UNIVERSITAT DE BARCELONA

Elucidating the molecular basis of Lynch-Like syndrome

Gardenia Maria Vargas Parra

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ELUCIDATING THE MOLECULAR BASIS OF LYNCH-LIKE SYNDROME

By

Gardenia María Vargas Parra

Barcelona, 2015

A thesis submitted for the degree of Doctor of Philosophy

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ELUCIDATING THE MOLECULAR BASIS OF LYNCH-LIKE SYNDROME

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LIST OF ABBREVIATURES

aa	aminoacids
ACI	Amsterdam Criteria (original version)
ACII	Amsterdam Criteria II
AFAP	Attenuated Familial Adenomatous Polyposis
Align-GVGD	Align-Grantham Variation Grantham Deviation
APC	Adenomatous Polyposis Coli
ASE	Allelic Specific Expression
ATP	Adenosine-5'-Triphosphate
ATPase	Adenosine-5'-Triphosphatase
BER	Base Excision Repair
bp	base pair
BRAF	V-Raf Murine Sarcoma Viral Oncogene Homolog B1
cDNA	complementary DNA
CIMP	CpG Island Methylator Phenotype
CIN	Chromosomal Instability
CMMR-D	Constitutional Mismatch Repair-Deficiency
CRC	Colorectal Cancer
CSCE	Conformation Sensitive Capillary Electrophoresis
DGGE	Denaturing Gradient Gel Electrophoresis
DHPLC	Denaturing High Performance Liquid Chromatography
DNA	Deoxyribonucleic acid
EC	Endometrial Cancer
EGFR	Epidermal Growth Factor Receptor
EPCAM	Epithelial Cell Adhesión Molecule
EXO I	Exonuclease 1
FAN1	FANCD2/FANCI-Associated Nuclease 1
FAP	Familial Adenomatous Polyposis
FBXW7	F-box and WD repeat domain containing 7
fCRC-X	Familial CRC type X
FDRs	First-Degree Relatives
FFPE	Formalin Fixed Paraffin Embedded
FOB	Fecal Occult Blood
GI	Gastrointestinal
HNPCC	Hereditary Non Polyposis Colorectal Cancer
ICL	Interstrand Cross-Links

ICO	Catalan Institut of Oncology; from the catalan, Institut Català d'Oncologia
IHC	Immunohistochemistry
InSiGHT	International Society for Gastrointestinal Hereditary Tumours
KRAS	Kirsten ras Sarcoma 2 Viral Oncogene Homolog
LLS	Lynch-Like Syndrome
LOH	Loss of Heterozygosity
LOVD	Leiden Open Variation Database
LS	Lynch Syndrome
MAP	MUTYH Associated Polyposis
MCA	Melting Curve Analysis
Mg	magnesium
MGMT	O-6-Methylguanine-DNA Methyltransferase
MLH1	Mutl Homolog 1 (E. Coli)
MLPA	Multiplex Ligation-dependent Probe Amplification
MMR	Mismatch Repair
mRNA	messenger RNA
MS	Methylation Specific
MSH2	Muts Homolog 2 (E. Coli)
MSH3	Muts Homolog 3 (E.Coli)
MSH6	Muts Homolog 6 (E.Coli)
MSI	Microsatellite Instability
MSI-H	MSI-High
MSI-L	MSI-Low
MS-MCA	Methylation-specific MCA
MS-MLPA	Methylation-specific MLPA
MSP	Methylation-specific PCR
MSS	Microsatellite Stability
MUTYH	Muty Homolog (E. Coli)
NF1	Neurofibromatosis type 1
NGS	Next Generation Sequencing
NMD	Nonsense Mediated Mrna Decay
NRAS	Neuroblastoma RAS Viral (v-Ras) Oncogene Homolog
PBL	Peripheral Blood Lymphocytes
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PI3KCA	Phosphatidyl Inositol 3-Kinase Catalytic Subunit
PMS1	Postmeiotic Segregation Increased 1 (S. Cerevisiae)
PMS2	Postmeiotic Segregation Increased 2 (S. Cerevisiae)
PolyPhen	Polymorphism Phenotyping
RFC	Replication Factor C

RNA	Ribonucleic Acid
RPA	Replication Protein A
RT-PCR	Retrotrancription Polymerase Chain Reaction
SIFT	Sorting Intolerant From Tolerant
SMAD4	SMAD family member 4
SNuPE	Single Nucleotide Primer Extension Analysis
TGFβR2	Transforming Growth Factor, Beta Receptor II
TILs	Tumor Infiltrating Lymphocytes
Tm	Melting temperature
TP53	Tumor protein p53
TSS	Transcription Start Site
USA	United States of America
UTRs	Untranslated Regions
VUS	Variant of Unknown Significance
WT	Wildtype

INTRODUCTION

1. COLON AND RECTUM

1.1. Anatomy

The colon or large bowel is a continuation of the small one, representing the last part of the gastrointestinal (GI) tract. It is a hollow muscular tube of about 1.5m in length and 6.5cm in diameter. At the cephalad end has an ileocecal valve and at the caudal the dentate line of the anus. Starting at the right side of the abdomen, the large bowel is connected to the ileum of the small intestine by the ileocecal sphincter. From where it forms a dead end segment called cecum. After here, the colon rises to reach the right lobe of the liver (ascending colon), where it turns to the left forming the hepatic flexure and run across the abdomen (transversal colon); this is the longest and most mobile segment of the colon. In the left of the body, after the splenic flexure is directed downwards (descending colon), and until it curves in an S-shape takes the name of sigma which has variable length, tortuosity and mobility, representing the narrowest part of the large intestine. At the peritoneal reflection, posteriorly, the sigma becomes the rectum, which ends in the anal canal, and finally opens to the outside through the anal sphincter (Fig. 1) (Moore, Agur, and Dalley 2013; Quiroz 2011).

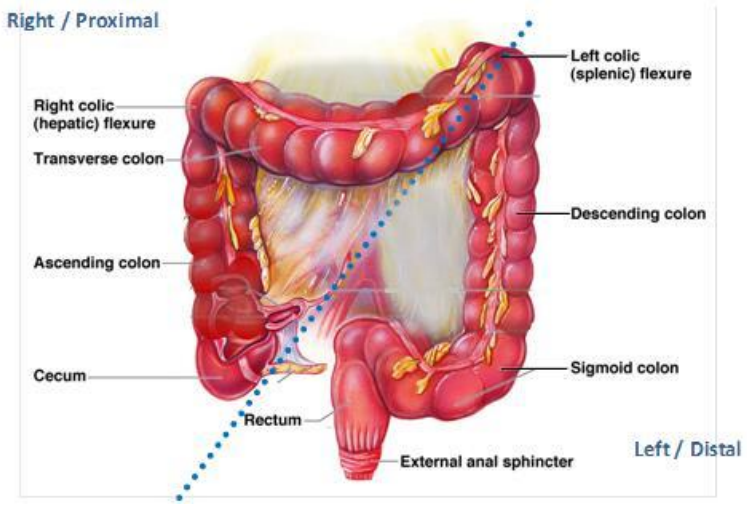

Figure 1. Anatomical and clinical segments of the colon.

1.2. Embriology

The GI tract is a three dimensional, complex and specialized organ system, derived from a simple tubal structure composed of the three embrionary layers (endoderm, mesoderm and ectoderm). Being that gut epithelium is a constitutively developing tissue, constantly differentiating from a stem cell in a progenitor pool throughout life, developmental pathways such as axes of development, and cell-cell "cross-talk" continue to be important in cell differentiation, homeostasis and apoptosis of the adult intestinal epithelium. The cecum, appendix, ascending and proximal portion of the transverse colon (right colon) are derived from the midgut, while the distal transverse, descending, sigmoid colon and rectum (left colon) are derived from the hindgut. The wide variation in patterns of gene expression, physiologic function, disease distribution, and variations in histology appearance between the right and left colon reflect the combined midgut and hindgut derivation.

The fundamental axis maintained in the adult is the radial (crypt to surface) axis (Fig. 2). Homeostasis of intestinal epithelium occurs throughout life along this axis. The epithelial and mesenchymal progenitor/proliferative cells are located in the depth of the radial axis. The differentiated functional cells and the apoptoic cells are located farther toward the villus and luminal portions.

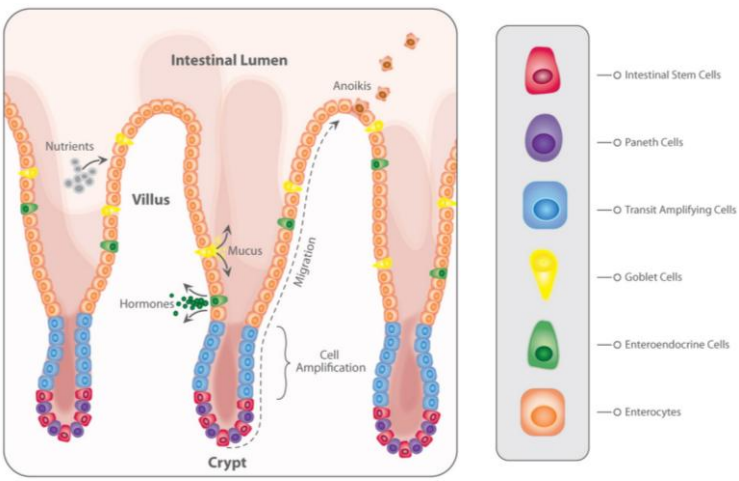

Figure 2. Intestinal crypt and villus epithelium diagram.

1.3. Histology

Microscopically, the wall of the colon is composed of four layers (Fig. 3). In the inner, it has a thick mucosa with deep undifferentiated crypt cells, tall columnar absorptive cells which retrieve water and sodium from the luminal content; these cells are sloughed into the lumen, and have to be replaced every 6 days. This glandular epitelium is also composed of goblet, Paneth, enteroendocrine, M cells and stem cells. It is supported by the lamina propria, formed by reticular connective tissue of elastin, reticulin and collagen fibers; here lymphocytes, plasma cells and eosinophilic granoluocytes act as guardians of immune response. Finally, a thin layer of muscle divides this innermost layer from the second layer, the submucosa. The submucosa is the second barrier of connective tissue, which confers flexibility for the mucosa to move during peristalsis. It contains blood and lymphatic vessels, and a nerve fiber plexus called Meissner's plexus, which has sympathetic and parasympathetic ganglion cells. A muscularis, responsible for contractility, is formed by internal circular fibers (haustra) and external longitudinal ones concentrated into three flat bands called teniae coli; this layer possess a myenteric plexus called Auerbach's. The outermost layer is composed of connective tissue and is called adventitia or serosa. (Anon n.d.; Mills 2007; Ross, Kaye, and Pawlina 2002)

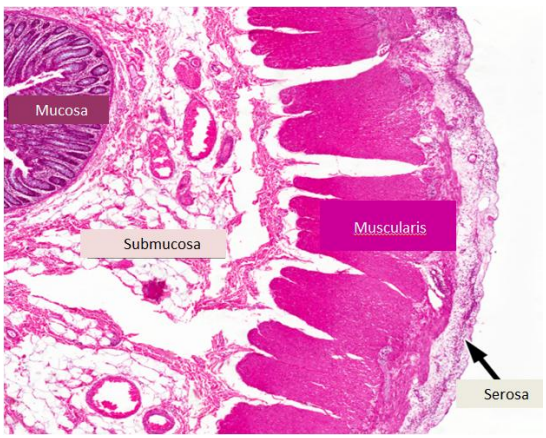

Figure 3. Longitudinal section of large bowel, stained with hematoxylin and eosin (HE).

2. COLORECTAL CANCER

2.1. Epidemiology

Worldwide, colorectal cancer (CRC) is the third most frequent cancer, with an annual incidence of 1.36 million. It maintains this place among men and goes up to the second among women, with 746 thousand new male cases per year and 614 thousand new females affected; representing 10 and 9.2% of all cancers in each gender, respectively. CRC together with lung, breast and prostate, represent over half of the cancer incidence (Fig. 4) (Ferlay et al. 2014). The incidence of CRC is thought to be related to the intensification of risk factors, such as smoking, poor diet and lifestyle and high caloric intake, so its higher in most developed countries, whereas in less developed ones, the most common cancers are related to infectious origin (Ferlay et al. 2014; Torre et al. 2015).

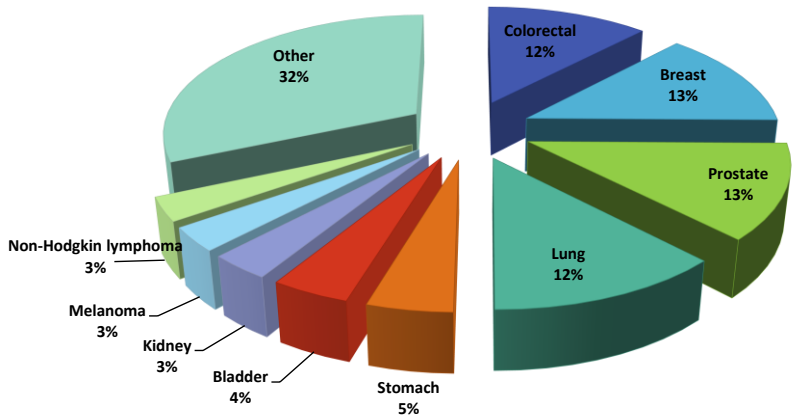

Figure 4. Proportion of estimated global number of new cancer cases in more developed regions, both genders combined (Statistic source: GLOBOCAN 2012).
Modified chart from Ferlay et al, 2014.

Over the world, mortality is lower than incidence, in both men and women (8.2%), although in less developed countries is higher (52% of the total cases) than in the more developed ones (~20%) (Fig. 5) (Ferlay et al. 2014). The discrepancy of survival and incidence in developed countries, beyond the apparently economic relationship and the possibility of better treatments, has been associated to the employment of proper CRC screening and surveillance (Sunkara and Hébert 2015)

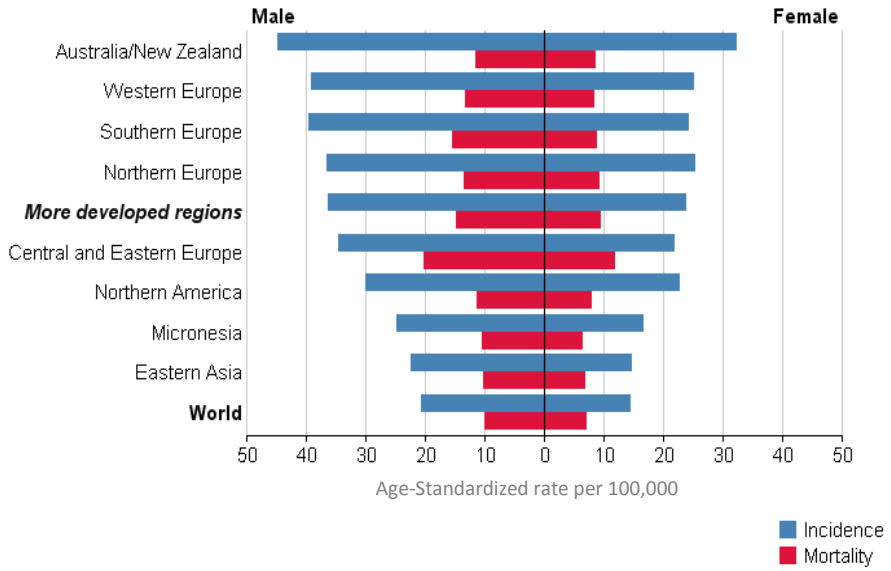

Figure 5. Colorectal cancer incidence and mortality rates in most developed regions. GLOBOCAN, 2012.

The cumulative lifetime risk for being diagnosed of CRC is 5.1% in some industrialized countries like the United States of America (USA). In Spain the estimated lifetime risk is 3.5% (Tarraga Lopez, Alberto, and Rodriguez-Montes 2014).

2.2. Molecular Basis of Colorectal Cancer

It is well known that CRC arises from the accumulation of genetic and epigenetic alterations in a colorectal epithelial cell, producing a transition from normal epithelium to a neoplastic state (Hanahan and Weinberg 2011). In 1990, Fearon and Vogelstein proposed a genetic model for CRC tumourigenesis that underlies the adenoma-carcinoma sequence (Fearon, E R and Vogelstein 1990). It postulates that only few (4 to 6) genetic alterations are required for growth advantage and clonal expansion of tumoral cells. These alterations can be activating oncogenes or inactivating tumor suppressor genes, and their accumulation is responsible for the carcinoma development (Fig. 6).

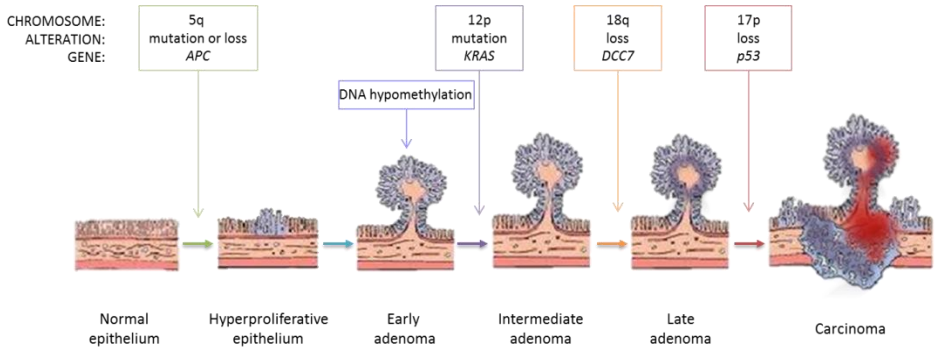

Figure 6. The genetic basis of the adenoma-carcinoma sequence.
Adapted from Fearon and Vogelstein, 1999.

In 2006, Sjöblom and collaborators sequenced more than 13,000 genes in breast and colorectal tumors, finding approximately 90 different genes mutated (~9 per colorectal tumor) further refining Fearon and Vogelstein's model. Sixty-nine of the detected mutations were recurrent, probably involved in cancer development. Furthermore, each tumor had a distinct mutational gene signature (Sjöblom et al. 2006). Even when these changes tend to appear in a lineal manner along time, the biological features of tumor are related to the pile of them and not to the sequence *per se*.

Since the postulation of adenoma-carcinoma genetic sequence theory, a wide progress has been made in understanding the underlying molecular mechanisms of neoplastic transformation. Hanahan and Weinberg defined six hallmarks in cell physiology that collectively dictate the behavior of malignant cells: self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000). In 2011, they included reprogramming of energy metabolism and evading immune destruction as "emerging hallmarks" since they are not validated, and pin-pointed two enabling characteristics, tumor promoting inflammation and genomic instability and mutation accumulation(Fig. 7) (Hanahan and Weinberg 2011).

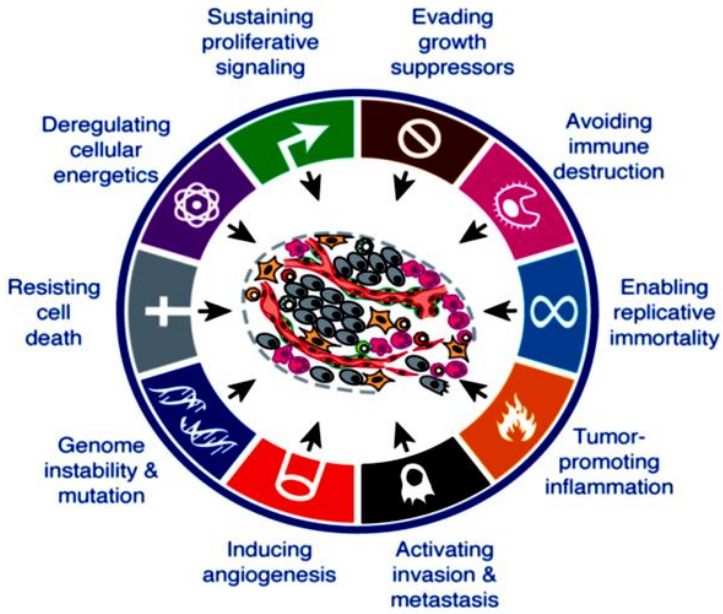

Figure 7. Colorectal cancer hallmarks.
Extracted from Hanahan and Weinberg, 2011.

Concerning genome instability, this enabling feature is postulated to be present in almost all types of cancers, but it has been widely described in colorectal ones. So far three distinct pathways have been identified: chromosomal instability (CIN), microsatellite instability (MSI) or CpG island methylator phenotype (CIMP) (Fig. 8).

CIN. Chromosomal instability is the most frequently observed and is present in 70 to 85% of all CRCs. It is characterized by aneuploidy (loss, gain or structural chromosomal rearrangements) and the loss of heterozygosity. An increased rate of chromosome missegregation leading to both, tumor promoter and tumor suppressor genes effects (Grady and Carethers 2008; Yuen and Desai 2008). Activation of *KRAS* and *MYC,* and inactivation of *APC*, *TP53*, *SMAD4* and *DCC* have been related to this pathway (Bloom 2012; Vogelstein et al. 1988). CIN is associated to poor prognosis (Popat, Hubner, and Houlston 2005), possibly because the anomalous mitosis contributes to tumor progression by increasing genetic diversity among malignant cells (Thompson, Bakhoum, and Compton 2010).

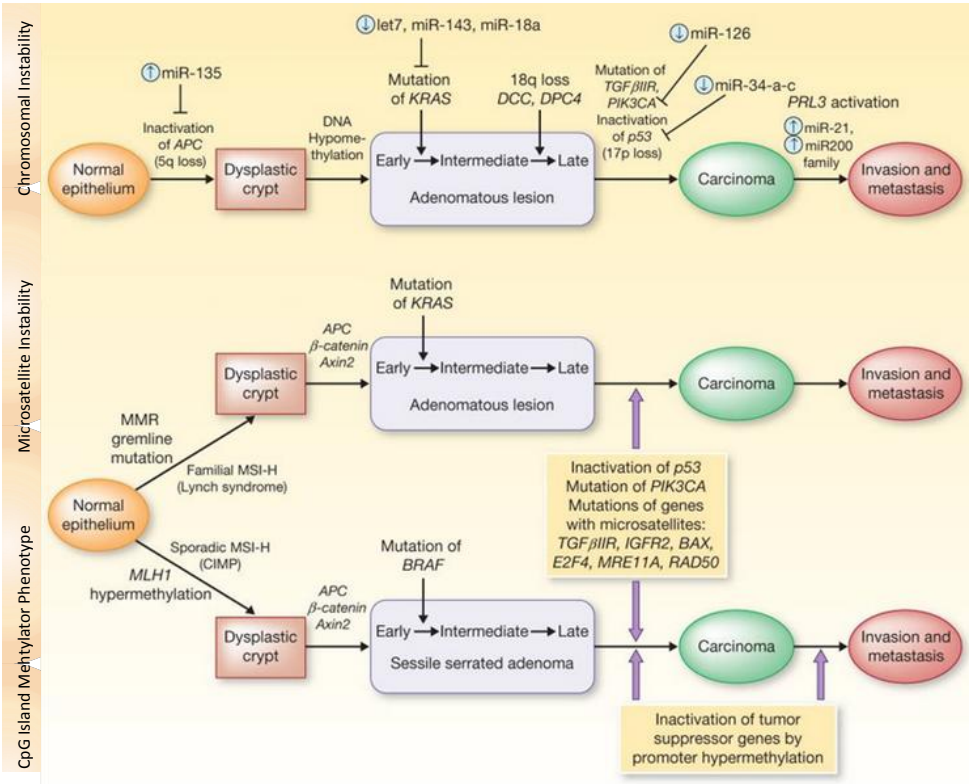

Figure 8. Molecular basis of adenoma-carcinoma sequence in CRC.
Adapted from Vilar et al, 2011.

MSI. It is implicated in around 15% of all CRC and the majority of the hereditary ones (Boland 2013; Vasen and de Vos Tot Nederveen Cappel 2013). It results from the accumulation of errors in short nucleotide repetitive DNA sequences, named microsatellites (Buecher et al. 2013; Imai and Yamamoto 2008). These could be either by losses or gains in length of a microsatellite with respect to its germline counterpart due to defective DNA mismatch repair (MMR) genes (Boland and Goel 2010; Vilar and Gruber 2010). MMR genes are implicated in the correction of errors that appear spontaneously during DNA replication, such as single base mismatches and short insertions or deletions. Failure of MMR function generates a hypermutability state, leading specially to frameshift mutations in cancer related genes (Fig. 9), providing a selective growth advantage for cells with defective MMR (Yamamoto and Imai 2015). This is followed by oncogenic mutations of *KRAS*, promoting the transition from early to intermediate adenomas, and inactivation of *TP53* as a late event (Kim et al. 2009; Vilar, Tabernero, and Gruber 2011). Moreover, recent data from multiple studies support the role of miRNA in the pathogenesis of MSI tumors (Sonia A Melo and Esteller 2011; Sonia A. Melo and

Esteller 2011; Yamamoto et al. 2012). However, MSI phenotype is associated to better prognosis since tumors are less prone to develop metastasis (Popat et al. 2005). This could be related to the fact that multiple mutations trigger production of more abnormal proteins in tumor cells and, in turn, promote the immune system to boost a bigger response against them (Le et al. 2015).

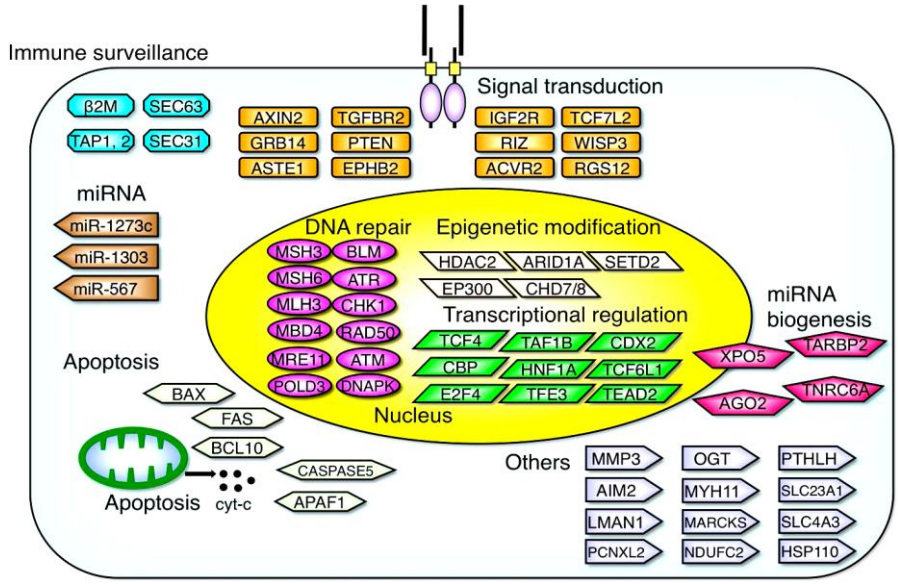

Figure 9. Representative target genes in MSI gastrointestinal cancers.
Yamamoto et al, 2015.

CIMP. This pathway is activated in 15-35% of CRCs (Goel et al. 2007; Ogino et al. 2006; Pritchard and Grady 2011) and is initiated by aberrant methylation of CpG rich regions in gene promoters, which leads to its transcriptional silencing and loss of function (Yamamoto et al. 2012). In CRC, such epigenetic alteration has been associated to environmental factors, like smoking (Samowitz et al. 2006), but the main cause remains elusive. The majority of these kinds of tumors have loss of MLH1 expression due to *MLH1* promoter methylation with a high frequency of *BRAF* mutations and low frequency of *APC* and *KRAS* mutations (Bloom 2012; Weisenberger et al. 2006). Other commonly methylated genes in CRC are *CDKN2A/p16*, *MGMT*, *THBS1*, *TIMP3*, *CDKN2A* (*p14ARF*) and *THSD* (Khamas et al. 2012; Toyota et al. 1999) Nevertheless, CIMP is also present in other type of tumors, having in each a quite different molecular profile, reason why it has been proposed that CIMP in CRC should be named C-CIMP to differentiate them from other molecular pathways (Fang et al. 2011; Hughes et al. 2013).

2.3. Diagnosis and Prevention of Colorectal Cancer

Signs and symptoms of CRC can vary from none to different degrees of rectal bleeding, changes in intestinal habits (diarrhea or constipation), mild discomfort or pain, tenesmus, vomiting, anemia, paleness, fatigue, or appetite and weight loss for no apparent reason (Esteva et al. 2014).

Primary prevention consists in avoiding smoking, maintaining a healthy diet: avoiding meals with high fat content or with high calories, lowering the intake of red meat and alcoholic drinks, increase fiber intake, vitamin C, calcium and selenium; as well as performing regular physical activity and normalizing the body mass index. Besides that, some chemical agents have been studied as preventive in CRC; within them are the acetylsalicylic acid and statins (Gonzalez and Riboli 2010).

Secondary prevention or early diagnosis is the most powerful tool to increase survival in CRC patients. It consists in screening population considered at risk. The ages and the techniques used for the early detection of CRC vary among countries, but the classical used to be detection of fecal occult blood (FOB), also named guaiac test. Nowadays, it is replaced by the immunological FOB test and fecal DNA analysis. Other assessment tool is the double contrast barium enema, in which the inner surface of the colon is delineated on X-rays by the contrast between liquid and air. Colonoscopy, on the other hand, searches for changes in the mucosal surface of the colon inserting an endoscope through the anus until reaching the cecum. Sigmoidoscopy is similar to colonoscopy, but reaching up to 60cm from the anal verge. More recently, virtual colonoscopy is performed using cross-sectional images of the colon and rectum by computed tomography. At least one of these assessment tools should be applied in general to all people 50 years old or older, whom do not have other risk factors (Provenzale et al. 2015).

2.4. Colorectal Cancer Staging

The determination of a specific diagnosis, the management of a CRC patient and its prognosis are based on the assessment of tumor invasion, its dissemination to regional lymph nodes and the presence of distant metastasis. Furthermore, cancer staging is vital for standardizing all aspects of clinical and translational research. There are various systems for ranking stage, and currently three staging systems are in use. The Dukes classification, consisting in three (A, B, C) categories, proposed in 1932 (Dukes 1932), with a further subdivision of the stage C (Gabriel et al. 1935), a subsequently modified version from Astler-Coller with the addition of one more stage (Stage D) (Astler and Coller 1954), and the most recent and widely accepted from the American Joint Committee on Cancer (AJCC) based on TNM classification, developed by Pierre Denoix in the 1940s (Table 1) (Edge and Compton 2010).

Recently, based on the differential gene expression profiles of tumors, three main molecular classifications of CRC have been proposed, one of 3 subgroups (Vermeulen et al. 2008, 2012), one of five (Sadanandam et al. 2013) and another of 6 subgroups (Marisa et al. 2013). Due to tumor cell heterogeneity, each gene product can be overrepresented by different cell types and, at the same time, each cell type can be overrepresented within a tumor.

Table 1. Left.- TNM classification. Right.- AJCC Stages - 2010 7th edition and equivalent stages from Dukes and Modified Astler-Coller systems.

T. Description
Tis = Carcinoma *in situ* : intraepithelial or invasion of lamina propria.
T1 = Tumor invades submucosa.
T2 = Tumor invades muscularis propria.
T3 = Tumor invades through the muscularis propria into pericolorectal tissues.
T4a = Tumor penetrates to the surface of the visceral peritoneum.
T4b = Tumor directly invades or is adherent to other organs or structures.
N. Description
N0 = No affection of lymph nodes.
N1 = Metastases in 1–3 regional lymph nodes.
N1a = Metastasis in 1 regional lymph node.
N1b = Metastases in 2–3 regional lymph nodes.
N1c = Tumor deposit(s) in the subserosa, mesentery, or nonperitonealized pericolic or perirectal tissues without regional nodal metastasis.
N2 = Metastases in ≥4 regional lymph nodes.
N2a = Metastases in 4–6 regional lymph nodes.
N2b = Metastases in ≥7 regional lymph nodes.
M. Description
M0 = No distant metastasis.
M1a = Metastasis confined to 1 organ or site (e.g., liver, lung, ovary, nonregional node).
M1b = Metastasis in >1 organ/site or the peritoneum.

