Risk of cancer in cases of suspected Lynch syndrome without germline mutation.

SHORT TITLE: Cancer risk in suspected Lynch syndrome.

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ABBREVIATIONS

CRC: colorectal cancer.

MSI: microsatellite instability.

MMR: mismatch repair.

LS: Lynch syndrome.

LLS: Lynch-like syndrome.

IHC: immunohistochemical.

SIR: standardized incidence ratios.

LSRC: Lynch syndrome related cancers.

SD: standard deviation.

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ABSTRACT

Background & Aims: Colorectal cancers (CRC) with microsatellite instability (MSI) and a mismatch repair (MMR) deficit without hypermethylation of the *MLH1* promoter are likely to be caused by Lynch syndrome. Some patients with these cancers have not been found to have pathogenic germline mutations, and are considered to have Lynch-like syndrome (LLS). The aim of this study was to determine the risk of cancer in families of patients with LLS.

Methods: We studied a population-based cohort of 1705 consecutive patients, performing MSI tests and immunohistochemical analyses of MMR proteins. Patients were diagnosed with Lynch syndrome when they were found to have pathogenic germline mutations. Patients with MSI and loss of MSH2 and/or MSH6 expression, isolated loss of PMS2, or loss of MLH1 without MLH1 promoter hypermethylation and no pathogenic mutation were considered to have LLS. The clinical characteristics of patients and the age- and sex-adjusted standardized incidence ratios (SIR) of cancer in families were compared between groups.

Results: The incidence of CRC was significantly lower in families of LLS patients than families with confirmed Lynch syndrome cases (SIR for Lynch syndrome=6.04; 95% confidence interval [CI], 3.58-9.54 and SIR for LLS=2.12; 95% CI, 1.16-3.56; *P*<.001). However, the incidence of CRC was higher in families of patients with LLS than in families with sporadic CRC (SIR for sporadic CRC=0.48; 95% CI, 0.27-0.79; *P*<.001).

Conclusions: The risk of cancer in families with LLS is lower that of families with Lynch syndrome, but higher than that of families with sporadic CRC. These results confirm the need for special screening and surveillance strategies for these patients and their relatives.

Keywords: inherited colon cancer; cancer risk; genetic testing; immunohistochemistry

INTRODUCTION

Lynch Syndrome (LS) is the most common inherited colon cancer susceptibility syndrome caused by germline mutations in one of several DNA mismatch repair (MMR) genes, mainly MLH1 and MSH2, but also MSH6 and PMS2.¹⁻³ Patients with LS have an increased risk of colorectal cancer (CRC), endometrial cancer, and several other cancers, including ovarian, upper urinary tract, gastric, small bowel, biliary/pancreatic, skin, and brain cancers. The molecular signature of LS is microsatellite instability (MSI), which is found in more than 95% of LS-associated CRCs.⁴ However, MSI is also present in up to 15% of sporadic CRCs, due to hypermethylation of the promoter region of MLH1 in tumor cells. Immunohistochemical (IHC) studies of MMR proteins have been shown to be equivalent to MSI in detecting MMR-defective CRC.⁵ CRC with MSI and a lack of staining of MSH2, MSH6, or MLH1 without promoter hypermethylation is a strong indicator of MSH2, MSH6, or MLH1 germline mutations.⁶ However, some of these CRC cases do not have pathogenic mutations in MMR genes. These cases are suspected to be non-sporadic because no mechanism of inactivation is known for these genes other than germline mutations in the context of LS. These patients are considered to have "probably non-sporadic" colorectal cancer or "Lynch-like" syndrome (LLS), and decisions about their management are not simple because of unconfirmed suspicions of hereditary cancer. These patients must be distinguished from familial CRC type X, where tumors do not show MMR deficiency. No studies have characterized these CRC patients, and the risk of cancer in this group of families is not known. Therefore, the surveillance strategy for these patients and their relatives is unclear.

We analyzed the clinical and familial characteristics of patients diagnosed with LLS, LS, or sporadic CRC. The main aim of this study was to determine the risk of cancer in families of patients with LLS.

