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Original Article

Molecular screening and clinicopathologic characteristics of Lynch-like syndrome in a Chinese colorectal cancer cohort

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Abstract: Colorectal cancers (CRC) with microsatellite instability (MSI) or mismatch repair-deficiency (dMMR), but without detectable MMR germline mutations are termed Lynch-like syndrome (LLS). We assess the clinicopathologic and molecular characteristics of LLS tumors and the proportion in LLS, which remain poorly investigated in China. We enrolled 404 CRC patients with surgery in our institution from 2014 to 2018. LLS tumors were detected by a molecular stratification based on MMR protein expression, MLH1 methylation and MMR gene mutation. LLS tumors were profiled for germline mutations in 425 cancer-relevant genes. Among 42 MMR-deficient tumors, 7 (16.7%) were attributable to MLH1 methylation and 7 (16.7%) to germline mutations, leaving 28 LLS cases (66.6%). LLS tumors were diagnosed at a mean age of 60.7 years, had an almost equivalent ratio among rectum, left colon and right colon, and had high rates of lymph node metastases (50%, 4/28 N2). Most MMR gene mutations (88.2%, 15/17) in LLS tumors were variants of unknown significance (VUS). Two novel frameshift mutations were detected in ATM and ARID1A, which are emerging as candidate responsible genes for LLS. In this study, 28 (66.6%) MMRd tumors were classified as LLS, which were significantly higher than reports of western countries. LLS tumors were more likely to carry lymph node metastases. However, it's hard to differentiated LLS tumors from LS through family history, tumor location, histological type of tumors, immunohistochemistry (IHC) for MMR proteins and MSI analysis.

Keywords: Colorectal cancer, Lynch-like syndrome (LLS), microsatellite instability, molecular screening, Chinese

Introduction

About 15% of colorectal cancers (CRCs) with more than 700 subtle mutations are considered as the hypermutated tumors [1]. While 75% of these hypermutated tumors are mainly caused by defects of DNA mismatch repair (MMR) system. These defects result in errors in repetitive DNA nucleotide sequences and mutations in the coding and non-coding microsatellites, which are termed microsatellite instability (MSI) [2]. Recent reports showing that MSI is a predictive biomarker for immunotherapy, which has increased the clinical gene test on CRCs with mismatch repair deficiency [3]. Identification and screening of MSI are of great significance, as patients can benefit from life-saving and cancer-related intensive surveillance [4].

Lynch syndrome (LS) is the most common CRC with MSI, accounting for approximately 1-4% of all cases [5]. *MSH2* and *MLH1* mutations account for most of the LS-associated CRCs, but *PMS2* and *MSH6* mutations are actually more prevalent on a population basis. China has the largest estimated number of LS carriers than any other country. The incidence of LS-related extraintestinal tumors has a clear upward trend [6]. Current clinical screening of LS is mainly based on the Amsterdam Standard (I/II). However, the family scale has decreased due to the family planning policy in China. This makes it a higher false negative rate when only

applied family history screening [5, 7]. Therefore, clinical diagnosis needs to combine molecular detection methods to improve the accuracy for LS screening. At present, most molecular tests of LS in China are lack of uniform standards and comprehensive molecular detection methods [8].

The rest MSI-CRCs traditionally were thought to be sporadic cancer through hypermethylation of its promoter region [9]. However, approximately 30% of CRCs with MSI carrying neither MLH1 methylation nor germline mutation of MMR gene are termed Lynch-like syndrome (LLS) [10]. By far, there is no consensus on management of patients with LLS because of unknown mechanism for the formation of MSI and unconfirmed suspicions of hereditary cancers [11]. Besides, there haven't been any related reports about LLS in China till now. It's significant to differentiate them from LS, assess their family risk and determine the appropriate surveillance approach. Hence, it is extremely urgent to strengthen the collection of clinical and genetic information of LLS patients in China.

In this study, we analyzed a large and well-annotated cohort of CRC patients in a tertiary referral center of China to assess the proportion and related clinicopathologic characteristics of LLS through the universal molecular testing strategies.

Materials and methods

Study design and patients

This retrospective study was conducted in Affiliated Hospital of Nanjing University of Chinese Medicine. Patients diagnosed with CRC were identified from the database of the Department of Pathology between October 2014 and October 2018. The overall analytical flow chart is shown in **Figure 1**. The ethics committee of the Affiliated Hospital of Nanjing University of Chinese Medicine approved this study (2014-NL-093-02). Informed consents were obtained from all patients.

Clinical characteristics and pathology review

Clinical information, such as sex, age, family history and tumor location, were collected from medical records. Patients and their first-degree or second-degree relatives diagnosed with CRC or LS-associated extraintestinal cancers were considered to have a family history [8]. Pathologic information was documented from pathology reports and assessed according to the AJCC classification atlas-2^{ed} edition [12], including the size and morphology of tumor, histological type and differentiation of tumor, stage at diagnosis (TNM), lymph nodes, vascular tumor emboli, nerve invasion and tumor deposit (TD).

Immunohistochemistry (IHC) for MMR proteins

All samples used for molecular studies were formalin-fixed paraffin embedded (FFPE) specimens. IHC analysis of 4 MMR proteins including MLH1 (ES05-IR079; Dako), MSH2 (FE11-IR085; Dako), MSH6 (EP49-IR086; Dako), and PMS2 (EP51-IR087; Dako) was performed according to the previous protocol [7]. Samples with deficient expression of one or more of the MMR proteins compared to positive controls were considered MMR deficient (dMMR), whereas tumors exhibiting normal positive staining for all four genes were MMR proficient (pMMR) [13].

MSI and BRAF V600E mutation analysis

MSI was detected on tumor-normal DNA samples using five microsatellite markers (BAT26, BAT25, D2S123, D5S346 and D17S250) [7]. Genomic DNA was extracted from paraffin-embedded tissue by Ex-DNA FFPE Genome (Cat No. T165; TIANLONG, Xian, China) following the manufacturer's instructions. Fragments were analyzed on the Hitachi 3500 Genetic Analyzer (Applied Biosystems, Tokyo, Japan). Tumors with two or more mutated markers were identified with MSI-H. Tumors with no instable makers was called MSS.

BRAF V600E analysis was used fluorescent polymerase chain reaction (PCR). Amplicons were detected on an Agilent Mx3000P (Applied Biosystems, Paloalto, CA, USA).

