CLINICAL AND MOLECULAR CHARACTERIZATION OF LYNCH-LIKE SYNDROME

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Abbreviations used in this paper: LS, Lynch syndrome ; LLS, Lynch-like; CRC, colorectal cancer; MMR, DNA mismatch repair genes; MSI, microsatellite instability; IHC, immunohistochemistry; PCR, polymerase chain reaction.

Competing Interests

The authors have declared that no competing interests exist.

ABSTRACT

BACKGROUND: Lynch syndrome (LS) is characterized by mismatch repair (MMR) deficiency. However, there is a group of patients where LS is suspected because of MMR deficiency but there is no germinal mutation in MMR genes. These patients are known as Lynch-like syndrome (LLS) and there is no consensus about their management. The aim of this study is to describe a large series of LLS patients and to analyze if there are clinical, pathology or molecular differences in patients with suspected hereditary or sporadic origin.

METHODS: Patients with colorectal cancer (CRC) were included in a national registry when their tumors show immunochemical loss of MSH2, MSH6, PMS2 or loss of MLH1 with BRAF-wild type and/or no MLH1 methylation and absence of pathogenic mutation in these genes. Demographic, clinical and pathological variables, as well as family history of neoplasms were registered.

RESULTS: We included 160 patients with LLS. Mean age at diagnosis of CRC was 55 years. A total of 66 patients were female (41%). Amsterdam I and II criteria were fulfilled by 11%, revised Bethesda guidelines by 65% of cases and 24% were diagnosed because of universal screening. There were no differences in sex, indication for colonoscopy, immunochemistry, pathology findings or personal history of CRC or other LS related tumors between patients fulfilling Amsterdam or Bethesda guidelines and patients diagnosed because of universal screening of LS without family history.

CONCLUSION: Patients with LLS show homogeneous clinical, demographic, molecular and pathology characteristics is spite of their suspected hereditary or sporadic origin.

KEYWORDS: familial colorectal cancer; cancer risk; Lynch syndrome; inmunochemistry.

BACKGROUND AND AIMS

Lynch syndrome (SL) is the most frequent cause of hereditary CRC. It is mainly characterized by high risk of developing CRC and endometrial cancer, as well as other neoplasms, namely ovarian, urinary tract, stomach, small intestine, pancreas, biliary tract, skin and brain [1-3]. LS is caused by germline mutations in one of the DNA mismatch repair (MMR) genes, having majority of cases mutations of the *MLH1* or *MSH2* genes, but also in *MSH6, PMS2* and *EPCAM*[4]. These genes are responsible for correcting errors that occur during DNA replication that result in structural anomalies involving unpaired bases. Therefore, the inactivation of these genes increases the rate of mutations during DNA synthesis, with the presence of an increase in these structural anomalies that tend to appear in repetitive DNA sequences. This characteristic is called microsatellite instability (MSI) and is observed in more than 95% of the tumors of patients with CRC or other tumors associated with Lynch syndrome [5]. The presence of MSI suggests a defect in the MMR genes, but its specificity is low because it also occurs in approximately 15% of sporadic CRC, usually due to hypermethylation of the promoter region of the *MLH1* gene in the tumor tissue [6]. On the other hand, immunohistochemistry (IHC) with antibodies against MMR proteins shows if there is loss of expression of these proteins and can be also useful to identify MMR [7]. However, in an increasing number of cases, the presence of microsatellite instability or loss of immunochemical expression of MMR genes is found, but the presence of germline pathogenic mutations in these genes or other cause for MMR proteins inactivation is not evident. These patients are considered to have "probably non sporadic" MMR defective CRC or Lynch-like syndrome (LLS) and represents approximately 30% of all patients

with unstable tumors [8]. A previous study from our group showed that these cases and their first degree relatives show a risk of CRC that is in between of that found in relatives of LS patients and sporadic cases. This result suggest that these LLS patients are probably a heterogeneous group that includes both patients with an unidentified hereditary syndrome and sporadic cases. Testing for somatic mutations in MMR genes has been proposed for differential diagnosis between hereditary and sporadic cases, however, use of this testing is not widely performed and there is no consensus about management of LLS cases and follow-up of patients and their relatives [9, 10].

The aim of this study is to describe the clinical and molecular features of a large nation-wide series of LLS patients as well as to analyze if patients with a suspected hereditary or sporadic origin show any different clinical, pathology or molecular characteristic.

