogenic and the high TML suggests this variant is pathogenic as well.

Besides a high TML, MMR-deficient tumors typically present with mutational profiles reflecting the loss of a particular MMR gene. Detecting contributions of mutational signatures associated with MMR deficiency provides an alternative strategy to detect loss of a functional MMR protein. In eight tumors, the number of SBSs was sufficient (≥200) to determine the contribution of COSMIC reference signatures to the mutational profile (Table S4). Seven of these tumors had contributions of COSMIC reference signatures associated with MMR deficiency: SBS6, SBS15, SBS21 and SBS26. Additionally, we found contributions of SBS1, which is not exclusively associated with MMR deficiency, but often found in MMR-deficient tumors.[35] The mutational profiles of all four LSS tumors were found to have high contributions of SBS1, SBS6 or SBS15 or a combination thereof, which are all dominated by C > T variants. The BCP-ALL and the t-AML mutational profiles (L07 and L04) had strong contributions of SBS26, a signature consisting mostly of T > C variants that is associated with *PMS2* deficiency.[36] The mutational profile of the relapsed OS of individual L14 showed high contributions of SBS21, another MMR-associated signature dominated by T > C variants. Finally, in the mutational profile of the t-AML of individual L04, we also found contributions of SBS6 (Table 2).

Six tumors had sufficient indels (≥150) for a reliable mutational profile, and all of these were found to have contributions of MMR-associated COSMIC reference signatures: ID1, ID2 and ID7 (Tables 2 and S5). The three DPHGGs all presented with high contributions of ID2, an indel signature associated with MSH2 and MSH6 deficiency,[37] consisting nearly exclusively of T deletions in large repeat regions. The

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TABLE 1 Description of the cohort of children with LS and pediatric cancer.

Individual ID[a]	Gene	Germline MMR variant	Tumor type[b] (age at diagnosis)	Additional germline variant	LS known in family
L01	*MLH1*	c.350C>T, p.(Thr117Met)	Desmoid type fibromatosis (10)		Yes
L02	*MSH6*	c.3261dup, p.(Phe1088Leufs*5)	Mature T-cell Lymphoma NOS (2)		Yes
L03	*PMS2*	c.736_741delinsTGTGTGTGAAG, p.(Pro246Cysfs*3)	Gardner Fibroma (1)		No
L04	*PMS2*	c.1882C>T, p.(Arg628*)	Precursor B-cell Acute Lymphoblastic Leukemia (5) Therapy-related Acute Myeloid Leukemia (9)		Yes
L05	*PMS2*	c.1164del, p.(His388Glnfs*10)	Ovarian Dysgerminoma (11)	PTEN c.920_921insTATA, p.(Glu307Aspfs*6)	No
L06	*PMS2*	c.137G>T, p.(Ser46Ile)	Wilms tumor (3)		No
L07	*PMS2*	c.1882C>T, p.(Arg628*)	Precursor B-cell Acute Lymphoblastic Leukemia (2)		No
L08	*MSH6*	g.47725638_47859715del[c]	Diffuse Large B-cell Lymphoma (5)		No
L09	*MLH1*	c.1845_1847del, p.(Lys618del)	Pilocytic Astrocytoma (4 m) Pilocytic Astrocytoma relapse 1 (5)		Yes
L10	*PMS2*	c.1A>T, p.?	Ependymoma PFA (1) Ependymoma PFA relapse 1 (2) Ependymoma PFA relapse 2 (4)		No
L11	*PMS2*	c.325del, p.(Glu109Lysfs*3)	Ewing Sarcoma (15) Ewing Sarcoma relapse 1 (17)		No
L12	*MSH6*	c.651dup, p.(Lys218*)	Pilocytic Astrocytoma (16)		Yes
L13	*MSH6*	c.3725G>A, p.(Arg1242His)	Sarcoma[d] (14) Sarcoma relapse 1[d] (19) Sarcoma relapse 2[e] (20) Sarcoma relapse 3[e] (22)		No
L14	*PMS2*	c.1076dup, p.(Leu359Phefs*6)	Osteosarcoma[d] (13) Osteosarcoma relapse 1[d] (15) Osteosarcoma relapse 2[f] (17)		No
L15	*MLH1*	c.2028_2032delinsT, p.(Ser677Lysfs*105)	Adenocarcinoma colon ascendens (17)		Yes
L16	*MSH2*	c.839dup, p.(Leu280Phefs*4)	Diffuse Pediatric High-Grade Glioma (15)		Yes
L17	*MSH6*	c.3959_3962del, p.(Ala1320Glufs*6)	Diffuse Pediatric High-Grade Glioma (15)		No
L18	*MSH2*	g.47466656A>G[g]	Diffuse Pediatric High-Grade Glioma (15)		Yes