T= primary tumor; N= regional lymph nodes; M= distant metastasis.

AJCC Stage	TNM	Dukes	MAC
0	Tis, N0, M0	–	–
I	T1, N0, M0	A	A
	T2, N0, M0	A	B1
IIA	T3, N0, M0	B	B2
IIB	T4a, N0, M0	B	B2
IIC	T4b, N0, M0	B	B3
IIIA	T1–T2, N1/N1c, M0	C	C1
	T1, N2a, M0	C	C1
IIIB	T3–T4a, N1/N1c, M0	C	C2
	T2–T3, N2a, M0	C	C1/C2
	T1–T2, N2b, M0	C	C1
IIIC	T4a, N2a, M0	C	C2
	T3–T4a, N2b, M0	C	C2
	T4b, N1–N2, M0	C	C3
IVA	Any T, Any N, M1a	–	–
IVB	Any T, Any N, M1b	–	–

AJCC: American Joint Comittee on Cancer
MAC: Modified Astler-Coller classification.

Colorectal cancer survival. At the time of diagnosis more than 20% of the cases already have distant metastasis, 37% have regional extension, in 37% the tumor is confined to the colon and the remaining 6% of patients lack staging. The 5-year survival is directly correlated with the stage at the moment of diagnosis (Table 2) (Tarraga Lopez et al. 2014).

Table 2. Approximate frequency and five year relative survival (%) by AJCC stage. Adapted from: Clinical guideline 2011. The diagnosis and management of colorectal cancer.

AJCC Stage	Approximate frequency at diagnosis	Approximate five-year survival
I	11%	83%
II	35%	64%
III	26%	38%
IV	28%	3%

2.5. Colorectal Cancer Treatment

Surgical management is considered the first-line treatment for resectable CRC. In stage 0, when cancer cells are fully contained in the epithelial layer, surgical removal is preferred, and is usually performed during colonoscopy. In stage I, when cancer has extended beyond the mucosa, tumor should be resected *en bloc* with part of the colon and regional lymph nodes, this is called colectomy. No adjuvant chemotherapy is indicated for either stage 0 or I (NCCN guidelines v1.2015: http://www.nccn.org/professionals/physician_gls/pdf/ colon.pdf).

For stage II CRC, when cancer cells have spread beyond the *muscularis propria* and without affecting the lymph nodes, adjuvant chemotherapy and radiation should be offered conjointly with surgery, in case of rectal localization. In case of stage II colonic localization, adjuvant chemotherapy is considered when high risk factors for recurrence are present. High risk factors are: poorly differentiated histology, lymphatic or vascular invasion, perineural invasion, bowel obstruction, localized perforation, <12 lymph nodes examined, endure of other co-morbidities and anticipated life expectancy (NCCN guidelines v1.2015: http://www.nccn.org/professionals/physician_gls/pdf/ colon.pdf).

Stage III CRC is referred to a cancer affecting lymph nodes and without distant metastasis. It is treated with surgery and FOLFOX regimen, which includes: 5-flourouacil (5-FU),

leucovorin and oxaliplatin, or optionally FOLFIRI (5-FU, leucovorin and irinotecan). Capecitabine is given instead of 5-FU to patients who do not tolerate an intravenous catheter. Rectal cancer patients are treated with radiation either before or after surgery (Praxi 2012; NCCN guidelines v1.2015: http://www.nccn.org/professionals/physician_gls/pdf/colon.pdf).

Finally, for stage IV or metastasized cancer, the recommendation is palliative surgery in case of obstruction, significant bleeding or for removal of distant metastasis in organs such as liver, ovaries or lung. Additionally, radiotherapy could be offered alone or in combination with chemo (NCCN guidelines v1.2015).

Treatment guidelines are flexible to variations and can be guided by the gene expression profiles and their associated risks. Regarding this matter, different tests have been developed to evaluate the risk of recurrence over other risk factors in patients with diagnosis of CRC. Some examples of these commercially available tests are Oncotype Dx (Colon Cancer Assay from Genomic Health, Inc.), ColoPrint (Agendia) and ColDx (Almac) (NCCN guidelines v1.2015).

2.5.1. Personalized treatment for colorectal cancer

The diagnostic landscape in oncology has changed due to high-complex genomic analyses (Stoffel 2015a). Molecular characterization of tumors allows the identification of markers that can be used to select more specific and personalized therapies (Jones et al. 2015).

Molecular biomarkers of cytotoxic chemotherapy response. As mentioned, the 5-FU and its prodrug, the capecitabine, are the cornerstones of CRC treatment. 5-FU is a direct inhibitor of thymidylate synthase (TS). The dihydropyrimidine dehydrogenase (DPD) is the constraint enzyme of 5-FU catabolism and along with TS, function as predictors of 5-FU response. The most frequent mutation that diminishes DPD activity is the IVS14+1G>A, present in ~25% of the patients showing 5-FU toxicity (Núñez Hernández et al. 2011).

Furthermore, different clinical trials have demonstrated that MSI CRCs in stages II and III do not respond to 5-FU. In contrast, these patients respond well to irinotecan (Shen 2015). The active metabolite of this agent, SN-38, inhibits the topoisomerase I leading to inhibition of

both DNA replication and transcription. This metabolite is then inactivated by the Uridine diphosphate Glucoronosyltransferase 1A1 (UGT1A1). Carriers of variants in *UGT1A1* show toxicity to this therapy. Additionally, patients with loss of 18q have also shown bad response to 5-FU(Núñez Hernández et al. 2011).

Biomarkers of anti-EGFR response. In an appropriate patient population, therapies targeting specific genetic alterations can be safer and more effective than traditional chemotherapies (Reichert and Dhimolea 2012). A wide variety of drugs have demonstrated successful at targeting specific gene products that are altered in cancer. In this context, one example is the development of agents targeting the epidermal growth factor receptor (EGFR) and the vascular endothelial growth factor (VEGF).

Cetuximab and panitumumab are monoclonal antibodies (MoAb) against the extracellular EGFR domain that have demonstrated to be effective in metastatic CRC (mCRC). EGFR is a tyrosine kinase frequently expressed in epithelial tumors. Its activation through an extracellular ligand triggers intracellular signaling in two different pathways: RAS/RAF/MAPK and PI3K/AKT, both involved in proliferation, adhesion, angiogenesis, cell migration and cell survival (Fig. 10). *EGFR* amplification by FISH or Chromosomal *In Situ* Hybridization, is related to treatment efficacy (Table 3). Thus, patients with elevated number of copies of *EGFR* show a better response to anti-EGFR agents.

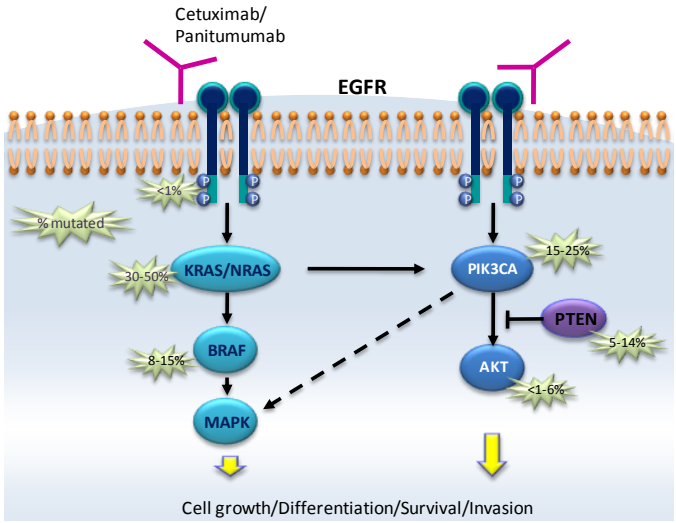

Figure 10. Common mutations affecting the response to MoAbs anti-EGFR. Modified from Núñez et al., 2011.

RAS/RAF/MAPK pathway. Several studies have shown that mCRC patients with *KRAS* wildtype show better response to MoAb anti-EGFR. This is due to the fact that *KRAS* is a proto-oncogene of RAS family and a major component of RAS/RAF/MAPK pathway. When this protein kinase is mutated, it activates MAPK, promoting cell growth and survival. Nevertheless, 40-60% of mCRCs with *KRAS* wildtype do not respond to this treatment. DeRoock *et al* observed that carriers of the specific p.G13D mutation in *KRAS* have a better outcome related to panitumumab or cetuximab than patients with other KRAS mutations (Roock et al. 2011). Although, no greater survival has been found in carriers of codon 13 mutations over carriers of codon 12 mutations (Shen 2015).

Table 3. Biomarkers and predictive value of anti-EGFR treatment.
Adapted from Núñez et al., 2011.

Biomarker	Prevalence	Predictive value for anti-EGFR treatment
EGFR	15% by IHC 20-40% ↑copy number	Only in case of copy number alteration
KRAS mutation	40% in codons 12 and 13; 2% in codons 61 and 146	Validated for codons 12 and 13
BRAF mutation	~10% in exon 15 (V600E)	Possible
NRAS mutation	5-8% in codon 61	Possible
PIK3CA mutation	15-25% in exons 9 and 20	Possible
PTEN loss of expression	20-40% by IHC	Possible

Mutations in *KRAS* and *BRAF* are mutually exclusive and the activation of any of them can initiate tumourigenesis through MAPK (Rajagopalan et al. 2002). *BRAF* gene codifies for a protein kinase that is a direct effector of KRAS in the RAS/RAF/MAPK pathway. *BRAF* mutations appear to be ligated to a chemorefractory response to MoAb anti-EGFR. In a similar fashion, carriers of *NRAS* mutations have shown a significant lower response to treatment than patients with *NRAS* wildtype (Núñez Hernández et al. 2011).

PI3K/AKT pathway. EGFR activation or PTEN loss of function produces PI3K/AKT pathway activation. It has been reported that mCRC carriers of *PIK3CA* mutations and/or loss of PTEN are resistant to MoAb anti-EGFR (Núñez Hernández et al. 2011). *PIK3CA* is mutated in 15-25% of CRCs (Sartore-Bianchi et al. 2009); the phosphatase and tensin homologue (PTEN) in ~30% (Frattini et al. 2007).

Frameshift peptides. Numerous researchers have tried to tackle the shifts of the translational reading frame in microsatellites, which lead not only to loss of protein function but also to the translation of numerous carboxy-terminal neopeptide sequences with immunological potential; these are called frameshift peptides (FSP). The presence of pronounced FSP-specific immune responses in TILs and the peripheral blood of LS patients have suggested that FSP antigens may represent promising target structures for immunotherapy (von Knebel Doeberitz and Kloor 2013). Recently, Le *et al* found that carriers of MMR mutations are prone to respond to immunotherapy directed to a molecule called Programmed Death-1 (PD-1) located on the surface of killer T cells, commonly overexpressed in cancer cells. They demonstrated that pembrolizumab, an antibody that binds and blocks activation of PD-1 is effective in tumors harboring MMR deficiency, significantly improving progression free survival and overall survival (Le et al. 2015).

3. HEREDITARY COLORECTAL CANCER

3.1. Classification According to the Hereditary Pattern

According to the hereditary pattern of CRC, it can be classified in:

◈ ***Sporadic,*** comprising patients without familial aggregation of cancer. They arise from an accumulation of aberrant changes in tumor suppressor genes and oncogenes, and are usually developed at a median age of 70-75 years. These are the majority of cases with CRC, representing between 70-80% (Fig. 11) (Watson and Collins 2011).

◈ ***Familial,*** considered when a special predisposition to develop cancer at young age is apparent, with at least two blood relatives diagnosed with CRC or adenoma. It is thought to be associated with genetic and/or environmental modifiers and represents around 25% of cases (Aaltonen et al. 2007; Joensuu et al. 2008; Kheirelseid, Miller, and Kerin 2013; Lichtenstein and Kisseljova 2001; Valle 2014).

◈ ***Hereditary,*** caused by high penetrance susceptibility genes and/or showing Mendelian inheritance patterns. Accounts for 2-6% of all CRC and can be classified according to its tendency to develop polyps, in polyposic and non polyposic CRC (Kinzler and Vogelstein 1996; H T Lynch et al. 2009).

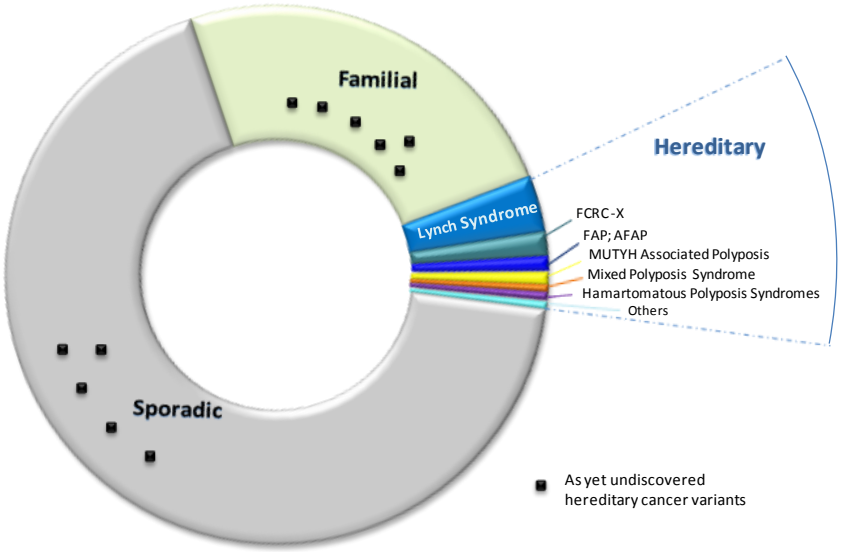

Figure 11. Proportions of sporadic, familial and hereditary colorectal cancer. Adapted from Lynch, 2009.

3.2. Hereditary Colorectal Cancer Syndromes

3.2.1. Polyposic syndromes

The adenomatous polyposis include the familiar adenomatous polyposis (classical and attenuated), the *MUTYH* associated syndrome, the polymerase proofreading associated polyposis and the hereditary mixed polyposis syndrome. Other types of polyposis are the hamartomatous, which include Peutz-Jeghers syndrome, juvenile polyposis, *PTEN* hamartoma tumor syndrome (Cronkhite-Canada Proteus and Cowden/Bannayan-Riley-Ruvalcaba syndrome), and the serrated polyposis (Valle 2014) (Table 4).

Table 4. Hereditary colorectal cancer syndromes, associated genes, type of inheritance, cumulative risk of colorectal cancer and average age at diagnosis.
Adapted from Valle, 2014 and Syngal, 2015.

Syndrome	Gene	Inheritance	Cumulative lifetime risk of CRC	Average age at diagnosis (years)
Sporadic Cancer			**4.8%**	**69**
Polyposic syndromes				
Familial adenomatous polyposis (FAP)	*APC*	Autosomal dominant De novo mutations Mosaicism	100%	38-41
Attenuated FAP			69%	54-58
MUTYH-associated polyposis	*MUTYH*	Autosomal recessive	43-100%	48-50
Polymerase proofreading associated polyposis	*POLE* *POLD1*	Autosomal dominant De novo mutations	Not estimated	Not estimated
Hereditary mixed polyposis	*GREM1*	Autosomal dominant	Not estimated	48
Peutz-Jeghers syndrome	*STK11*	Autosomal dominant	39%	42-46
Juvenile polyposis	*BMPR1A* *SMAD4* *ENG*	Autosomal dominant	38-68%	34-44
PTEN hamartoma tumour syndrome	*PTEN*	Autosomal dominant	9-16%	44-48
Serrated polyposis syndrome	Not known	Not defined	->50%	48
Non-polyposic syndromes				
FCRC-X	Unknown	Autosomal dominant	->20%	61
Lynch syndrome	*MLH1/MSH2*	Autosomal dominant	M:27-74% F: 22-61%	27-60
	MSH6	Autosomal dominant	M: 22-69% F: 10-30%	50-63
	PMS2	Autosomal dominant	M: 20% F: 15%	47-66

Familial Adenomatous Polyposis (FAP) is the second most frequent CRC syndrome, accounting for less than 1% of the total CRC cases (de la Chapelle 2004; Henry T Lynch and A de la Chapelle 2003; Rustgi 2007). A proportion of cases (~18%) arise from *de novo* mutations, but predominantly is an autosomal dominant condition caused by germline frameshift and nonsense mutations in *APC* gene. It encodes for a tumor suppressor protein that acts as an antagonist of the Wnt signaling pathway, implicated in cell proliferation and migration (Galiatsatos and Foulkes 2006; Segditsas and Tomlinson 2006). The classical FAP is characterized by the presence of more than 100 adenomas at a median age of diagnosis of 36 years (Galiatsatos and Foulkes 2006; Vasen et al. 2008), conferring them a statistical increased

risk to develop CRC (H T Lynch and de la Chapelle 2003). When a less aggressive phenotype is present, with 10-99 adenomas at older age than the classical FAP, the syndrome is called Attenuated FAP (AFAP) (Bouguen et al. 2007; Burt et al. 2004; Knudsen, Bisgaard, and Bulow 2003). Both presentations are susceptible to extracolonic manifestations such as cutaneous lesions, gastroduodenal adenomas, osteomas, dental anomalies, retinal hypertrophy, desmoids tumors and cancer of stomach, pancreas, liver, small bowel, thyroid and central nervous system; although in AFAP are less frequent events (Lynch et al. 1995).

MUTYH Associated Polyposis (MAP) is a recessive autosomal condition, caused by biallelic germline mutations in *MUTYH* gene, which belongs to the Base Excision Repair (BER) family and is responsible for preventing G:C → T:A transversions by removing adenines from mispairs with 8-oxoguanine during oxidative DNA damage (Al-Tassan et al. 2002; Jones et al. 2002). There are two predominant variants that account for ~70% of MAP cases, the c.536A>G (p.Tyr179Cys) and c.1187G>A (p.G396D) (reference sequences NM_001128425.1 and NP_001121897) (Nielsen et al. 2009). These are missense mutations found in 1-2% of Caucasian European population. In Spain, another common MAP mutation is the c.1227_1228dup (p.Glu410Glyfs*43) (Gomez-Fernandez et al. 2009). Clinically, MAP patients usually have few to hundreds of polyps at the moment of diagnosis, which typically appear around the age of 50 (Out et al. 2012), however while 60% debut with CRC, up to half of them will have 0 to less than 10 polyps at the time of diagnosis (Cleary et al. 2009; Morak et al. 2010; Nielsen et al. 2009). MAP CRCs usually have proximal localization, lymphocytic infiltration, mucinous histology and extracolonic manifestations, such as duodenal and endometrial adenomas, as well as malignancies of bladder, ovaries and skin (Aretz et al. 2006; Morak et al. 2010; Nielsen et al. 2009). Tumors typically show *KRAS* c.34G>T transversion in codon 12 (64% prevalence) (Lipton et al. 2003; Nielsen et al. 2011; van Puijenbroek et al. 2008).

Polymerase Proofreading Associated Polyposis is a recently described syndrome, caused by germline mutations in DNA polymerase ε (*POLE*) and δ (*POLD1*) genes. It conveys an autosomal dominant predisposition to develop multiple adenomas, large adenomas, early onset CRC and multiple CRC tumors. *POLD1* mutations have been reported also in patients with endometrial tumors (Briggs and Tomlinson 2013; Palles et al. 2012; Valle et al. 2014). So far, all pathogenic mutations found, are localized in the exonuclease domain of the respective enzyme, suggesting a deficient proofreading during DNA replication. It has been reported that

the arising tumors accumulate a median of 5000 somatic base substitutions with a high number of G:C>T:A and A:T>C:G transversions (Cerami et al. 2012). Little evidence has been reported about somatic *POLD1* mutations as compelled for CRC, nonetheless *POLE* somatic mutations have been found in both colorectal and endometrial tumors (Bloom 2012).

Hereditary Mixed Polyposis is a rare syndrome that shows an autosomal dominant inheritance pattern with variable penetrance. It is associated to heterozygous duplications spanning the 3' end of the *SCG5* gene until a region immediately upstream the *GREM1* locus. It has been proposed that *BMPR1A* and *CRAC1* mutations are also related to this syndrome (Cheah et al. 2009; Jaeger et al. 2003, 2012; O'Riordan et al. 2010). Phenotypically, is characterized by a mixture of colorectal lesions (including Peutz-Jeghers polyps, juvenile polyps, hyperplastic or serrated lesions, classic adenomas and CRC), as well as polyps containing mixed patterns; without any other extracolonic manifestation (Whitelaw et al. 1997).

Peutz-Jeghers Syndrome (PJS) is an autosomal dominant syndrome caused by germline mutations in *STK11* gene, which is involved in cell cycle regulation, cellular polarity and apoptosis (Lindor 2009b). Patients usually develop cutaneous lesions and hamartomatous polyps in childhood or adolescence that affects the entire GI tract. These polyps usually have a strong mucinous component, abundant connective tissue retaining cysts and chronic eosinophilia. Hamartomas could be malignant precursors as adenomas, as well as adenomatous component within hamartomatous polyps may be responsible for malignancy, but the truth is these patients have a high cumulative risk of GI cancer (Hearle et al. 2006; van Lier et al. 2012; Patel and Ahnen 2012). Extra-GI cancer has been reported in pancreas, breast, ovaries, lung, cervix, endometrium and testicles (Beggs et al. 2010).

Juvenile Polyposis Syndrome (JPS) is the most common hamartomatous syndrome affecting 1 of 100,000 persons (Burt et al. 1990). Is an autosomal dominant condition, associated with germline mutations in one of three genes related to the transforming growth factor-beta (*TGF-β*), *SMAD4*, *BMPR1A* or *ENG*. It is characterized by the presence of some (3-10) juvenile polyps (polyps with abundant edematous lamina propria, inflammatory cells and cystically dilated glands lined by cuboidal to columnar epithelium with reactive changes) at young age, with a cumulative lifetime risk of 38-68% (van Hattem et al. 2011; Syngal et al.

2015) and it is usually associated to strong family history of the disease (Howe, Mitros, and Summers 1998).

PTEN hamartoma tumor syndrome (PHTS) includes both Cowden syndrome (CS) and Bannayan-Riley-Ruvalcaba syndrome (BRRS). It is caused by germline mutations in *PTEN*. They account for rare autosomal dominant conditions in adulthood and childhood, respectively. PHTS has a high penetrant pattern of a clinically variable spectrum, with predisposition to develop hamartomatous, hyperplastic, adenomatous, ganglioneuromatous and inflammatory polyps in the colon at young age (Heald et al. 2010; Ngeow et al. 2013). Carriers have a higher risk for CRC, EC, melanoma, thyroid, renal cell and breast cancer, as well as developmental disorders and macrocephaly (Nieuwenhuis et al. 2014; Tan et al. 2012).

Serrated Polyposis Syndrome (SPS) is thought to be a hereditary condition as prevalent as JPS (1 in 100,000) (Snover 2011), but the genetic basis remains unidentified. The revised criteria from the World Health Organization are: at least five serrated polyps proximal to the sigmoid colon with ≥2 of these being >10 mm; any number of serrated polyps proximal to the sigmoid colon in an individual who has a first-degree relative (FDR) with serrated polyposis; and more than 20 serrated polyps of any size, but distributed throughout the colon (Snover 2011). Furthermore, three different phenotypes within this syndrome have been related to different molecular features: large polyps in the right colon associated to *BRAF* mutations and higher risk of CRC; small polyps in the left colon associated to *KRAS* mutations; or, a mixture of the above mentioned (Boparai et al. 2010; Carvajal-Carmona et al. 2007; Kalady et al. 2011).

3.2.2. Non polyposic syndromes

There are two main inherited syndromes whose affected show no special predisposition to form polyps, familial CRC type X (fCRC-X) and Lynch syndrome (LS) (Table 3). LS will be further explained in the next topic.

FCRC-X. It is an autosomal dominant inherited syndrome from an unestablished genetic basis. Lindor *et al* coined the name of this disease in 2005 to portray families that meet Amsterdam I criteria, reflecting a strong familial aggregation, and have microsatellite stable (MSS) CRCs (Lindor et al. 2005). FCRC-X tumors appear mostly in the distal colon and rectum at younger age than sporadic affects (~10 years earlier diagnosis) and patients have no apparent

propensity to exhibit extracolonic tumors (Francisco et al. 2011; Koh et al. 2011; Lindor 2009a). Histological features and mutational tumor profiles indicate that this is a very heterogeneous disease. Recently, germline alterations in *POLE*, *POLD1*, *SEMA4A, RPS20* and *FAN1* have been reported as responsible of a small number of fCRC-X families (Bellido et al. 2015; Nieminen et al. 2014; Palles et al. 2012; Schulz et al. 2014; Seguí, Mina, et al. 2015; Spier et al. 2015; Valle et al. 2014). Moreover, 28 genes have been reported likely involved in fCRC-X, among them is *BARD1* (Esteban-Jurado et al. 2015). Other alternative explanations for the CRC predisposition in these families are accumulation of low penetrance alleles, epigenetic mechanisms or common environmental factors among these CRC susceptible families (Valle 2014).

4. LYNCH SYNDROME

4.1. History

Dr. Aldred Scott Warthin, from the University of Michigan, published in 1913 the first known case report of a large pedigree including multiple cases of CRC in the absence of polyposis, as well as cases of gastric and endometrial cancer (EC), under the name of Family G (Warthin A. S. 1913, 1925). Fifty-three years later, Dr. Henry Lynch reported two American Midwestern large families (Families N and M) whose members had very similar spectrum of tumors to Family G's, so he proposed that this affection could be associated to an autosomal dominant cancer family syndrome. It wasn't until 1984 that this syndrome was coined as Lynch Syndrome (LS), and had a subdivision named Lynch I, whose patients had only CRCs and Lynch II, referring to those families with additional extracolonic tumors (Boland and Troncale 1984; Lynch et al. 1985).

In 1991, the term hereditary non polyposis colorectal cancer (HNPCC) was forged by an international collaborative group of researchers to distinguish them from the FAP ones, and Amsterdam clinical criteria arise to ease its diagnosis (Topic 5.1) (Vasen et al. 1991). Later on Amsterdam criteria II (ACII) (Topic 5.1) were broadened to recognize a diagnostic role for extracolonic tumors (Vasen et al. 1999a).

Ulterior advances in molecular genetics, led to the identification of two loci on chromosomes 2p and 3p by means of genome-wide search and linkage analysis (Lindblom et al. 1993; Peltomaki et al. 1993) linking the genes *MSH2* and *MLH1* to LS. Within the same period, it was reported that LS tumors had distinct histopathologic and molecular features, as somatic mutations in simple repetitive sequences that were named replication error phenotype (RER) (Aaltonen et al. 1993; Ionov et al. 1993), now known as microsatellite instabillity. Defective DNA repair was associated to this special characteristic allowing the recognition of MMR gene mutations as responsible for the disease (Bronner et al. 1994; Fishel et al. 1993; Leach et al. 1993; Papadopoulos et al. 1994). Within the same year, Nicolaides *et al* described two LS cases harboring mutations in *PMS1* and *PMS2*, each, being recognized at that time as MMR genes involved in LS pathogenesis (Nicolaides et al. 1994). Later on, a family without Amsterdam

criteria but multiple members affected of LS non-CRC tumors was reported in association to a *MSH6* deletion (Miyaki et al. 1997).

MSI association to LS tumors transformed the diagnosis of the disease. In 1997, the US National Cancer Institute hosted an expertise committee to develop standard methods for MSI testing, and Bethesda guidelines emerged (Boland et al. 1998; Rodriguez-Bigas et al. 1997). These criteria were further modified in 2004, to include clinicopathological features for patient selection and a consensus MSI testing panel (Topics 5.1 and 5.2) (Umar et al. 2004).

Importantly, in 2002 Gazzoli *et al* reported an alternative cause for LS, describing a case with constitutional *MLH1* methylation (Gazzoli et al. 2002). Later on, Hitchins showed that these epimutations could be transmitted to the next generation (Hitchins et al. 2007). Furthermore, heritable *MSH2* epimutations were described in 2006 (Chan et al. 2006) and three years later *MSH2* promoter methylation in LS families was associated to germline deletions of the 3' end of *EPCAM* gene, contiguous to *MSH2* (Ligtenberg et al. 2009).

In an international meeting in 2013, a series of LS patients manifesting more than 10 adenomatous polyps was described, grieving the name of HNPCC, reason why now Lynch syndrome is the accepted term to designate families affected with germline heterozygous mutations in MMR genes (Kastrinos and Stoffel 2014).

4.2. Prevalence

LS is the most common inherited CRC syndrome, accounting for 2-6% of all CRC cases (D'Emilia, Rodriguez-Bigas, and Petrelli 1995; Jasperson et al. 2010; Henry T Lynch and Albert de la Chapelle 2003; Lynch and Smyrk 1996; H T Lynch et al. 2009; Tomoda, Baba, and Oshiro 1996). In an international pooled data analysis, comprising 10,206 unrelated CRC patients from the Colon Cancer Family Registry, EPICOLON, the Ohio State University, and the University of Helsinki, the prevalence of LS was of 3.1% (L Moreira et al. 2012). The EPICOLON consortium, comprised by 1872 CRC patients, estimated a prevalence of LS in Spain of 2.5% (Pinol et al. 2005).

4.3. Genetic Characteristics

Mutational Spectrum. LS is an autosomal dominant condition caused by germline mutations in MMR genes, specifically *MLH1*, *MSH2*, *MSH6* and *PMS2* (Table 5), as well as epimutations in *MLH1* and *MSH2*, the later associated to *EPCAM* deletions.

Table 5. Summary of the main characteristics of MMR genes involved in LS.

Gene symbol	Name	Reference number	Chromosomal position	Strand	Coding exons	DNA length (bp)	RNA length (bp)	Protein length (aa)	Protein domains
MLH1	MutL homolog 1	NM_00249.3	3p21.3	FW	19	75,557	2,752	756	ATPase domain Interaction domain for MSH2, MSH3, MSH6 Interaction domain for PM2, MLH3, PMS1
MSH2	MutS homolog 2	NM_000251.2	2p21	FW	16	80,259	3,307	934	DNA binding domain Interaction domain for MSH3 and MSH6 Interaction domain for MLH1 and PMS2
MSH6	MutS homolog 6	NM_000179.2	2p16	FW	10	23,871	7,476	1360	Helix-turn-helix domain associated with a Walker-A motif (adenone with Mg binding motif) with ATPase activity PCNA biniding motif PWWP domain that bound to dsDNA
PMS2	Postmeiotic segregation increased 2	NM_000535.5	7q22.1	RV	15	35,886	2,855	862	ATP interaction domain MLH1 binding domain Exonuclease domain

FW: forward; RV: reverse; bp: base pairs; aa: aminoacids; ATP: adenosine triphosphate; PCNA: proliferating cell nuclear antigen; PWWP: proline-tryptophan-tryptophan-proline; ds: double-stranded.

MLH1 and *MSH2* mutations account for more than 80% of LS cases. *MSH6* represents ~9%, *PMS2* less than 5% and *EPCAM* mutations are responsible for about 1-3% of LS families (Fig. 12). MMR gene mutations affect all races, although frequencies vary among genders and geographic regions (Duraturo et al. 2011; Genuardi et al. 1998; Henry T Lynch, Lynch, and Attard 2009; Nicolaides et al. 1994; Niessen et al. 2009; Peltomaki and Vasen 2004; Talseth-Palmer et al. 2010).