PATIENTS AND METHODS

Data have been extracted from a descriptive, observational, multicenter nation-wide registry (EPICOLON-III) on familial CRC, involving 25 Spanish hospitals. Patients with CRC were included when their tumors show immunochemical loss of MSH2, MSH6, PMS2, or loss of MLH1 with BRAF-wild type and/or no MLH1 methylation and absence of pathogenic mutation in these genes or in *EPCAM*. Immunochemical study of the tumors was performed because of fulfilment of revised Bethesda Guidelines[11] or because of universal molecular screening for LS[12]. These patients were included in the national registry EPICOLON-III and demographic, clinical and pathological variables, as well as family history of neoplasms were registered.

MSI, immunohistochemical staining, and detection of germline mutations.

MSI and/or IHC analysis was performed in all patients. MSI status was analyzed using multiplexed polymerase chain reaction (PCR) patterns at the monomorphic repetitive markers: BAT26, BAT25, NR21, NR24 and NR27[13, 14]. Amplicon detection and analysis were performed using an ABI Prism 3130 Genetic Analyzer, and Genotyper software (Life Technologies, Carlsbad, CA, USA), respectively. A diagnosis of MSI was considered positive when two or more markers showed an altered pattern.

Immunohistochemical analysis of MLH1, MSH2, MSH6, and PMS2 was performed in formalin-fixed, paraffin-embedded tumor tissue as previously described [15].

In patients with a loss of MLH1, methylation of *MLH1* and somatic *BRAF* mutation status was analyzed. *MLH1* methylation analysis was performed using methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) according to the manufacturer's protocol using SALSA MS-MLPA Kit ME011 Mismatch Repair Genes (MRC-Holland, Amsterdam, The Netherlands)[16]. The V600E *BRAF* mutation was detected using specific TaqMan probes in real-time polymerase chain reaction (ABI Prism 7500; Applied Biosystems, Foster City, CA) and allelic discrimination software as described previously[17].

Germline mutation analysis was performed in accordance with the results of IHC analysis as described previously[8]. Patients with loss of MSH2 expression

with no detected mutation were analyzed for *EPCAM* rearrangements using MLPA according to the manufacturer's recommended protocol.

DNA sequencing was performed to characterize the deletion breakpoints[18]. Large rearrangements (deletions and insertions) were tested using MLPA according to the manufacturer's protocol. The results of genetic analysis were interpreted based on the ACMG Recommendations for Standards for Interpretation of Sequence Variations (2000) and the InSIGHT database[19].

Statistical Analysis

The statistical analysis was carried out with the SPSS program (SPSS 19.0, Chicago, IL, USA). Regarding the descriptive analysis, the qualitative variables are presented as percentages. Continuous quantitative variables are described from the mean and the standard deviation or from the median and the interquartile range, depending on whether they follow a normal distribution or not. To analyze the association between qualitative variables, the chi-square test was used, followed by Fisher's exact test and Student's t-test or Mann-Whitney U test for quantitative variables, according to or not a normal distribution. For the contrast of the hypotheses described above, a confidence level (p) <0.05 was used.

RESULTS

We included 160 patients diagnosed with CRC who meet the diagnostic criteria of LLS. The characteristics of patients with LLS are shown in **table 1**. Mean age at diagnosis of CRC was 55 years and 53 of them (36%) were diagnosed under the age of 50. A 41% of them were females. Majority of cases

were diagnosed because of symptoms (81%). The most frequent IHC finding was lack of MLH1/PMS2 expression in 52% of cases, followed by lack of MSH2 expression (27%). Isolated loss of MSH6 (11.5%) or PMS2 (9.5%) was less frequent. Regarding family history, 65% of cases fulfilled revised Bethesda guidelines and 11% fulfilled Amsterdam criteria for LS diagnosis. A 50% of patients reported any family history of CRC and 39% family history of other LS-related cancer. On the other hand, in a 24% of cases IHC of MMR proteins was performed in the context of universal screening of LS. Five patients (3%) developed a second CRC with a median length time of 7 years (SD 3.9 years), a 17% had a previous history of CRC and 3% a previous history of other LS-related neoplasms.