[a]L05,[15] L06,[44] L08,[45] L13,[14] and L18[15] were included in previous studies.
[b]Further specification of genetic characteristics in Table S3.
[c]134-kb deletion in chr2p16.3, including *MSH6* and exon 2–23 of *FBXO11*. Breakpoints detected through SNP-array.
[d]Subtype not specified.
[e]BCOR-driven small-blue-round-cell tumor.
[f]High-grade osteosarcoma.
[g]Variant commonly known as c.1511-2A>G, p.(IVS9-2A>G).

CRC of L15 presented with contributions of ID2 and ID7, the latter consists of C and T deletions in larger repeats. The BCP-ALL and the relapsed OS (L07 and L14) both exclusively had contributions of ID1, dominated by T insertions in large repeat regions (Table 2). ID1 is known to contribute strongly to mutational profiles of *PMS2*-deficient tumors, consistent with the underlying *PMS2* defect in these tumors.[37]

3.4 | Immunohistochemistry of the MMR proteins

For 11 tumors, IHC for the four MMR proteins was performed to study the effect of the MMR variants. As expected, MSH2 and MSH6 were (partially) lost in all three DPHGGs. In six non-LSS tumors, nuclear staining of all MMR proteins was retained. In the two

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TABLE 2 Molecular characterization of 20 malignant tumors of children with LS and pediatric cancer.

Individual ID	Gene	Tumors	Second hit MMR	TML	MMR SBS signatures[a,b]	MMR indel signatures[a,b]	Immunohisto-chemistry[a]
L01	*MLH1*	Desmoid type fibromatosis	No	0.2	np	np	Retained
L02	*MSH6*	Mature T-cell Lymphoma NOS	No	0.4	np	np	Inconclusive[c]
L03	*PMS2*	Gardner Fibroma	No	0.2	np	np	Inconclusive[c]
L04	*PMS2*	Precursor B-cell Acute Lymphoblastic Leukemia	No	0.3	np	np	np
L04	*PMS2*	Therapy-related Acute Myeloid Leukemia	Monosomy chr7	2.7	SBS26/SBS6	np	np
L05	*PMS2*	Ovarian Dysgerminoma	No	0.6	np	np	Retained
L06	*PMS2*	Wilms tumor	No	2.2	None	np	Retained
L07	*PMS2*	Precursor B-cell Acute Lymphoblastic Leukemia	Monosomy chr7	11.5	SBS26	ID1	np
L08	*MSH6*	Diffuse Large B-cell Lymphoma	No	1.2	np	np	Retained
L09	*MLH1*	Pilocytic Astrocytoma relapse 1	No	0.7	np	np	Retained
L10	*PMS2*	Ependymoma PFA relapse 2	No	0.4	np	np	np
L11	*PMS2*	Ewing Sarcoma relapse 1	No	0.8	np	np	np
L12	*MSH6*	Pilocytic Astrocytoma	No	0.2	np	np	Retained
L13	*MSH6*	Sarcoma relapse 2	No	0.9	np	np	np
L13	*MSH6*	Sarcoma relapse 3	No	1.6	np	np	np
L14	*PMS2*	Osteosarcoma relapse 2	Loss chr7p	15	SBS21/SBS15	ID1	np
L15	*MLH1*	Adenocarcinoma colon ascendens	CN-LOH chr3p26.3-p22.2	17.1	SBS6	ID7/ID2	np
L16	*MSH2*	Diffuse Pediatric High-Grade Glioma	Monosomy chr2	14.8	SBS1[d]/SBS15	ID2/ID1	MSH2-MSH6 partially-
L17	*MSH6*	Diffuse Pediatric High-Grade Glioma	CN-LOH chr2p25.3-p16.2	52.5	SBS15	ID2	MSH2 partially-MSH6 partially-
L18	*MSH2*	Diffuse Pediatric High-Grade Glioma	MSH2 c.2435-2436dup, p.(Met813Leufs*6)	15.3	SBS1[d]	ID2	MSH2-MSH6-

[a]np = not performed.
[b]SBS and indel MMR-associated signatures that contribute to the mutational profile of the tumor are shown, ordered from the highest contribution to the lowest. For tumors with less than 200 SBS or 150 indels, no reliable mutational profile can be constructed, and no strict refit approach was performed (np). One tumor had sufficient SBS, but no contributions of MMR signatures (None).
[c]Immunohistochemistry was inconclusive due to low cellularity in the leftover tumor material.
[d]SBS1 is not specifically linked to MMR deficiency, but is often found in MMR deficient tumors.[35]

remaining non-LSS tumors, the presence of the MMR proteins was inconclusive due to a low cellularity in leftover tumor material (Table 2).