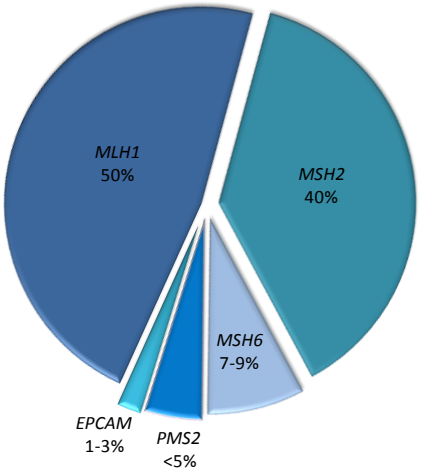

Figure 12. Proportion of MMR gene pathogenic mutations found in Lynch syndrome.
Data from (Kohlmann and Gruber 2014).

LS pathogenic genetic mutations usually are nonsense, frameshift or splicing variants. Gross rearrangements are also causative and especially frequent in *MSH2* gene, due to the high content of Alu elements. In Figure 13, the proportions found in MMR genes of each type of variant are schematized (Auclair et al. 2006; Lastella et al. 2006; Spurdle 2010; Tournier et al. 2008; Woods et al. 2007).

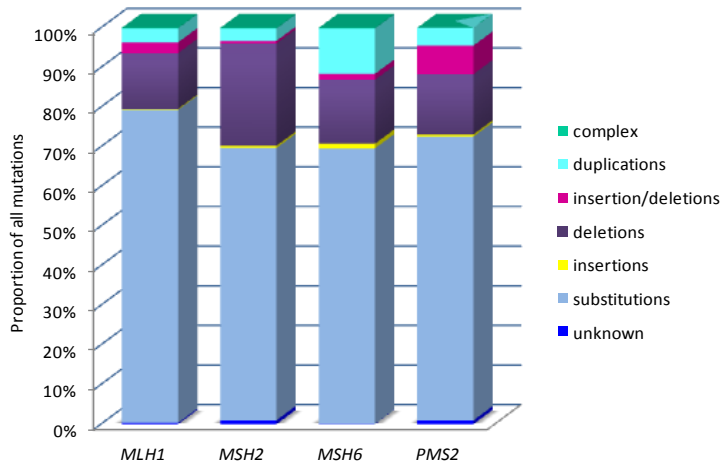

Figure 13. Proportion of type of DNA variants found in MMR genes according to LOVD classification.
Source: LOVD (August 2015); (Fokkema et al. 2011).

Interestingly, there are recurrent mutations that reappear due to genetic circumstances or other factors, like the A>T transversion in a splice site of intron 5 of *MSH2* (c.942+3A>T), explained by the fact that this adenine is the first of 26 adenines in a stretch, creating a hotspot for this particular change, possibly by *de novo* mutation produced by polymerase slippage during replication (Desai et al. 2000). Moreover, there are also mutations shared by ostensibly unrelated cases inherited form a common ancestor many generations before, recognized as founder mutations. The likelihood for them to become common is greater in isolated or rapidly grown populations. Several founder mutations have been detected in MMR genes, in specific populations (Ponti et al. 2015). In Spain, two founder mutations in *MLH1* have been reported, these are: c.306+5G>A and c.1865T>A. Besides, there have been found 5 founder mutations in *MSH2*: c.2063T>G, c.[2635-3T>C; 2635-5C>T], deletion of exons 4 to 6, deletion of exon 7 and the deletion of exons 8 to 9.

Epimutations. As mentioned, constitutional methylation refers to an epigenetic alteration present throughout normal tissues, which result in silencing of normally expressed genes or activation of otherwise silent genes. In LS, constitutional methylation of *MLH1* and *MSH2* genes is responsible for a small proportion of cases (Chan et al. 2006; M P Hitchins, Owens, C.-T. T. Kwok, et al. 2011; Ligtenberg et al. 2009; Suter, Martin, and Ward 2004; Robyn L. Ward et al. 2013).

Hereditary patterns of MMR epimutations can differ depending upon their underlying origin. Primary epimutations, of an unknown cause, are usually responsible for dense *MLH1* hemiallelic promoter methylation (Goel et al. 2011; M P Hitchins, Owens, C.-T. T. Kwok, et al. 2011); and are not usually inherited in a Mendelian fashion (Hitchins and Ward 2009; Hitchins et al. 2007; Morak et al. 2008). The last, does not apply for secondary constitutional epimutations, that cosegregate with an in *cis* genetic change, giving an autosomal dominant pattern of inheritance. Example of this genetically facilitated epimutations is the one that appear after 3'*EPCAM* deletions that conduce to *MSH2* promoter methylation; they seem to be specific of EPCAM-expressing tissues (Chan et al. 2006; Ligtenberg et al. 2009). Moreover, different in *cis* genetic alterations have been reported in *MLH1* as responsible of its promoter methylation:

- Gross rearrangements as the deletion of c.-67 to the intron 2 or the complete duplication of *MLH1* (Gylling et al. 2009; Morak et al. 2011).
- The variants c.-27C>A/c.85G>T in the promoter region (Megan P Hitchins et al. 2011). Reduced transcriptional activity has been associated only to c.-27C>A by reporter assays (Robyn L. Ward et al. 2013). The compound of these variants is a European founder haplotype (C.-T. Kwok et al. 2014).

4.4. DNA Mismatch Repair Pathway

MMR genes are involved in different cellular processes. They modulate DNA recombination, DNA damage signaling, and have a role in apoptosis regulation (Altieri et al. 2008; Jun, Kim, and Ban 2006; Kolas and Cohen 2004). However, their most important function is to restore replication fidelity when the polymerase fails. Polymerase errors occur during DNA replication when this enzyme unpaired nucleotides in the DNA chain. This can happen either by simple mismatches or by strand slippage, that convey small insertions or deletions in the newly synthetized DNA strand (Chung and Rustgi 2003).

MMR is a well conserved pathway, fundamental to maintain genome integrity by correcting replication or recombination base-base errors and small insertion deletion loops (Kim, Laird, and Park 2013). There are four basic steps to repair a mismatch: 1) When an error escapes polymerase proofreading, the heterodimers MSH2/MSH6 (MutSα) and MSH2/MSH3 (MutSβ) recognize the mismatch; MutSα focuses on mismatches and single-base loops, whereas the second dimer recognizes indels (Kunkel and Erie 2005). It is thought that MutSα is formerly charged before replication starts through MSH6-PWWP interaction with histone marks, and SETD2 is required for the interaction (Li et al. 2013). 2) MutS heterodimers recruit MLH1/PMS2 (MutLα) dimmers and slide as a clamp on DNA. 3) The tetramer formation in presence of RFC and PCNA, stimulates endonuclease activity of MutLα leading to many incisions on the newly made strand, generating entry points for EXO1, which degrades the error stretch, so resynthesis is initiated. 4) Finally, MutS/MutL complex dissociates from DNA (Genschel and Modrich 2003) (Fig. 14).

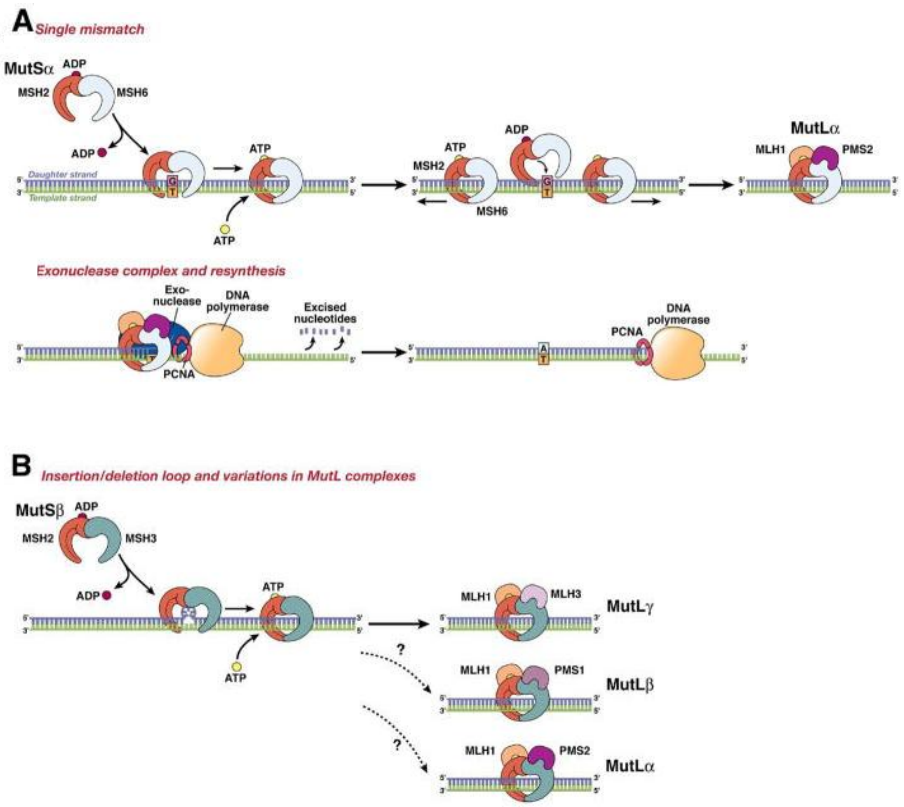

Figure 14. Schematization of DNA mismatch repair pathway. A) Stages after recognition of single base-pair mismatches and B) Variations on the DNA MMR theme.
Modified from Boland and Goel, 2010.

There are additional MMR genes proposed to play a role in LS predisposition. Some studies have reported germline *MLH3*, *MSH3*, *EXO1*, *PMS1*, or *TGFBR2* variants in LS families, but the clinical significance of mutations in these genes is unclear (Duraturo et al. 2011; Lu et al. 1998; Peltomäki 2003; Thompson et al. 2004).

4.5. Molecular Characteristics of LS Tumors

Considering that MMR genes act as tumor suppressor genes, germline mutations in one allele confer a predisposition to be affected of LS. At the molecular level, they require alterations in both alleles in order to lose MMR protein function; this event is known as Knudson's two hits theory (Knudson 2001). This second event could be due to loss of heterozygosity (LOH), somatic mutations or methylation of gene promoter regions (de la

Chapelle 2004). Biallelic inactivation of MMR genes lead to loss of MMR protein expression in tumor tissue.

Polymerase errors are relatively common in microsatellites (Chung and Rustgi 2003). Failure of DNA mismatch repair protein activity in LS, results in the accumulation of errors especially in these repetitive sequences, therefore producing microsatellite instability. It has been acknowledge that MSI increase the mutation rate in the order of 100 to 1,000 fold (Le et al. 2015; Pawlik, Raut, and Rodriguez-Bigas 2004; Shibata et al. 1994). This high mutation rate entails an increase probability of other tumor suppressor genes or oncogenes to be also affected; when this occur tumorigenesis is prompted (Lynch et al. 2010).

MMR deficiency (loss of expression of MMR proteins and/or MSI) is a typical feature of LS tumors, being found in 77% of *MSH6* and *PMS2* affected cases to 89% of the *MLH1* or *MSH2* cases (Aaltonen et al. 1993; EGAPP 2009; Tannergård et al. 1997). However, MMR deficiency is not pathognomonic of LS since 10-15% of sporadic CRCs also exhibit MSI (Hampel, Frankel, et al. 2005; Hutchins et al. 2011; Perez-Carbonell et al. 2012; Samowitz et al. 2001; Sinicrope et al. 2011). As commented in topic 2.2, this event is not related to MMR germline mutations, but rather to *MLH1* inactivation by its promoter methylation. Furthermore, this cases are thought to be associated with *BRAF* p.V600E mutation in 50 to 68%, while is almost disjointed of LS tumors (G Deng et al. 2004; M Gausachs et al. 2012; Y. H. Kim et al. 2008; Loughrey et al. 2007). In Figure 15 the independent molecular pathways leading to MSI in both LS and sporadic CRCs, are schematized.

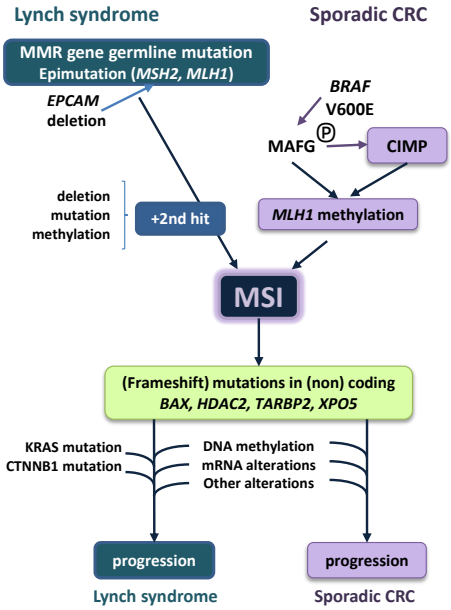

Figure 15. Different MSI pathways for Lynch syndrome and sporadic colorectal cancers.
Modified from Yamamoto *et al*, 2015.

4.6. Clinical Characteristics of Lynch Syndrome

Despite its heterogeneous nature, LS has a clinical signature that eases its identification. Affected individuals generally develop tumors at a young age of onset. In fact, the median age of the first tumor diagnosis is of 45 years, 24 years earlier than general population.

Cumulative lifetime risk of CRC found in a recent meta-analysis varies from 10 to 74%, conditional not only upon affected MMR gene but also by gender and geographical region (Table 3, in topic 3.2) (Syngal et al. 2015). In a French study, comprising 537 families with *MLH1*, *MSH2* and *MSH6* segregating mutations, the cumulative risk of colorectal cancer at 70 years of age was of 41% for *MLH1* mutation carriers, 48% for *MSH2*, and 12% for *MSH6* (Bonadona et al. 2011).

LS colorectal tumors are predominantly right sided. Patients have an elevated risk to develop multiple synchronic neoplasias (diagnosed at once) and metachronic ones (more than 6 months after previous tumor resection). At the histopathological level, CRCs generally are

poorly differentiated, have a mucinous component, with signet ring cells, tumor infiltrating lymphocytes (TILs) and intense lymphocytic reaction (Crohn-like) (Risio et al. 1996).

CRC in LS patients is associated with a better prognosis than sporadic colon cancers (Watson et al. 1998). It has been demonstrated that the typical MMR deficient status in these tumors, is a favorable prognostic marker for stage II and III colon cancer patients treated with surgical approaches (Clark et al. 2004). In contrast, they do not seem to benefit from adjuvant fluorouracil (FU) based chemotherapy (commented in topic 2.5) (Arnold, Goel, and Boland 2003; Sargent et al. 2010; Tajima et al. 2004).

Furthermore, although LS individuals usually form less adenomas and at an older age than FAP cases, precursor lesions evolve to malignancy in a highly accelerated manner, with adenoma- carcinoma sequences lasting less than 3 years, in contrast with CRCs from sporadic origin where usually carcinomas arise after 7 to 10 years (Johnson and Fleet 2013; H T Lynch et al. 2009; Leticia Moreira et al. 2012).

Patients with LS also have an increased risk of EC, between 14-71% depending on the affected gene, compared to the general population of 2.7% (Table 4), and several other cancers, such as ovarian, upper urinary tract, gastric, small bowel, biliary/pancreatic tracts, sebaceous and central nervous system tumors (Umar et al. 2004). The last two when associated to LS, are called Muir-Torre and Turcot syndrome, respectively, and are considered clinical variants of LS, accounting for less than 1% of the extracolonic LS manifestations (Hamilton et al. 1995; Koornstra et al. 2009; Schwartz and Torre 1995). It is worth mentioning that Turcot syndrome could be caused also by *APC* mutations, when so, affected cases develop different tumor features from the MMR gene mutated, with a special predisposition to manifest polyps (Hamilton et al. 1995). More recently, sarcomas, breast and prostate tumors, have been found in LS families, but are not still officially accounted within the LS spectrum of tumors (den Bakker et al. 2003; Geary et al. 2008; Harkness et al. 2015; Hirata et al. 2006; Soravia et al. 2003; Westenend et al. 2005). Preference in tumor spectrum regardless of all tissues carrying equally the predisposing mutation, remains unclear.

LS shows an incomplete penetrance pattern, therefore some carriers of MMR monoallelic pathogenic mutations may never develop cancer (Hampel, Stephens, et al. 2005;

Stoffel et al. 2009, 2010). The other side of the coin is genetic anticipation reported in some LS families (Bozzao, Lastella, and Stella 2011; Gruber and Mukherjee 2009). In this phenomenon, diagnosis is made at an earlier age as it is passed on to the next generation; in most cases, a more aggressive phenotype is also noted (Strachan and Read 1999). The molecular mechanism responsible for this event in MMR mutation carriers remains unclear.

Table 6. Genotype-phenotype correlation. Cancer risk up to 70 years of age in LS individuals with different affected MMR genes, compared to the general population.

Extracted from NCCN Guidelines v1.2015

Cancer	General population risk (%)	*MLH1* or *MSH2*		*MSH6*		*PMS2*	
		Risk (%)	Mean age of onset (years)	Risk (%)	Mean age of onset (years)	Risk (%)	Mean age of onset (years)
Colon	5.5	40-80	44-61	10-22	54	15-20	61-66
Endometrium	2.7	25-60	48-62	16-26	55	15	49
Stomach	<1	1-13	56	≤3	63	∫	70-78
Ovary	1.6	4-24	42.5	1-11	46	∫	42
Hepatobiliary tract	<1	1.4-24	50-57	NR	NR	∫	NR
Urinary tract	<1	1-4	54-60	<1	65	∫	NR
Small bowel	<1	3-6	47-49	NR	54	∫	59
Bran/CNS	<1	1-3	~50	NR	NR	∫	45
Sebaceous neoplasm:	<1	1-9	NR	NR	NR	NR	NR
Pancreas	<1	1-9	NR	NR	NR	NR	NR

∫ The combined risk for renal, pelvic, stomach, ovary, smal bowell, ureter, and brain is 6% (Senter *et al*, 2008)

LS, caused by heterozygous mutations in MMR genes must be differentiated from ***constitutional MMR deficiency (CMMR-D)***, caused by biallelic germline mutations in one of the MMR genes. In this condition, the most frequently reported malignancies are haematological and primary brain tumors, usually arising in childhood around 5.5 and 8 years of age, respectively; as well as very early onset (mean age 16 years) CRC (Wimmer and Etzler 2008). CRC and other LS associated tumors are commonly found in patients who survive the first neoplasia. While their tumors are MSI and ultrahypermutated (Shlien et al. 2015), MSI and loss of expression of MMR proteins are evident both in normal and tumor tissue. Furthermore, most of the cases have *café au lait* spots usually related to neurofibromatosis type 1 (NF1); although so far, no CMMR-D tumor has been found to have NF1 genotype, one explanation stated for this event is probable somatic mosaicism (Wimmer et al. 2014).

4.7. Genetic Counseling in Lynch Syndrome

Genetic counseling is the process of helping to understand and adapt to health, medical and psychological consequences of having a special cancer risk due to a hereditary

predisposition (Resta et al. 2006). Patients are referred to the genetic counseling unit in order to receive education about inheritance, genetic testing, cancer prevention and ongoing research. Additionally, genetic counseling includes interpreting medical and family history to assess the risk of occurrence or recurrence of cancer or multiple associated pathologies within the syndrome. It also offers appropriate advice to promote informed decisions and adaptation to the elevated risk.

Genetic counseling should ensure the monitoring of the person and the family also in the long term in order to: update the family history and assess changes, review the medical monitoring regularly, assess and promote adherence to preventive measures and early detection, and provide psychological help if needed.

In LS, since associated tumors tend to develop at early ages and progress faster than sporadic tumors, specialized surveillance must be warranted not only for mutation carriers but also for their at-risk relatives. As noted, surveillance has proven effective in reducing CRC incidence and mortality in LS families (Järvinen et al. 2000; Mecklin et al. 2007; Stoffel et al. 2010; Vasen et al. 2010) and consensus guidelines recommend colonoscopy every 1 to 2 years starting at 20 to 25 years of age (Giardiello et al. 2014).

Surgical treatment of CRC in LS patients depends on the location of the primary tumor, location of the synchronous lesions if any, extension, co-morbidities, and patient agreement to the secondary risks of each type of management. Given the elevated risk of developing metachronous CRC, from 40 to 72% at 10 and 40 years after initial surgical resection respectively (Rodriguez-Bigas and Möeslein 2013), optional subtotal colectomy should be considered (Giardiello et al. 2014; Win et al. 2013). For risk reduction of endometrial cancer, possibly the most effective strategy is prophylactic hysterectomy (Schmeler et al. 2006), however for women with incomplete parity at age 30 to 35 years, recommendations are to start performing annual transvaginal ultrasounds with endometrial biopsies (Giardiello et al. 2014).

If molecular diagnosis has not been performed in a suspicious high-risk candidate, then first this risk must be estimated upon correct and thorough family and personal cancer history.

5. MOLECULAR DIAGNOSIS OF LYNCH SYNDROME

5.1. Selection Criteria

Identification of Lynch patients and families has significant effect on their clinical management and may impact the surgical approach, recurrence of cancer surveillance and screening for extracolonic malignancies. The diagnostic algorithm starts with the suspicion of LS and this is usually based upon clinical evidence (Fig. 16). As previously commented Amsterdam I and II were the first consensus to clinically enlist LS criteria for research purposes; while they are too stringent for clinical use (Table 7). Both of them imply special familiar aggregation and identify around 60 to 80% of LS patients (Llor et al. 2005; Vasen et al. 1991, 1999b, 2013). As commented, due to the correlation of LS and its MSI tumor phenotype, Bethesda criteria were proposed to select tumors for MSI testing and afterward they were modified (named "revised") in order to maximize specificity, without losing sensitivity (Table 7) (Umar et al. 2004).

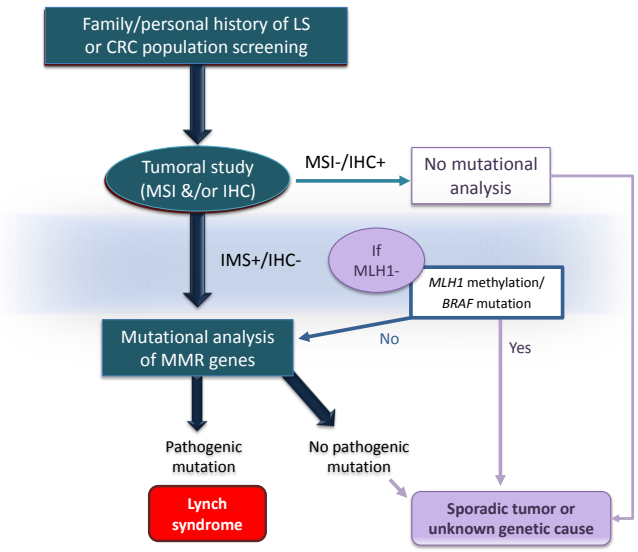

Figure 16. Diagnostic algorithm of Lynch syndrome.

Table 7. Breakdown of the Amsterdam Criteria I+II and Revised Bethesda Guidelines.
Adapted from Sehgal, 2014.

Amsterdam criteria I
At least three relatives with histologically verified colorectal cancer:
1. One is a first-degree relative of the other two;
2. At least two successive generations affected;
3. At least one of the relatives with colorectal cancer diagnosed at <50 years of age;
4. Familial adenomatous polyposis has been excluded.

Amsterdam criteria II
At least three relatives with a Lynch syndrome associated cancer [Φ]
1. One is a first-degree relative of the other two;
2. At least two successive generations affected;
3. At least one of the syndrome-associated cancers should be diagnosed at <50 years of age;
4. FAP should be excluded in any colorectal cancer cases;
5. Tumors should be verified whenever possible.

Revised Bethesda guidelines
Colorectal tumors from individuals should be tested for MSI in the following:
1. Colorectal cancer diagnosed in a patient who is <50 years of age.
2. Presence of synchronous or metachronous colorectal, or other LS-associated tumors[Ω] regardless of age.
3. Colorectal cancer with MSI-H histology‡ diagnosed in a patient who is <60 years of age.
4. Colorectal cancer diagnosed in one or more first-degree relatives with an HNPCC-related tumor, with one of the cancers being diagnosed under age 50 years.
5. Colorectal cancer diagnosed in two or more first- or second-degree relatives with HNPCC-related tumors, regardless of age.

[Φ] In Amsterdam II, LS-associated tumors are: large bowel, endometrium, small bowel, ureter or renal pelvis, ovary, brain, hepatobiliary tract and skin (sebaceous tumors).

[Ω] In the revsied Bethesda guidelines, LS-related tumors include colorectal, endometrial, stomach, ovarian, pancreas, ureter and renal pelvis, biliary tract, and brain (usually glioblastoma as seen in Turcot syndrome) tumors, sebaceous gland adenomas and keratoacanthomas in Muir–Torre syndrome, and carcinoma of the small bowel.

‡ Presence of tumor infiltrating lymphocytes, Crohn's-like lymphocytic reaction, mucinous/ signet-ring differentiation, or medullary growth pattern.

In 2006, three different groups proposed the use of predictive models, such as PREMM, MMRpro and MMRpredict. Prediction of these models relies on the oncological history, at personal and family level, to determine who should undergo genetic analysis (Balmana et al. 2006; Barnetson et al. 2006; Chen et al. 2006; Farrington et al. 2005).

The Evaluation of Genomic Applications in Practice and Prevention Working Group proposed in 2009 universal screening for all newly diagnosed CRCs (EGAPP 2009). and most recently all ECs (Batte et al. 2014), independently of personal or family history of LS associated tumors, in order to identify the most of LS patients that will benefit from genetic counselling and germline testing (Hampel and de la Chapelle 2011; Hampel 2010; Hampel et al. 2008). Many authors have proved the feasibility of universal screening for MMR deficiency detection

(Hampel et al. 2008; Heald et al. 2013; de la Chapelle, Palomaki, and Hampel 2009; Leticia Moreira et al. 2012; Robyn L Ward, Hicks, and Hawkins 2013), entailing higher diagnose costs but also lower morbidity and mortality among LS relatives (EGAPP 2009). The Jerusalem workshop in 2009, proposed the application of an age-of-diagnosis cut-off, testing all CRCs diagnosed at the age of 70 or younger, and older cases with at least 1 revised Bethesda criterion. This approach has a sensitivity of 95.1% and a specificity of 95.5% (Boland and Shike 2010; Leticia Moreira et al. 2012).

5.2. Molecular Tumor Testing

Analysis of microsatellite instability. In view of the high prevalence of MMR deficiency in LS, the first test in suspected patients is analysis of tumor for MMR activity, either by direct PCR of microsatellite repeats and/or by immunohistochemistry (IHC) of MMR proteins (Fig. 17) (Perez-Carbonell et al. 2012; Poulogiannis, Frayling, and Arends 2010).

After DNA extraction either from fresh tissue or formalin fixed paraffin embedded (FFPE) sections, MSI testing can be performed. The USA National Cancer Institute (NCI) recommended the use of Bethesda panel composed of five markers (three dinucleotide and two mononucleotide repeats) (Boland et al. 1998) (Table 8). When 30% or more of the repeats are unstable, tumors are classified as MSI-high (MSI-H). If fewer than 30% of them are unstable, are classified as MSI-low (MSI-L), and if no repeats are unstable, the tumor is considered as MSS. Being that MSI-L does not appear to predict LS, is often accounted as MSS and the LS algorithm ends for them.

Table 8. Microsatellite markers for MSI.
Extracted from Hegde *et al*, 2014.

Marker name	Length (base pair)	Forward sequence	Reverse sequence	Position (chr)	Gene near marker	MS repeat
NCI panel markers						
BAT25	110-130 (122)	VIC 5'-TCGCCTCCAAGAATGTAAGT-3'	5'-TCTGCATTTTAACTATGGCTC-3'	4q11-12	*KIT*	A (25)
BAT26	112-120 (117)	NED 5'-TGACTACTTTTGACTTCAGCC-3'	5'-AACCATTCAACATTTTTAACCC-3'	2p	*MSH2*	A (26)
D2S123	197-227	VIC 5'-AAACAGGATGCCTGCCTTTA-3'	5'-GGACTTTCCACCTATGGGAC-3'	2p16	*MSH2*	CA (n)
D17S250	130-170	FAM 5'-GGAAGAATCAAATAGACAAT-3'	5'-GCTGGCCATATATATATTTAAACC-3'	17q11.2-q12	*BRCA1*	CA (n)
D5S346	96-129	FAM 5'-ACTCACTCTAGTGATAAATCGGG-3'	5'-AGCAGATAAGACAGTATTACTAGTT-3'	5q21	*APC*	CA (n)
Quasimonomorphic mononucleotide markers						
BAT25	110-130 (122)	VIC 5'-TCGCCTCCAAGAATGTAAGT-3'	5'-TCTGCATTTTAACTATGGCTC-3'	4q11-12	*KIT*	A (25)
BAT26	112-120 (117)	NED 5'-TGACTACTTTTGACTTCAGCC-3'	5'-AACCATTCAACATTTTTAACCC-3'	2p	*MSH2*	A (26)
NR-21	103	5'-TAAATGTATGTCTCCCCTGG-3'	VIC 5'-ATTCCTACTCCGCATTCACA-3'	14q11.2	*SLC1A8*	T (21)
NR-22	142	5'-GAGGCTTGTCAAGGACATAA-3'	FAM 5'-AATTCGGATGCCATCCAGTT-3'	11q24-q25	*STT3A*	T (22)
NR-24	132	5'-CCATTGCTGAATTTTACCTC-3'	VIC 5'-ATTGTGCCATTGCATTCCAA-3'	2q11.2	*ZNF-2*	A (24)

However, some limitations appeared with the use of dinucleotide markers that showed lower sensitivity and specificity compared with mononucleotide ones (Perucho 1999; Suraweera et al. 2002). For this reason, quasimonomorphic panel, consisting in five mononucleotide repeats (Table 8) was proposed. It enhance sensitivity, especially in MSH6 deficient tumors and allows the use of only tumor tissue DNA without matched normal (Buhard et al. 2004; Ebinger et al. 2006; Goel et al. 2010; Umar et al. 2004; Wong et al. 2006; Xicola et al. 2007; You et al. 2010).

Both panels are highly concordant with the designation of MSI-H (Buhard et al. 2004; Ebinger et al. 2006; Pyatt et al. 1999; Søreide 2007). Nevertheless, BAT26 mononucleotide appears to be the marker with better MSI-H correlation with respect to MSS tumors. However, its isolated use is not recommended, because in some MSI LS tumors can be unaffected (Bartley et al. 2012; Laghi, Bianchi, and Malesci 2008). Furthermore, in ~28% of African Americans tumors, BAT25 and BAT26 can be polymorphic at one of the loci and therefore could be incorrectly classified as instable (Pyatt et al. 1999).

Immunohistochemistry analysis. Loss of MMR protein expression assessed by IHC is reported evident in more than 90% of CRC tumors with clearly pathogenic mutations in the unstained gene product, and correlates with >90-95% of MSI-H phenotype (Cicek et al. 2011).

The analysis is performed on tissue sections that are incubated with monoclonal antibodies against MLH1, MSH2, MSH6 and PMS2. Further examination of MMR proteins expression in the nucleus of tumor cells and adjacent tissue is made by a pathologist, who

defines presence or absence of these proteins in tumor tissue (Fig. 17) (Debniak et al. 2000; Dietmaier et al. 1997; Thibodeau et al. 1998).