METHODS

Patients and data collection

The present study was a population-based observational cohort study including 1,705 patients with CRC from two nationwide multicenter studies, EPICOLON I and EPICOLON II. EPICOLON I included consecutive patients with a new diagnosis of CRC between November 2000 and October 2001 with the main goal of estimating the incidence of LS in Spain.⁷ EPICOLON II also included consecutive newly diagnosed CRC patients between March 2006 and December 2007 with the aim of investigating different aspects related to the diagnosis of hereditary CRC.⁸ All of the patients provided written informed consent. Both studies were approved by the institutional review boards of the participating hospitals.

Patients were divided into three groups based on genetic data: 1) LS group, patients with a confirmed pathogenic mutation in *MLH1*, *MSH2*, *MSH6*, or *PMS2*; 2) LLS group, patients with MSI and loss of MSH2/MSH6 expression, isolated loss of PMS2, or loss of expression of MLH1 without *MLH1* promoter hypermethylation in which no germline mutation was found; and 3) sporadic group, patients with CRC and MSS tumors showing normal expression of MMR genes or a loss of MLH1 expression with *MLH1* promoter hypermethylation.

Demographic, clinical, and pathology data were collected at the time of diagnosis. Cancer pedigrees were built at diagnosis for CRC cases in the EPICOLON I and II studies. The pedigrees were traced backward and laterally as far as possible. This information was verified by reviewing medical records when available. Standardized incidence ratios (SIR) for cancer were calculated as the ratio of the observed to expected number of cases diagnosed in the families at the time of inclusion in the EPICOLON I or II cohorts. In order to avoid recall bias, only cancer cases found in first-

degree relatives were included in the calculation of SIR. We considered tumors in the endometrium, ovaries, upper urinary tract, stomach, small intestine, and hepatobiliary system as non-colorectal LS-related cancers (LSRC). The index case was excluded for the analysis of family history at the time of diagnosis. Calculation of the SIR was only possible in families with complete pedigrees and information about the ages of all family members, including relatives without cancer.

In 2011, the pedigrees were updated by asking patients and/or relatives about new cancer cases after diagnosis of the index case. We include the index case for this analysis, and the appearance of metachronous CRC or a new case of LSRC in the index case was considered a new case in the family.

Microsatellite instability, immunohistochemical staining, and detection of germline mutations

MSI analysis was performed in all patients. We ascertained MSI status using BAT26 and NR24 quasimonomorphic markers as described previously.⁹ MSI was present when one of the two markers was unstable. IHC analysis of the four mismatch repair proteins MLH1, MSH2, MSH6, and PMS2 in tumor tissue was performed in all patients using tissue microarrays (TMAs) as described previously.¹⁰ In patients with a loss of MLH1, methylation of *MLH1* and *BRAF* mutation status were analyzed. *MLH1* methylation analysis was performed using methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) according to the manufacturer's protocol using the SALSA MS-MLPA kit ME011 Mismatch Repair Genes (MRC-Holland, Amsterdam, the Netherlands).¹¹ The V600E *BRAF* mutation was detected using specific TaqMan probes in real-time PCR (ABI PRISM 7500, Applied Biosystems, Foster City, CA, USA) and allelic discrimination software as described previously.¹²

Germline mutation analysis was performed in accordance with the results of the IHC analysis as described previously.¹⁰ Patients with loss of MSH2 expression with no detected mutation were analyzed for *EPCAM* rearrangements using MLPA (MRC-Holland, Amsterdam, the Netherlands) according to the manufacturer's recommended protocol. DNA sequencing was performed to characterize the deletion breakpoints.¹³ Large rearrangements (deletions and insertions) were tested using MLPA (MRC-Holland) according to the manufacturer's protocol. The genetic analysis results were interpreted based on the ACMG Recommendations for Standards for Interpretation of Sequence Variations (2000) and the InSIGHT database.¹⁴

Statistical analysis

Continuous variables are reported as mean ± standard deviation (SD) or medians and 25th and 75th percentiles for non-normally distributed data. Categorical variables are reported as frequencies or percentages. Significant differences between groups were analyzed using the chi-square test for categorical data and the non-parametric Mann-Whitney U test for guantitative data.

The SIR of each cancer was calculated as the ratio of the observed to expected number of cases among relatives. Person-years were calculated from 20 years of age until the earliest cancer diagnosis or death. The expected number of cases was calculated as the sum of the products of the number of person-years for each 5-year age/sex group and the corresponding age/sex-specific incidence rates in Spanish regional registers.¹⁵ The confidence limits were based on the Byar's approximation of the exact Poisson distribution, which is accurate even with small numbers.¹⁶ All reported p-values are two-sided, and p < 0.05 was considered significant. All calculations were performed using the SPSS 19.0 software.