Targeted panel sequencing (TPS) and data analysis

Tumors with dMMR and/or MSI were considered as MMRd-CRCs. Samples with MMRd were screened for germline variants in TPS. Sequencing was conducted in Shihe Gene

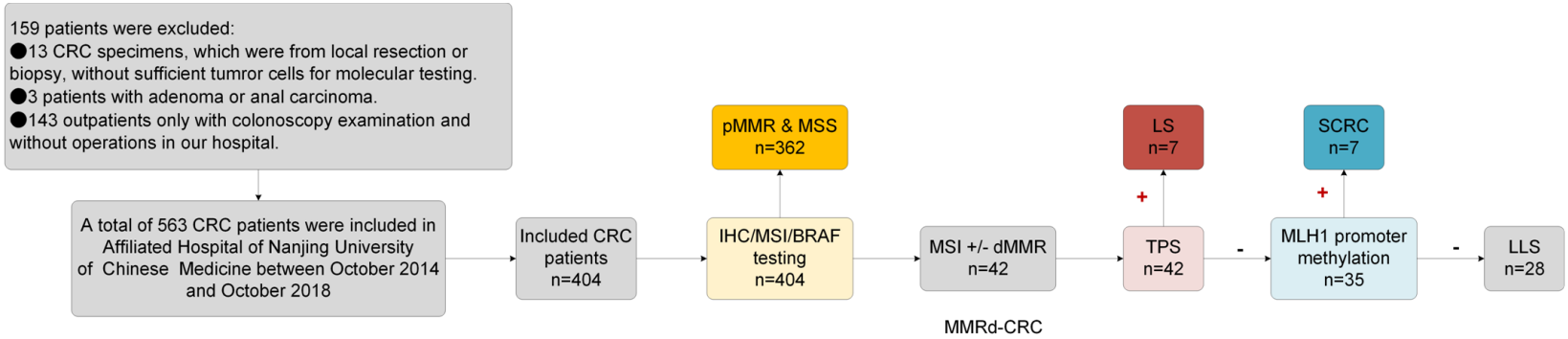

Figure 1. Flow chart of analytical strategy. A flow chart to stratify 404 colorectal carcinomas first into pMMR & MMR (SCRC) and MSI +/- dMMR groups and the latter further into SCRC, LS, and LLS subgroups.

Biotechnology Ltd (Nanjing, China). Hybridization-based target enrichment was carried out with 425 cancer-related genes (GeneseeqOne-TM pan-cancer gene panel), including 7 MMR genes (MLH1, MLH3, MSH2, MSH6, PMS1, PMS2, EPCAM) and 418 non-MMR genes (see Table S1). FFPE samples were de-paraffinized with xylene followed by genomic DNA extraction using QIAamp DNA FFPE Tissue Kit (Qiagen) following the manufacturer's instruction. In brief, 1 μg of fragmented genomic DNA underwent end-repairing, A-tailing and ligation with indexed adapters sequentially, followed by size selection using Agencourt AMPure XP beads (Beckman Coulter). Libraries were prepared using the KAPA Hyper Prep kit (KAPA Biosystems). Captured libraries by Dynabeads M-270 (Life Technologies) were amplified in KAPA HiFi HotStart ReadyMix (KAPA Biosystems) and quantified by qPCR using the KAPA Library Quantification 160 kit (KAPA Biosystems) for sequencing. Paired-end sequencing was on Illumina HiSeq4000 NGS platforms (Illumina) according to the manufacturer's instructions. The mean coverage depth was >100× for tumor tissues. Trimmomatic [14] was used for FASTQ file quality control (below 15 or N bases were removed). Reads were then mapped to the reference genes using Burrows-Wheeler Aligner (BWA-mem, v0.7.12) (https://github.com/lh3/bwa/tree/master/bwakit). Local realignment around the indels and base quality score recalibration was applied with the Genome Analysis Toolkit (GATK 3.4.0) (https://software.broadinstitute.org/gatk/), which was also applied to detect germline mutations. Detected variants were subsequently annotated using ANNOVAR [15]. Interpretation of sequence variants was according to ACMG guidelines [16]. Mutations in genes with class5 & 4 were considered as pathogenic variants.

MLH1 promoter methylation analysis

MMRd tumors without germline mutations were accepted bisulfite-specific PCR (BSP) [17] to rule out *MLH1* promoter methylation at the Cyagen commercial laboratory. Samples with cutoff percentage of 10% were considered to be methylated [17]. Genomic DNA was extracted and bisulfite treatment was followed by EpiTect Fast FFPE Bisulfite Kit (Cat No./ID: 59844; QIAGEN). BSP was carried out with validated PCR primers specific to MLH1 promoter sequences: ME022-hMLH1-F2: 5'-TTTTTTTTAGGAGTGAAGGAGGTTA-3'; ME022-hMLH1-R1: 5'-TTAACCCTACTCTTATAACCTCCC-3'. Forty-cycle PCR was performed as follows: denaturation 94°, 30 s; annealing 55°, 30 s; extension 40 s, 5 min. The PCR products were analyzed using a 1.5% agarose gel.

Statistical analysis

Data are analyzed by IBM SPSS statistics software 23.0 (Chicago, IL, USA). Fisher exact test, Chi-Square test and Bonferroni test are used to analyze the categorical variables for comparison of clinicopathological characteristics among LLS, LS and SCRC subgroups, while continuous variables among different groups are compared using Kruskal-Wallis test or Median test. All *p*-values less than 0.05 are considered to be statistically significant.

Results

A total of 563 patients with CRC were identified. One hundred and fifty-nine patients were excluded: 1) 143 outpatients only received colonoscopy examination without surgical operations in our center; 2) 13 patients received local resection without enough tissue for molecular testing; 3) the postoperative pathology of 3 patients manifested to be adenoma or anal carcinoma. Finally, 404 patients were included (**Figure 1**).

Molecular stratification of colorectal carcinomas

After molecular testing of IHC and MSI, 362 (89.6%) tumors with MSS & pMMR were classified as sporadic colorectal cancer (SCRC). Thirty-five tumors showed abnormal expression of MMR proteins by IHC: 9 (25.7%) failed to express MLH1 and PMS2; 3 (8.6%) showed isolated loss of MLH1; 13 (37.1%) evidenced isolated loss of PMS2; 1 (2.9%) carried the loss of MSH2 and MSH6; 1 (2.9%) evidenced isolated loss of MSH2; 5 (14.3%) showed isolated loss of MSH6; 2 (5.7%) showed loss of MSH2 and MLH1. Specially, 1 tumor (2.9%) was observed with loss of MLH1, PMS2 and MSH6. Among 35 dMMR tumors, 21 tumors were identified with MSI-H and 14 tumors with MSS. Besides, 7 MSI-H CRCs showed normal expression of MMR proteins (**Figure 2**). Totally, 42 tumors were classified as MMRd-CRCs.