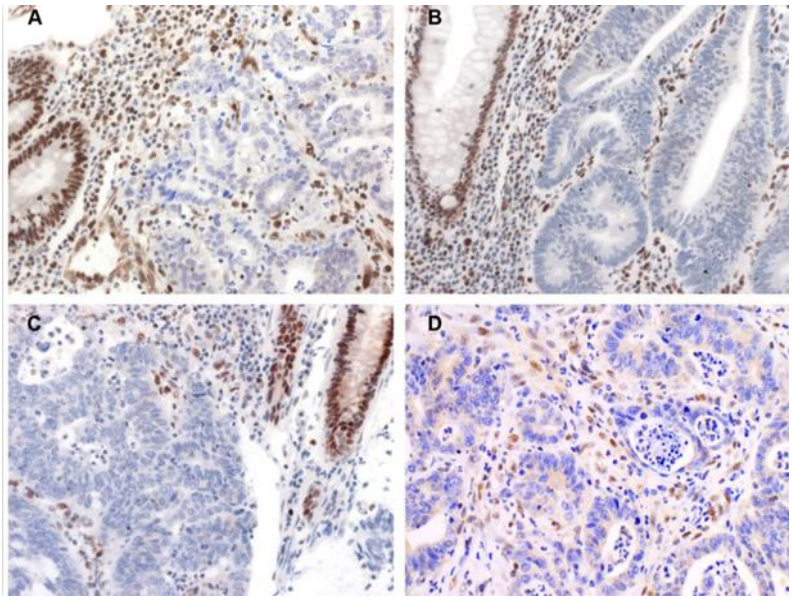

Figure 17. Examples of immunostains showing loss of MMR proteins.
Positive nuclear staining in normal colonic epithelium or stromal cells and loss of expression in colorectal cancer of MLH1 (A), MSH2 (B), MSH6 (C) and PMS2 (D). Source: Yangun Liu, 2014.

IHC can be also used to choose the MMR gene of interest for germline testing. In tumors in which only one protein is not stained (typically MSH6 or PMS2), the correspondent gene can be evaluated. In contrast, If two gene products are not expressed (usually MLH1/PMS2 or MSH2/MSH6), then either *MLH1* or *MSH2* are the likely responsible (Vasen et al. 2007). This happens because of PMS2 stability depends on its ability to form a complex with MLH1 (a similar situation takes place with MSH6 and MSH2). The opposite, however, does not usually apply because tumors with defects in PMS2 or MSH6 may maintain expression of MLH1 or MSH2, respectively. Besides, a tumor with MLH1/PMS2 loss of expression may be either sporadic or LS associated since promoter methylation or a germline mutation in *MLH1* will lead to the same IHC profile. In contrast, lack of expression of MSH2, MSH6, or PMS2 (with maintenance of MLH1 expression) is less common in sporadic MSI tumors. IHC of each MMR protein has a sensitivity of 74, 91, 55 and 77% for the detection of mutation carriers in *MLH1*, *MSH2*, *MSH6* and *PMS2*, respectively (Shia and Zhang 2008). The specificity varies from 80 to

100% depending on the antibody and the panel used (Barrow et al. 2010; Hall et al. 2010; Shia and Zhang 2008).

Screening for BRAF mutations has been widely used to discriminate between LS-associated and sporadic cancers. Somatic *BRAF* mutations are found in ~10% of sporadic CRCs. When restricted to only sporadic tumors with MSI, the prevalence is of more than half (50–68%). In contrast, *BRAF* p.V600E is rarely detected in LS–associated cancers (~4%) (Guoren Deng et al. 2004; Y. S. Y. H. Kim et al. 2008; Parsons et al. 2012).

Somatic *BRAF* mutations can be detected using different techniques, such as: direct sequencing (dideoxy sequencing and pyrosequencing), Single Stranded Conformation Polypormphism (SSCP), heteroduplex analysis and High Resolution Melting (HRM) analysis. Detection of the specific loci of the most prevalent mutation (p.V600E) can be accomplished by allele-specific primer extension, restriction enzyme digestion or real-time PCR (Hegde et al. 2014; Pineda et al. 2010). The sensitivity of these analyses varies from 96-100% and the specificity for depiction of LS MLH1-negative tumors is around 34% (Mireia Gausachs et al. 2012; Perez-Carbonell, Cristina Alenda, et al. 2010).

MLH1 promoter methylation analysis can be performed in MLH1 and PMS2 deficient tumors, in order to differentiate cases derived from germline predisposition from the somatically acquired. This association is commented in topic 4.5 (MMR deficiency). *MLH1* promoter regions that are susceptible for methylation have been widely studied. Region "C" of Deng is a small region (−248 to −178 relative to the transcription start site) (Fig. 18) in which the methylation status invariably correlates with the loss of MLH1 and is accepted that at least this region should be tested. However, in some laboratories also the "D" region is studied (Capel et al. 2007; Mireia Gausachs et al. 2012; Perez-Carbonell, Cristina Alenda, et al. 2010).

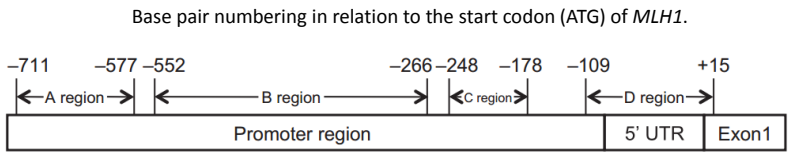

Figure 18. Schematic representation of *MLH1* promoter regions, proposed by Deng *et al*, 1999.
Source: Parsons *et al*, 2012.

Briefly, technologies for DNA methylation analysis are based on three different approaches to discriminate the methylated and unmethylated cytosines (Zhang and Jeltsch 2010).

1) Techniques based on methylation sensitive restriction enzyme digestion. An example of these is MS-MLPA technique, which uses restriction endonucleases that are sensitive to methylation since they contain a *Hha*I recognition site. If the site contains a CpG methylated cytosine, digestion is prevented, undigested probes are amplified during a PCR and a peak is observed in an electropherogram. Comparison of MLPA peak patterns of an unmethylated and a methylated control detects relative differences between them. MS-MLPA has the advantage of needing small amounts of DNA (50-100ng), on-hands protocol last less than 2 days and provides information on copy number and methylation status of multiple loci in a single experiment. The greatest limitation of this technique is that it provides methylation data only at the restriction enzyme recognition sites (Zhang and Jeltsch 2010). SALSA MS-MLPA Kit ME011 MMR (MRC-Holland) allows the identification of methylation at 6 MMR gene promoters *(MLH1, MSH2, MSH6, MSH3, MLH3, PMS2*) at the same time. Besides, it contains 11 reference probes which are not influenced by *Hha*I activity and act as amplification controls.

2) Affinity purification. By the use of antibodies against methylated cytosine, methylated or unmethylated fractions of genomic DNA can be immunoprecipitated (Illingworth et al. 2008; Keshet et al. 2006; Rakyan et al. 2008; Weber et al. 2005, 2007). In this method, coverage is limited by the hybridization array and the distribution of the potential affinity targets in the genome. Moreover, the exact methylation state of individual CpG sites cannot be determined.

3) Bisulfite conversion of DNA. The method is based on the selective deamination of cytosine but not 5-methylcytosine by treatment with sodium bisulfite (Clark et al. 1994; Frommer et al. 1992). Sodium bisulfite converts unmethylated cytosines into uracils, and during the process of PCR amplification, they become thymines, while methylated cytosines remain as such (Fig. 19). Therefore, methylated and unmethylated cytosines can be distinguished according to the sequence changes. The bisulfite conversion efficiency is critical for the accuracy and the reliability of the results, especially for non-CpG methylation analysis (Genereux et al. 2008). On the opposite side, these techniques have the advantage of interrogate more CpG sites then usually MS-MLPA does.

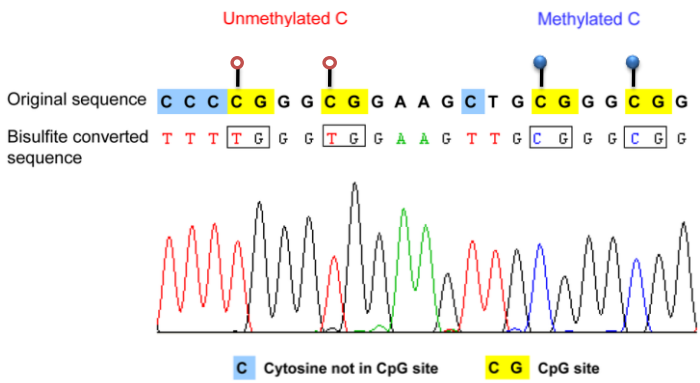

Figure 19. Sequence changes after bisulfite conversion in both unmethylated and methylated DNA. Modified from Zhang, 2010.

Different methods are used to read out the DNA methylation information after bisulfite treatment, such us MS-MCA, pyrosequencing and bisulfite sequencing. In MS-MCA, bisulfite treated DNA intercalates with a fluorescent dye and an amplification reaction is made in a thermal cycler with a fluorometer (Light Cylcer), since methylated DNA after bisulfite treatment contains more GCs than unmethyated DNA, it will require higher melting temperature (Tm), thus MS-MCA differentiate methylated and unmethylated alleles when compares the differences in Tm. Bisulfite sequencing is referred to the use of Sanger in bisulfite amplified DNA fragments. This method has been often used for specific loci or for the validation of results obtained by other DNA methylation analysis methods (Zhang and Jeltsch 2010). Furthermore, pyrosequencing has the advantage of being quantitative, allowing to directly measure the levels of methylation. In this technique, bisulfite converted DNA amplifies using biotinylated primers, and sequencing allows to take measures of pyrophosphate upon nucleotide incorporation using ATP-coupled luciferase reaction (Tost and Gut 2007).

Techniques used to test *MLH1* promoter methylation include bisulfite sequencing, pyrosequencing, methylation specific PCR (MSP), methylation specific multiplex ligation-dependent probe amplification (MS-MLPA) and methylation specific melting curve analysis (MS-MCA) (Mireia Gausachs et al. 2012; Goel et al. 2011; Hitchins and Ward 2007, 2008, 2009; M P Hitchins, Owens, C.-T. T. Kwok, et al. 2011; Moreira et al. 2015; Ogino et al. 2006).

Analysis of *MLH1* promoter methylation using MS-MLPA has proved to be more specific and cost-effective than *BRAF* mutational analysis. The specificity ranges from 66 to 78, depending on the technique and the criteria utilized for case selection (Mireia Gausachs et al. 2012; Perez-Carbonell, Cristina Alenda, et al. 2010). The results using pyrosequencing have been controversial (Moreira et al. 2015; Newton et al. 2014). The obtained results must be examined thoroughly and in global, since frequency of *MLH1* promoter methylation in sporadic CRC varies from 0% (Belshaw et al. 2008) to 67% (Kumar et al. 2009), but is also present in 0% (Menigatti et al. 2001) to 21% of LS CRCs (Nagasaka et al. 2004; Rahner et al. 2008).

5.3. Molecular Germline Testing

Patients whose tumors result MMR deficient and, MLH1-negative cases that do not have *MLH1* promoter methylation or *BRAF* mutation, should undergo genetic testing of MMR genes at germline level. This is done by point mutation and gross rearrangement analysis. Over years, the study of MMR genes has been guided by the IHC pattern.

Study of the whole coding region plus regulatory sequences of MMR gene is mandatory for diagnosis purposes. Complete Sanger sequencing of all coding regions and intron-exon boundaries of the relevant MMR gene is considered the gold standard for mutation detection, but it results expensive. Different screening methods can be used in addition to sequencing. Most screening methods are based on properties of heteroduplex (such as denaturing gradient gel electrophoresis, conformation sensitive capillary electrophoresis and denaturing high performance liquid chromatography); properties of ssDNA (like the SSCP assay); real-time instruments to scan for mutations using the HRM approach based on the ability to record and evaluate fluorescence intensities in function of the temperature of dissociation; and methods designed to detect truncated proteins produced by frameshift or nonsense DNA mutations (like the protein truncation test) (Pineda et al. 2010).

Neither of the techniques above mentioned identify deletions or duplications of single or multiple consecutive exons. To overcome this technical limitation several quantitative approaches have been developed. Quantitative real-time PCR of the region of interest has demonstrated useful in some instances. In this sense, MLPA increase the accuracy of simple

quantitative PCR and it is very popular due to its simplicity, relatively low cost, possibility of high-throughput and robustness. A similar approach, quantitative multiplex PCR of short fluorescent fragments (QMPSF), has demonstrated to be useful to detect copy number variations (CNV) in CRC genes as well. In the diagnostic routine, it is advantageous to confirm any CNV using a different method and, if possible, to establish the exact molecular nature of the deletion. Conventional Southern blotting, fluorescence *In Situ* hybridization (FISH), array-comparative genomic hybridization (aCGH) or SNP-arrays are used to detect and confirm CNV (Pineda et al. 2010).

Next Generation Sequencing (NGS) has been having a drop in the costs, promoting a clear tendency to incorporate it as a diagnostic tool (Huddy et al. 2015; Pritchard and Grady 2011; Pritchard et al. 2012; Stoffel 2015b).

Three different NGS approaches can be applied in the diagnostic of heterogeneous diseases: targeted enrichment of a set of genes, also called multiplex or gene panel (list of examples in Table 9), whole-exome sequencing (WES), and whole-genome sequencing (WGS). As cost-effectiveness is an important factor in healthcare, the choice of a particular approach must be justified, and differences in costs may limit implementation of the "superior" approach. Depth of coverage is a critical factor as well, and depends on the desired mutational sensitivity, the sequencing platform used and the individual sensitivity of the bioinformatic pipeline (Sun et al. 2015).

Table 9. Commercially available multiplex gene panels specific for colorectal cancer.
Modified from (Stadler et al. 2014).

Panel	Genes included
ColoNext; Ambry Genetics	APC, BMPR1A, CDH1, CHEK2, EPCAM, MLH1, MSH2, MSH6, MUTYH, PMS2, PTEN, SMAD4, STK11, TP53.
ColoSeq; University ofWashington LaboratorySeattle, WA	APC, BMPR1A, CDH1, EPCAM, MLH1, MSH2, MSH6, MUTYH, PMS2, PTEN, SMAD4, STK11, TP53.
Mayo Medical Laboratories; Rochester, MN	APC, AXIN2, BMPR1A, CDH1, CHEK2,EPCAM, GREM1, MLH1, MLH3,MSH2, MSH6, MUTYH, PMS2,PTEN, SMAD4, STK11, TP53.
Oto-ColoCa; Otogenetics, Norcross, GA	APC, BMPR1A, MLH1, MSH2, MSH6, MUTYH, P16(CDKN2A), PTEN, SMAD4, STK11, TP53

There are different methods for target enrichment that can be used (Fig. 20). Selection can be done either by PCR-based methods, such as highly multiplex PCR or digital PCR, or by in-solution hybridization–based methods. Circularization is another method suitable for targeting small to medium sized regions of interest, is based on padlock and molecular inversion probes containing universal sequences; target molecules can be selected and circularized in a single reaction and subsequently amplified either by PCR or hybridization (Moorthie, Mattocks, and Wright 2011).

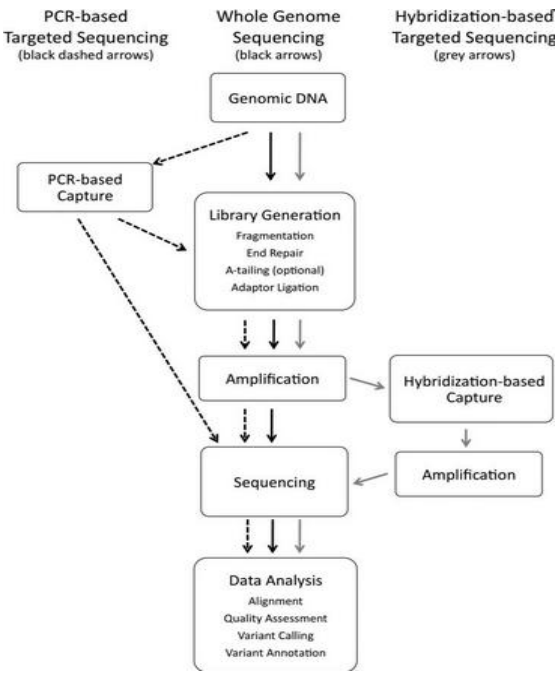

Figure 20. Next generation sequencing components.
Extracted from Rehm et al, 2013.

Afterwards, sequencing can be performed with short or long read technologies platform dependent (Hegde et al. 2014). The choice of platform, test design, and read length should be based on the type of variation that must be detected and the length of the fragment to be analyzed (Rehm et al. 2013).

General types of sequencing include single-end sequencing and paired-end sequencing. Paired-end sequencing increases the ability to map reads unambiguously, particularly in repetitive regions, and has the added advantage of increasing coverage and stringency. A variation of paired-end sequencing is mate-pair sequencing, which can be useful for structural variant detection (Rehm et al. 2013).

The sequencing process in NGS is a stepwise reaction consisting in nucleotide addition, determination of the incorporated nucleotides identity on each fragment focus being sequenced, and a wash step that may include chemistry to remove fluorescent labels or blocking groups. NGS instruments conduce sequencing and detection simultaneously, one of which is completed before the other takes place (in parallel). Moreover, these steps are

executed in a setup that allows hundreds of thousands to billions of reaction foci to be sequenced during each run, producing massive data sets (Mardis 2013), which require complex analysis. It is important to know that, regions of interest may be out of rich and will need further conventional sequencing to complete clinical testing. Moreover, single-exon and multiexon deletions and duplications could not be detected and other methodologies may be required as well (Hegde et al. 2014).

5.4. Variants of Unknown Significance

While MMR gene variants identified in LS suspected patients that result in premature truncation of the protein (nonsense, frameshift) are easily classified as pathogenic, mutation analysis also identifies many variants whose biological significance may be unknown (i.e. silent, missense, and intronic variants or small in-frame insertions/deletions). They are called variants of Unknown Significance (VUS), and represent around 30% of the mutations found in MMR genes (Peltomaki and Vasen 2004; B. a. Thompson, Martins, and Spurdle 2014). Nowadays, with the use of NGS technologies for diagnostic routine, VUS detection is increasing in a substantial manner (Valle 2014). Assumptions about the biological effect and clinical implications of these kinds of changes are often difficult to make (Auclair et al. 2006; Winawer et al. 1997).

Since the identification of MMR genes as responsible of LS, pathogenicity of variants has been assessed based on different levels of evidence, such as cosegregation of the mutation with the disease, MSI and IHC tumor profile, concomitant deleterious mutations *in trans*, frequency of the variant among unaffected individuals, aminoacid polarity, size and evolutionary conservation. Notwithstanding, MMR variants can have different phenotypes within different families, and data about cosegregation is not always accessible (Barnetson et al. 2008; Genuardi et al. 1999). Later, functional assays were developed.

Functional assays can evaluate RNA processing and protein functionality. When possible, assays at RNA level should be performed using lymphocyte RNA from the variant carrier. Most used techniques are real time polymerase chain reaction (RT-PCR) and allelic specific expression (ASE) (Arnold et al. 2009; Castellsague et al. 2010; Perera et al. 2010;

Santibanez Koref et al. 2010; Sharp et al. 2004; Tournier et al. 2008). At the protein level, the capacity to reconstitute the MMR pathway *in vitro* has provided a crucial and reliable tool for studying the functional repercussion of variants (Betz et al. 2010; Lastella et al. 2006; Naruse et al. 2009; Tournier et al. 2008). Human cell lines are commonly used in these assays, nevertheless, the evolutionary conservation of repair proteins facilitates the use of yeast for MMR pathway studies as well (Ou et al. 2007). Protein expression and subcellular location are also commonly evaluated in the pathogenicity assessment of variants.

Besides experimental assays, computational tools, also called *in silico* assays, that assess the functional effect on transcription or protein function, can give predictive information on a particular variant (Arnold et al. 2009; Chao et al. 2008; Spurdle et al. 2008).

Standardized variant classification system. A multidisciplinary expert committee of the International Society for Gastrointestinal Hereditary Tumours gathered in 2013 to refine the scheme for classification of MMR variants identified in suspected LS individuals (B. A. Thompson et al. 2014). They developed and applied a standardized classification scheme for MMR variants, based on multiple lines of evidence including clinical and functional data. Variants were classified according to the five class IARC scheme as pathogenic (class 5), likely pathogenic (class 4), uncertain (class 3), likely non-pathogenic (class 2) and non-pathogenic (class 1) (Table 10) (Plon et al. 2008). A summary of the classification rules is schematized in Figure 21.

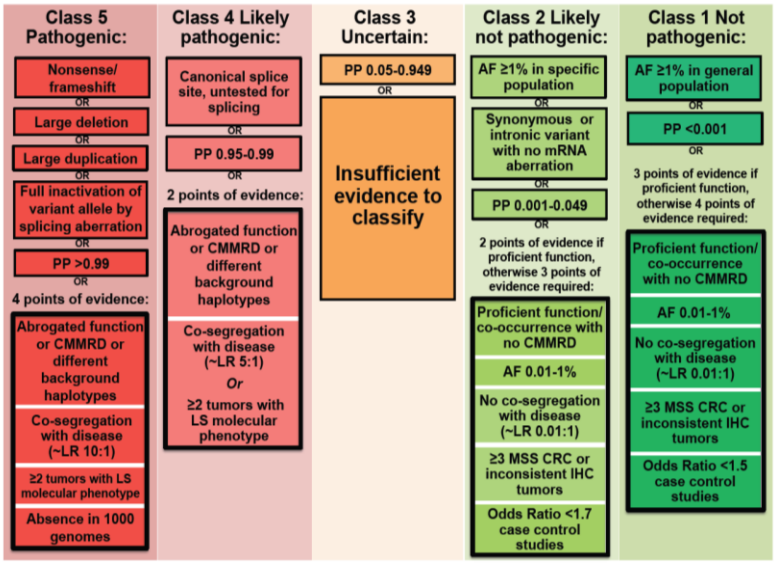

Figure 21. Overview of 5-tiered InSiGHT classification guidelines.
Simplified guidelines describing levels and types of evidence required to reach different classes.
Extracted from Thompson, Nature genetics, 2014.

Furthermore, this system has been linked to clinical recommendations for all classes: predictive testing and full high-risk surveillance guidelines for carriers of Class 5 and Class 4 variants; advice to treat as "no mutation detected for this disorder" for carriers of Class 1 and Class 2 variants; and acquisition of additional data to provide more robust classifications for Class 2, Class 4 and Class 3 (Table 10).

Table 10. Testing recommendations associated with each class of variant.
Modified from Plon et al, 2008.

Class	Clinical testing	Surveillance if at-risk relative is positive	Research testing of family members
5	Test at-risk relatives for variant	Full high-risk surveillance guidelines	Not indicated
4	Test at-risk relatives for variant*	Full high-risk surveillance guidelines	May be helpful to further classify variant
3	Do not use for predictive testing in at-risk relatives*	Based on family history (and other risk factors)	May be helpful to further classify variant
2	Do not use for predictive testing in at-risk relatives*	Treat as "no mutation detected" for this disorder	May be helpful to further classify variant
1	Do not use for predictive testing in at-risk relatives*	Treat as "no mutation detected" for this disorder	Not indicated

* Recommended continued testing of proband for any additional available testing modalities, *i.e.* rearrangements.

Nowadays (June, 2015), an international database that collects MMR gene variants to support research and clinical management (www.insight-group.org/mutations) lists around 1,000 different VUS; they are thought to be just a proportion of the real total.

5.5. Diagnostic Yield

The diagnostic yield of the LS diagnostic algorithm (Fig. 16) is good (Leticia Moreira et al. 2012), but it can certainly be improved. The overall mutation detection rate in pre-selected patients, ranges from 30 to 78%, depending on the inclusion criteria applied (Lipton et al. 2004; Lynch, Lynch, and Lynch 2007; Mangold et al. 2005; Leticia Moreira et al. 2012; Syngal et al. 1999). Only in highly selected series of Amsterdam families with MSI, the percentage of mutation detection may be as high as 95% (Mueller et al. 2009). When published data from Win et al, 2014, Hampel et al, 2005 and Rodríguez-Soler et al, 2013 is combined, 59% (95% confidence interval [CI]: 55-64%) of CRCs remain with no mutation identified; and when published data from endometrial cancer cohorts from Buchanan et al, 2014, Moline et al, 2013, Leenen et al, 2012 and Hampel et al 2006, is combined, 52% (95% CI: 41-62%) of endometrial cancer patients remain undiagnosed (Buchanan et al. 2014).

6. LYNCH-LIKE SYNDROME

Individuals with MMR deficient LS spectrum tumors (in the absence of MLH1 methylation), in which no pathogenic germline mutation has been identified are known as having “Lynch-like syndrome (LLS)” (Rodriguez-Soler et al. 2013), also called “suspected Lynch syndrome”. However, failure in the identification of pathogenic germline mutations in MMR genes among patients with MMR deficient tumors does not exclude an inherited predisposition to cancer.

6.1. Cancer Risk

The mean age at diagnosis of CRC in LLS cases has been reported similar to LS (Overbeek et al. 2007) or in-between LS and sporadic MMR deficient individuals (Rodriguez-Soler et al. 2013; Win et al. 2015). In 2007, the first approximation by implication of cancer risk among Lynch-like cases was made, this group observed in a Dutch cohort, that 66% (50/75) of LS families fulfilled the Amsterdam II criteria, in contrast, only 11% (2/18) of Lynch-like fulfilled them (*P*=0.001) (Overbeek et al. 2007). Later on, a study comprising 25 LLS families quantified the risk of CRC in their 177 FDRs and found that MMR gene mutation carriers had the highest risk, LLS cases an intermediate risk, and the MMR deficient cases due to *MLH1* promoter methylation the lowest (Rodriguez-Soler et al. 2013). Recently, a bigger cohort comprising 271 LLS CRC cases and 1,799 FDRs, confirmed these findings (Win et al. 2015). Up-to-date, there are no published data about the FDRs risk of other tumors within the LS spectrum.

6.2. Current Clinical Management Recommendation

Given the intermediate risk of CRC found among LLS, intermediate surveillance could be the best approach (Rodríguez-Soler, 2013). However these cases are most probably a heterogeneous group of different molecular and family background, so as a result no optimal screening can be generalized until specific diagnosis is made.

The inability to define evidence-based screening and management guidelines for LLS cases, makes difficult their medical care. Therefore, LLS individuals and their relatives could be

receiving different shades of cancer surveillance, ranging between low and high risk individuals, which mean that some of them are being subjected to unnecessarily over-screening and emotional distress, while others lack proper examination (Geurts-Giele, 2014). This is a problem that aggravates families, physicians and that also affects health care system.

6.3. Potential Causes of MMR Deficient Tumors

Unidentified germline MMR gene mutations and somatic MMR inactivation have been reported as causes of MMR deficiency in some LLS cases.

6.3.1. Germline inactivation

Unidentified germline MMR gene mutations. Current mutational analysis techniques could be missing complex or cryptic mutations in MMR genes (Ligtenberg 2004; Clendening, 2011; Morak, 2011). An example of deep intronic mutations that could be overlooked with current strategies is the one found within the first intron of *MSH2*, at position c.212-553_c.212-479 (Clendening, 2011). This change creates a canonical donor splice site at the 3' end of the insertion cointaining a stop codon, which is predicted to truncate the protein.

Other examples of unidentified mutations are complex structural variations comprising MMR genes. Fusions of *MLH1* with *ITGA9* gene has been found in cases with interstitial deletion on chromosome 3p21.3 (Meyer, 2009) or with *LRRFIP2* after paracentric inversion on chromosome 3p22.2 (Morak, 2011). Also, inversion of exons 1-7 in *MSH2* are not an uncommon cause of LLS (Wagner, 2002; Chen, 2008; Rhees, 2014).

Besides, LLS individuals could be carriers of undetected low penetrant mutations in regulatory regions of MMR genes (Dowty, 2013). The 5' and 3' untranslated regions (UTRs) of most genes contain regulatory sequences that control mRNA processing and message stability. Germline 3'UTR mutations in *MLH1* have been related to loss of its protein expression as well (Wilding, 2010). Likewise, miRNA anomalous regulation has been proposed as possible responsible for low expression, such is the case of miR-21 and miR-155. (Valeri, Gasparini, Fabbri, Proc Natl Acad Sci USA, 2010).

MMR mosaicism could be also a cause of misdiagnosis of LS. It has been reported only in two LS suspected cases. Somatic mosaicism was found in a woman with synchronous endometrioid adenocarcinomas of the ovary and endometrium at 44 years old. Her family had Amsterdam II clinical criteria, and *MLH1* c.1050delA mutation was identified in her sister's blood, which had been affected with EC as well. The same mutation was found in the reported case but with a wildtype allele fraction of around 20% in normal tissue from different organs. This was attributable to revertant somatic mosaicism since their father had been affected with 4 tumors within the LS-spectrum (Pastrello, 2009). Surrouille *et al* described a CRC case with MSI and a frameshift mutation in *MSH2* (c.2541delA) in his blood lymphocyte DNA, whose mother had history of a colorectal tumor showing the same mutation in tumoral tissue but without it at blood lymphocyte DNA. Mutational analysis at normal colon DNA from her mother revealed a weak signal for c.2541delA mutation, evidencing the presence of somatic mosaicism. The fact that she passed the mutation to her son demonstrates that she had germinal mosaicism (Surrouille, 2013).

6.3.2. Somatic inactivation

Recent studies have confirmed that somatic mutations are responsible of MMR loss of expression in a proportion of LLS cases. Sourrouille *et al* performed mutation analysis of 17 MSI CRCs with loss of MLH1 or MSH2 immunoexpression, and detected two somatic mutations in each of four tumors (1/7 in MLH1 and 3/8 in MSH2). Mensenkamp *et al* combined mutation and LOH analysis in 7 MSH2 deficient cases and 18 MLH1, and identified two somatic hits in each of 13 tumors (8/18 in *MLH1* and 5/7 in *MSH2*). Geurts-Giele *et al* combined mutation, copy number and LOH analysis to study 40 LLS cases, finding 21 of them (16/24 in *MLH1* and 5/12 in *MSH2)* as carriers of double somatic hits (Geurts-Giele et al. 2014; Mensenkamp et al. 2014; Sourrouille et al. 2013).

Furthermore MMR genes could be targets of somatic methylation. As commented in topic 2.2, MMR gene inactivation caused by promoter hypermethylation has been reported at somatic level for *MLH1* (Herman, 1998; Hitchins, Gastroenterology, 2005) and *MSH2* (Rumilla, 2011). In contrast, no evidence has been reported about *MSH6* or *PMS2* inactivation by promoter methylation in CRCs.

HYPOTHESIS

MMR deficiency is a hallmark of tumors from Lynch syndrome patients, who harbor germline mutations in MMR genes. Besides, in tumors from Lynch syndrome suspected patients without identified germline MMR gene mutation, somatic *MLH1* methylation and, recently, double somatic mutations have been described as responsible causes of MMR deficiency.

Our hypothesis is that in Lynch syndrome suspected patients there may be other responsible causes for the MMR deficiency in tumors, such as unidentified germline mutations or epimutations in MMR genes, or mutations in other CRC predisposing genes (either germline or somatic).

AIMS AND OBJECTIVES

Main aim:

To elucidate the molecular basis of MMR deficiency in suspected Lynch syndrome cases without identified germline MMR mutation.

Specific aims:

- To refine the analysis of MMR genes in this selected set of cases by means of:
  1. Evaluating the contribution of (epi)mutations in the promoter region of MMR genes.
  2. Studying the relative contribution of constitutional epimutations to suspected Lynch syndrome cases.
  3. Searching for cryptic mutations in the *MSH2* gene and assessing pathogenicity of *MSH2* VUS.

- To study the contribution of germline mutations in *MUTYH* gene to Lynch-like syndrome.

- To study the relative contribution of mutations in other CRC-associated genes to Lynch-like syndrome.

To do this we have analyzed a series of 260 Lynch syndrome-suspected patients, 160 identified at five different Catalonian hospitals and the remaining 100 at Valencian hospitals. Out of the 160 Catalonian patients, thirty-four harbored *MLH1*-methylated tumors and 126 were classified as Lynch-like (without identified germline MMR mutation and absence of somatic *MLH1* methylation or *BRAF* mutation). For a part of the above mentioned specific aims we have restricted the analysis to MSH2/MSH6 deficient LLS cases.