RESULTS

A total of 1,705 patients with CRC were included in the study. The median age was 71 years (range: 27-101 years) and 59% of patients were male. Sixteen patients were excluded because of discrepancies between the IHC and MSI analyses; no mutation was found in these patients. Therefore, data from 1,689 patients were analyzed. **Tumors from all patients were analyzed.** A total of 135 patients (8%) exhibited **in their tumors** MSI and loss of expression of any of the MMR proteins. In 104 patients (6.1%), loss of MLH1 expression was found in IHC analysis. Of these patients, 25 (1.4%) did not exhibit hypermethylation of the promoter region. We also used *BRAF* mutation as a sporadic CRC marker in these 25 cases, but no case of *BRAF* mutation indicating sporadic origin was found. Loss of MSH2 expression was seen in the IHC analysis of 22 patients (1.3%). Three patients (0.2%) had an isolated loss of MSH2 was found in 6 (0.3%) patients. Finally, an isolated loss of PMS2 was found in 3 patients (0.2%).

A germline pathogenic mutation in any of the MMR genes was found in 16 patients (0.9%) and considered to have LS. Three of these patients exhibited MSI with non-valuable expression of MMR proteins in IHC analysis. Four of the LS patients were found to have a pathogenic mutation in *MLH1*, eight in *MSH2*, three in *MSH6*, and one in *PMS2*. All of these patients exhibited MSI. No case was found with deletions in *EPCAM*. Variants of uncertain significance were found in five patients (**Supplementary Table 1**). Forty-three patients (2.5%) exhibited MSI and loss of MSH2/MSH6, PMS2, or MLH1 expression without promoter hypermethylation, but no pathogenic germline mutation was found. These patients were considered to have LLS. Among the LLS patients, 21 were found to have a loss of MLH1 protein expression, and 22 loss of

MSH2, MSH6, or PMS2 expression (14 with loss of MSH2 and MSH6, 6 with isolated loss of MSH6, and 2 with isolated loss of PMS2). Finally, 1,630 patients (96%) were considered to have sporadic CRC.

Demographic, clinical, and pathological characteristics of patients with LLS

The characteristics of the LLS group (n=43) were compared to the LS (n=16) and sporadic CRC (n=1630) groups (**Table 1**). Fewer LLS patients fulfilled the revised Bethesda guidelines than LS patients, and LLS patients less often had a personal history of non-colorectal LSRC compared to LS patients. No differences were found in the presence of metachronous CRC, median age at diagnosis of CRC, sex and tumor characteristics, such as location or TNM classification. When we compared LLS patients to sporadic CRC patients, patients with LLS were younger at diagnosis, predominantly female, and more frequently fulfilled the revised Bethesda guidelines. Personal history of non-colorectal LSRC was more frequent in patients with LLS without differences in the presence of metachronous or synchronous CRC (**Table 1**).

Familial cancer risk

A total of 13 families with LS and 25 families with LLS had complete pedigrees including the ages of relatives without cancer. A random sample of 115 families with sporadic CRC was used for a comparison. A total of 1,102 first-degree relatives were included: 80 from LS families, 177 from LLS families, and 845 from sporadic CRC families. The mean number of first-degree relatives was 6.1 for LS families, 7.0 in LLS families, and 7.3 in sporadic CRC families.

In LS families we identified 18 cases of CRC and six cases of non-colorectal LSRC. Only in 4 (30.7%) families there was no cancer case other than the index case. In LLS

families, we found 14 cases of CRC and eight cases of non-colorectal LSRC. In 12 out of 25 families (48%), no case of cancer was found other than the index case. The characteristics of patients and distribution of cancer cases in LLS families are provided in **Table 2**. Finally, in sporadic CRC families, 15 first-degree relatives had CRC and 27 had non-colorectal LSRC. In 85 (79.9%) families no cancer cases were identified other than the index case.