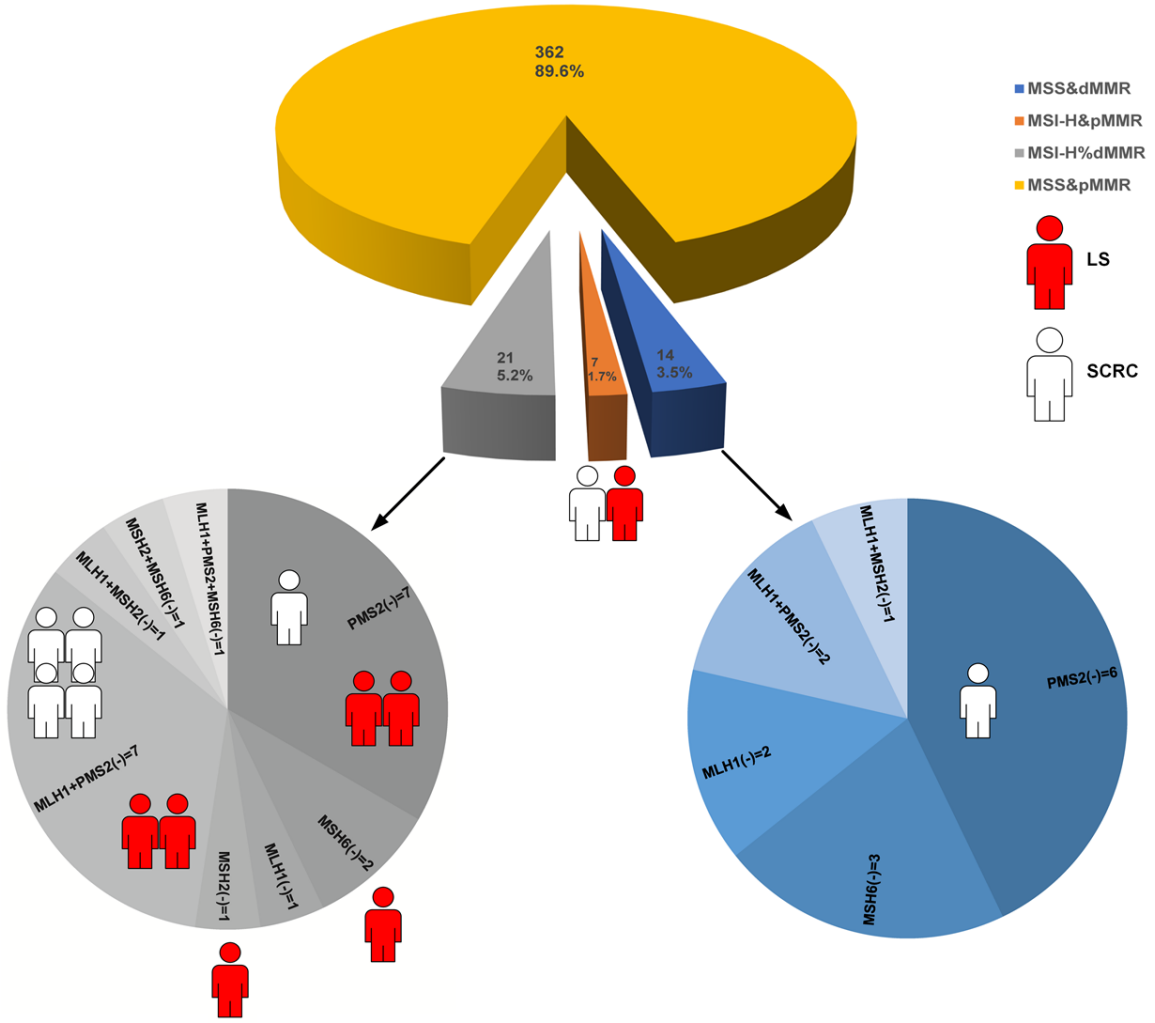

Figure 2. Results of universal molecular screening in 42 MMRd-tumors. Three hundred and sixty-two (89.6%) tumors were identified with MSS & pMMR; 14 (3.5%) tumors were classified as MSS & dMMR, 1 tumor with loss of MLH1 protein and MLH1 promoter methylation was diagnosed as SCRC; 7 (1.7%) tumors were diagnosed as MSI-H & pMMR, 1 tumor was identified with SCRC and 1 tumor were identified with MSH6 germline mutation; 21 (5.2%) tumors were diagnosed as MSI-H & dMMR, 5 tumors were diagnosed as SCRC, 6 tumors were identified with germline mutations (LS), including 2 MLH1 genes, 2 PMS2 genes, 1 MSH6 gene and 1 EPCAM gene respectively.

All MMRd tumors were checked by the TPS, resulting in the diagnosis of LS in seven (7/42, 16.7%). Seven (7/42, 16.7%) tumors with MLH1 methylation were also classified as SCRC, including 4 tumors with MLH1-deficiency, 1 MMR-proficient tumor with BRAF V600E mutation and 2 PMS2-deficient tumors with wild BRAF V600E. The remaining 28 (66.7%) MMRd-CRCs without MMR/EPCAM germline mutations and MLH1 promoter methylation were classified as LLS (**Figures 1** and **2**).

Clinicopathological characteristics of LLS tumors

There were some clinicopathological characteristics which were significantly different among patients with LLS, LS and SCRCs (**Table 1**). LLS were diagnosed at the mean age of 60.7 (SD 11.6) years vs 44.7 (SD 11.1) years in the LS (P=0.035), and 62.3 (SD 11.3) years in the SCRCs (P=0.246). The cumulative percentage of patients against their age of onset of CRC in LLS, LS and SCRC was summarized in **Figure 3**. Obviously, the diagnostic age of LLS patients was older than that of LS patients, but younger than SCRC patients. Two patients in LS (2/7, 28.6%) were found to have family history. While LLS patients with family history were six (6/28, 21.4%), which were significantly more than SCRC patients (30/362, 8.1%) (P=0.013). LLS tumors had an almost equivalent ratio among rectum, left colon and right colon, whereas LS tumors occurred in left colon (4/7, 57.1%) and