3.5 | Timing of the second hit in tumor development of MMR-deficient tumors

Causality of the germline MMR variant implies that the MMR deficiency has occurred early in tumor development and has resulted in crucial driver variants. By comparing tumor purity and the allele frequency of somatic MMR aberrations in the MMR-deficient tumors, we found that in at least six of the seven tumors, including the four LSS tumors, these second hit abberations were clonal, suggesting they occurred before clonal expansion (Table S6 and Figure S1). In the t-AML sample (L04), the loss of chromosome 7 appeared to be present in a subfraction of the tumor cells, but these estimates were less accurate due to low tumor percentage.

Next, we explored the likelihood that potential driver variants identified in the seven tumors arose as a consequence of the MMR deficiency. To do this, we calculated the a posteriori probability that these driver variants were caused by each of the assigned COSMIC

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reference signatures using a previously published method.[29] Due to the low number of COSMIC signatures contributing in most of the MMR-deficient tumors, we additionally assessed whether the sequence context of these driver variants also represented a prominent peak in the assigned COSMIC reference signature (Table S7 and Figure S2). Using this dual approach, we identified potential driver variants in all seven tumors that were likely caused by MMR-associated mutational processes, including a *PHF6* variant in the t-AML of L04, a *PAX5* variant in the BCP-ALL of L07, and a *RB1* variant in the relapsed OS of L14. These findings indicate that in both the LSS and non-LSS tumors with MMR deficiency, the second hit abberations precede clonal expansion of the malignancy and may cause driver variants that contribute to tumorigenesis.

4 | DISCUSSION

Recent studies have shed light on the prevalence of adult CPS, such as LS, in children with cancer.[4–7] While for LSS tumors, clues supporting causality have been presented in multiple studies,[6,11,12] further research is required to determine whether there is an association between the underlying LS and other pediatric tumors. In this study, we present a cohort of 14 children with LS who developed 16 non-LSS tumors, which we compare to four children with LS who developed LSS tumors. By molecular characterization of the individual tumors, we found that all LSS tumors display molecular evidence for MMR deficiency as a consequence of acquired somatic second hit mutations, while this was the case for only three out of 16 non-LSS tumors. These results show that in the majority of non-LSS tumors in this cohort, the molecular tumor data do not indicate a causal relation between tumor development and the underlying LS.

There is a difference in the prevalence of heterozygous germline P/LP MMR gene variants between LS families and the general population that directly reflects penetrance of the different MMR genes. Whereas LS families with a positive history for cancer are often heterozygous for the more penetrant *MLH1* and *MSH2* germline P/LP variants,[38] germline P/LP variants in *PMS2* and *MSH6* are more prevalent in the general population.[39] In our cohort of children with LS and non-LSS tumors, the distribution of P/LP germline MMR variants is similar to that in the population, suggesting that there is no selection toward more penetrant MMR genes. Furthermore, three out of five children with *MLH1* or *MSH2* germline P/LP variants developed LSS tumors, while all children with *PMS2* germline P/LP variants developed non-LSS tumors. These data indicate that for the lower penetrance MMR genes, causality between LS and childhood cancer is less likely than for high penetrance MMR genes.

Despite the apparent weaker association between LS and the development of non-LSS tumors, we found that three non-LSS tumors, a relapsed OS, a t-AML and a BCP-ALL, presented with *PMS2* deficiency, suggesting a causal relation in initiation or progression. In all tumors, the wildtype *PMS2* allele was lost through (partial) loss of chromosome 7, which was a clonal event in at least two out of three tumors. Furthermore, driver variants in these non-LSS tumors appeared to be caused by MMR-associated mutational processes, indicating that these driver variants originated as a result of MMR deficiency. Therefore, it is likely that loss of the wildtype allele occurred early during tumor development. On the other hand, the loss of chromosome 7 itself is a recurrent aberration in each of these tumor types, also in the absence of *PMS2* germline variants. In fact, monosomy 7 is found in 23% of treatment related AML,[40] and the loss of the wildtype *PMS2* allele in the relapsed OS might be a consequence of the typical complex karyotype that can affect all chromosomes.[41] Thus, although there is molecular evidence supporting causality in these three non-LSS tumors, a coincidental side effect during tumor development cannot be excluded.