The SIR of CRC and non-colorectal LSRC are found in **Table 3**. The incidence of CRC was significantly lower in LLS families compared to confirmed LS families (SIR in LS: 6.04, 95% CI 3.58-9.54; SIR in LLS: 2.12, 95% CI 1.16-3.56; p<0.001). However, the incidence of CRC was significantly greater in the LLS families than in sporadic CRC families (SIR in sporadic CRC: 0.48, 95% CI 0.27-0.79; p<0.001). The SIR for non-colorectal LSRC was <u>non-significantly</u> higher in LS families (SIR 2.81, 95% CI 1.03-6.12) compared to LLS families (SIR 1.69, 95% CI 0.73-3.34; p=0.09). <u>There were no differences in SIR for non-colorectal LSRC between LLS families and sporadic CRC families (SIR 1.20, 95% CI 0.79-1.74; p=0.5).</u> Taken together, the results indicate that, for CRC and non-colorectal LSRC, the highest risk is for LS families (SIR 4.69; 95% CI 3.00-6.98), followed by LLS families (SIR 1.94; 95% CI 1.22-2.94; p<0.001). The risk in LLS families was significantly higher than the risk in relatives of patients with sporadic CRC (SIR 0.78; 95% CI 0.56-1.05; p<0.001).

Figure 1 shows the cumulative age-of-onset curves for CRC among all relatives in the LS, LLS, and sporadic CRC groups. The relatives of patients with LLS developed CRC at an earlier mean age (53.71 ± 16.8 years) than those with sporadic CRC (68.8 ± 9 years; p=0.004), but was similar to the mean age in LS patients (48.5 ± 14.13 ; p=0.23).

After a median 8.3 years of prospective follow-up, cancer pedigrees were updated in 93 families: 10 in the LS group, 16 in the LLS group, and 67 in the sporadic CRC. A total of 533 first-degree relatives were included (including the index case): 41 from LS

families, 89 from LLS families, and 403 from sporadic CRC families. During this period, 7 (17.1%) new cases of CRC or non-colorectal LSRC appeared in LS families, 4 (4.5%) new cases in LLS families, and 4 (1.0%) in families with sporadic CRC **(Table 4)**.

DISCUSSION

The main finding of our study is that the risk of CRC is lower in LLS families than among patients with genetically confirmed LS but significantly higher than in cases of sporadic CRC. The results confirm the need for a special surveillance strategy for these patients and their relatives. In addition, the age of onset for CRC in LLS families was similar to that of LS families. Differences between LLS families and families with sporadic CRC cases were more prominent in regards to CRC risk than for the risk of other LSRCs.

Recent studies have shown that MSI testing and IHC analysis of MMR genes in all patients with CRC improves the detection of patients with LS.^{10, 17, 18} Because of the generalization of this universal strategy following Jerusalem guidelines,¹⁹ an increasing number of patients exhibit a loss of MMR protein expression with no pathogenic mutation. In CRC cases with a loss of MSH2, MSH6, PMS2, or MLH1 without hypermethylation, no cause of MMR gene inactivation is known other than germline mutation. In these cases, when a germline mutation is not found, a high suspicion of LS persists, and these patients and their relatives should be followed-up appropriately. The clinical characteristics of some of these patients suggest that they are clear hereditary cases, even though we were not able to find a genetic defect. Some of the pedigrees of patients with LLS showed a significant history of CRC with metachronous and synchronous tumors and fulfillment of the Amsterdam criteria. In these cases, the genetic defect was not found, probably due to it being located in a still unknown part of the gene, or simply that our technical capacity is not yet able to detect the pathogenic mutation. Some of these cases have been explained in the literature by alterations in other genes, such as in cases of EPCAM deletions or MLH1 constitutional epimutations. Other mechanisms, including inversions and duplications, could also explain some of these cases.²⁰⁻²⁶ However, some cases do not show any specific characteristics that suggest a hereditary origin. A notable proportion of LLS families do