Table 1. Clinicopathological characteristics of LLS, LS and SCRC subgroups

Characteristics	LLS (n=28, 6.9%)	LS (n=7, 1.8%)	SCRC (n=369, 91.3%)	P¶
	No. of cases (%)	No. of cases (%)	No. of cases (%)	
Sex				0.328
Male	15 (53.6)	3 (42.9)	234 (63.4)	
Female	13 (46.4)	4 (57.1)	135 (36.6)	
Age at diagnosis, y				0.002§
<50	3 (10.7)[a]	5 (71.4)[b]	48 (13)[a]	
50-60	11 (39.3)[a]	1 (14.3)[b]	93 (25.2)[a]	
≥60	14 (50)[a]	1 (14.3)[b]	228 (61.8)[a]	
Mean (SD)	60.7 (11.6)	44.7 (11.1)	62.3 (11.3)	0.246♯
Median (range)	59 (52, 68.5)	46 (36, 51)	63 (24, 91)	0.158£
Family history				0.013§
yes	6 (21.4)[a]	2 (28.6)[a,b]	30 (8.1)[b]	
no	22 (78.6)[a]	5 (71.4)[a,b]	339 (91.9)[b]	
Tumor location				<0.001§
Rectum	11 (39.3)[a]	0 (0)[a]	256 (69.4)[b]	
Left colon	7 (25)[a]	4 (57.1)[a]	81 (22)[b]	
Right colon	10 (35.7)[a]	3 (42.9)[a]	32 (8.6)[b]	
Tumor size				0.322
<5 cm	18 (64.3)	4 (57.1)	274 (74.3)	
≥5 cm	10 (35.7)	3 (42.9)	95 (25.7)	
Tumor morphology				0.163
Protrude type	13 (46.4)	4 (57.1)	104 (28.2)	
Ulcerative type	14 (50)	3 (42.9)	240 (65)	
Infiltrative type	1 (3.6)	0 (0)	25 (6.8)	
Histological differentiation				0.452
Low	13 (46.4)	3 (42.9)	107 (29)	
Moderate	15 (53.6)	4 (57.1)	252 (68.3)	
High	0 (0)	0 (0)	6 (1.6)	
Missing	0 (0)	0 (0)	4 (1.1)	
Histological type				0.002§
Adenocarcinoma	18 (64.3)[a]	3 (42.9)[a,b]	314 (85.1)[b]	
Mucinous carcinoma/Signet ring cell carcinoma	10 (35.7)[a]	4 (57.1)[a,b]	54 (14.6)[b]	
Neruoendocrine neoplasm	0 (0)[a]	0 (0)[a,b]	1 (0.3)[b]	
Stage at diagnosis (TNM)				0.585
I+II	14 (50)	5 (71.4)	206 (55.8)	
III+IV	14 (50)	2 (28.6)	163 (44.2)	
Serosa infiltration				0.513
Present	2 (7.1)	0 (0)	15 (4.1)	
Absent	26 (92.9)	7 (100)	354 (95.9)	
Lymph nodes				<0.001§
N0	14 (50)[a]	5 (71.4)[b]	224 (60.7)[b]	
N1	0 (0)[a]	2 (28.6)[b]	77 (20.9)[b]	
N2	14 (50)[a]	0 (0)[b]	68 (18.4)[b]	
Vascular tumor emboli				0.282
Present	8 (28.6)	0 (0)	92 (24.9)	
Absent	20 (71.4)	7 (100)	277 (75.1)	
Nerve invasion				0.218
Present	3 (10.7)	0 (0)	72 (19.5)	
Absent	25 (89.3)	7 (100)	297 (80.5)	
Tumor deposit (TD)				0.312

Present	3 (10.7)	0 (0)	65 (17.6)
Absent	25 (89.3)	7 (100)	304 (82.4)

[¶]Comparison among groups is by Fisher's exact test or Chi-square test. [§]Comparison between two groups respectively among variables with significantly different *P*-vales is by Fisher's exact test or Chi-Square Test and adjusted by Bonferroni test. Statistically significant differences between two groups are identified if the superscript letters are a and b different, vice versa. [ƒ] Comparison among groups is by Kruskal-Wallis test. [£] Comparison among groups is by Median test. Abbreviations: LS, Lynch syndrome; LLS, Lynch-like syndrome; SCRC, sporadic colorectal cancer.

right colon (3/7, 42.9%), and SCRC tumors occurred mainly in the rectum (256/369, 69.4%) (P<0.001). Mucinous carcinoma or signet ring cell carcinoma was more often in LS tumors (4/7, 57.1%), compared with 35.7% in LLS (10/28) and 14.6% in SCRC (54/369) (P=0.002). 50% of patients with LLS had lymph node metastases (14/28 N2), which were significantly higher than LS and SCRC (28.6%, 2/7 N1 and 39.3%, 145/369 N^+) (P<0.001).

Variants of unknown significance (VUS) of MMR genes in LLS tumors

A total of 15 VUS in MMR genes were seen in 10 LLS tumors. The most common MMR genes in which VUS were identified was MSH2 (n=5), followed by MSH6 (n=3), MLH1 (n=3), MLH3 (n=3), PMS2 (n=1). Specially, a fusion of *PMS2* with *USP42* on chromosome 7 (6160707) had been identified (USP42: exon3~PMS2: exon10) (**Table 2**).

Pathogenic mutations of non-MMR genes in LLS tumors

Nine (32.1%) of 28 LLS patients were found to have 13 pathogenic mutations in non-MMR genes (**Figure 4**). Two had mutations in TP53 (Hereditary cancer-predisposing syndrome), 6 in APC (familial adenomatous polyposis), 1 in ATM (DNA damage signaling regulator), 1 in ARID1A (chromatin remodeling), 1 in NSD1 (Sotos syndrome 1), 1 in PTEN (Hereditary cancer-predisposing syndrome) and 1 in TSC2 (Tuberous sclerosis syndrome). Frameshift duplication of c.8432dupA in ATM and frameshift deletion c.19_44delCCCGCCGCCGCCAGCAGCCTGGGCAA in ARID1A were 2 novel mutations, which are emerging as candidate responsible genes for LLS. The exonuclease domain indicating the location of ATM and ARID1A variants was showed in **Figure 5**. The clinicopathological characteristics of tumor in patient (No. 13 **Table 2**) with ATM mutation showed low differentiation, lymph nodes invasion and MSH6 protein deficiency, while that of patient (No. 14 **Table 2**) with ARID1A mutation was moderate differentiation, no lymph nodes metastasis and loss of MLH1/MSH2 proteins. Both tumors were carried with MSS and wild BRAF phenotype.

Discussion

To best of our knowledge, although with a relatively small sample size, this is the first study in a cohort of Chinese LLS patients with TP sequencing. In this study, we determine the frequency of LS, LLS and SCRCs among 404 CRCs in a tertiary-referral center of China during 2014-2018. Forty-two patients (10.4%, 42/404) were diagnosed with MMRd. Seven patients (7/42, 16.7%) were diagnosed with LS, which was also consistent with the previous studies in China, but lower than western countries [18, 19]. Interestingly, we found 66.7% (28/42) CRC patients with MMRd were diagnosed with LLS, and this incidence was significantly higher than the literature reports. In some population-based studies of western countries, the prevalence of LLS ranged from 13% to 56% [20, 21]. This demonstrates that the proportion of LLS in Chinese people may be rather higher than western countries.

So far, the preventive management of LLS patients and their relatives are uncertain. There hasn't been any consensus about whether LLS should be considered as a likely hereditary disease or a sporadic condition. Besides, there were no hereditary clinical origin, molecular markers or germline mutations, which were able to differentiate LLS patients from other patients with MSI or loss of expression of MMR proteins. Buchanan pointed that LS had the highest risk of CRC; LLS patients together with their first-degree relatives were considered to have an intermediate risk of developing CRC compared with sporadic MMR deficient CRC [22]. In this study, we found that patients with family history in LLS, LS or SCRCs were consistent with previous studies (21.4%, 28.6% and 8.1%, P=0.013).