RESULTS

Outline

The present thesis comprises five segments of results. For the purpose of clarity, this section begins with unpublished results on promoter (epi)mutational analyses of MMR genes that address sub-objectives 1 and 2 of the first specific aim. Afterwards, published or submitted articles are enclosed with a specific mention of the contribution of the PhD candidate to each article.

Article 1

"*MLH1* methylation screening is effective in identifying epimutation carriers"

European Journal for Human Genetics, 2012.

Article 2

"Prevalence of germline *MUTYH* mutations among Lynch-like syndrome patients"

European Journal of Cancer, 2014.

Article 3

"Identification of germline *FAN1* variants in MSH2-deficient Lynch-like syndrome patients"

Submitted for publication.

Article 4

"Elucidating the molecular basis of MSH2-deficient tumors in Lynch syndrome suspected patients"

Submitted for publication.

ANALYSIS OF GERMLINE MUTATIONS AND TUMOR METHYLATION AT MISMATCH REPAIR GENE PROMOTER REGIONS OF LYNCH-LIKE SYNDROME PATIENTS

The promoter region of a gene, corresponding to the DNA sequence located upstream the transcription start site, holds specific sites for transcription factors and RNA polymerase binding and, therefore, is essential for the regulation of gene expression (Levine and Tjian 2003). Genetic and epigenetic modifications at promoter regions can lead to transcriptional silencing/activation of a gene. The most studied epigenetic modification is DNA methylation (Ficz 2015).

With the aim of identifying (epi)genetic modifications at the MMR gene promoters as the responsible cause for the MMR-deficiency in tumors from Lynch syndrome suspected patients, we sequenced promoter regions of candidate MMR genes in DNA isolated from Peripheral Blood Lymphocytes (PBL) of LLS patients and analyzed the methylation status of MMR promoter regions in available FFPE tumors from the Catalonian LLS series.

A total of 126 LLS cases were identified at the Catalan Institute of Oncology at Duran i Reynals, Germans Trias i Pujol and Dr. Josep Trueta hospitals, Vall d'Hebrón Hospital and Santa Creu i Sant Pau Hospital. Inclusion criteria were: i) diagnosis of LS-associated tumors showing loss of MMR protein expression ii) absence of *BRAF* p.V600E mutation or *MLH1* promoter methylation in *MLH1* deficient tumors and iii) absence of germline pathogenic variants in MMR genes (mutational analyses guided by IHC results). Fourteen LLS patients with MSI tumors without evidence of MMR protein loss by IHC were excluded from these analyses. Finally, 112 LLS cases were included. Informed consent was obtained from all cases and the study was approved by the respective IRBs.

The results on the promoter analysis of MMR genes of 88 LLS patients are the object of this section while the results concerning the analysis of the 24 patients from the Catalan Institute of Oncology with tumors showing loss of expression of MSH2/MSH6 are described in the fourth article. Fifty-eight of the 88 patients had tumors with loss of expression in the IHC analysis of MLH1/PMS2, 5 in PMS2 only, 12 in MSH2/MSH6 and 13 in MSH6 only.

1. Promoter analyses in patients with tumors lacking MLH1 and/or PMS2 expression

The promoter region of *MLH1* gene was analyzed in 58 probands with tumors lacking either MLH1/PMS2 or PMS2 protein alone. The mean age at first tumor diagnosis was 51.7 years (range 16-82). Forty-one cases harbored at least one Bethesda criterion, three families fulfilled Amsterdam criteria, and the remaining 14 were referred from the Pathology Department because of suspected MMR deficiency. Seven percent of the cases (4 out of 58) had a personal history of multiple primary tumors within the LS spectrum. More than 90% (53 out of 58) of the analyzed MMR deficient tumors were colorectal, 4 were endometrial tumors and 1 was a sebaceous adenoma (Table 11). No pathogenic mutations were identified in the *MLH1* coding region.

Five patients harbored tumors lacking PMS2 expression with a mean age at diagnosis of 51 (range 45-59). Three had Bethesda criteria, one fulfilled Amsterdam criteria, and 1 was referred from the Pathology Department. None had personal history of previous tumors. Four of the 5 tumors were colorectal and one was ovarian. Mutational analysis of *PMS2* coding region did not identify any pathogenic mutation (Table 11).

1.1 Mutational analysis of MLH1 *and* PMS2 *promoter regions*

We sequenced 1,469bp upstream the transcription start site of *MLH1*, comprising the region containing conserved transcription factor binding sites (according to UCSC), which contains a CpG island region upstream exon 1 in In PBL DNA of all included patients (Fig. 22). Two uncommon variants at *MLH1* promoter: c.-1018G>A and c.-574T>A were found. The carrier of the *MLH1* c.-1018G>A (rs190305737) variant was a female diagnosed of CRC at 42 years of age; her tumor lacked f MLH1 and PMS2 staining by IHC and was MSI (Table 11). The variant has a MAF of 0.0002, being detected in heterozygosis in 11 out of 4119 Europeans (1000 Genomes: Abecasis et al. 2012). Furthermore this variant is part of 21 transcripts, one of them is the *EPM2AIP1*, corresponding to the change c.548C>T p.(Ala183Val) which is predicted to affect the EPM2AIP1 product by SIFT and Polyphen tools.

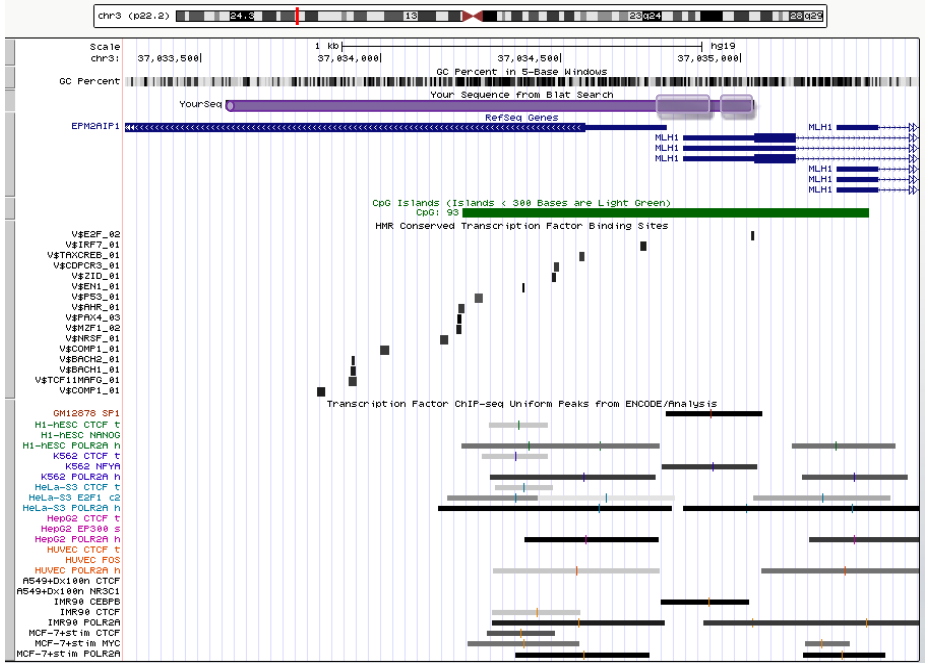

Figure 22. UCSC blat of the selected region for *MLH1* promoter sequencing.
Promoter region sequenced by conventional method in PBL is marked in purple and regions analyzed by MS-MCA are colored in light purple. CpG island is colored in green; transcription factor bind sites from HMR conserved and ENCODE are shown in the lower part.

The second case was heterozygous for the novel *MLH1* c.-574T>A variant. The carrier is a male patient diagnosed of CRC at 70 years of age; his tumor had lost MLH1 protein expression and was MSI (Table 11). He met Bethesda 4 criteria in light of a CRC affected daughter at the age of 36 years. Her daughter's tumor did not lack MLH1 expression and was stable.

None of the 5 cases with PMS2 deficiency in tumors harbored pathogenic variants at *MLH1* gene promoter. It is worth mentioning that the majority of cases harboring PMS2 deficient tumors are usually studied for germline mutations at both, *MLH1* and *PMS2* genes. Nevertheless, case 168 lack *MLH1* mutational analysis of codifying regions, and is being evaluated (Table 11).

1.2 Somatic methylation analysis at MLH1 *and* PMS2 *promoter regions.*

In order to determine the methylation status of the *MLH1* promoter we studied the promoter region in 13 available tumor samples that were *BRAF* negative and MLH1/PMS2 deficient. Methylation-specific Melting Curve Analysis (MS-MCA) of regions C and D of MLH1 promoter was performed as described (Mireia Gausachs et al. 2012). This technique offers an analytical sensitivity of 25% and 1% for C and D regions, respectively (Mireia Gausachs et al. 2012; Article 1).

Eleven of the 13 samples analyzed were informative and four of them were found methylated (Table 11). One sample analyzed by both methods corresponded to a colorectal tumor from a female diagnosed at 76 years old that was referred by the pathologist. Figure 23 shows the MS-MCA results (Fig. 23). Methylation levels of 33.9 and 35.4% in *MLH1* C and D promoter regions were estimated by MS-MLPA. .

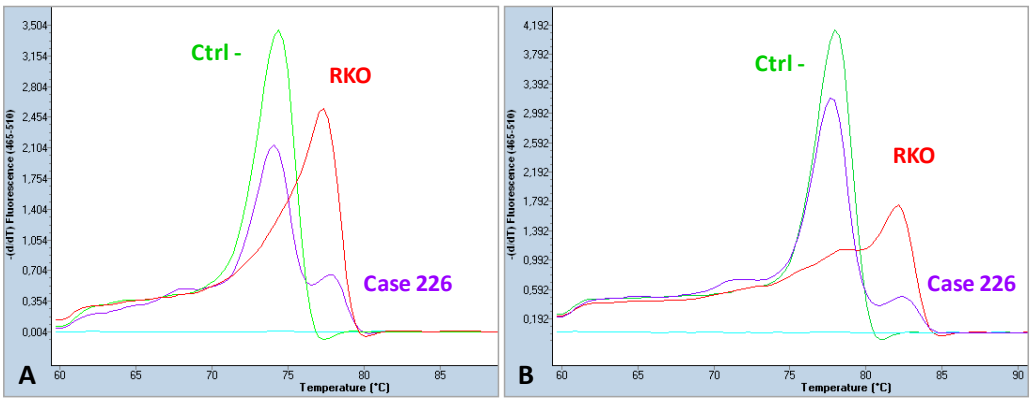

Figure 23. MS-MCA of case 226, positive for methylation at *MLH1* promoter.
A) Region C; B) region D.

Table 11. Clinicopathological and molecular features of LLS cases harboring MLH1 and/or PMS2

PATIENT INFORMATION				GERMLINE DATA								SOM						
				MMR MUTATIONAL ANALYSIS								CLINICAL DATA						
Patient ID	SEX	DATE OF BIRTH	CLINICAL CRITERIA	TECHNIQUE	*MLH1*	*MSH2*	*MSH6*	*PMS2*	VUS Presence (Insight classification)	*MLH1* promoter sequencing	*MLH1* c.-93	TYPE/ ORGAN	AGE AT DIAGNOSIS	HISTOLOGICAL TYPE	ANATOMIC LOCALIZATION	TNM STAGE	AJCC STAGE	H DIF
Patients harboring MLH1 (at least) deficient tumors																		
132	2	10/08/1954	B1-3	MLPA; SEQ	NM	NM	NP	NP	-	NM	GG	CRC	44	ADK	RIGHT	T3N0M0	II-A	
133	2	12/03/1963	B1	MLPA; SEQ	NM	NM	NP	NP	-	NM	GA	CRC	31	ADK	LEFT	T3N0M0	II-A	
134	1	16/05/1953	B1	MLPA; SEQ	NM	NM	NP	NP	-	NM	GA	CRC	49	ADK	RIIGHT	T3N0M0	II-A	
135	2	22/05/1953	B1	MLPA; SEQ	NM	NM	NP	NP	-	NM	GG	CRC	48	ADK	RIGHT	T3N0M0	II-A	
136	1	16/06/1966	B1	MLPA; SEQ	NM	NP	NP	NP	-	NM	GG	CRC	40	ADK	RIGHT	T3N1M0	III-B	
226	2	03/03/1931	AP	MLPA; SEQ	NM	NP	NP	NP		NP	GG	CRC	76	NI	NI	NI	NI	
137	2	12/12/1976	B1	MLPA; SEQ	NM	NP	NP	NP	-	NM	GG	CRC	30	ADK	RIGHT	NS	IV	
138	2	04/08/1971	B1	MLPA; SEQ	NM	NP	NP	NP	-	NM	GA	CRC	35	ADK	RIGHT	NS	NS	
139	1	27/05/1961	B1	MLPA; SEQ	NM	NP	NP	NP	-	NM	GG	CRC	48	CA	RIGHT	T3N0M0	II-A	
140	1	07/06/1925	AP	MLPA; SEQ	NM	NP	NP	NP	-	NM	GG	CRC	66	ADK	NS	TXN0M0	NS	
141	1	18/07/1937	B4	MLPA; SEQ	NM	NP	NP	NP	-	*MLH1* c.-574T>TA	AA	CRC	70	ADK	RIGHT	T3N0M0	II-A	
142	1	26/10/1946	B3	MLPA; SEQ	NM	NP	NP	NP	-	NM	GG	CRC	59	ADK	RIGHT	T3N0M0	II-A	
143	1	30/06/1943	AP	MLPA; SEQ	NM	NP	NP	NP	-	NM	GG	CRC	63	ADK	RIGHT	T3N0M0	II-A	
144	2	13/02/1936	B4	MLPA; SEQ	NM	NP	NP	NP	-	NM	GA	CRC	71	ADK	LEFT	T2N0M0	I	
145	2	25/04/1944	AP	MLPA; SEQ	NM	NP	NP	NP	-	NM	GG	CRC	61	ADK	RIGHT	T3N0M0	II-A	
146	2	12/07/1974	B1	MLPA; SEQ	NM	NP	NP	NP	-	NM	GG	CRC	32	ADK	RIGHT	T3N0M0	II-A	
147	1	31/08/1935	AP	MLPA; SEQ	NM	NP	NP	NP	-	NM	GA	CRC	73	ADK	RIGHT	T3N1M0	III-B	
148	1	18/04/1956	B3	MLPA; SEQ	NM	NP	NP	NP	-	NM	GG	CRC	54	ADK	LEFT	T2N0M0	I	
149	1	24/07/1954	B3	MLPA; SEQ	NM	NP	NP	NP	-	NM	GA	CRC	55	ADK	RIGHT	T3N0M0	II-A	
150	1	24/03/1928	AP	MLPA; SEQ	NM	NP	NP	NP	-	NM	GA	CRC	82	ADK	RIGHT	T3N0M0	II-A	
151	1	18/08/1944	AP	MLPA; SEQ	NM	NP	NP	NP	-	NM	GG	CRC	66	ADK	RIGHT	T2N0M0	I	
152	2	22/07/1962	B1	MLPA; SEQ	NM	NP	NP	NP	-	NM	GA	CRC	41	ADK	RIGHT	T3N0M0	II-A	
227	1	01/01/1940	B2	MLPA; SEQ	NM	NP	NP	NP	-	NM	GG	CRC	61	ADK	LEFT	T3N0M0	II-A	
												CRC	68	ADK	RIGHT	T4N1M1	III-C	
153	2	29/10/1965	B1	MLPA; SEQ	NM	NP	NP	NP	-	NM	GG	CRC	42	NS	NS	NS	NS	
154	2	20/07/1963	B1	MLPA; SEQ	NM	NP	NP	NP	-	NM	GG	CRC	44	ADK	LEFT	T4N0M0	II-B	
155	2	12/06/1983	B1	MLPA; SEQ	NM	NP	NP	NP	-	NM	GG	CRC	25	ADK	LEFT	NS	NS	
156	1	18/12/1956	AP	MLPA; SEQ	NM	NP	NP	NP	-	NM	GG	CRC	52	ADK	RIGHT	T3N0M0	II-A	
157	1	29/10/1952	AP	MLPA; SEQ	NM	NP	NP	NP	-	NM	AA	CRC	57	ADK	LEFT	NS	NS	
158	1	07/07/1979	B1	MLPA; SEQ	NM	NP	NP	NP	-	NM	AA	SBC	30	NS	Duodenum	T4N1M0	III-B	
159	2	12/03/1954	B3	MLPA; SEQ	NM	NP	NP	NP	-	NM	GG	CRC	55	ADK	RIGHT	T3N0M0	II-A	
160	1	19/05/1954	B1	MLPA; SEQ	NM	NP	NP	NP	-	NM	GG	CRC	41	ADK	RIGHT	B Dukes	II-A	
161	1	25/08/1946	B3	MLPA; SEQ	NM	NP	NP	NP	*MLH1* c.1852_1853delAA insGC; p.K618A (Class1)	NM	GG	CRC	39	ADK	RIGHT	T3N0M0	II-A	
162	2	11/01/1956	B1	MLPA; SEQ	VUS	NP	NP	NP	*MLH1* c.702G>A; p.= (Class 2)	NM	GG	CRC	52	ADK	RIGHT	T2N0M0	I	
163	2	16/06/1938	AP	MLPA; SEQ	NM	NP	NP	NP	*MLH1* c.307-29C>A; p.= (Class 1)	NM	GG	CRC	70	ADK	RIGHT	T4N1M0	III-B	
164	1	26/08/1956	B1	MLPA; SEQ	NM	NP	NP	NP	*MLH1* c.2146G>A ; p.V716M (Class 1)	NM	GG	CRC	48	ADK	RIGHT	T3N2M0	III-C	
177	2	06/12/1926	B5	MLPA; SEQ	NM	NP	NP	NP	*MLH1* c.307-29C>A; p.= (Class 1)	NM	GG	CRC	78	ADK	RIGHT	T3N0M0	II-A	
178	1	06/04/1952	AII	EXSEQ	NM	NP	NP	NP		NM	NI	CRC	40	ADK	LEFT	T3N1M0	III-B	
179	1	20/12/1949	B1	EXSEQ	NM	NM	NP	NP	-	NA	AA	CRC	50	ADK	RIGHT	T3N0M0	II-A	
185	2	09/01/1957	B1	MLPA; SEQ	NM	NP	NP	NM	-	NM	GG	EC	50	ADK	LUS	NS	III-C	
186	1	16/03/1962	AII	MLPA; SEQ	NM	NP	NP	NP	-	NM	GG	CRC	45	ADK	RIGHT	T2N0M0	I	
188	2	17/12/1966	B1	MLPA; SEQ	NM	NP	NP	NM	-	NM	GG	CRC	42	ADK	RECTAL	T2N0M0	I	
201	2	12/12/1944	AII	EXSEQ	NM	NP	NP	NP	-	NM	GG	EC	60	ADK	NS	T1bN0M0	I	
202	2	06/10/1940	AP	EXSEQ	NM	NP	NP	NP	-	NM	GG	CRC	66	ADK	RIGHT	T2N1M0	III-A	

Table 11. Continued.

PATIENT INFORMATION				GERMLINE DATA								SOM						
				MMR MUTATIONAL ANALYSIS								CLINICAL DATA						
Patient ID	SEX	DATE OF BIRTH	CLINICAL CRITERIA	TECHNIQUE	*MLH1*	*MSH2*	*MSH6*	*PMS2*	VUS Presence (Insight classification)	*MLH1* promoter sequencing	*MLH1* c.-93	TYPE/ ORGAN	AGE AT DIAGNOSIS	HISTOLOGICAL TYPE	ANATOMIC LOCALIZATION	TNM STAGE	AJCC STAGE	H DIF
Continuation: Patients harboring MLH1 (at least) deficient tumors																		
203	2	06/12/1956	B5	MLPA; SEQ	NM	NP	NP	NP	-	NM	GG	CRC	51	ADK	RIGHT	T4N2M0	III-C	
204	1	21/10/1988	B1	MLPA; SEQ	NM	NP	NP	NP	-	NM	GA	CRC	16	NI	NI	NS		
205	2	16/07/1938	AP	MLPA; SEQ	NM	NP	NP	NP	-	NM	GA	CRC	69	ADK	RIGHT	T3N0M0	II-A	
206	2	26/08/1965	B1	MLPA; SEQ	NM	NP	NP	NP	-	*MLH1* c.-1018G>GA	GA	CRC	42	ADK	NI	NS	NS	
207	2	15/10/1932	B2	MLPA; SEQ	NM	NP	NP	NP	-	NM	GG	CRC	69	ADK	LEFT	NS	NS	
												EC	69	EN	NS	NS	NS	
208	2	06/11/1961	B1	MLPA; SEQ	NM	NP	NP	NP	-	NM	GG	CRC	43	ADK	LEFT	T3N0M0	II-A	
210	2	29/01/1944	B2	MLPA; SEQ	NM	NP	NP	NP	-	NM	GG	CRC	64	ADK	RIGHT	NS	NS	
												CRC	64	ADK	LEFT	NS	NS	
212	1	10/07/196	B1	MLPA; SEQ	NM	NP	NP	NP	-	NM	GG	CRC	41	ADK	RIGHT	T3N0M0	II-A	
215	2	15/06/1951	AP	MLPA; SEQ	NM	NP	NP	NP	-	NM	GA	EC	56	EN	NS	NS	NS	
216	2	05/11/1954	B2	MLPA; SEQ	NM	NP	NP	NP	-	NM	GA	EC	50	NS	NS	NS	NS	
220	1	21/10/1928	B2	MLPA; SEQ	NP	NM	NP	NP	-	NM	GG	CRC	51	ADK	RECTAL	T4N0M0	II-B	
												CRC	81	NS	NS	NS	NS	
												CRC	81	NS	NS	NS	NS	
221	1	26/03/1944	AP	MLPA; SEQ	NM	NP	NP	NM	-	NM	GA	CRC	65	ADK	RIGHT	T4N2M0	III-C	
223	1	24/09/1960	B1,3	MLPA; SEQ	NM	NP	NP	NP	-	NM	GG	CRC	47	ADK	RIGHT	T4N0m0	II-B	
225	1	26/06/1962	B1,5	MLPA; SEQ	NM	NP	NP	NP	-	NM	GA	CRC	49	ADK	LEFT	T3N0M0	II-A	
190	2	01/03/1944	B1	MLPA; SEQ	NM	NP	NP	NP	-	NM	GG	CRC	49	ADK	LEFT	T3N1M0	III-B	
Patients harboring PMS2 (only) deficient tumors																		
165	2	09/03/1964	B1	MLPA; SEQ	NM	NM	NM	NM	-	NM	GG	45	CRC	ADK	RIGHT	T3N0M0	II-A	
166	1	17/04/1947	B1	MLPA; SEQ	NM	NM	NP	NM	-	NM	NI	47	CRC	ADK	RECTAL	T3N0M0	II-A	
167	2	03/08/1950	AP	MLPA; SEQ	NM	NP	NP	NM	*MLH1* c.2146G>A ; p.V716M (Class1)	NM	GG	57	CRC	ADK	NS	T2N0M0	I	
168	1	05/03/1947	B5	MLPA; SEQ	NP	NP	NP	NM	*PMS2* c.59G>A, p.R20Q (Class 1); c.*17G>C, p.=(Class 1)	NM	NI	59	CRC	ADK	RIGHT	T3N1M0	III-B	
180	2	07/09/1946	AII	MLPA; SEQ	NM	NM	NP	NM	-	NM	GA	47	OC	ADK	LEFT OVARY	T1N0M0	I	

Abbreviations: AP: anatomo-pathological, AII: Amsterdam Criteria II, B: Bethesda Criteria, MLPA: Multiplex Ligation-dependent Probe Amplification, NP: not performed, NM: non mutated, WT: wildtype, NS: not specified, NI: no information, M: methylated, UM:un
cancer, EC: endometrial cancer, BC: breast cancer, ADK: adenocarcinoma, EN: endometrioid.

2. Promoter analyses in patients with tumors lacking MSH2 and/or MSH6 expression.

A total of 25 patients were analyzed (Table 12 and 13). Twelve displayed loss of MSH2/MSH6 proteins (not included in article 4) and 13 displayed loss of MSH6 protein. Of the 12 MSH2/MSH6 deficient (Table 12), eight fulfilled at least one Bethesda criterion and 4 fulfilled Amsterdam. Mutational analysis of *MSH2* coding region had previously been performed in all the cases. Two cases were carriers of germline class 3 *MSH2* variants (according to Insight classification in December 2013). Germline mutations in coding regions of *MSH6* were analyzed in 9 cases with no mutations found. Three have had multiple primary LS-associated tumors, and the mean age of first tumor diagnosis was 44.9 years old (range 21-74) (Table 12). Microsatellite analysis showed instability in the 11 analyzed tumors (9 were colorectal, 1 endometrial and 1 corresponded to an ovarian cancer).

The series of patients with tumors lacking MSH6 protein expression alone was composed of 7 females and 6 males. Eight had at least 1 Bethesda criterion, 3 fulfilled Amsterdam criteria and the remaining 2 were deferred by the Pathology Department. Mutational analysis of *MSH6* coding region was previously performed in all the cases. Seven cases were carriers of germline class 3 *MSH6* variants (according to Insight classification rules in December 2013). No germline mutations in coding region of *MSH2* were identified in 5 analyzed patients. Two of them had personal history of multiple primary LS-associated tumors and the mean age at first tumor diagnosis was of 53.8 years (range 36-85). MSH6 deficient tumors were either CRC (n=11) or endometrial (n=2). Microsatellite instability was detected in 10 analyzed tumors.

Table 12. Clinicopathological and molecular features of LLS cases harboring MSH2/MSH6 def

PATIENT INFORMATION				GERMLINE DATA						PROMOTER SEQUENCING				TUMOR				
				MMR MUTATIONAL ANALYSIS						*MSH2*		*MSH6*						
Patient ID	SEX	DATE OF BIRTH	CLINICAL CRITERIA	TECHNIQUE	*MLH1*	*MSH2*	*MSH6*	*PMS2*	VUS Presence (Insight classification)	rs1863332 c.-433T>G	rs2303425 c.-118T>C	rs3136228 c.-557T>G	rs3136229 c.-448G>A	TYPE/ ORGAN	AGE AT DIAGNOSIS	HISTOLOGICAL TYPE	LOCALIZATION	TNM STAGE
181	2	06/01/1974	AII	MLPA; SEQ	NM	NM	NM	NP	-	TT	TT	TG	GG	OC	25	NI	NI	NI
183	1	08/04/1938	B2	MLPA; SEQ	NM	VUS	NM	NP	*MSH2* c.2045C>G; p.T682S (Class 3*)	TG	TT	TG	GG	CRC	58	ADK	RIGHT	T4N1M0
														CRC	61	ADK	RIGHT	T3N0M0
187	1	23/06/1960	AII	MLPA; SEQ	NP	VUS	NM	NP	*MSH2* c.561_569delTGAGGCTCT; p.E188_L190del (Class 3), and *MSH2* c.965G>A; p.G322D (Class 1)	TT	TT	TG	GG	CRC	45	ADK	RIGHT	TisN0M0
189	1	26/05/1988	B1,4	MLPA; SEQ	NP	NM	NM	NP	*MSH2* c.965G>A; p.G322D (Class 1)	TT	TT	TG	GG	CRC	21	ADK	LEFT	T2N0M0
191	1	19/02/1947	B2	MLPA; SEQ	NP	NM	NP	NP	*MSH2* c.1666T>C; p.= (Class 1)	TG	TT	TT	GG	CRC	58	ADK	LEFT	T4N0M0
198	1	10/09/1956	AII	MLPA; SEQ	NP	NM	NM	NP	*MSH2* c.965G>A; p.322D(Class 1)	TT	TT	TT	GG	CRC	44	ADK	RIGHT	T4N2M0
211	1	23/04/1962	B1,B4	MLPA; SEQ	NP	NM	NM	NP	-	TG	TT	TG	GA	CRC	45	ADK	LEFT	NS
214	2	15/09/1959	AII	MLPA; SEQ	NP	VUS	NP	NP	*MSH2* deletion of exon 16; p=? (Class 3*)	TT	TT	TG	GG	EC	40	ADK	NS	NS
														CRC	47	ADK	NS	NS
218	2	20/07/1978	B1	MLPA; SEQ	NP	VUS	NP	NP	*MSH2* c.2702A>T; p.E901V (Class 3*)	TT	TT	TT	GG	CRC	31	ADK	RIGHT	NS
219	2	09/01/1936	B1	MLPA; SEQ	NP	NM	NM	NP	-	TT	TT	TG	GG	CRC	50	NS	RIGHT	NS
														CRC	63	NS	NS	NS
														BC	78	NS	NS	NS
222	2	27/02/1950	B1,4	MLPA; SEQ	NP	VUS	NM	NP	*MSH2* c.518T>G; p.L173R (Class 3)	TT	TC	TG	GG	CRC	48	ADK	RECTAL	NS
224	1	16/08/1936	B5	MLPA; SEQ	NP	NM	NM	NP	-	TT	TT	TT	GG	CRC	74	ADK	RIGHT	T3N0M0

Class 3 *: Variant has not been reported but corresponds to a Class 3 accoridng to the rules.

Abbreviations: AP: anatomo-pathological, AII: Amsterdam Criteria II, B: Bethesda Criteria, MLPA: Multiplex Ligation-dependent Probe Amplification, NP: not performed, NM: non mutated, WT: wildtype, NS: not specified, NI: no information, M: methylated, U colorectal cancer, EC: endometrial cancer, BC: breast cancer, ADK: adenocarcinoma, EN: endometrioid.

Table 13. Clinicopathological and molecular features of LLS cases harboring MSH6 deficie

PATIENT INFORMATION				GERMLINE DATA						PROMOTER SEQUENCING				TUMOR					
				MMR MUTATIONAL ANALYSIS						*MSH2*		*MSH6*							
Patient ID	SEX	DATE OF BIRTH	CLINICAL CRITERIA	TECHNIQUE	*MLH1*	*MSH2*	*MSH6*	*PMS2*	VUS Presence (Insight classification)	rs1863332 c.-433T>G	rs2303425 c.-118T>C	rs3136228 c.-557T>G	rs3136229 c.-448G>A	TYPE OF TUMOR	AGE AT DIAGNOSIS	HISTOLOGICAL TYPE	LOCALIZATION	TNM STAGE	AJCC
125	1	13/04/1922	AP	MLPA; SEQ	NP	NP	NM	NP	-	TT	TC	TG	GA	CRC	85	ADK	RIGHT	T4N0M0	I
126	1	06/06/1952	AP	MLPA; SEQ	NP	NP	NM	NP	-	TT	TC	TT	GG	CRC	56	ADK	RECTAL	T2N0M0	
127	1	29/12/1948	AII	MLPA; SEQ	NP	NM	NM	NP	-	TT	TT	TG	GA	CRC	58	ADK	RECTAL	T3N0M0	I
128	1	15/05/1930	AII	MLPA; SEQ	NM	NM	VUS	NP	*MSH6* c.1439T>A; p.V480E (Class 3 *)	TT	TT	TT	GG	CRC	61	ADK	RECTAL	T3N0M0	I
129	2	12/12/1954	B2,5	MLPA; SEQ	NP	NP	VUS	NP	*MSH6* c.1153_1155delAGG p.R385del (Class 3 *)	TT	TT	GG	GA	CRC	53	ADK	RIGHT	T3N0M0	I
130	2	01/03/1959	B1,4	MLPA; SEQ	NP	NP	VUS	NP	*MSH6* c.1618_1620delCTT; p.L540del (Class 3 *)	TT	TT	TT	GG	CRC	46	ADK	RIGHT	T3N0M0	I
131	1	03/02/1959	B1,5	MLPA; SEQ	NM	NM	VUS	NP	*MSH6* c.1439T>A; p.V480E (Class 3 *)	TT	TC	TT	GG	CRC	39	ADK	LEFT	NS	II
184	2	15/09/1948	B1	MLPA; SEQ	NM	NM	NM	NP	*MSH2* c.965G>A; p.G322D (Class 1)	TG	TT	TG	GA	CRC	36	NI	RIGHT	NI	
199	1	18/05/1940	B2	MLPA; SEQ	NP	NP	VUS	NP	*MSH6* c.1450G>A; p.E487K (Class 3 *)	TT	TT	TG	GG	CRC	65	ADK	RIGHT	T3N0M0	I
200	2	22/12/1951	B2	MLPA; SEQ	NP	NP	VUS	NP	*MSH6* c.3296T>A; p.I1099N(Class 3 *)	TG	TT	TG	GA	EC	52	EN	NS	T1aN0M0	I
														CRC	53	NS	RIGHT	T4b N0 M1	
209	2	20/04/1951	AII	MLPA; SEQ	NP	NP	NM	NP	-	TT	TT	GG	GA	CRC	56	ADK	RIGHT	TisN0M0	
213	2	11/07/1963	B1	MLPA; SEQ	NP	NM	VUS	NP	*MSH6* c.1618_1620delCTT p.L540del (Class 3 *)	TT	TT	TT	GG	CRC	45	ADK	RIGHT	T3N0M0	I
217	2	19/04/1939	B1,4	MLPA; SEQ	NP	NP	NM	NP	-	TT	TC	TG	GA	EC	48	EN	NS	NS	I
														BC	69	NS	NS	NS	I

Class 3 *: Variant has not been reported but corresponds to a Class 3 accoridng to the rules.