not have a history of other cancers, and the only reason to suspect LS is the presence of MSI and loss of MMR protein expression. In these cases, determining the appropriate counseling for patients and their relatives is difficult. It is possible that some of these LLS patients could be cases with CRC who may have false positive results of IHC and/or MSI or sporadic MMR-deficient CRC, and LLS patients would be a mixture of true LS patients with non-detected germline mutation and sporadic CRC cases. However, the high risk of CRC found in our study suggests that, in its entirety, LLS cases should be considered as high-risk cases and strategies for cancer prevention must be implemented in this group of patients and their relatives. The SIR of CRC for LLS families was similar to that described in a group of families with familial colorectal cancer syndrome type X, but in this syndrome no molecular alteration has been found.²⁷ Even though LLS is a completely different entity because of the presence of MSI, the similar risk of CRC cancer should guarantee at least a similar surveillance program, even in cases without previous family history. Our results can contribute some rationale for designing follow-up strategies and, together with family history, can help clinicians appropriately schedule surveillance for these patients and their relatives. In our study, the age of CRC onset was similar to that of LS, and therefore surveillance strategies should start at the same age as recommended for LS cases. On the other hand, the frequency of CRC screening should be individualized. Given that the risk of CRC is lower in LLS families than in LS families, longer surveillance intervals for LLS cases and relatives without a prominent family history of CRC may be recommended. We have not found higher risk for noncolorectal LSRC in LLS families compared to sporadic CRC families. However, that can be due to the small number of cases detected in our series. For this reason, specific recommendations for endometrial and other non-colorectal LSRC cannot be appropriately supported by our data.

The limitations of our study are the possibility of underreporting or misreporting cancers because our information was not always confirmed with objective clinical and pathological data. However, we think this limitation is minor because it would affect the LLS group to the same extent as the other groups. Another limitation is the relatively small number of families, especially in the prospectively followed cases, which precludes finding clear differences between groups. Moreover, the follow-up time for these cases could be considered too short.

The main strength of our study is its population-based approach with cases ascertained from general clinics and not from specialized high-risk clinics. This approach provides robustness to our data in terms of potential applicability to general practice. Cancer risk can be overestimated in studies coming from select populations in genetic high-risk clinics. Studies based on recruitment through cancer genetics clinics do not usually correct for the selection bias caused by the over-representation of families with multiple cases in the data set.^{28, 29} Our results attempt to provide a rationale for follow-up and surveillance of this growing group of patients that will mostly be seen in the general clinics and not in high-risk clinics. New research is necessary to refine the classification of these patients in order to distinguish between sporadic and true hereditary cases.

Figure 1. Cumulative age of onset of colorectal cancer in first-degree relatives of Lynch syndrome, Lynch-like syndrome, and sporadic CRC.

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Table 1. Demographic, clinical, and pathology data comparing LLS, LS, and

sporadic CRC.

	LS	LLS	Sporadic CRC
	(n=16)	(n=43)	(n=1630)
• Sex, % women (n)	62.5% (10)	55.8% (24) [¶]	40.1% (654)
 Median age, years (interquartile range) 	69 [51-75]	66 [55-73] [¶]	71 [64-78]
 Revised Bethesda Guidelines, % fulfillment (n) 	81.3% (13)	51.2% (22) ^{§¶}	22.0% (358)
• Location, % right colon (n)	56.3% (9)	55.8% (24) [¶]	26.9% (438)
• TNM Stage II, % (n)	50.0% (8)	59.0% (25) [¶]	39.6% (645)
 Histology, % (n) 			
Poor differentiation	25.0% (4)	4.9% (2) [§]	8.3% (135)
Lymphocytic infiltration	25.0% (4)	28.6% (12)	29.2% (475)
Mucinous	20.0% (3)	36.6% (15) [¶]	13.0% (212)
• Metachronous CRC, % (n)	12.5% (2)	0% (0)	1.2% (20)
 Personal history of non- colorectal LS cancer, % (n) 	43.8% (7)	11.6% (5) ^{§¶}	3.3% (54)
• Synchronous CRC, % (n)	12.5% (2)	9.3% (4)	5.6% (91)

§ p < 0.05 LS patients vs LLS patients.

 $\P\ p$ < 0.05 LLS patients vs sporadic CRC patients.