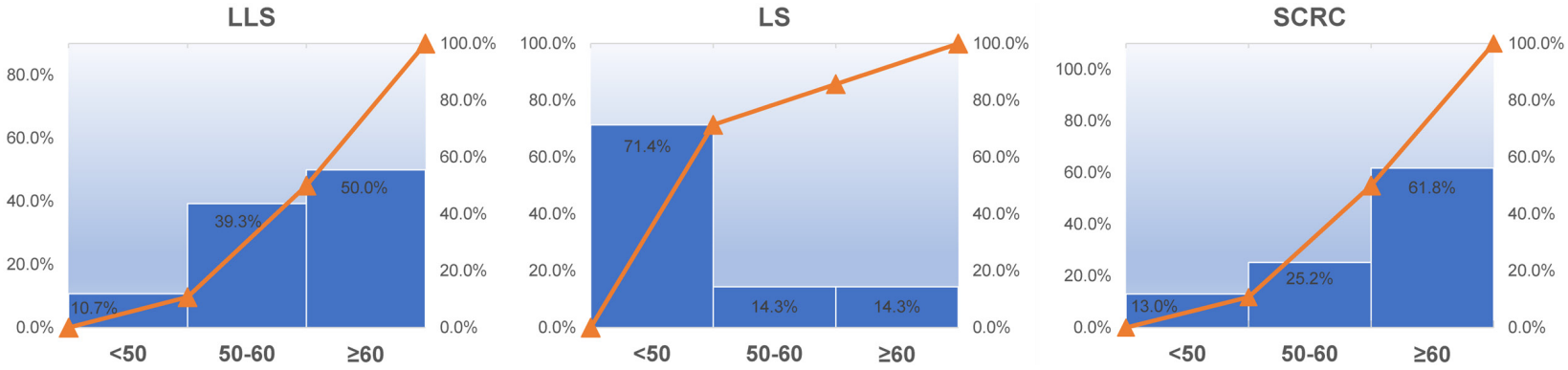

Figure 3. The cumulative percentage of patients against their age of onset of CRC in patients with LLS, LS and SCRC.

Table 2. Molecular characteristics and distribution of MMR genes mutations in tumors of LLS patients

NO.	Sex	Age	Family history	Tumor location	Stage	Histological differentiation	Expression of MMR proteins by IHC	MSI status	BRAF V600E Mutation analysis	MLH1 Methylation (%)	MMR gene mutations	ACMG	AF
1	Male	48	No	Rectum	I	Adenocarcinoma	pMMR	MSI-H	WT	1.9	NR	NR	NR
2	Female	53	No	Rectum	I	Adenocarcinoma	PMS2 (-)	MSS	WT	6.5	NR	NR	NR
3	Male	57	No	Right colon	II	Adenocarcinoma	PMS2 (-)	MSI-H	WT	0	NR	NR	NR
4	Male	72	No	Rectum	III	Mucinous carcinoma	pMMR	MSI-H	WT	0.3	NR	NR	NR
5	Male	52	Yes	Rectum	III	Adenocarcinoma	PMS2 (-)	MSS	WT	2.6	NR	NR	NR
6	Female	60	No	Right colon	II	Adenocarcinoma	PMS2 (-)	MSI-H	WT	0.3	NR	NR	NR
7	Female	64	No	Rectum	I	Adenocarcinoma	PMS2 (-)	MSS	WT	0.6	NR	NR	NR
8	Male	88	No	Left colon	II	Adenocarcinoma	MLH1, MSH2 (-)	MSI-H	MT	0	NR	NR	NR
9	Female	51	No	Right colon	II	Mucinous carcinoma	MLH1, MSH6, PMS2 (-)	MSI-H	WT	3.5	MLH1: Intron11 (c.1039-1G>T)	VUS	47.37%
											MSH6: exon5 p.P1082S (c.3244C>T)	VUS	47.99%
10	Male	58	Yes	Rectum	III	Adenocarcinoma	MLH1, PMS2 (-)	MSS	WT	3.2	NR	NR	NR
11	Female	62	No	Rectum	III	Adenocarcinoma	MLH1 (-)	MSS	WT	1.3	MSH2: exon1 p.P5Q (c.14C>A)	VUS	46.67%
12	Male	53	Yes	Right colon	II	Mucinous carcinoma	MSH6 (-)	MSS	WT	1	MLH3: exon2 p.V207I (c.619G>A)	VUS	1.00%
13*	Male	73	No	Right colon	III	Mucinous carcinoma	MSH6 (-)	MSS	WT	0.6	MSH6: exon5 p.G1105E (c.3314G>A)	VUS	46.63%
14#	Female	79	No	Left colon	I	Adenocarcinoma	MLH1, MSH2 (-)	MSS	WT	0.3	MLH1: exon19 p.R725C (c.2173C>T)	VUS	50.37%
											MLH1: intron13 (c.1558+1G>A)	VUS	1.36%
											MSH2: exon13 p.R680X (c.2038C>T)	VUS	1.97%
											MSH6: exon4 p.V215I (c.643G>A)	Likely benign	13.37%

15	Male	69	Yes	Left colon	I	Mucinous carcinoma	MSH6 (-)	MSI-H	WT	1.9	MLH3: exon2 p.F627L (c.1879T>C)	VUS	52.17%
											MSH2: exon3 p.V161F (c.481G>T)	VUS	50.69%
16	Female	67	Yes	Right colon	I	Signet ring cell carcinoma	MSH2, MSH6 (-)	MSI-H	WT	1.6	MLH3: exon10 p.L1315F (c.3943C>T)	VUS	48.89%
											MSH6: exon5 p.P1082S (c.3244C>T)	VUS	47.83%
17	Male	60	No	Right colon	II	Mucinous carcinoma	PMS2 (-)	MSI-H	WT	0	USP42: exon3~PMS2: exon10	SV	3.92%
18	Male	80	No	Rectum	I	Adenocarcinoma	pMMR	MSI-H	WT	1	NR	NR	NR
19	Female	60	No	Rectum	III	Mucinous carcinoma	Pmmr	MSI-H	WT	1.3	NR	NR	NR
20	Male	67	No	Rectum	III	Adenocarcinoma	PMS2 (-)	MSI-H	WT	0.6	NR	NR	NR
21	Female	79	No	Right colon	III	Adenocarcinoma	pMMR	MSI-H	WT	0.3	NR	NR	NR
22	Male	46	No	Right colon	III	Signet ring cell carcinoma	MLH1 (-)	MSS	WT	3.2	NR	NR	NR
23	Female	54	No	Left colon	III	Adenocarcinoma	PMS2 (-)	MSS	WT	0	MSH2: exon12 p.V655I (c.1963G>A)	VUS	1.30%
24	Female	50	No	Right colon	III	Adenocarcinoma	MLH1, PMS2 (-)	MSS	WT	0	NR	NR	NR
25	Female	52	Yes	Left colon	III	Mucinous carcinoma	MLH1 (-)	MSI-H	WT	0.6	NR	NR	NR
26	Male	43	No	Left colon	III	Adenocarcinoma	MSH6 (-)	MSS	WT	0.6	PMS2: exon11 p.E667K (c.1999G>A)	VUS	52.89%
27	Male	52	No	Rectum	III	Adenocarcinoma	PMS2 (-)	MSS	WT	1.6	NR	NR	NR
28	Female	51	Yes	Left colon	II	Adenocarcinoma	MLH1, PMS2 (-)	MSI-H	WT	8.4	MSH2: exon12 p.V655I (c.1963G>A)	VUS	1.26%