With the aim of identifying if there are any difference between patients suspected to have LLS with suspected hereditary origin and those with probable sporadic origin, an analysis was performed comparing cases with LLS who met the Amsterdam or Bethesda criteria, with those who did not meet these criteria and in whom the diagnosis was made due to the realization of universal screening for the diagnosis of LS. In this case, the only differences we found were related to the definition of cases, with a mean age at diagnosis of CRC of 65.5 (SD 10.1) in the group of patients diagnosed by universal screening versus 51.6 (SD 13.7) in the group who fullfilled Amsterdam or Bethesda criteria (p 0.02). We also observed significant differences related to a higher percentage of patients with a family history of CRC in the group that met criteria of Amsterdam or Bethesda criteria, that was of 57% versus 28% (p 0.00) in the group of universal screening. However, we did not find any statistical differences between both groups related to other variables like sex, indication

for colonoscopy, immunohistochemical findings, characteristics related to the tumor (location, size, TNM stage, pathology), personal history CRC or other LS associated cancer, either family history of non-colorectal cancer associated with LS (**Table 2**).

On the other hand, a second analysis was carried out, comparing the characteristics of the patients based on the age at diagnosis of CRC (before the age of 50 years) and/or the presence of a family history of tumors associated with LS versus those patients with diagnosis of CRC with age equal to or higher than 50 years and absence of family history of tumors associated with LS. In this case, we also did not observe any significant difference regarding to sex, vital status, indication for colonoscopy, immunohistochemistry, characteristics related to the tumor (location, size, TNM stage, histology) or personal history CCR or other LS associated cancer between both groups (**Table 3**).

DISCUSSION

In this study, that includes the largest published cohort of patients with Lynch-like syndrome (LLS), we describe clinical, and molecular characteristics of these patients and we found that cases with suspected hereditary origin due to family history or young age onset are similar to cases with suspected sporadic origin in terms of clinical, molecular or pathological characteristics. These results support that, in the absence of a molecular marker able to differentiate both groups, these patients should be managed homogeneously.

The implementation of universal LS screening has led to an increase in the percentage of tumors that exhibit microsatellite instability or loss of expression of the MMR proteins, but in which no germline pathogenic mutation

or any other cause of MMR deficiency is found[8]. This situation, called LLS or MMR tumors of unknown origin is associated with uncertainties for the preventive management of patients and their relatives, because there is no consensus about considering that as a probably hereditary or sporadic condition. There are different mechanisms that could cause this phenotype **(Figure 1**). The first possible cause is the presence of atypical germline alterations in MMR genes (regulatory regions, inversions or traslocations), that could provoke somatic alteration of the remaining allele in MMR genes. So this group of patients are actually, unidentified patients with Lynch syndrome. Other possible cause is the presence of germline alterations in another genes (eg. MUTYH, POLD1, POLE) that could also alter the MMR system, being these patients genetically real Lynch-like syndrome. Finally, we can also observe sporadic tumors with MMR biallelic alterations secondary to somatic alterations in cancer genes (tumor suppressor genes, oncogenes, repair genes), somatic biallelic alterations in MMR genes or a combination of both findings.

Under a clinical point of view, patients with LLS probably represent at least two different subsets. The first group includes cases in which the clinical characteristics strongly suggest a hereditary origin, but in which the genetic defect has not yet been identified through the protocols that are routinely performed. These patients probably have an undiagnosed hereditary condition with high risk of CRC for them and their first-degree relatives. The second subset includes a significant proportion of families with LLS who do not have a history of cancer and the only element to suspect LS is the presence of MSI or the loss of expression of some of the MMR proteins. In this latter group, probably a double somatic mutation in MMR genes is the underlying cause of

the MSI phenotype. This second group of patients have sporadic tumors and specific preventive measures are not necessary for them and their relatives.