Based on the results of this study, there is no strong indication for screening for germline P/LP variants in MMR genes among all children with cancer. Particularly since there is great unanimity in medical genetics that predictive genetic testing for adult onset conditions should be delayed until adulthood, except for disorders for which preventive actions or therapeutic interventions could be initiated before that time.[42] However, the presence of MMR deficiency can influence treatment decisions independent of underlying germline conditions, since MMR deficiency positively affects the response to immune checkpoint inhibitors.[43] To utilize the full treatment potential, all tumors should be screened for a high TML or any other sign of MMR deficiency. Subsequently, screening for germline P/LP variants in MMR genes could be limited to children who develop tumors with signs of MMR deficiency. For all families of children with LS, including the four children developing LSS tumors, we follow the standard recommendations for siblings, as we did not observe a more severe phenotype than in other LS families, nor did we find evidence for digenic inheritance.

In conclusion, by systematic molecular characterization of tumors in children with LS, we show that the likelihood that a germline MMR variant is causative for cancer development depends on tumor type. In contrast to LSS tumors, molecular evidence for MMR deficiency is absent in most non-LSS tumors, illustrating that the causal relation in this group is much weaker compared to LSS tumors. These findings indicate the need for larger cohorts to further study causal associations between germline P/LP MMR variants and cancer development in children in a tumor type-specific context.

AUTHOR CONTRIBUTIONS

The work reported in the paper has been performed by the authors, unless clearly specified in the text. *Conceptualization*: Dilys D. Weijers, Marjolijn C. J. Jongmans, Roland P. Kuiper. *Data curation*: Dilys D. Weijers, Steffen Hirsch, Jette J. Bakhuizen, Nienke van Engelen, Robert J. Autry, Natalie Jäger, Marjolijn C. J. Jongmans. *Formal analysis*: Dilys D. Weijers. *Funding acquisition*: Kristian W. Pajtler, Marjolijn C. J. Jongmans, Roland P. Kuiper. *Investigation*: Dilys D. Weijers. *Methodology*: Dilys D. Weijers, Marjolijn C. J. Jongmans, Roland P. Kuiper. *Project administration*: Dilys D. Weijers, Marjolijn C. J. Jongmans, Roland P. Kuiper. *Resources*: Steffen Hirsch, Jette J. Bakhuizen, Nienke van Engelen, Lennart A. Kester, Mariëtte E. G. Kranendonk, Laura S. Hiemcke-Jiwa, Evelien de Vos-Kerkhof, Jan L. C.

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Loeffen, Natalie Jäger, Marjolijn C. J. Jongmans, Roland P. Kuiper. *Software*: NA. *Supervision*: Marjolijn C. J. Jongmans, Roland P. Kuiper. *Validation*: Lennart A. Kester, Robert J. Autry, Natalie Jäger. *Visualization*: Dilys D. Weijers. *Writing-original draft*: Dilys D. Weijers, Marjolijn C. J. Jongmans, Roland P. Kuiper. *Writing-review & editing*: Dilys D. Weijers, Steffen Hirsch, Jette J. Bakhuizen, Nienke van Engelen, Lennart A. Kester, Mariëtte E. G. Kranendonk, Laura S. Hiemcke-Jiwa, Evelien de Vos-Kerkhof, Jan L. C. Loeffen, Robert J. Autry, Kristian W. Pajtler, Natalie Jäger, Marjolijn C. J. Jongmans, Roland P. Kuiper.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All next-generation sequencing data in this article has been deposited at the European Genome-phenome Archive (EGA), which is hosted by the EBI and the CRG, under accession numbers EGAS00001006992 and EGAS00001007146. Other data that support the findings of this study are available from the corresponding author upon request.

ETHICS STATEMENT

All studies were conducted in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki. Patient data was de-identified. For all data originating from the Princess Máxima Center, informed consent has been obtained for all individuals involved in this study through the Máxima biobank (https://research.prinsesmaximacentrum.nl/en/core-facilities/scientific-committee) informed consent procedure and corresponding protocol. The Máxima biobank protocol has been approved by the Medical Ethics Committee of the Erasmus Medical Center in Rotterdam, The Netherlands, under reference number MEC-2016-739. Approval for use of the individual's data within the context of this study has been granted by the Máxima biobank and data access committee, biobank request nr. PMCLAB2021-269. For all data collected through the INFORM study, all individuals or their legally acceptable representative, or both (if possible), provided written informed consent. Approvals for the study protocol (and any modifications thereof) were obtained from independent ethics committees and the institutional review board at each participating center. The study was registered with the German Clinical Trial Register, number DRKS00007623.

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