Abbreviations: IHC, immunohistochemistry; WT, wild type; MT, mutation type; AF, allele frequency; VUS, variants of unknown significance; SV, structural variation; NR, not reported. *The patient carried frameshift duplication of c.8432dupA in chr11:108216476 of ATM gene. #The patient carried frameshift deletion c.19_44delCCCGCCGCCGCCAGCAGCCTGGGCAA in chr1:27022913 of ARID1A gene.

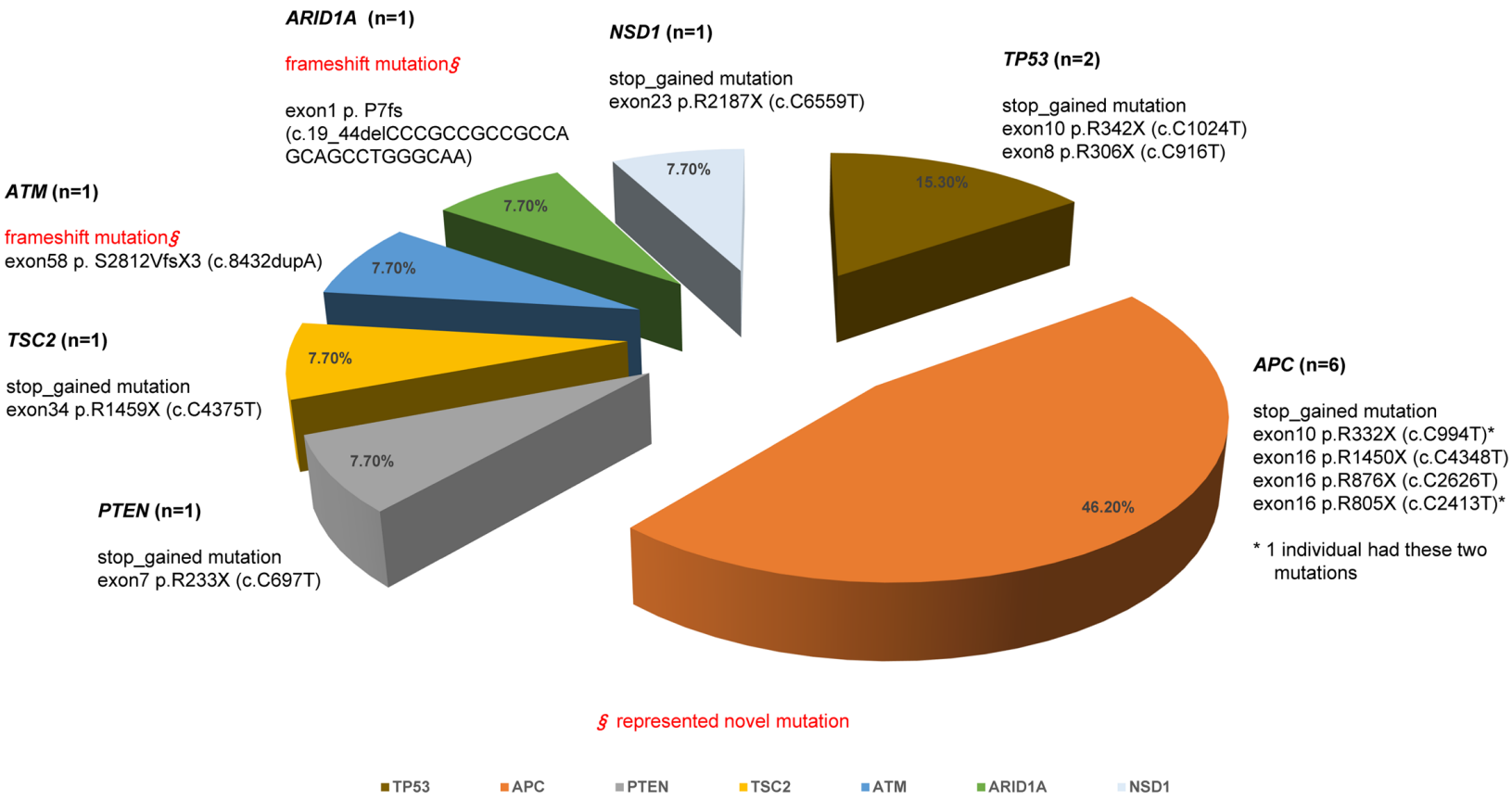

Figure 4. Distribution of 13 pathogenic mutations in 7 non-MMR genes of LLS tumors. Interpretation of sequence variants was according to ACMG guidelines. Mutations in genes with class5 classification (ACMG) were considered as pathogenic variants.