Abbreviations: AP: anatomo-pathological, AII: Amsterdam Criteria II, B: Bethesda Criteria, MLPA: Multiplex Ligation-dependent Probe Amplification, NP: not performed, NM: non mutated, WT: wildtype, NS: not specified, NI: no information, M: methylated, UM:u endometrial cancer, BC: breast cancer, ADK: adenocarcinoma, EN: endometrioid.

2.1 Mutational analysis of MSH2 and MSH6 promoters

MSH2 and *MSH6* promoter regions were analyzed in patients with tumors lacking either MSH2/MSH6 or MSH6 protein alone. A region encompassing 662bp and 915bp upstream the TSS (Transcription Start Site) of *MSH2* and *MSH6* genes, respectively was amplified and sequenced (Fig. 24).

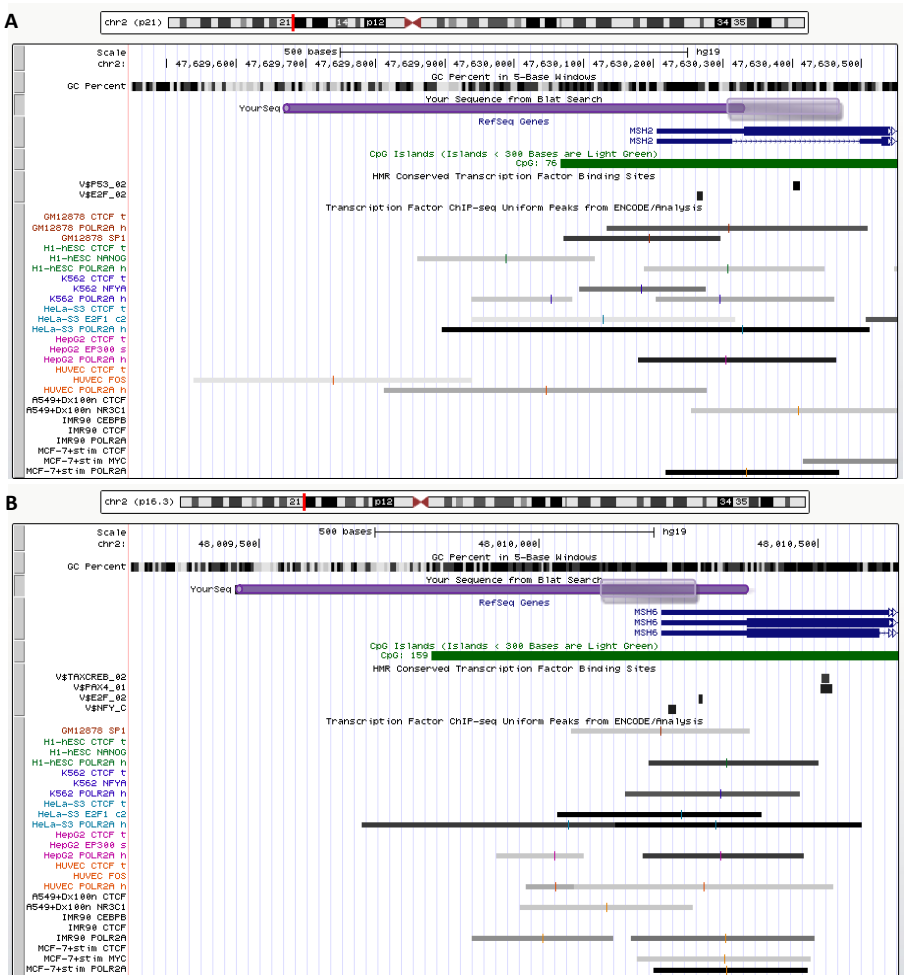

Figure 24. UCSC blat of the selected region for promoter sequencing of: A) *MSH2* promoter; B) *MSH6* promoter.

Promoter region sequenced by conventional method in PBL is marked in purple and regions analyzed by MS-MCA are colored in light purple. CpG island is colored in green; transcription factor bind sites from HMR conserved and ENCODE are shown in the lower part.

Only known polymorphisms were found: two in the promoter region of *MSH2* and two in the *MSH6*. Three out of 12 of patients with MSH2/MSH6 deficient tumors were carriers of rs1863332 (c.-433T>G) and 1 of rs2303425 (c.-118T>C). Among the 13 cases with MSH6 deficient tumors, 2 were carriers of rs1863332 (15.4%) and 4 of rs2303425 (30.8%).

Eight (67%) of the 12 cases with MSH2/MSH6 deficient tumors were heterozygous for rs3136228 (c.-557T>G) and one (8.3%) case for rs3136229 (c.-448G>A) of the MSH6 gene. Six out of 13 MSH6 deficient tumors were heterozygous carriers of c.-557T>G and 15.4% (2 out of 13) were homozygous. For *MSH6* c.-448G>A, all 7 carriers (53.8%) were heterozygous. However, the mutational analysis of the *MSH6* promoter is not completed, because the region comprised between c.-220 to c.-23 was not covered with the design used. This region is being currently analyzed.

2.2 Somatic methylation analysis at MSH2 and MSH6 promoters

Methylation analyses of *MSH2* and *MSH6* genes in tumor samples were performed by MS-MCA. The amplified region in *MSH2* covered 13 CpGs containing MS-MLPA probe +126 (ME011-B1 kit). We achieved 10% detection sensitivity of methylation at *MSH2* promoter region. For *MSH6* promoter analysis, the MS-MCA design covered 18 CpGs (containing the *Hha*I enzyme target of MS-MLPA probe 208 of ME011-B1 kit, with a sensitivity of 25%. *In vitro* methylated DNA from Jurkat cell line and a CRC sample from an *EPCAM* deletion carrier were used as methylated controls in these experiments.

No methylation in *MSH2* or *MSH6* promoters was detected in available MSH2/MSH6 deficient tumors (see Article 4). In the series of 13 patients with MSH6-deficient tumors, we collected 5 tumor blocks. In the analysis of methylation, two of them did not amplify the promoter region of *MSH2* and one of those neither amplified *MSH6* promoter (Table 13). Therefore, we discarded the presence of hypermethylation at the promoter regions of *MSH2* and *MSH6* in 3 and 4 samples, respectively.

Additionally, as methylation has been detected by COBRA in tumors from *MSH2* mutation carriers (Nagasaka et al. 2010), we evaluated 8 tumor samples from this kind of patients from our LS series, not detecting *MSH2* methylation in any of them (Article 4).

ARTICLE 1

***MLH1* methylation screening is effective in identifying epimutation carriers.**

Hypothesis: Constitutional epimutations in the *MLH1* gene have been identified as a potential cause of LS. Germline methylation analysis of LS suspected patients with *MLH1* methylated tumors may be of help in its identification.

Aim: To investigate the prevalence of *MLH1* epimutations in a series of 34 patients with *MLH1*-methylated CRC and no detected germline *MLH1* mutations and to characterize *MLH1*-epimutation carriers.

Summary of the obtained results: We identified two *bona fide MLH1* epimutation carriers in a series of 34 patients (5.9%) with MLH1-deficient tumors, in whom no germline *MLH1* mutation was identified. In one of the cases, the identified *MLH1* constitutional methylation was monoallelic and resulted in *MLH1* and *EPM2AIP1* allele-specific transcriptional silencing. It was present in normal somatic tissues and absent in spermatozoa. The methylated *MLH1* allele was maternally transmitted and methylation was reversed in a daughter who inherited the same allele. In the other epimutant case, average methylation levels in blood were ~20% and Single-nucleotide primer extension analysis evidenced partial silencing of *EPM2AIP1* G allele. The study adds further evidence to the emerging entity of soma-wide *MLH1* epimutation and its heritability.

Contribution of the PhD candidate: Molecular characterization of the second epimutant reported in this work (case 34) by means of: (i) Direct sequencing of *EPM2A1P1* gene and *MLH1* gene promoter; (ii) *EPM2AIP1* allele-specific expression analysis by SNuPe; (iii) Bisulfite sequencing of the promoter region of *MLH1* gene of PBL DNA; (iv) Methylation-specific melting curve analysis (MS-MCA) and pyrosequencing of regions C and D of *MLH1* promoter; (v) Methylation-specific

multiplex ligation-dependent probe amplification (MS-MLPA) of PBL and tumoral DNA of case 34; and (vi) Preparation of all figures and sections of tables related to the characterization of case 34 and contributing to writing of the article.

MLH1 methylation screening is effective in identif epimutation carriers

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Recently, constitutional *MLH1* epimutations have been identified in a subset of Lynch syndrome (LS) cases. The aim study was the identification of patients harboring constitutional *MLH1* epimutations in a set of 34 patients with a cli suspicion of LS, *MLH1*-methylated tumors and non-detected germline mutations in mismatch repair (*MMR*) genes. *M* promoter methylation was analyzed in lymphocyte DNA samples by MS-MLPA (Methylation-specific multiplex ligation- probe amplification). Confirmation of *MLH1* constitutional methylation was performed by MS-MCA (Methylation-specif curve analysis), bisulfite sequencing and pyrosequencing in different biological samples. Allelic expression was deterr using heterozygous polymorphisms. Vertical transmission was evaluated by MS-MLPA and haplotype analyses. MS-ML analysis detected constitutional *MLH1* methylation in 2 of the 34 individuals whose colorectal cancers showed *MLH1* methylation (5.9%). These results were confirmed by bisulfite-based methods. Both epimutation carriers had develope metachronous early-onset LS tumors, with no family history of LS-associated cancers in their first-degree relatives. In cases, the identified *MLH1* constitutional methylation was monoallelic and results in *MLH1* and *EPM2AIP1* allele-spe transcriptional silencing. It was present in normal somatic tissues and absent in spermatozoa. The methylated *MLH1* maternally transmitted and methylation was reversed in a daughter who inherited the same allele. *MLH1* methylation in lymphocyte DNA from patients with early-onset *MLH1*-methylated LS-associated tumors allows the identification of epimutation carriers. The present study adds further evidence to the emerging entity of soma-wide *MLH1* epimutation heritability.

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Keywords: Lynch syndrome; constitutional epimutation; *MLH1*; methylation; MS-MLPA; pyrosequencing

INTRODUCTION

Lynch syndrome (LS) is characterized by an autosomal dominant inheritance of early-onset colorectal cancer (CRC) and increased risk of other cancers.[1,2] It is caused by germline mutations in DNA mismatch repair (*MMR*) genes. *MLH1* or *MSH2* are the most commonly mutated *MMR* genes in LS, whereas mutations in *MSH6* or *PMS2* are significantly less common.[3,4] Occasionally, the presence of constitutional epimutations in *MSH2* and *MLH1* has been reported (reviewed in Hitchins and Ward[5] and Kuiper *et al*[6]).

Constitutional epimutations are those stable changes in gene expression that do not affect DNA sequence and that are present in normal tissues of a given individual.[7] An epimutation that occurs in the germline or early embryo can affect all or most of the soma, and phenocopy genetic disease. *MSH2* epimutations, associated with a strong heritability, have been shown secondary to the presence of deletions in the neighboring *EPCAM* gene.[6] The mutations lead to mosaic methylation of *MSH2* in EPCAM-expressing cells.[8]

Approximately 40 index cases of constitutional *MLH1* methylation have been reported.[9–23] However, the prevalence of *MLH1* constitutional epimutations is still unknown. Most studies addressing th enriched their sampling with patients affected with CRC sho MLH1 protein expression.[13,17,20,22] In other cases, series w for patients with CRC at an age of onset below 50 years.[9,

In a very few cases genetic alterations in *cis* (gross rea and variants in the promoter region) have been id responsible for the methylation.[13,16,19] In these cases, an dominant pattern is readily observed. However, in mo genetic cause for the epimutation has been identified (Hitchins and Ward[5]). In this context, the inherita epimutation has only been experimentally confirme cases.[10,17,20] The functional impact of these epimuta clear. In the few cases analyzed, methylation has l to allele-specific silencing of *MLH1* and *EPM2AIP1*.[12 associates with an allele-specific methylation pattern.[11,17,2 cases, methylation seems to be widespread affecting all layers being mosaicism reported.[10,12,20]

The aim of our study was to investigate the pr *MLH1* epimutations in a series of 34 patients with *MLH1*

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CRC and no detected germline *MLH1* mutations. We identified two *bona fide MLH1* epimutation carriers and extensively characterized one of them. The epimutated allele is maternally transmitted, methylation is present in all embryonic layers, erased in spermatozoa and not transmitted to the next generation.

MATERIALS AND METHODS

Patients and samples

Patients were assessed through Cancer Genetic Counselling Units of the Institut Català d'Oncologia (ICO) and the University of Michigan (UM) from 1998 to 2010. A total of 34 individuals (30 ICO, 4 UM) presenting *MLH1*-methylated tumors (methylation levels above 20% in C or D regions) were included in this study (Table 1). The ICO patients were selected from a series of 56 individuals with *MLH1*-deficient CRC and no germline mutations identified in *MLH1*.[24] In all, 29 patients met Bethesda criteria, 1 case met Amsterdam criteria and 4 cases showed other types of CRC familial aggregation. Clinico-pathological information was recorded. Informed consent was obtained from all individuals, and ethics committee a this study. Sample processing is detailed in Supplementary Methods.

MLH1 promoter methylation analyses

DNA from RKO colorectal tumor cell line (American Type Culture Co Manassas, VA, USA) was used as a biallelic *MLH1* methylation To generate unmethylated DNA, peripheral blood lymphocyte (PBL) I amplified using the REPLI-g kit (Qiagen, Valencia, CA, USA). A sa CEPH DNA from the Coriell Institute was used as an unmethylated c pyrosequencing analyses.[25]

Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA)

SALSA MS-MLPA ME011 kit (MRC Holland, Amsterdam) is based o of probes that contain a digestion site specific for the methylation-*Hha*I enzyme. All reactions were carried out using 100 ng of DNA. includes five probe pairs in *MLH1* promoter (with the respective *H*

Table 1 Clinical and molecular features of patients with *MLH1*-methylated CRC

									% somatic MLH1 methylation		
Case	*Gender*	*Clinical criteria*	*CRC age of onset*	*CRC location*	*TNM*	*Grade*	*Mucinous component*	*Other tumors (age of onset)*	*BRAF*	*C region (−246)*	*D region (−13)*	*rs1 (c.*
1	M	BC	32	L	T3N0M0	G1	No	CRC (34)	wt	57.6	59.7	
2	F	BC	49	R	T2N2M0	G3	Yes		V600E	24.9	36.9	
3	M	BC	37	L	T3N0M0	G2	No		wt	29.3	31.7	
4	F	BC	73	R	T4aN0M0	na	Yes		wt	73.5	70.5	
5	M	BC	50	R	T3N1M0	G2	No		wt	28.6	33.6	
6	F	FA	62	R	T3N0M0	G3	No		wt	61.5	78.5	
7	M	BC	42	R	T4N2M0	G2	No	CRC (synch)	wt	24.1	25.2	
8	M	BC	29	R	T3N0M0	G2	No		wt	25.1	27.6	
9	F	BC	47	L	T3N1M0	G2	Yes		wt	38.5	34.9	
10	F	BC	77	R	T3N0M0	na	Yes		wt	38.2	24.1	
11	M	BC	52	R	T3N0M0	G2	No		V600E	35.4	4.4	
12	F	BC	62	L	T3N0M0	G2	No		wt	53.7	76.7	
13	F	BC	59	R	T3N0M0	G2	No		V600E	39.4	45.8	
14	F	BC	77	R	T3N0M0	G2	No		V600E	34.5	28.4	
15	F	BC	52	R	T4aN0M0	G2	Yes		V600E	22.9	41.4	
16	F	BC	24	R	T3N0M0	G3	No		V600E	57.5	75.1	
17	M	FA	78	R	T3N0M0	G2	No		wt	12.5	24.0	
18	M	BC	48	R	na	na	No		wt	32.8	34.8	
19	M	FA	73	R	T3N0M0	G3	Yes		V600E	19.4	31.2	
20	F	BC	50	R	T3N0M0	G2	Yes		V600E	35.8	27.0	
21	F	BC	58	R	T3N0M0	G2	No	3 CRC (synch)	V600E	40.6	66.6	
22	M	FA	85	R	T4bN0M0	G3	No		V600E	41.4	42.5	
23	F	BC	47	L	T3N0M0	G3	Yes		V600E	20.3	39.3	
24	F	BC	59	R	T1N0M0	G2	No	CRC (29)	V600E	11.4	20.6	
25	M	BC	69	R	T4N0M0	G3	Yes	CRC (synch)	wt	50.3	43.1	
26	F	BC	75	R	T2N0M0	G2	No	CRC (64)	V600E	27.1	30.3	
27	M	BC	47	L	T3N0M0	G1	No		wt	40.1	21.6	
28	M	BC	31	L	T4N0M0	G2	Yes		wt	26.2	32.7	
29	F	BC	23	L	T4N1M0	G2	No	GC (26)	wt	79.8	50.4	
30	M	BC	86	R	T3N0M0	na	na	BrC (69); RC (78)	wt	na	na	
31	M	AMS	68	R	T3N0M0	na	na	M (80)	wt	na	na	
32	F	BC	55	R	T2N0M0	na	na		wt	na	na	
33	F	BC	52	R	T3N1M0	G3	na		wt	na	na	
34	F	BC	47	R	T1N0M0	na	No	CRC (29), EC (49)	wt	26.1	37.3	

Abbreviations: AMS, Amsterdam criteria; BC, Bethesda criteria; FA, Familial aggregation; M, male; F, female; R, right; L, left; CRC, colorectal cancer; EC, endometrial cancer; GC, gastric BrC, breast cancer; RC, renal cancer; M, mesothelioma; synch, synchronous; wt, wild-type; na, not available.

located at −659, −383, −246, −13 and +208 relative to the start codon; GenBank accession number U26559) that cover five independent regions: regions A to D of the promoter and intron 1.[26]

Methylation-specific melting curve analysis

Methylation-specific melting curve analysis method consists in a real-time PCR followed by temperature dissociation on DNA previously treated with sodium bisulfite,[27] using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA). Experimental conditions and primers are detailed in Supplementary Methods, Figure S1 and Table S1.

Bisulfite sequencing

A total of 1 μl of bisulfite-converted DNA was used in a PCR reaction for the amplification and subsequently sequencing of *MLH1* promoter regions C and D.[26] Experimental conditions and primers are detailed in Supplementary Methods, Figure S1 and Table S1.

Clonal bisulfite sequencing

A total of 1 μl of bisulfite-modified DNA was amplified, cloned and sequenced. Experimental conditions and primers are detailed in Supplementary Methods, Figure S1 and Table S1.

Pyrosequencing

In all, 2 μl of bisulfite-converted DNA were used in a PCR reaction for the amplification of regions C and D of the *MLH1* promoter[26] using HotStarTaq master mix (Qiagen) and biotinylated primers (Supplementary Table S1 and Figure S1). Primers were designed using the Pyromark Assay Design Software 2.0 (Qiagen). Experimental conditions are detailed in Supplementary Methods.

MLH1 allelic expression analyses

For allelic expression analyses at the c.655A>G SNP (rs1799977) within *MLH1* exon 8, the relative levels of the A/G alleles were determined in genomic DNA and cDNA by single-nucleotide primer extension (SNuPE) and pyrosequencing, as described in Supplementary Methods.

EPM2AIP1 allelic expression analysis

Amplification and sequencing of rs9311149 flanking region, within *EPM2AIP1* gene, was performed as previously described.[12] For allelic expression analysis at rs9311149, the relative levels of G/T alleles were determined in genomic DNA and cDNA by SNuPE as described in Supplementary Methods, using primers listed in Supplementary Table S1.

Direct sequencing of *MLH1* promoter

Screening for mutations within the *MLH1* promoter was performed by PCR amplification and sequencing as described.[28] One reverse amplification primer has been modified (Supplementary Table S1).

Haplotype analysis

Haplotype analysis was performed using four intragenic *MLH1* single-nucleotide polymorphisms (rs1800734, rs9876116, rs1799977 and rs4234259) and seven microsatellite markers (D3S1609, D3S1612, D3S2369, D3S1611, D3S3623, D3S1298, D3S3564) covering 12 Mb around *MLH1*, as previously described.[29] To deduce the methylation-associated haplotype, intrafamilial segregation analysis was performed under the assumption that the number of crossovers between adjacent markers was minimal.

Second hit analysis

Loss-of-heterozygosity (LOH) analysis was performed on DNA extracted from paraffin-embedded tumor tissue and compared with PBL DNA at informative microsatellites (see haplotype analysis) and SNP rs1799977, either by genotyping or SNuPE (see Supplementary Methods), respectively. *MLH1* somatic mutation status was assessed in tumor DNA by direct sequencing and multiplex ligation-dependent probe amplification (SALSA MLPA P003-B1; MRC Holland).

BRAF V600E screening

A 196-bp region of human *BRAF* gene spanning the hotsp c.1799T>A (V600E) was amplified by PCR (Supplementary described.[24] The PCR products were purified using Illustra GFX Band Purification kit (GE Healthcare, Buckinghamshire, UK). mutation detection was performed by SNuPE using the ABI PRI Multiplex Kit (Applied Biosystems, Foster City, CA, USA) a primer.

RESULTS

Clinical and molecular features of patients with *MLH1* CRC

In all, 34 patients (15 males; 19 females) were analyze Mean age at diagnosis was 55 (range 23–86 years). Twenty (76%) were located in the right colon and ten (33%) were mucinous. Only six patients (18%) had lymph node invo none of them had distal metastasis. *BRAF* mutations were 13 tumors (38%). A common SNP rs1800734 (c.−93G the *MLH1* promoter was found to be heterozygote in 10 and homozygote A in 5 (19%). In eight individuals (24% LS-associated tumors were diagnosed, three synchrono metachronous (Table 1). Molecular characterization of tional tumors (Table 2) allowed demonstrating the exist *MLH1*-methylated tumors in four individuals (cases 1, 7,

Identification of new LS cases harboring a constitution epimutation

The methylation status of *MLH1* promoter was analyz MLPA in DNA extracted from PBLs. Constitutional met only detected in 2 individuals (cases 1 and 34) of the included (5.9%). It represented 2 out of 100 LS cases in (2%). In both cases, methylation in *MLH1* promoter was the five regions analyzed, including C and D promoter re was correlated with transcriptional silencing[26] (Table 3).

Sequencing analysis of the whole *MLH1* promoter (fro to intron 1) in PBL DNA from cases 1 and 34 did no variant affecting the binding of MLPA probes nor *Hha* sites. Likewise, it revealed that case 1 was heterozygo rs1800734 (c.−93G>A) and case 34 was heterozygo rs34566456 (c.−607G>C). No other variants – c.−27C>A and c.85G>T[16] – were identified within th region.

Case 1 is a 47-year-old male who underwent urgent sig due to intestinal occlusion secondary to a sigmoid aden (pT3N0M0, stage II) at the age of 32. After 2 years, the diagnosed with an adenocarcinoma of the hepatic flexure stage II) and a subtotal colectomy was carried out. M analysis showed MSI, loss of MLH1 and PMS2 expression *BRAF* V600E mutation and somatic *MLH1* methylati tumors (Table 2). The patient had no family history of c first-degree relatives as it is shown in his pedigree (Figur

Case 34 is a 55-year-old female who was diagnosed c adenocarcinoma (pT3N1M0, stage III) at the age of 2 underwent a sigmoidectomy. After 15 years, the patient wa with an adenocarcinoma of the hepatic flexure (pT1N0M At the age of 49 years, she was diagnosed of an adenocarcinoma (pT1N0M0). Microsatellite analysis show ity of the five analyzed markers in the second CRC, and i bat26 and MONO-27 in the endometrial cancer. B colorectal and endometrial tumors showed loss of

Table 2 Molecular features of tumors from patients affected by multiple LS-associated tumors

				IHC					% somatic MLH1 methy	
Case	Tumor type	Age of onset	MSI analysis	MLH1	MSH2	MSH6	PMS2	BRAF	C region (−246)	D (
1	CRC[a]	32	+	−	+	+	−	wt	57.6	
	CRC	34	+	−	+	+	−	wt	60.5	
7	CRC[a]	42	+	−	+	+	ND	wt	24.1	
	CRC	42	+	−	+	+	−	wt	28.9	
21	CRC[a]	58	+	−	+	+	ND	V600E	40.6	
	CRC	58	−	+	+	+	+	ND	ND	
	CRC	58	−	+	+	+	+	ND	ND	
	CRC	58	−	ND	ND	ND	ND	ND	ND	
24	CRC	29	NA	NA	NA	NA	NA	NA	NA	
	CRC[a]	59	+	−	+	+	ND	V600E	11.4	
25	CRC[a]	69	+	−	+	+	ND	wt	50.3	
	CRC	69	NA	NA	NA	NA	NA	NA	NA	
26	CRC	64	NA	NA	NA	NA	NA	NA	NA	
	CRC[a]	75	+	−	+	+	−	V600E	27.1	
29	CRC[a]	23	+	−	+	+	ND	ND	79.8	
	GC	26	+	−	+	+	ND	ND	63.0	
34	CRC	29	NA	NA	NA	NA	NA	NA	NA	
	CRC[a]	47	ND	−	ND	ND	−	wt	55.5	
	EC	49	+	−	+	+	ND	wt	26.1	

Abbreviations: NA, not available; ND, not done.
[a]Tumors included in the initial series listed in Table 1.

Table 3 Analysis of *MLH1* methylation using MS-MLPA in samples from the proband and relatives

			% MLH1 methylation				
Family	Individual	Sample	A region (−659)	B region (−383)	C region (−246)	D region (−13)	Intron 1 (+208)
A	I.1	PBL	0	0	0	0	0
	II.1 (case 1)	CRC 1	**61.2**	**83.7**	**57.6**	**59.7**	**60.9**
		CRC 2	**62.3**	**86.9**	**60.5**	**62.8**	**63.5**
		PBL	**60.5**	**76.7**	**56.0**	**56.2**	**60.2**
		fibroblasts	**55.8**	**53.2**	**64.0**	**52.4**	**63.0**
		colonic mucosa	**52.3**	**78.9**	**58.3**	**48.5**	**62.6**
		sperm	0	0	0	0	0
	II.2	PBL	0	0	0	0	0
	III.1	PBL	0	0	0	0	0
	III.2	PBL	0	0	0	0	0
B	case 34	EC	**33.6**	**59.4**	**26.1**	**37.3**	**28.5**
		CRC	**58.0**	**56.3**	**55.5**	**48.8**	**56.4**
		PBL	**35.9**	**45.3**	**25.1**	**27.6**	**27.7**
		RKO	**110.1**	**113.2**	**103.0**	**88.2**	**103.4**

Peripheral blood lymphocytes (PBL), skin fibroblasts, colorectal tumors (CRC 1 and 2), normal adjacent mucosa and sperm from case 1 (II.1), PBL from his relatives, and PBL, CRC and endometrial cancer (EC) from case 34, were analyzed. DNA from RKO cell line (methylated in *MLH1*) is used as a positive control. Representative data from two independent experiments is shown. Methylation levels above 20% are shown in bold.

PMS2 expression, absence of *BRAF* V600E mutation and somatic *MLH1* methylation (Table 2). Patient's mother was affected by a breast cancer at the age of 77 years (Figure 1b).

Methylation-specific melting curve analysis confirmed the presence of a methylated peak in C and D promoter regions in both cases (Figure 2a and Supplementary Figure S2). Likewise, bisulfite cing showed the presence of both methylated C as well methylated T (bisulfite-converted non-methylated C) alleles CpG site in the samples of interest (Figure 2a and Suppler Figure S2). Average methylation levels in PBL of the case 1 w and 39% in C and D regions, respectively, as assesed by pyro cing (Figure 2a; Table 4). Clonal bisulfite sequence analysis co hemiallelic methylation in PBL DNA confined to allele A rs1800734 (Figure 2b). In case 34, average methylation levels were 20% and 19% in C and D regions, respectively (Suppler Figure S2; Table 4).

Functional impact of the *MLH1* epimutations

The *MLH1* promoter is bi-directional for transcription of *ML EPM2AIP1* genes. In case 1, the neutral heterozygous polymo c.655G>A (rs1799977) within *MLH1* exon 8 was used to de the effect of the epimutation on *MLH1* transcriptional Monoallelic expression of *MLH1* transcript, associated to was demonstrated by pyrosequencing and SNuPE (Figure (allele-specific expression) values obtained in patient and sample were 0.05 and 1.17 when analyzed by pyrosequenci 0.02 and 0.98 by SNuPE, respectively. In case 34, the abs coding heterozygous polymorphisms in *MLH1* prevented i scriptional analysis.

SNuPE analysis at rs9311149 of *EPM2AIP1* evidenced c silencing of *EPM2AIP1* G allele in case 1 (Figure 3b, right pa partial silencing of the same allele in case 34 (Supplementary S2b), further reinforcing the functional impact of the consti methylation. The obtained ASE values were 0.02 in case 1, 0.48 34 and 1.00 in control sample.