It has been proposed that current LS diagnostic strategy should be complemented with algorithms that integrate other molecular data of the tumors that allow the differential diagnosis between sporadic and hereditary origin in LLS cases[20]. In that sense, different authors have proposed the investigation of somatic mutations in mismatch repair genes and other genes that could explain sporadic CRC cases with LLS. In a previous study carried out by Sourrouille et al,[21] they included 18 patients with MSI CRCs and loss of expression of a MMR protein with absence of germline pathogenic mutation. They analyzed tumors by sequencing and large rearrangement analysis and also looked for mosaicism. Finally, they found 4 patients (22%) with double somatic mutations. In other study, Messenkamp et al [22], analyzed 25 CRCs or endometrial carcinomas with MMR-deficient tumors and absence of germline pathogenic mutations in these genes, and without somatic MLH1 promoter methylation. They were screened for somatic mutations and loss of heterozygosity in MLH1 and MSH2. In this study, they were able to identify that more than a half (52%) had two bi-allelic somatic events in MLH1 or MSH2. These authors proposed that the higher percentage of detection of somatic bi-allelic mutation in cases with previously negative tested for germline MMR gene mutations was secondary to the addition of the analysis for loss of heterozygosity (LOH). In their study they showed that LOH of MLH1 represented a high proportion of somatic events (8 of 15 tumors). On the other hand, Haraldsdottir et al analyzed blood and tumor samples, using somatic multigene panel testing in a group of 32 patients with colorectal or endometrial cancer with MMR deficiency but no germline

mutations in MMR genes. They found that twenty-two out of 32 patients (69%) were found to have two somatic mutations in MMR genes and all had an hypermutated phenotype[23].

Recent studies, using somatic mutations for classifying LLS patients in hereditary and sporadic also did not find clinical or pathological characteristics able to differentiate between both populations. In a recently published study by Hemminger et al [24], the presence of double somatic mutation in the MMR was observed in 69% of patients with unexplained MMR deficiency with lack MLH1 methylation and germline mutation. They analyzed whether histomorphology could distinguish patients with double somatic mutations from those with LS, but no significant differences in histologic features were found between tumors in LS patients and tumors with double somatic mutations. This similar tumor histology could be secondary to a similar underlying oncogenesis involving defective MMR function, leading to a hypermutated phenotype. Also, in a previous study, Mas-Moya et al[25], compared clinicopathological differences in colorectal carcinomas between patients with LS and a group of 21 patients with LLS. Curiously, they found a higher percentage of CRC in the right colon in the group of LLS than in the LS (93% versus 45%; $P < .002$) but there were no significant differences related to tumor stage, tumor grade, size, tumor infiltrating lymphocytes, Crohn-like lymphocytic reaction, mucinous differentiation, signet ring cell differentiation, or medullary differentiation (24).

However, the use of multigene panel testing or other tools for performing this diagnosis has not been yet routinely implemented in the majority of centers due to discrepancies about the appropriate somatic gene analysis. Moreover, the high cost of this approach, the need of next-generation sequencing technology and the difficulties for applying this technology in

paraffin samples are barriers for the implementation of this diagnostic tool for the adequate classification of LLS patients as sporadic or probably hereditary cases. Moreover, the addition of somatic mutations to the diagnostic algorithm of Lynch syndrome has not yet been validated out of research studies. Finally, it has not yet been fully ruled out that these somatic mutations would be related to germline inactivation of still unknown genes related to MMR deficiency (**figure1**) and only a germline exome approach or a clinical follow-up validation could finally confirm the sporadic behavior of these LLS tumors with somatic mutations. For these reasons, the majority of cases of LLS remains unclassified and patients and their relatives are heterogeneously followed-up. If we consider LLS patients as a group, risk of CRC in patients and their first-degree relatives is in between of that found in LS syndrome and sporadic CRC, with incidence of CRC in families of patients with LLS significantly lower than that found in families with confirmed LS but higher than in families with sporadic CRC [10], and because of that, some preventive measures should be guaranteed in this population.

In summary, we found that there are no clinical, molecular or pathological features differentiating tumors with a suspected hereditary or sporadic origin. These data support that, if we do not have any molecular or genetic tool that help us in the classification of this group of patients, we should consider them as a homogeneous group, applying preventive measures with periodic colonoscopies for patients and their relatives, even considering to apply the same follow-up that is recommended in patients diagnosed with Lynch syndrome and their relatives. Moreover, validation studies aimed to know if family history or age of CRC onset could be of help for identifying cases needing more or less intensive surveillance protocol should be granted. Our findings also support the need of increasing the study of the pathogenesis of CRC in these patients as well as the appropriate way for identifying them as really hereditary or sporadic cases.