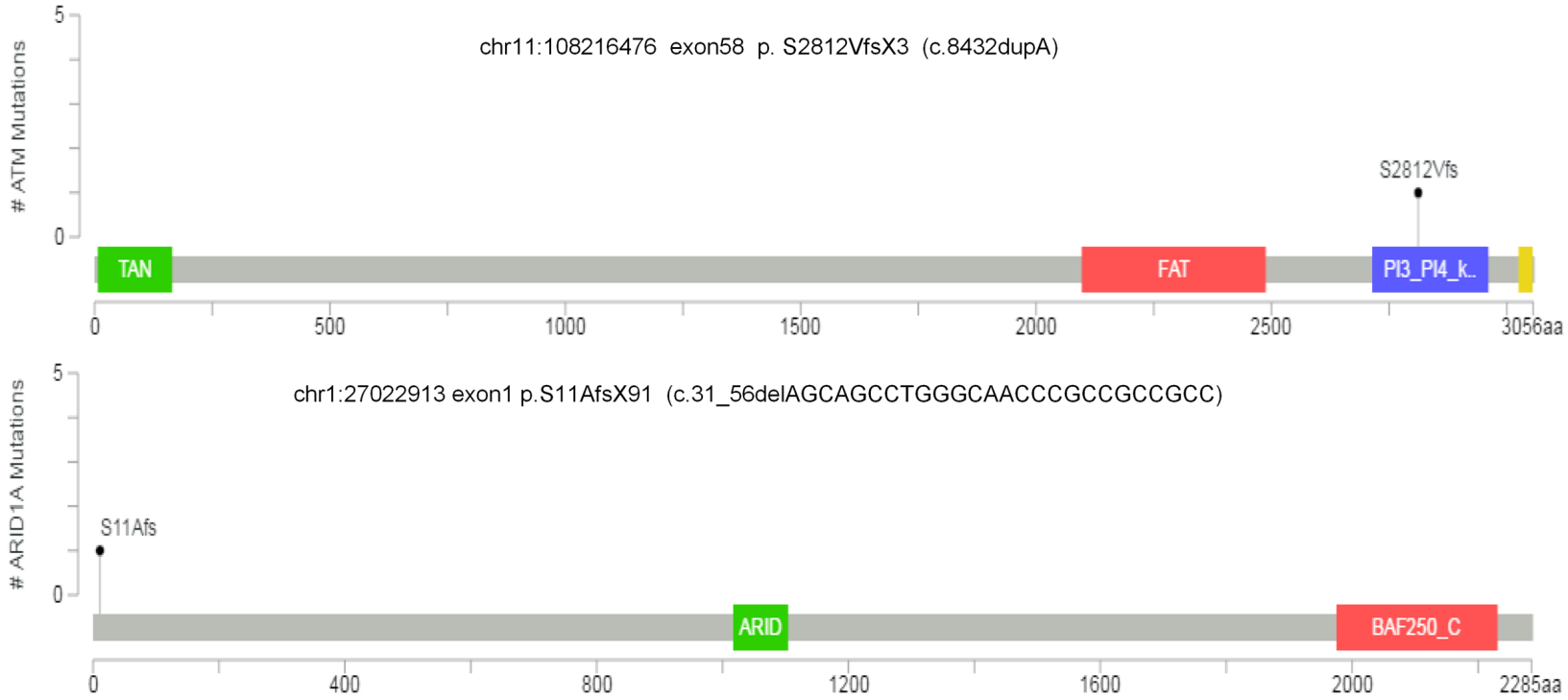

Figure 5. Location of variants in the ATM and ARID1A gene.

In a previous study, Mas-Moya found LLS patients only had a higher percentage of right-colon location than LS patients (93% versus 45%; P<0.002) [23]. However, we found that most patients with LLS had an almost equivalent distribution among rectum, left colon and right colon, compared to patients with LS in colon (100%) and SCRC in rectum (69.4%, P< 0.001). According to Shia J, mucinous type appeared to be more frequent in HNPCC tumors other than LLS tumors, which is consistent with our study [24]. Surgical resection remains the most effective therapy for colorectal cancer. The presence of lymph node metastases in surgical resection specimens is inextricably linked to the prognosis of the disease [25, 26]. Positive lymph nodes have been identified as a highly effective predictor of adverse outcomes. MSI tumors are distinct from MSS tumors, and have an immunologically active phenotype which could reduce positive rates of lymph nodes and distant metastases [26]. However, compared to the patients with LS and SCRCs, we found that patients with LLS had a higher rate of lymphatic metastasis (50%), which was much higher than the patients with LS (28.6%) and MSS (39.8%) in our dataset.

In our study, 65.4% mutations of MMR genes were VUS (17/26) (see **Tables 2** and S2), which was more than that in literature with the prevalence ranged from 10% to 43.2% [27, 28]. But the variant interpretation and classification benefit from quantitative and qualitative analyses. So, in the future, the final proportion of VUS still need to combine functional characterization and the actual level of evidence that the scientific community will deem as acceptable to classify a variant as causal.

Noteworthy, we identified 2 novel frameshift mutations in non-MMR genes in this cohort, including 1 frameshift duplication in ATM and 1 frameshift deletion in ARID1A. ATM, as a DNA damage sensor, could regulate the phosphorylation of ARP8 to maintain the fidelity of DNA repair and to prevent chromosome abnormalities. Besides, it may suppress tumor neoplasia [29]. According to Sriramulu S, complete loss of ATM expression could lead to the poor survival, but ATM gene mutation cannot change the age of onset in HNPCC patients [30]. Xiong H used quantitative real-time PCR to detect the expression of ATM in tumor tissues of CRC patients [31]. They found the level of ATM mRNA was higher expressed in tumor tissues than in normal mucosa tissues. So, it might consider the mutated ATM gene as the predicted biomarker in LLS patients. ARID1A, as a novel tumor suppressor gene, was related to transcription regulation and repression of select genes in the way of chromatin remodeling. The frequency of ARID1A mutation in CRC patients ranged from 8.9%-39% [32-35]. Some reports pointed out that ARID1A mutations and the deficiency of its protein expression were significantly involved in advanced tumor depth, poor differentiation, lymphatic metastasis, BRAF V600E mutation, MMR deficiency and MSI phenotype in tumors of CRC patients, which were similar in our research. However, there was no significance of overall survival in CRC patients with ARID1A mutation [33-35]. Specially, Chou A found ARID1A gene was mutated in all LS patients [33]. Niedermaier reported depletion of ARID1B increased radiosensitivity and may improve outcomes in ARID1A-mutat CRC patients [36]. So far, non-related studies had reported ATM and ARID1A mutations in LLS tumors. In the future, it will be critical to explore molecular mechanism of ATM and ARID1A mutation in LLS tumor transformation. Besides, high penetrance cancer predisposition gene mutations in this study, including TP53, APC, PTEN, implied alternative hereditary genes that would impact on genetic counselling and subsequent management.

This study had certain limitations. First, this study is composed of hospital-based patients other than population-based patients, which may introduce patient selection bias. Second, this is a single-center study with a relatively small sample size which can't fully represent the Chinese population. Third and most important, we performed a clinical description of these cases with LLS but lack of molecular information about somatic mutations in the LLS cases, In the future, we expect well-designed, multi-center and population-based studies to find out the applicable and valid screening of LS and explore the real world of LLS in China.

Conclusions

In this study, 28 (66.6%) MMRd tumors were classified as LLS, which were significantly high-

er than reports of western countries. LLS tumors were more likely to carry lymph node metastases. Two novel mutations in ARID1A and ATM genes of LLS carriers were identified. However, it was hard to differentiated LLS tumors from LS through family history, tumor location, histological type of tumors, immunohistochemistry (IHC) for MMR proteins and MSI analysis.

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Disclosure of conflict of interest

None.