Characterization of the *MLH1* epimutation

MLH1 methylation pattern. Follow-up of case 34 and her fa proved difficult. Thus, for the purpose of detailed characteriza

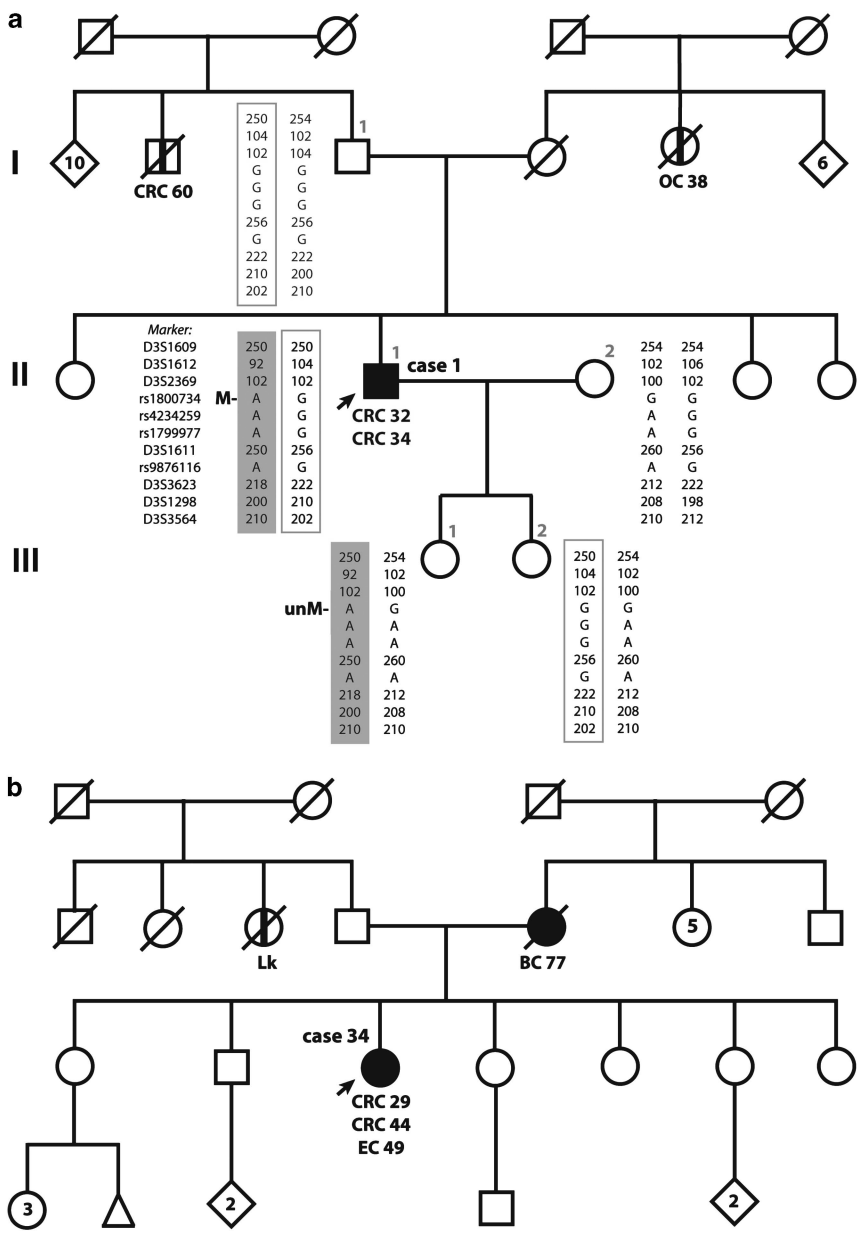

Figure 1 Family pedigree of the epimutation carriers. Circles, females; squares, males; filled, cancer affected; vertical line at center, non-cont affected. Cancer localization (CRC, colorectal cancer; OC, ovarian cancer; EC, endometrial cancer; BC, breast cancer; Lk, leukemia) and age are indicated. (**a**) Pedigree and haplotypes of case 1. The epimutation carrier (II.1) is indicated by an arrow. Generations are indicated on the l Roman numerals and analyzed relatives are identified by numbers. Haplotypes, generated by analyzing SNP and microsatellite markers flank *MLH1*, are detailed according to the key indicated in individual II.1. The paternally inherited allele in II.1 is in a square and the maternally de highlighted in dark gray. The presence of methylation (M) or its absence (unM) is indicated. (**b**) Pedigree of case 34. The epimutation carrier is an arrow.

have focused in the characterization of case 1. First, we wanted to explore whether methylation was present in all embryonic layers and in the germline of case 1. MS-MLPA analysis in skin fibroblasts (ectoderm) and colorectal mucosa (endoderm) revealed similar levels of *MLH1* methylation than in PBL (Table 3), indicating hemiallelic methylation in all embryonic layers. In contrast, no methylation was detected in patient sperm as evidenced by MS-MLPA and pyrosequencing analyses (Tables 3 and 4). Direct sequencing of the PBL and sperm for *MLH1* promoter C region evidenced the presence of both alleles at rs1800734 in both samples (data not shown). T indicate the reversion of the epimutation in patient sper

Inheritance pattern of the epimutant allele. To further in inheritance pattern of the allele harboring the epim analyzed the *MLH1* promoter methylation status as well as of 12 Mb around *MLH1* in available PBL DNA from pa degree relatives. MS-MLPA analysis showed no evidenc methylation in relatives (Table 3). Haplotype analysis reve

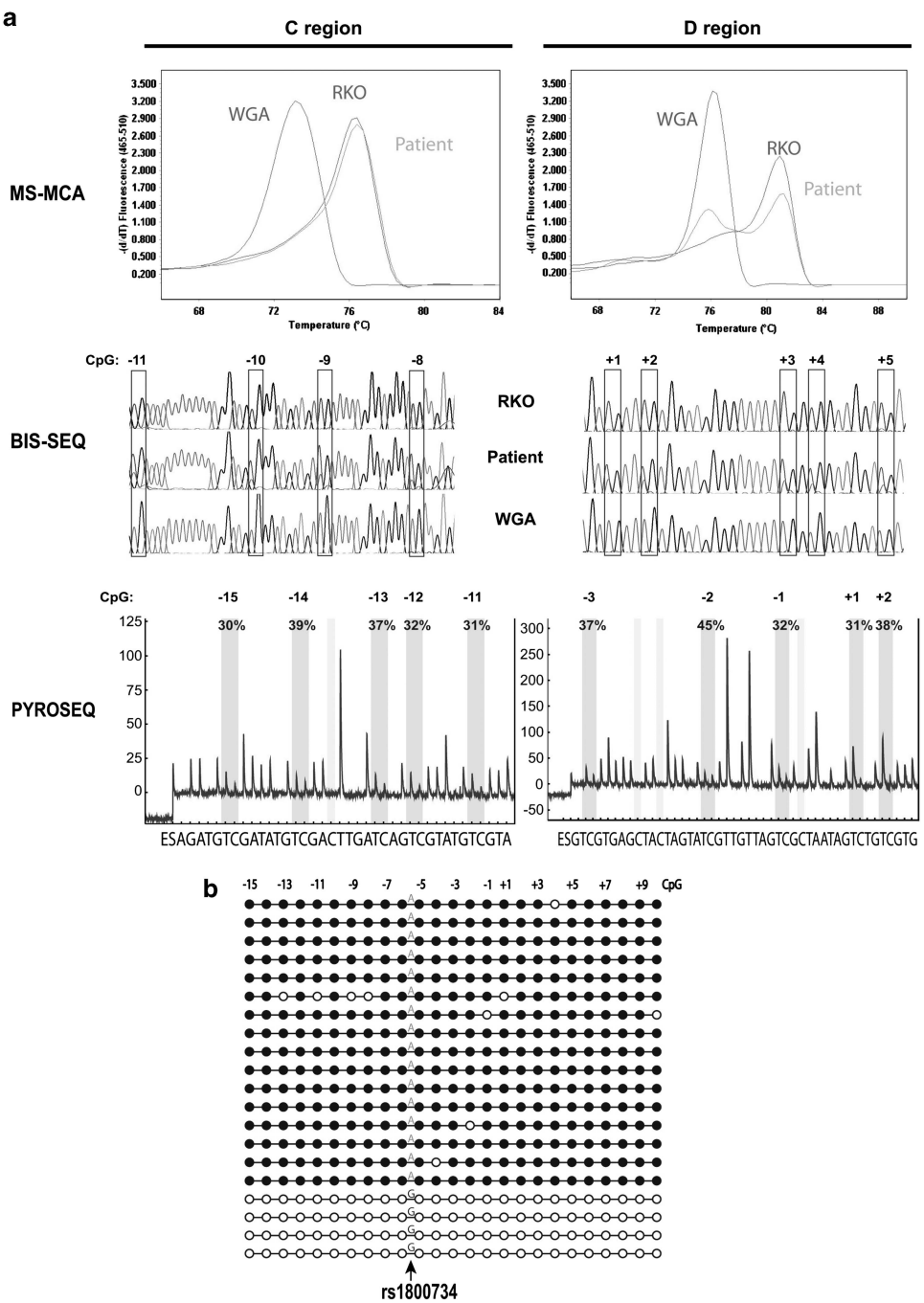

Figure 2 Confirmation of the constitutional *MLH1* epimutation of case 1. (**a**) Analysis of the *MLH1* promoter C and D regions by methylation-specific curve analysis (MS-MCA), bisulfite sequencing (BIS-SEQ) and pyrosequencing (PYROSEQ). Top panel: MS-MCA of *MLH1* promoter. In the analysis of C WGA DNA (unmethylated control) and RKO DNA (methylated control) show single melting peaks at 73 and 77°C, respectively. In D region, WGA a melting peaks temperature are 76 and 82°C, respectively. Analysis by MS-MCA in PBL DNA from the patient 1 (green line) shows the presenc methylated peak in both regions. Middle panel: sequence analysis of bisulfite-converted DNA. WGA DNA shows T at each CpG analyzed, consist complete modification of the DNA. RKO DNA shows C at each CpG. Patient DNA shows a mixture of T and C at CpG sites, attributable to partial met Bottom panel: representative pyrograms obtained in the analysis of C and D *MLH1* promoter regions in PBL DNA from the patient. The peaks within th area of the pyrogram correspond to the CpG interrogated. Percentage methylation at each site is calculated as the C:T ratio of peak heights (rep methylated:unmethylated cytosine). *x* axis represents the nucleotide dispensation order. *y* axis units are arbitrary representing light intensity. (**b**) Clonal sequencing of the *MLH1* promoter in PBL DNA from the epimutation carrier 1. Each horizontal line represents a single allele. CpG dinucleotides are dep circles. Black and white circles indicate methylated and unmethylated CpG, respectively. The allele at rs1800734 (c.−93G>A) is indicated as Methylation is confined to the A allele. Each CpG analyzed is numbered according to its position relative to the translation initiation codon.

epimutated allele is only shared by the patient and one of his daughters (Figure 1a). The lack of availability of biological material from the mother has precluded us from analyzing the presence of the epimutation in her. These results confirmed that the epimutat is maternally inherited in the patient, and that methylation is e the patient's daughter who inherited the same allele.

Table 4 Quantification of *MLH1* promoter methylation by pyrosequencing

MLH1 *promoter C region*

			CpG position							
Family	*Individual*	*Sample*	*−15*	*−14*	*−13*	*−12*	*−11*	*Mean*	*SD*	*Min*
A	II.1 (case 1)	PBL	32.0	38.1	36.1	31.7	33.6	34.3	2.8	31.7
		sperm	2.1	0.0	3.8	2.1	1.4	1.9	1.4	0.0
B	case 34	PBL	22.1	21.6	20.1	17.1	17.7	19.7	2.3	17.1
		RKO	95.5	96.5	94.2	92.6	95.9	94.9	1.6	92.6
		CEPH	2.2	2.15	3.6	2.55	2.3	2.6	0.6	2.2

MLH1 *promoter D region*

			CpG position									
Family	*Individual*	*Sample*	*−6*	*−5*	*−4*	*−3*	*−2*	*−1*	*1*	*2*	*Mean*	*SD*	*M*
A	II.1 (case 1)	PBL	39.0	50.0	38.9	36.4	43.8	33.3	32.1	39.4	39.1	5.8	3
		sperm	0.0	5.3	0.0	1.6	6.5	2.9	0.0	1.7	2.3	2.5	
B	case 34	PBL	19.7	20.5	19.1	19.2	17.2	18.3	19.8	19.2	19.1	1.0	1
		RKO	95.5	92.6	84.0	90.2	76.1	72.7	81.0	93.7	85.7	8.6	7
		CEPH	3.4	5.7	0.0	0.0	9.4	3.8	2.7	2.9	3.5	3.0	

Each sample was run in triplicates. Methylation at each specific CpG was calculated as the mean of the triplicates. Values for each specific CpG within the region are given in perc percentage of methylation of the whole region was calculated as the mean for the five CpGs analyzed in C region and the eight CpGs in the D region. Both peripheral blood lympho sperm from the proband (II.1) were analyzed. DNA from the colorectal cancer cell line RKO was used as positive control. CEPH DNA was used as negative control. Each CpG analy according to its position relative to the translation initiation codon.

Inactivation of the non-methylated allele in tumor tissue. We explored the nature of the putative second hit in the patient's sigmoid colon cancer. Full exonic sequencing of the *MLH1*-coding region did not identify any additional mutation. LOH was evidenced at *MLH1* rs1799977 and D3S1611 (data not shown). Retention of heterozygosity was observed at the distal marker D3S3564, whereas LOH was not evaluable at markers D3S1612, D3S3623 and D3S1298 due to their instability. These results point to the loss of the wild-type *MLH1* allele in tumor DNA. MLPA analysis in tumor DNA was not conclusive, probably owing to the poor quality of tumor FFPE-DNA.

DISCUSSION

We identified two *bona fide MLH1* epimutations and one of them has been extensively characterized. In previous reports, *MLH1* epimutations were detected in 8–13% of patients with tumors showing MLH1 loss of expression.[13,17,20,22] We have detected this alteration in 2 out of 30 patients with *MLH1*-methylated CRC meeting Bethesda or Amsterdam criteria (6.7%) and in 2 of 14 patients with an age of onset below 50 years (14.2%), in whom no germline *MLH1* mutation was identified. This is in line with the prevalence reported by van Roon *et al*[23] in patients with *MLH1*-methylated tumors enriched for cases with an early age of onset. If we take into consideration only the ICO series, *MLH1* epimutations represent so far 2% of all LS cases.

In accordance with previous reports (reviewed in Hitchins and Ward[5]), the cases identified in this study had developed multiple LS tumors at an early age. This may not only reflect the phenotype associated with the epimutation but also the selection criteria used so far in most studies. Of note, methylation was not only detected in metachronous colon tumors but also in endometrial carcinomas as well. *BRAF* mutation was absent in four analyzed tumors from the identified epimutation carriers. However, the presence of somatic *BRAF* V600E mutation has been previously reported in tumors from three epimutation carriers,[10,12,23] representing 15.8% (3/22) of the reported cases. In our set of cases, the degree of *MLH1* methylation is highly variable among tumors from both epimutation carriers and the remaining patients. Epimutations have been detected in two of four cases where multiple tumors showed son hypermethylation.

PBL methylation levels correlated with the observed tra silencing, suggesting the presence of mosaicism in case of the methylated allele is important. In line with prev vations, approximately 50% of the alleles were me case 1.[10,11,14,17,20,21] As reported, the functional imp epimutation seems clear, as it associates with monoalleli of *MLH1* and *EPM2AIP1* transcripts[12,13,15,17] and an a methylation pattern.[14,17,20,21] LOH in an intrage microsatellite marker was detected, consistent with son the unmethylated allele. In fact, LOH has been found to frequent mechanism of inactivation of wild-type allele in t epimutation carriers.[12]

So far, in all cases identified but one, the methylated maternal origin.[10,12,15,17,20] The epimutation was fou maternally inherited allele. Although we were unable to demonstrate whether the epimutation was inherited or may further support the notion that this type of aberrat likely to accumulate during the oogenesis. We were able t more detailed study of the index case and descendants. W methylation was present in every embryonic layer of the i complete erasure was observed in the spermatozoa, by Hitchins *et al*.[17,30] The lack of methylation in sperm not necessarily mean that inheritance cannot occur. I was clearly demonstrated in one descendant who in epimutation out of three harboring the same allele.[17] I the epimutated allele was transmitted unmethylated to daughters.

In spite of an extensive search, we have not been able genetic alteration underlying the epimutated allele. Gen tions in *cis* (gross rearrangements in two cases (one deletio exons 1 and 2, and one duplication involving the whole g a third one the variant c. −27C>A within the promoter been identified as responsible for *MLH1* methyl Dominant transmission pattern is observed in t

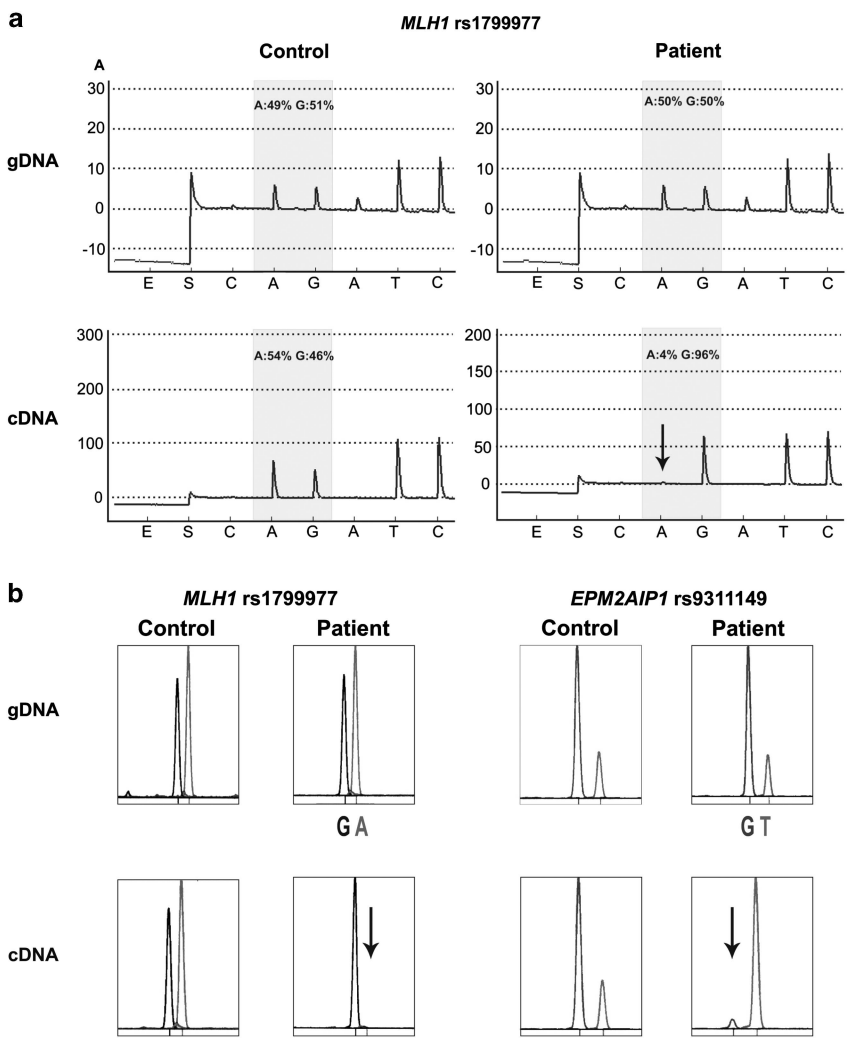

Figure 3 Transcriptional inactivation of *MLH1* and *EPM2AIP1* alleles. (**a**) Illustrative example of the pyrogram across the expressible *MLH1* rs1 (c.655A>G) in genomic DNA (gDNA) (top panels) and cDNA (bottom panels) derived from a heterozygous healthy control (left panels) and the epi carrier (right panels). The peaks within the shaded area of the pyrogram are the nucleotides at the SNP site, quantified with respect to nei nucleotides. Their relative values are given as percentage values above the pyrogram trace. There was a transcriptional inactivation of the ' (indicated with a downward arrow) in the cDNA of the patient with the *MLH1* epimutation. *x* axis represents the nucleotide dispensation order. *y* a are arbitrary representing light intensity. (**b**) Representative results of the SNuPE analysis at *MLH1* rs1799977 (c.655A>G) (left panel) and *EP* rs9311149 (right panel) in gDNA and cDNA derived from a heterozygous control and the epimutation carrier. Transcriptional silencing of the A *MLH1* rs1799977 and T allele at *EPM2AIP1* rs9311149 in the cDNA of the patient was observed.

Dominant inheritance has been also observed in cases where no genetic alterations are detected.[10,12,15,17,20] In these cases, methylation was mosaic and associated to a shared haplotype.

Although we cannot completely rule out that aberrations have been missed, the lack of family history and the lack of vertical transmission are compatible with a *de novo* methylation occurred in the early embryo, where there is no apparent predisposing genetic mechanism that would allow for the restoration of methylation after the gametogenesis. However, this is an unsettled issue. The epimutation carrier identified in this study showed methylation confined to the A allele at rs1800734, although allele-specific methylation is not restricted to either A or G allele in other reported cases.[14,17,20,21] It is intriguing that the A allele at rs1800734 associates with somatic *MLH1* promoter methylation and increased risk of MSI CRC.[23,31–35] In addition, it has been shown that this polymorphism modifies the efficiency of *MLH1*/*EPM2AIP1* transcription.[36]

It is difficult to translate these findings into specific recom tions for these patients and their relatives. At this time ca mandatory. In the presence of a detected constitutional epim genetic screening of descendants is important. However, presence of an inherited non-methylated allele in lym DNA, two options are available. On the one hand, descenda be counseled as relatives of a LS case where direct genetic has been non-informative. In this setting, it is assum lack of methylation in the inherited allele does not rule ou mosaic status is present in the patient or that a non- genetic alteration predisposing to a late acquisition of met is present in this family. Alternatively, recommendati be made based on the degree of personal and familial his cancer. Further knowledge is needed to translate these findings into useful information for management of patie families.

The increasing detection of epimutations has lead to the suggestion that the diagnostic algorithm of LS might be improved. So far, the detection of somatic *MLH1* hypermethylation is often used to exclude patients from further MMR mutation analysis, based on cost effectiveness considerations.[24,37] The patients with somatic *MLH1* hypermethylation could now be considered as candidates to screen for constitutional *MLH1* epimutations. Based on the clinical presentation of the reported cases[5] and our experience, this screening could be restricted to those diagnosed earlier than 50 years or with multiple tumors the first one before the age of 60. If this was the case, MS-MLPA could be a good methodological approach. The robustness and informativeness already shown for paraffin-embedded tissues[24] has been confirmed when being used in the germline. In any case, confirmation with at least another technique (ie, pyrosequencing) would be mandatory.

In summary, *MLH1* methylation screening in PBL from patients with early-onset *MLH1*-methylated CRC allows the identification of epimutation carriers. Using this strategy we have identified two *bona fide MLH1* epimutations. In one of them, the methylated allele is from maternal origin, is present in all embryonic layers and is absent in spermatozoa. The characterization of these cases provides further evidence of the emerging entity of soma-wide MLH1 epimutation and its heritability.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on European Journal of Human Genetics website (http://www.nature.com/ej

Table S1. Primers used in the current study. (*) Biotinylated labeled primer. The location
methylation is shown in Figure S1.

Gene	Analysis	Primer name	Forward primer (5'-3')
MLH1			
	MS-MCA	MLH1C_PCR_ext	TATTTTTGTTTTTATTGGTTGG
		MLH1C_PCR_int	TGTTTTTATTGGTTGGATATTT
		MLH1D_PCR_ext	AGGTATTGAGGTGATTGGTTG
		MLH1D_PCR_int	GGTGATTGGTTGAAGGTATTTT
	Promoter bisulfite sequencing	MLH1C_BS	TTTTAAAAAYGAATTAATAGGA
		MLH1D_BS	AAATTTGATTGGTATTTAAGTT
	Clonal promoter bisulfite sequencing	MLH1C-D_BS	TTTTAAAAAYGAATTAATAGGA
	Promoter bisulfite pyrosequencing	MLH1C_PCR	GGTATTTTTGTTTTTATTGGTTG
		MLH1C_Seq	TAAAAAGAATTAATAGGAA
		MLH1D_PCR	TTGAGAAATTTGATTGGTATTTA
		MLH1D1_Seq	TGAAGGGTGGGGTTG
		MLH1D2_Seq	GATTGGTTGAAGGTATTTT
	Promoter sequencing	MLH1promoter_PCR	AACCCTTTCACCATGCTCTG
		MLH1promoter_Seq1	TACATGCTCGGGCAGTACCT
		MLH1promoter_Seq2	TGAAGAGAGAGCTGCTCGTG
	ASE (SNuPE)	rs179997_PCR_cDNA	CACAATGCAGGCATTAGTTTCT
		rs179997_PCR_gDNA	GTTTCAGTCTCAGCCATGAG
		rs179997_snupe	
	ASE (pyrosequencing)	rs179997_Pyr_cDNA	GCCTCAACCGTGGACAATATTC
		rs179997_Pyr_gDNA	GCCTCAACCGTGGACAATATTC

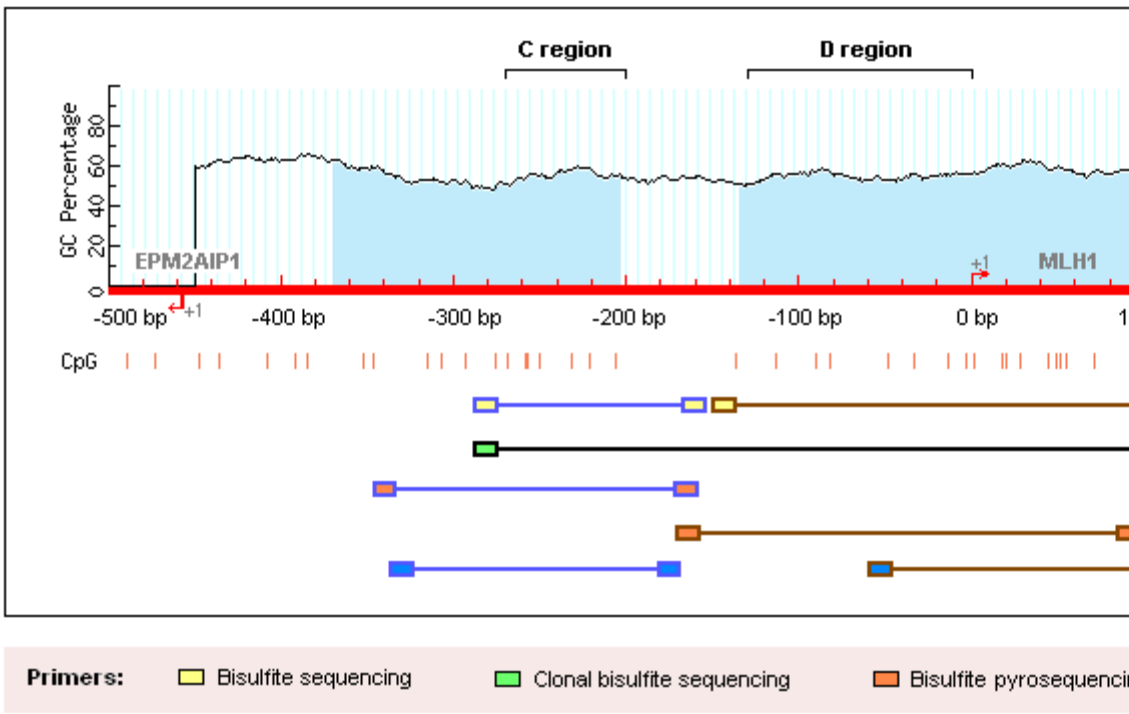
		rs179997_Pyr_Seq	GGACAATATTCGCTCC
EPM2AIP1			
	ASE (SNuPE)	rs9311149_PCR	GTCCTGTTGTAGCAGTGAATAT
		rs9311149_Seq1	TAGGTCCTTACCAGTTACTG
		rs9311149_Seq2	
		rs9311149_snupe	TCCTTGAAACACTTGAACACTTC
BRAF			
	BRAF V600E screening (SNuPE)	BRAF_PCR	CCTAAACTCTTCATAATGCTT
		BRAF_snupe	TAAAAATAGGTGATTTTGGTCTA

Figure S1. Location of primers used in the study of *MLH1* promoter methylation. Ma

EPM2AIP1 promoters (adapted from MethPrimer program). Two CpG islands are identified

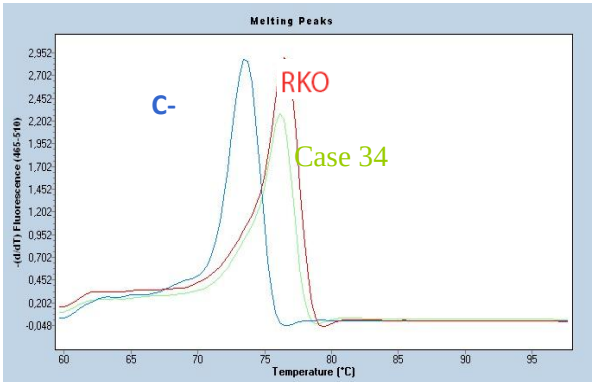
Each small vertical red line represents a CpG site. Primer position is indicated by squares.

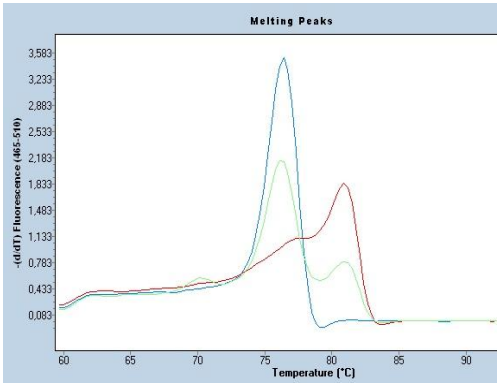
shown as blue, brown and black lines, respectively. The translation start sites of *MLH1* and *E*



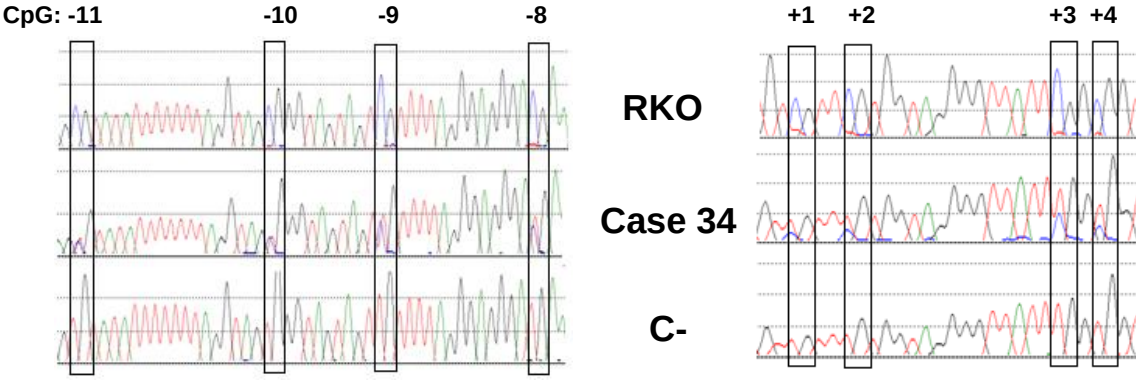
In the analysis of C region, unmethylated control (C-) and RKO DNA (methylated control) show single melting peaks at 73ºC and 77ºC, respectively. In D region, unmethylated control and RKO melting peaks temperature are 76ºC and 82ºC, respectively. Analysis by MS-MCA in PBL DNA from the patient 34 (green line) shows the presence of the methylated peak in both regions. Middle panel: Sequence analysis of bisulfite converted DNA. Unmethylated control shows T at each CpG analyzed, consistent with complete modification of the DNA. RKO DNA shows C at each CpG. Patient DNA shows a mixture of T and C at CpG sites, attributable to partial methylation. Bottom panel: Representative pyrograms obtained in the analysis of C and D *MLH1* promoter regions in PBL DNA from the patient. The peaks within the shaded area of the pyrogram correspond to the CpG interrogated. Percentage methylation at each site is calculated as the C:T ratio of peak heights (representing methylated:unmethylated cytosine). *X*-axis represents the nucleotide dispensation order. *Y*-axis units are arbitrary representing light intensity. **B. Transcriptional inactivation of *EPM2AIP1* allele.** Representative results of the SNuPE analysis at *EPM2AIP1* rs9311149 in genomic DNA and cDNA derived from a heterozygous control and the epimutation carrier. Partial transcriptional silencing of the T allele at *EPM2AIP1* rs9311149 in the cDNA of the patient was observed.