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Lynch-like Syndrome	**n=160**
Female sex, n (%)	66 (41.2%)
Mean age (SD)	64 (14.4)
Mean age at CRC diagnosis (SD)	55 (14.2)
Indication for colonoscopy, n (%)	
Symptoms	118 (87.4%)
CRC screening	17 (12.6%)
Immunohistochemistry, n (%)	
Loss of MLH1 and PMS2	77 (50%)
Loss of MSH2 and MSH6	43 (27.9%)
Isolated loss of MSH6	20 (12.9%)
Isolated loss of PMS2	14 (9.1%)
Reason for IHC, n (%)	
Amsterdam I and II criteria	18 (11.2%)
Revised Bethesda guidelines	103 (64.3%)
Universal screening	39 (24.3%)

Location, n (%) Right colon Left colon and rectum	 89 (61.3%) 56 (38.6%)
TNM, n (%) Stage I and II	 81 (60.4%)
Size of the tumor, Median cm (range)	5 (0.6-30)
Histology, n (%) Poor differentiation Lymphocytic infiltration Mucinous tumor Vascular invasion	 33 (24.8%) 37 (25.8%) 46 (31.9%) 18 (12.5%)
Metachronous CRC, n (%)	5 (3.1%)
Personal history of non-CRC tumors, n (%)	27 (16.8%)
Personal history of non-CRC LS associated tumors, n (%)	5 (3.1%)
Family history of CRC, n (%)	80 (50%)
Family history of non-CRC LS associated tumors, n (%)	62 (38.7%)

Table 1: characteristics of patients with LLS.

SD: standard deviation; CRC: colorectal cancer; LS: Lynch syndrome

	Amsterdam or Bethesda n=121 (76%)	**Universal screening n=39 (24%)**

Age at CRC diagnosis, median (SD)	51.62 (13.7)	65.54*(10.1)
Female sex, n (%)	47 (38.8%)	19 (48.7%)
Indication for colonoscopy, n (%)		
Symptomatic	89 (87.2%)	29 (87.8%)
CRC screening	13 (12.7%)	4 (12.1%)
Immunohistochemistry, n (%)		
MLH1 and PMS2	57 (49.1%)	20 (52.6%)
MSH2 and MSH6	30 (25.8%)	13 (34.2%)
MSH6	16 (13.8%)	4 (10.5%)
PMS2	13 (11.2%)	1 (2.6%)
Location, n (%)		
Right colon	66 (60.5%)	23 (63.8%)
Rectum and left colon	43 (39.4%)	13 (36.1%)
TNM, n (%)		
Stage I-II	62 (59.6%)	19 (63.3%)
Stage III-IV	42 (40.3%)	11 (36.6%)
Size of CRC Median cm (range)	5.88 (4.9-6.8)	4.5 (3.7-5.2)
Histology, n (%)		
Poor differentiation	25 (27.1%)	8 (30.7%)
Lymphocytic infiltration	30 (40.5%)	7 (29.1%)
Mucinous	36 (46.7%)	10 (37%)
Vascular infiltration	17 (23.6%)	1 (4%)

Personal history, **n (%)**		
CRC or other LS associated cancer	11 (9.1%)	1 (2.5%)
metachronous CRC	4 (3.3%)	1 (2.5%)
synchronous CRC	2 (1.6%)	0 (0%)
non-CRC LS tumor	5 (4.1%)	0 (0%)
Family history of CRC, n (%)	69 (57%)	11 (28.2%)*
Family history of non-CRC LS associated tumor, n (%)	51 (42.1%)	11 (28.2%)

Table 2: characteristics of patients based on the presence of a family history of neoplasms associated or not with LS.

* $p < 0.05$

	CRC diagnosed under 50 years and/or family history of LS-related cancer n=128 (80%)	**CRC diagnosed ≥ 50 years and no family history of LS-related cancer n=32 (20%)**
Age at CRC diagnosis, median (SD)	52.05 (14)	65.71* (9)
Female sex, n (%)	52 (40.6%)	14 (43.7%)
Indication for colonoscopy, n (%)		
Symptomatic	100 (92.5%)	18 (66.6%)
CRC screening	8(7.4%)	9 (33.3%)
Immunohistochemistry, n (%)		
MLH1 and PMS2	58 (47.5%)	19 (59.3%)
MSH2 and MSH6	34 (27.8%)	9 (28.1%)