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Table S1. List of 425 cancer-related genes (GeneseeqOneTM pan-cancer gene panel)

Categorization	Gene names
MMR genes	MLH1, MLH3, MSH2, MSH6, PMS1, PMS2, EPCAM
Non-MMR genes	ABCB1 (MDR1), ABCB4, ABCC2 (MRP2), ADH1A, ADH1B, ADH1C, AIP, AKT1, AKT2, AKT3, ALDH2, ALK, AMER1, AR, ARAF, ARID1B, ARID2, ARID5B, ASCL4, ASXL1, ATF1, ATIC, ATRX, AURKA, AURKB, AXIN2, AXL, APC, ARID1A, ATM, ATR, B2M, BAD, BAI3, BAK1, BAP1, BARD1, BAX, BCL2, BCL2L11 (BIM), BCR, BIRC3, BMPR1A, BRAF, BRD4, BTG2, BTK, BUB1B, BLM, BRCA1, BRCA2, BRIP1, CDH1, CHEK1, CHEK2, CDC73, CDK10, CDK12, CDK4, CDK6, CDK8, CDKN1A, CDKN1B, CDKN1C, CDKN2A, CDKN2B, CDKN2C, CEBPA, CEP57, CHD4, CREBBP, CRKL, CSF1R, CTCF, CTLA4, CTNNB1, CUL3, CUX1, CXCR4, CYLD, CYP19A1, CYP2A13, CYP2A6, CYP2A7, CYP2B6*6, CYP2C19*2, CYP2C9*3, CYP2D6, CYP3A4*4, CYP3A5, c11orf30, CASP8, CBL, CBLB, CCND1, CCNE1, CD274 (PD-L1), CD74, CDA, DAXX, DDR2, DENND1A, DHFR, DICER1, DLL3, DNMT3A, DPYD, DUSP2, ESR1, ETV1, ETV4, ETV6, EXT1, EXT2, EZH2, EWSR1, EGFR, EML4, EP300, EPAS1, EPHA2, EPHA3, ERBB2 (HER2), ERBB2IP, ERBB3, ERBB4, EPHA5, EPCAM, EPHB2, ERCC1, ERCC2, ERCC3, ERCC4, ERCC5, FANCA, FANCC, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCL, FANCM, FH, FLCN, FOXA1, FOXP1, FAT1, FBXW7, FGF19, FGFR1, FGFR2, FGFR3, FLT1 (VEGFR1), FGFR4, FLT3, FRG1, FLT4, GATA1, GATA2, GATA3, GATA4, GATA6, GNA11, GNAQ, GNAS, GRIN2A, GSTM1, GSTM4, GSTM5, GSTP1, GSTT1, GRM3, GRM8, HDAC9, HDAC2, HGF, HLAA, HNF1A, HNF1B, HRAS, HSD3B1, IDH2, IFNG, IFNGR1, IGF1R, IGF2, IKBKE, IKZF1, IL7R, INPP4B, IRF2, IDH1, JAK1, JAK2, JAK3, JARID2, JUN, KDM5A, KDM6A, KDR (VEGFR2), KEAP1, KIF1B, KIF5B, KIT, KITLG, KLLN, KMT2A (MLL), KMT2B, KMT2C, KMT2D (MLL2), KRAS, LHCGR, LMO1, LRP1B, LZTR1, LYN, MEN1, MPL, MTOR, MYC, MYCL, MYCN, MYD88, MYH9, MAP2K1 (MEK1), MAP2K2 (MEK2), MAP2K4, MAP3K1, MAP3K4, MAP4K3, MAX, MCL1, MDM2, MDM4, MECOM, MET, MGMT, MITF, MED12, MEF2B, MLLT1, MLLT3, MLLT4, MTHFR, MRE11A, MUTYH, NAT1, NRAS, NOTCH2, NOTCH3, NPM1, NQO1, NBN, NFKBIA, NRG1, NCOR1, NF1, NF2, NFE2L2, NKX21, NKX24, NOTCH1, NSD1, NTRK1, NTRK2, NTRK3, PALB2, PBRM1, PDE11A, POLE, POLH, PTCH1, PTEN, PTPN13, PRKACA, PRKACG, PRKAR1A, PRKCI, PRKDC, PRSS1, PRSS3, PTK2, PTPN11, PTPRD, PAK3, PALLD, PARK2, PARP1, PARP2, PAX5, PDCD1 (PD1), PDGFRA, PDGFRB, PDK1, PGR, PHOX2B, PIK3C3, PIK3CA, PIK3R1, PLAG1, POLD1, POLD3, POT1, PPARD, PKHD1, PPP2R1A, PRDM1, PRF1, PIK3R2, PLK1, PDCD1LG2 (PDL2), QKI, RAD50, RAD51, RB1, RECQL4, RET, RAC1, RAC3, RAD51B, RAD54L, RAF1, RARA, RARG, RELN, RASGEF1A, RHOA, RNF43, ROS1, RICTOR, RPTOR, RRM1, RUNX1, RUNX1T1, SBDS, SDHA, SDHB, SDHC, SDHD, SMAD4, STK1, SOX14, SOX2, SOX21, SPOP, SPRY4, SRC, SRY, STAG2, STAT3, STMN1, SDC4, STT3A, SUFU, SEPT9, SETBP1, SETD2, SF3B1, SGK1, SLC34A2, SLC3A2, SLC7A8, SMAD2, SMAD3, SMAD7, SMARCA4, SMARCB1, SMO, SOS1, SOX1, TGFBR2, THADA, TP53, TP63, TSC1, TSC2, TSHR, TAP1, TAP2, TEK, TEKT4, TERC, TERT, TET2, TMEM127, TMPRSS2, TNFAIP3, TNFRSF11A, TNFRSF14, TNFRSF19, TNFSF11, TOP1, TOP2A, TPMT, TTF1, TUBB3, TUBB4A, TUBB4B, TUBB6, TYMS, U2AF1, UGT1A1, VHL, VAMP2, VEGFA, WRN, WT1, WAS, WISP3, XPA, XPC, XRCC1, YAP1, ZNF2, ZNF217, ZNF703

Table S2. MMR gene mutations in LS tumors

Sample ID	MMR gene mutations			
	Mutated genes†	AF (%)	Exonic function	ACMG
F180505117512-KY546	PMS2: exon8 p.R287fs (c.861_864delACAG)	40.18	Frameshift	class5
	PMS2: exon2 p.R20P (c.G59C)	48.98	Missense	class3
	PMS1: exon3 p.G58R (c.G172A)	1.04	Missense	class3
F180505117518-KY546	MLH1: exon12 p.A353fs (c.1057_1060delGCTG)	50.00	Frameshift	class5
F180505117525-KY546	MSH6: exon1 p.P57fs (c.169delC)	49.20	Frameshift	class5
F180505117531-KY546	MSH6: exon5 p.E1119fs (c.3357_3358delAG)	47.50	Frameshift	class5
F180505117532-KY546	EPCAM: exon 8 & 9 deletion	-	LGR	class5
F180505117538-KY546	MLH1: exon8 p.R226Q (c.G677A)	50.11	Missense; Splice-region-variant	class5
F180926149479-KY546-1	PMS2: exon4 p.F104fs (c.312delT)	46.26	Frameshift	class5
	PMS2: exon11 p.E489X (c.G1465T)	17.23	stop_gained	class4

†*MLH1, MLH3, MSH2, MSH6, PMS1, PMS2* and *EPCAM* gene were tested and mutations with ACMG class 5 were identified.