Patient	Protein lost	Age index case	No relatives with CRC §	No relatives with LSRC 1	Total of first- degree	% of CRC ‡	% of LSRC *
			(n=14)	(n=8)	(p=177)		
					(1=177)		
1	PMS2	71	0	1 (pancreas)	10	0	10
2	MLH1/PMS2	81	0	0	9	0	0
3	MLH1/PMS2	73	0	0	7	0	0
4	MLH1/PMS2	75	1	1 (stomach)	7	14.2	14.2
5	MLH1/PMS2	45	3	1 (ovary)	10	30	10
6	MSH2/MSH6	38	0	0	5	0	0
7	MSH2/MSH6	74	0	0	6	0	0
8	MSH2/MSH6	32	2	0	3	66.6	0
9	MLH1/PMS2	72	2	3 (uterus)	11	18.1	27.2
10	MLH1/PMS2	51	0	0	4	0	0
11	MLH1/PMS2	46	1	1 (pancreas)	3	33.3	33.3
12	MSH2/MSH6	69	1	0	9	11.1	0
13	MSH6	55	0	0	6	0	0
14	MSH6	56	0	0	4	0	0
15	MLH1/PMS2	72	1	0	7	14.2	0
16	MSH2/MSH6	69	0	0	5	0	0
17	MSH2/MSH6	66	0	1 (stomach)	6	0	16.6
18	MSH2/MSH6	66	0	0	9	0	0
19	MLH1/PMS2	63	1	0	8	12.5	0
20	MLH1/PMS2	46	0	0	11	0	0
21	MLH1/PMS2	71	0	0	4	0	0
22	MLH1/PMS2	69	1	0	12	8.3	0
23	MLH1/PMS2	59	0	0	4	0	0
24	MSH6	79	1	0	8	12.5	0
25	MSH6	54	0	0	9	0	0

Table 2. Characteristics and family history of LLS patients.

§ Number of first-degree relatives diagnosed with CRC at any time until diagnosis of the index case.

+ Number of first-degree relatives diagnosed with LSRC at any time until diagnosis of the index case.

‡ Percentage of family members diagnosed with CRC at any time until diagnosis of the index case.

* Percentage of family members diagnosed with LSRC at any time until diagnosis of the index case.

NA: Not available

Table 3. Standardized incidence ratios between LLS families and LS/sporadic

CRC families.

	LS (n=80)			<u>LLS</u> (n=177)			Sporadic CRC (n=845)	
	No tumors	SIR (95%CI)	P-value §	No tumors	SIR (95%CI)	P-value ‡	No tumors	SIR (95%CI)
CRC	18	6.04	<0.001	14	2.12	<0.001	15	0.48
		(3.58-9.54)			(1.16-3.56)			(0.27-0.79)
	6	2.81	0.09	8	1.69	0.5	27	1.20
LORC		(1.03-6.12)		(0.73-3.34)				(0.79-1.74)
Total	24	4.69	<0.001	22	1.94	<0.001	42	0.78
		(3.00-6.98)			(1.22-2.94)			(0.56-1.05)

§ comparing the SIR of the LS and LLS groups.

‡ comparing the SIR of the LLS and sporadic groups.

Table 4. Differences in the prospective appearance of new cancer cases betweenLLS families and LS/sporadic CRC families during follow-up.

	LS (n=41)	P-value §	LLS (n=89)	P-value ‡	Sporadic CRC (n=403)
CRC (%)	3 (7.3%)	0.16	2 (2.2%)	0.2	3 (0.7%)
No CRC LSRC (%)	4 (9.8%)	0.05	2 (2.2%)	0.02	1 (0.2%)
Total cancers (%)	7 (17.1%)	0.01	4 (4.5%)	0.01	4 (0.9%)

§: comparing percentage of LS and LLS groups.

‡: comparing percentage of LLS and sporadic groups.

Supplementary Table 1. Variants of uncertain significance.

Patient	Sex	Age	MSI-h	MMR	Gene	VUS	Protein	Reported	Mainly
				protein loss		(DNA change)	change	insight (times)	reported as
1	Female	80	No	MLH1/PMS2	MLH1	c.1013 A>G	p.Asn338Ser	14	-? (10/14)
2	Female	80	Yes	MLH1/PMS2	MLH1	c.1959 G>T	p.Leu653Leu	20	? (11/20)
3	Female	72	Yes	MLH1/PMS2	MLH1	c.1331 A>G	p.Asn444Ser	Not reported	
					MLH1	c.2401 G>A	p.Ala681Thr	71	? (43/71)
4	Female	83	Yes	MSH2/MSH6	MSH2	c.1021 C>G	p.Leu341Val	Not reported	
5	Male	66	Yes	MSH2/MSH6	MSH2	c.366 +6 T>C		Not reported	

? variant of uncertain clinical significance

-? Likely not pathogenic

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