MSH6	18 (14.7%)	2 (6.2%)
PMS2	12 (9.8%)	2 (6.2%)
Location, n (%)		
Right colon	71 (61.7%)	18 (60%)
Rectum and left colon	44 (38.2%)	12 (40%)
TNM, n (%)		
Stage I-II	63 (58.8%)	18 (66.6%)
Stage III-IV	44 (41.1%)	9 (33.3%)
Size of CRC Median cm (range)	5.97 (5-6.9)	3.98 (3.1-4.8)
Histology, n (%)		
Poor differentiation	27 (28.1%)	6 (27.2%)
Lymphocytic infiltration	27 (35%)	10 (47.6%)
Mucinous	33 (41.2%)	13 (54.1%)
Vascular infiltration	15 (19.7%)	3 (14.2%)
Personal history, n (%)		
CRC or other LS associated cancer	10 (7.8%)	2 (6.2%)
metachronous CRC	4 (3.1%)	1 (3.1%)
synchronous CRC	1 (0.7%)	1 (3.1%)
non-CRC LS tumor	5 (3.9%)	0 (0%)

Table 3: characteristics of patients based on the age of diagnosis of CRC and family history of neoplasms associated with SL.

* $p < 0.05$

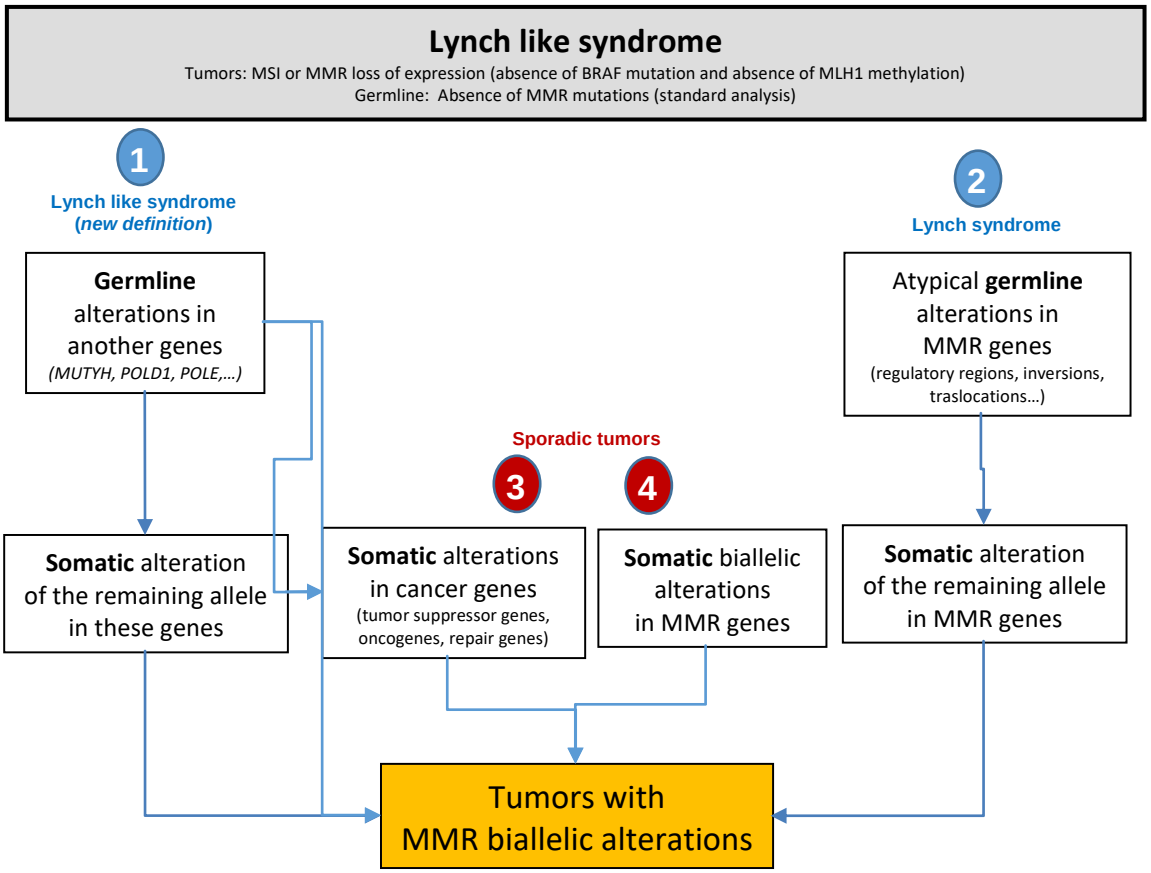

Figure 1. Potential mechanisms for Lynch-like syndrome