MS-MCA

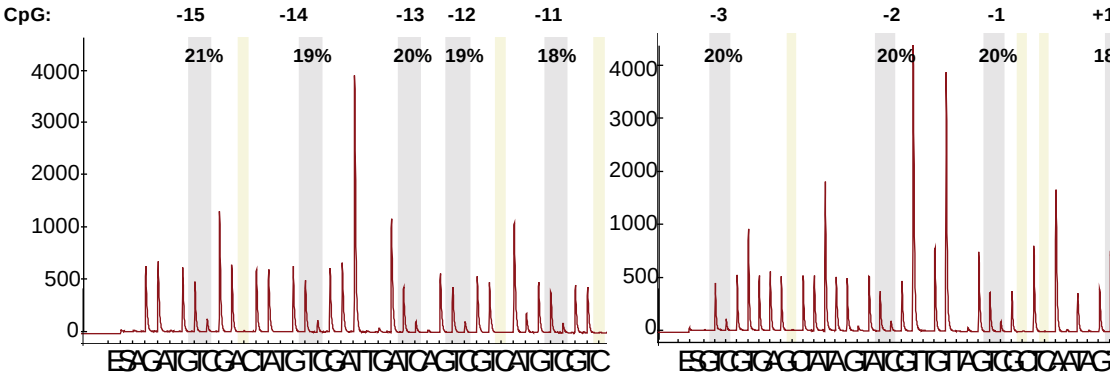




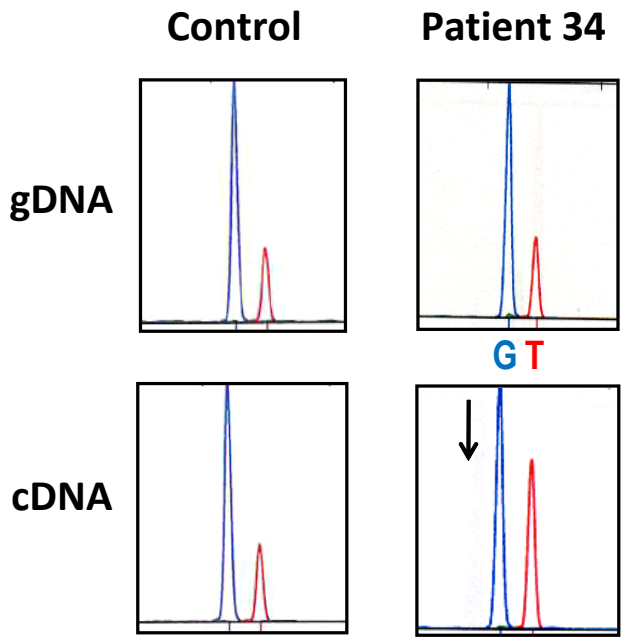
BIS-SEQ



PYROSEQ



EPM2AIP1 rs9311149



DNA extraction of colorectal mucosa and tumour tissue from paraffin-embedded material was done after enrichment for normal and tumour cells using the QIAmp DNA Mini Kit (Qiagen). Microsatellite instability testing was performed in tumor DNA using the MSI Analysis System (Promega). Genomic DNA was extracted from peripheral blood lymphocytes (PBL) using the FlexiGene DNA kit (Qiagen, Hilden, Germany). Different samples were acquired from the epimutation carrier: skin fibroblasts, peripheral blood lymphocytes, colorectal tumor and normal adjacent mucosa, and sperm. For fibroblast isolation, a skin biopsy was cut into small pieces and digested with 160 U/ml collagenase type 1 (Sigma, St. Louis, MO) and 0.8 U/ml dispase grade 1 (Roche Diagnostics, Penzberg, Germany).[1] Fibroblasts were grown with Dulbecco's modified Eagle's medium (Gibco, Invitrogen), 10% fetal bovine serum (Gibco, Invitrogen), and penicillin/streptomycin (Gibco, Invitrogen) at 37ºC and 5% CO2. DNA from cultured fibroblasts was extracted using the Gentra Puregene Cell Kit. Sperm was washed twice in 1x SSC/ 1% SDS, then washed in 1x SSC and incubated in 0.2x SSC/ 1% SDS/ 1M 2-mercaptoethanol for 1 hr at room temperature. DNA was extracted from spermatozoa using a standard phenol-chloroform method and ethanol precipitation. Total RNA was extracted from PBL using Trizol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) and random primers (Invitrogen).

***MLH1* promoter methylation analyses**

MS-MCA. Bisulfite converted DNA was used in a nested PCR reaction for the amplification of regions C and D of the *MLH1* promoter.[2] Each promoter region was preamplified using external primers (Table S1). Eighty ng of bisulfite modified DNA

was carried out in a LightCycler 480 II (Roche) using 1μl of amplified *MLH1* promoter fragments in 9μl of Light Cycler 480 SYBR Green I Master Kit (Roche) containing 0.5μM of internal primers. The amplification protocol was: 95ºC for 10min, followed by 40 cycles of 95ºC for 10s, 50°C for 20s, and 72ºC for 25s. Melting curve analysis was performed by heating the PCR products from 60ºC to 98ºC with an increase of 0.2°C/s whereas fluorescence was monitored continuously.

Bisulfite-Sequencing. One μl of bisulfite converted DNA was used in a 10ul-PCR reaction for the amplification of *MLH1* promoter regions C and D^2 using Double Megamix (Microzone Ltd., UK) and 0,2μM of primers (Table S1). The cycling program included 10 min at 94ºC, 35 cycles of 30s at 95°C, 30s at 50°C and 30s at 72ºC and final extension at 72ºC for 10 min. PCR products were purified using ExoSAP-it (Affymetrix, Inc.) and sequenced using the amplification primers and BigDye Terminator v.3.1 Sequencing Kit (Applied Biosystems, Carlsbad, CA).

Clonal Bisulfite-Sequencing. One μl of bisulfite modified DNA was amplified in a PCR reaction using EcoStar DNA polymerase (Ecogen, Spain) and 0,3μM of primers (Table S1). PCR products were purified by ExoSAP-it (Affymetrix, Inc.) and cloned into pGEM-T vector (PromegaCorp, Madison, WI). In order to confirm that transformed cells contained the fragment of interest we performed a colony-PCR using M13 primers. Amplification conditions were: 10 min at 94ºC; and 35 cycles of 1min at 94°C, 1min at 55°C, 1min at 72ºC and final extension at 72ºC for 10 min. The PCR products were analyzed by agarose gel electrophoresis. Twenty individual clones were sequenced using M13 primers and BigDye Terminator v.3.1 Sequencing Kit (Applied Biosystems).

was: 95°C for 15 min, 35 cycles of 94°C for 1 min, 1 min at the annealing temperature (Table S1), 72°C for 1 min and a final extension at 72°C for 10 min. Five µl of PCR product were evaluated for % methylation using the PyroMark Q96 MD pyrosequencer (Qiagen,Valencia, CA). If the sample failed at more than one site, it was repeated using 10 µl of PCR product. Purification and subsequent processing of the biotinylated single-stranded DNA was performed according to the manufacter's recommendations at the PyroMark Q96 Vacuum Prep Workstation (Qiagen). The pyrosequencing primers were used in a final concentration of 0.3µM. The pyrosequencing reaction was performed using each specific sequencing primer on a PyroMark Q96 MD pyrosequencer system with the Pyromark Gold Q96 reagents kit. The sequences interrogated were GAGYGGATAGYGATTTTTAAYGYGTAAGYGTATATTTTTTTAGGTAG for promoter C region, GATGGYGTAAGTTATAGTTGAAGGAAGAAYGTGAGTAYGAGGTATTGAGGT GATTGGTTGAAGG for promoter "D1" region, and YGTTGAGTATTTAGAYGTTTTTTTGGTTTTTTTGGYGTTAAAATGTYGTTYGT GGTAGGGGTTATT for promoter "D2" region. The relative levels of the C (representing methylated) and T (representing unmethylated) nucleotides at Y positions of target CpGs sites were determined using the Pyro Q-CpG Software (Qiagen). Each sample was run in triplicates. Methylation at each specific CpG was calculated as the mean of all triplicates. Average % methylation of the whole region was calculated as the mean for the 5 CpGs in C region and the 8 CpGs at the D region.

amplification conditions were: 5 min at 94ºC, 35 cycles of 1 min at 94°C, 1 min at 59°C and 1 min at 72ºC and final extension at 72ºC for 7 min. For genomic DNA the cycling program included 10 min at 94ºC, 35 cycles of 30s at 95°C, 30s at 55°C and 30s at 72ºC and final extension at 72ºC for 10 min. PCR products were purified using ExoSAP-it (Affymetrix, Inc.) and sequenced if necessary using amplification primers and BigDye Terminator v.3.1 Sequencing Kit (Applied Biosystems). The amplified band was analyzed using the ABI PRISM SNaPshot kit (Applied Biosystems) and a specific primer (Table S1). SNaPshot reactions were carried out in a 10μl volume containing SNaPshot Multiplex Ready Reaction Mix, specific primer (0.2μM) and the purified PCR product. The cycling program included 25 cycles of 96°C for 10s, 50°C for 5s and 60ºC for 30s. Extension products were purified with 1U of shrimp alkaline phosphatase (Amersham, UK) for 15 min at 37ºC and 15min at 75ºC. The purified products were run in an ABI Prism 3130 DNA sequencer and analyzed by GeneMapper v4.0 (Applied Biosystems).

Pyrosequencing: Quantitative pyrosequencing assays were designed as previously described.[3] PCR and sequencing primers are shown in Table S1. After PCR amplification of genomic DNA and cDNA, products were sequenced on a PyroMark Q24 pyrosequencing instrument (Qiagen, Valencia, CA). A control in which the template was omitted was used to detect background signal. A nucleotide dispensation order of CAGATCTGA was used to interrogate the sequence of interest A/GTCTTTGGAAA. The proportion of A allele versus G alleles of rs1799977 were obtained using PyroMark Q24 AQ software calculations. The mean of triplicates for both DNA and cDNA were calculated for each sample.

ASE value was calculated as the mean of the ASE values obtained for the triplicates studied in each sample.[4] ASE values of 1.0 indicate equal levels of expression form both alleles. ASE values <<1.0 indicate reduced expression.

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ARTICLE 2:

Prevalence of germline *MUTYH* mutations among Lynch-like syndrome patients.

Hypothesis: Overlapping phenotypes between *MUTYH*-associated polyposis and Lynch syndrome has been reported. A subset of Lynch-like syndrome individuals may harbor germline *MUTYH* biallelic mutations.

Aim: To investigate the prevalence of germline *MUTYH* mutations in a Spanish series of patients considered as having Lynch-like syndrome, with MMR-deficient tumors without identified germline MMR mutations.

Summary of the obtained results: We found a prevalence of 3.1% of MAP syndrome in the whole series of LLS (7/225). Patients with *MUTYH* biallelic mutations had more adenomas than monoallelic (P=0.02) and wildtype patients (P<0.0001). Six out of nine analyzed tumors from six biallelic *MUTYH* carriers harbored *KRAS* p.G12C mutation. The obtained results justify the inclusion of *MUTYH* in the diagnostic strategy for Lynch syndrome-suspected patients.

Contribution of the PhD candidate: Selection of cases for analysis and collection of the samples from five different Catalonian hospitals. Collection of clinico-pathological information from the set of all Catalonian patients. Analysis of *MUTYH* mutations, interpretation of results and drafting the article. Dr. Adela Castillejo, who shares first co-authorship of this article, was responsible of the study of *MUTYH* variants in the Valencian series of cases. Both first authors were active in preparing the final version of the manuscript.

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Prevalence of germline *MUTYH* mutations among Lynch-like syndrome patients

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KEYWORDS
Lynch syndrome
MAP syndrome
MUTYH
KRAS mutations

Abstract ***Background and aims:*** Individuals with tumours showing mismatch repair deficiency not linked to germline mutations or somatic methylation of MMR genes h recently referred as having 'Lynch-like syndrome' (LLS). The genetic basis of these L is unknown. *MUTYH*-associated polyposis patients show some phenotypic simila Lynch syndrome patients. The aim of this study was to investigate the prevalence of *MUTYH* mutations in a large series of LLS patients.
Methods: Two hundred and twenty-five probands fulfilling LLS criteria were include study. Screening of *MUTYH* recurrent mutations, whole coding sequencing and a la rangement analysis were undertaken. Age, sex, clinical, pathological and molecular c istics of tumours including *KRAS* mutations were assessed.
Results: We found a prevalence of 3.1% of MAP syndrome in the whole series of LL and 3.9% when only cases fulfilling clinical criteria were considered (7/178). Patie *MUTYH* biallelic mutations had more adenomas than monoallelic ($P = 0.02$) and patients ($P < 0.0001$). Six out of nine analysed tumours from six biallelic *MUTYH* harboured *KRAS*-p.G12C mutation. This mutation was found to be associated with *MUTYH* germline mutation when compared with reported series of unselected colore cer cohorts ($P < 0.0001$).
Conclusions: A proportion of unexplained LLS cases is caused by biallelic *MUTY* tions. The obtained results further justify the inclusion of *MUTYH* in the diagnostic for Lynch syndrome-suspected patients.
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1. Introduction

About 1–5% of colorectal cancers (CRCs) are caused by germline mutations or epimutations in mismatch repair (MMR) genes: *MLH1*, *MSH2*, *MSH6* and *PMS2* [1]. This disorder is named the Lynch syndrome (LS) and is characterised by an autosomal dominant inheritance, a predisposition to early onset CRC and an increased risk of other cancers [1].

Molecular diagnosis of LS is well established and is mainly based in the use of clinical criteria to identify those patients with CRC candidate for molecular analysis [2]. Tumours of candidate patients are analysed for the presence of microsatellite instability (MSI) and/or loss of expression of MMR proteins by immunohistochemistry (IHC) as a screening method to evidence MMR deficiency. Whenever MSI or MMR protein loss is present in the absence of *BRAF* mutation or *MLH1* methylation, germline mutational analysis is offered [3,4]. While the diagnostic yield of the molecular diagnosis of LS is good [5], it can certainly be improved. The overall mutation detection rate in pre-selected patients ranges from 30% to 78%, depending on the inclusion criteria applied [5–9]. In a highly selected series of Amsterdam families with MSI, the percentage of mutation detection may be as high as 95% [10]. However, failure to identify a pathogenic germline mutation in MMR genes does not exclude a hereditary cancer predisposition. Individuals with tumours showing MMR deficiency not linked to germline mutations or somatic methylation of MMR genes have been recently to as having 'Lynch-like syndrome' (LLS) [11].

MUTYH (OMIM*604933) encodes for excision repair DNA glycosylase [12]. Mutation gene cause the *MUTYH*-associated polyposis syndrome, an autosomal recessive inherited co commonly characterised by the presence of few dreds of colonic adenomatous polyps and an in CRC risk at young age [12].

It has been reported that MAP patients sho phenotypic similarities to LS patients. In this the extracolonic tumour spectrum is similar groups and CRC can be diagnosed in the abs polyps or associated with a small number of (reviewed in [13]). Moreover, MAP CRCs sha histological similarities with LS carcinomas a associated with better prognosis [13]. At the level, human MUTYH is physically associat MSH2/MSH6, and the MSH2/MSH6 c stimulates the DNA binding and glycosylase a of MUTYH with oxoG:A mispairs [14]. H deficiency on MMR system is not frequently i in MAP tumours [15–18], and MSI has been r in very few CRCs from biallelic *MUTYH* [15,18–21].

The aim of this study was to investigate the lence of germline *MUTYH* mutations in a Spanis of patients considered as having LLS, with deficient tumours without identified germline mutations. Our study confirms that biallelic g

centage of patients with MMR-deficient tumours.

2. Materials and methods

2.1. Patients and specimens

A total number of 225 probands were studied. Inclusion criteria were: (i) diagnosis of LS-associated tumours showing MSI phenotype and/or loss of MMR protein expression; (ii) absence of BRAF p.V600E mutation or MLH1 promoter methylation in those tumours with loss of MLH1 expression; and (iii) absence of germline pathogenic variants in the MMR genes (*MLH1*, *MSH2*, *MSH6 and PMS2*). Analyses of these genes were guided by IHC results when available. One hundred patients were assessed at the Cancer Genetic Counseling Units of the Hereditary Cancer Programme from the Valencian Region in Spain from 2005 to 2013, and 125 were recruited at Cancer Genetic Counseling Units from Catalonia from 1999 to 2012 (Table 1 and Table A.1). All patients referred for MMR mutation analysis were suspected of having LS because they fulfilled LS clinical criteria [2] (Amsterdam or revised Bethesda guidelines) or of having tumours showing loss of MMR proteins and/or MSI at an age of diagnosis over 59 y. Patients enrolled in this study gave written informed consent and this study was approved by the Internal Ethics Committee of the participant hospitals. Clinical and pathological information was collected (Table A.1).

DNA extraction of tumour tissue from paraffin-embedded material was conducted after enrichment for tumour cells using the QIAmp DNA Mini Kit (Qiagen, Hilden, Germany). Genomic DNA from peripheral blood lymphocytes was extracted using the same kit or the FlexiGene DNA kit (Qiagen, Hilden, Germany). *BRAF* p.V600E mutation and *MLH1* promoter methylation were performed as previously described [3,4].

2.2. Analysis of MMR genes

MMR genes were studied using established recommendations and consensus algorithms for screening and mutation analysis [2]. Variants were classified using InSiGHT classification criteria for MMR genes (version 1.9, August 2013) [22]. For the purpose of this study, cases harbouring variants of unknown clinical significance in MMR genes were considered.

2.3. MUTYH mutational analysis

A three-stage approach was performed to identify patients with pathogenic variants at the *MUTYH* gene. First, we analysed for the presence of the three most recurrent *MUTYH* pathogenic variants in the Spanish population: c.536A>G (p.Y179C); c.1187G>A Mutations p.Y179C and p.G396D were analysed bidirectional Sanger sequencing (149 cases) or rea PCR allelic discrimination assay (76 cases). Analy the c.1227_1228dup (p.E410Gfs*43) variant performed by Sanger sequencing. Suspected mut positive patterns from the allelic discrimination were confirmed by sequence analysis. Mutation ca were confirmed in independent experiments. Seco heterozygous mutations carriers, the whole c sequence and exon–intron boundaries of the *MU* gene were amplified and sequenced (primers and c tions available upon request). Third, heterozygou apparent homozygous mutation carriers were screened for large rearrangements by multiplex tion-dependent probe amplification technique, Salsa MLPA P378 MUTYH Kit (MRC-Holland Netherlands) according to the manufacturer's pro Mutation nomenclature of *MUTYH* gene is acco to GenBank accession NM_001128425.1 and H recommendations.

2.4. Somatic KRAS mutation analysis

Available tumours from *MUTYH* carriers screened for *KRAS* somatic mutations in codo and 13 using real-time PCR (LightCycler® 480; I Applied Science, Indianapolis, IN, United Stat America) as reported [24] or KRAS Strip (ViennaLab, VienaLab, Austria) according to manufacturer.

2.5. Statistical analysis

Continuous variables are reported as m standard deviation for normally distributed data. gorical variables are reported as frequencies or pe ages. Analysis of variance (ANOVA) tests were a to analyse the differences between group means. S cant differences between groups were analysed usi χ^2 test for categorical data and the non-para Mann–Whitney U test for quantitative data. Wilcoxon rank test was applied to identify signi differences between the two groups of patient reported P values are two sided, and $P < 0.05$ wa sidered significant. All calculations were perfo using SPSS 19.0 (IBM, Armonk, NY).

3. Results

Biallelic *MUTYH* mutations were found in sev 225 LLS cases (3.1%), corresponding to 3.9% (of patients fulfilling clinical criteria of LS (Table Fig. 1). Frequency of *MUTYH* biallelic mutation significantly higher in our LLS series when com with a Spanish control population ($n = 934$) a

Clinicopathological features of included patients.

Characteristic	Total *n* (%)	MUTYH biallelic	MUTYH monoallelic	MUTYH wildtype	*P*-value (comparison between biallelic and monoallelic groups)	*P*-value (co between bi wildptype
Number of cases	225	7	8	210		
Sex					$P = 1$	$P = 0.46$
Female	129 (57.3)	3	3	123		
Male	96 (42.7)	4	5	87		
Age at first diagnosis, mean (SD)	52.5 (13.8)	47.4 (7.3)	56 (11.8)	52.5 (14)	$P = 0.12$	$P = 0.12$
Clinical criteria						
Amsterdam	28 (12.4)	1	2	26		
Bethesda	151 (67.1)	6	5	141		
Anatomo-pathological	46 (20.4)	0	1	46		
Malignant tumours diagnosed (organ)[a]					$P = 1$[e]	$P = 0.35$[e]
Colorectal	210 (74.7)	9	10	191		
Endometrial	37 (13.2)	2	2	33		
Ovarian	7 (2.5)	0	0	7		
Small bowell	4 (1.4)	1	0	3		
Gastric	5 (1.8)	1	0	4		
Other LS related	7 (2.5)	0	1	6		
Others non-LS related	11 (3.9)	1	1	9		
Multiple primary tumours	45 (20)	4	4	35	$P = 1$	$P = \mathbf{0.02}$
Presence of colorectal polyps (any type)[b]					$P = 0.06$[f]	$P = \mathbf{0.001}$[f]
At colorectal cancer (CRC) diagnosis[c]					$P = 0.08$[f]	$P = \mathbf{0.03}$[f]
0	87 (38.7)	1	5	81		
1–10	46 (20.4)	1	1	44		
>10	3 (1.3)	2	0	1		
Not specified number	5 (2.2)	2	0	3		
No information/no CRC	84 (37.3)	1	2	81		
During follow-up[d]					$P = 1$[f]	$P = 0.02$[f]
0	48 (21.3)	0	1	47		
1–10	37 (16.4)	2	4	31		
>10	3 (1.3)	2	0	1		
Not specified number	3 (1.3)	1	0	2		
No information/no follow-up	134 (59.6)	2	3	129		
Presence of colorectal adenomas					$P = \mathbf{0.02}$[f]	$P < \mathbf{0.001}$[f]
At CRC diagnosis[c]					$P = 0.08$[f]	$P = \mathbf{0.003}$[f]
0	97 (43.1)	1	5	91		
1–10	38 (16.9)	3	1	34		
>10	1 (0.4)	0	0	1		
Not specified number	4 (1.8)	2	0	2		
No information/no CRC	85 (37.8)	1	2	82		
During follow-up[d]					$P = 0.38$[f]	$P = \mathbf{0.003}$[f]
0	57 (25.3)	0	1	56		
1–10	25 (11.1)	3	2	20		
>10	3 (1.3)	2	0	1		
Not specified number	2 (0.9)	0	0	2		
No information/no follow-up	138 (61.3)	2	5	131		
Characteristics of MMR-deficient tumours						
Result of MSI analysis						
MSI	164 (72.9)	5	7	152		
MSS	19 (8.4)	1	0	18		
Inconclusive	2 (0.9)	0	0	2		
Not studied	40 (17.8)	1	1	38		
Result of IHC analysis						
MLH1–/PMS2– or NP	91 (40.4)	3	3	85		
MSH2–/MSH6– or NP	55 (24.4)	0	1	54		
MSH6–	26 (11.6)	0	1	25		
PMS2–	7 (3.1)	0	1	6		
Other loss of expression patterns	11 (4.9)	1	0	10		
No loss of expression	22 (9.8)	3	1	18		
Not performed/not informative	13 (5.8)	0	1	12		

SD, standard deviation; MMR, mismatch repair; LS, Lynch syndrome; VUS, variant of unknown significance; IHC, immunohistochemi
microsatellite instability; MSS, microsatellite stability; NP, not performed.

[a] All the tumours diagnosed are considered.

[b] Adenomatous, serrated, hyperplastic or not biopsied polyps.

[c] Polyps found at the time of CRC diagnosis (in case of multiple CRC, taking the first into account).

[d] Polyps found after surgical CRC intervention.

[e] CRC versus other tumours.

[f] Presence versus absence of polyps (any type) or adenomas.

Table 2
Clinicopathological and molecular features of biallelic *MUTYH* mutation carriers.

Case	Sex	Clinical criteria	Family history*	*MUTYH* mutation	*MUTYH* polymorphism	MMR VUS	Tumour/ lesion location	Age at diagnosis	Stage	Degree of differentiation	Mucinous production	Infiltrating lymphocytes	MSI status	MMR IHC	KRAS status	Number of colorectal polyps	
																Surgical removal	Follow-up(age)
49	M	Bethesda/ Muir–Torre	Gastric cancer(mother, 72; maternal uncle, 60); Cervix and skin cancer (maternal aunt); Breast cancer (paternal aunt); Hepatic cancer (paternal aunt); Sarcoma (nephew, 8)	Y179C homo	ND	NI	Gastric	52	T2N1M0	G3	ND	ND	MSI	Normal	WT		11 adenomas (58–69 y)
							Left colon	57	T3N0M0	G2	ND	ND	ND	ND	ND		
							Left colon	57	T2N0M0	G1	ND	ND	MSS	Normal	G12C	3 adenomas	
							Right colon	57	T2N0M0	G1	ND	ND	ND	ND	ND		
							Sebaceous adenoma (face)	63	–	–	–	–	MSS	Normal	WT		
							Jejunum	68	T2N0M0	G1	YES	ND	ND	ND	ND		
51	F	Bethesda	3 synchronous colorectal cancers (sister, 54); Endometrial cancer(maternal aunt , 50)	G396D homo	ND	NI	Left colon	36	TXNXM0	GX	ND	ND	MSS	Normal	G12C	NSN	5 adenom (37–58 y)
							Endometrial	51	T1bN1M0	G2	ND	ND	MSS	Loss of MLH1	G12C		
61	F	Bethesda	Thyroid cancer (nephew, 29)	Y179C hetero/ R368QfsX164	V22M	NI	Left colon	46	T3N0M0	GX	ND	ND	MSI	Normal	G12C	0	3 adenom
							Breast	67	ND	ND	ND	ND	ND	ND	ND		NSN of hyperplas (47–59 y)
73	M	Bethesda	Colorectal cancer (maternal aunt, 70)	Y179C hetero/ G396D hetero	ND	NI	Left colon	45	T4N2M0	G2	ND	ND	MSI	Normal	WT	6 adenomas, 5 hyperplastic	34 adenomas (46–53 y)
95	F	Bethesda	Endometrial cancer (sister, 55); leukaemia (daughter, 3); Sarcoma (mother)	G396D hetero/Del Ex 4–16 hetero	ND	NI	Endometrial	60	T1N0M0	G1	ND	ND	MSI	Loss of MLH1 and PMS2	G12C		No follow up
							Right colon	65	T3N0M0	G2	ND	ND	ND	ND	ND	ND	
164	M	Bethesda	Colorectal cancer (paternal uncle, 72), Endometrial cancer (paternal aunt, 48); Bladder cancer (father, 75)Thyroid cancer (nephew, 25)	G396D homo	ND	MLH1 c.2146G>A (p.V716M)	Right colon	48	T3N2MX	ND	YES	ND	MSI	Loss of MLH1	G12C	3 adenomas, 8 NS	No follow up
186	M	Amsterdam Criteria I	Colorectal cancer (father, 75; paternal uncle; paternal aunt, 71; sister 46)	Y179C homo	ND	NI	Right colon	45	T2N0M0	G2	ND	ND	ND	Loss of MLH1, MSH6 and PMS2	ND	NSN	1 adenom (45–52 y)

M, Male; F, female; ND, no data; NI, not identified; homo, homozygous; hetero, heterozygous; Del Ex, deletion of exons; MMR, mismatch repair; IHC, immunohistochemistry; NS, not specifi
NSN, not specified number; MSI, microsatellite instability; MSS, microsatellite stability; WT, wildtype; G, grade; Stage is given by TNM Classification of malignant tumours.
* Affected relative and age at diagnosis are indicated between parentheses.

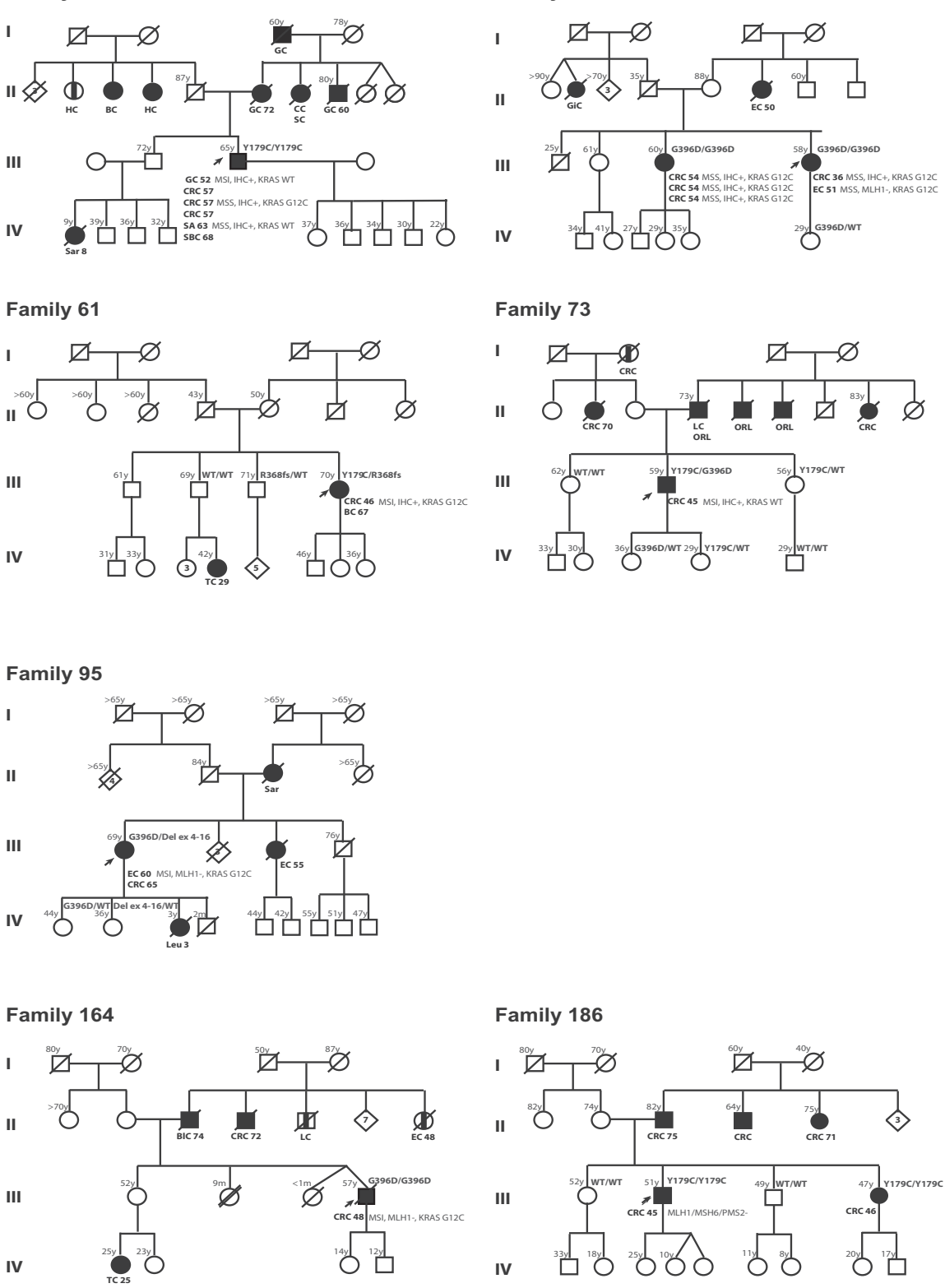

Fig. 1. Pedigrees from biallelic MUTYH carriers. Circles, females; squares, males; filled, cancer affected; vertical line at centre, non-cancer affected. Tumours, age at diagnosis and the result of the molecular analysis are depicted below the affected individuals. MUTYH status and current age (or age at deceased) are depicted above right and above left, respectively. Probands are indicated by arrows. CRC, cancer; GC, gastric cancer; SBC, small bowel cancer; HC, hepatic cancer; BlC, bladder cancer; LC, lung cancer; TC, thyroid canc otorhinolaryngological cancer; BC, breast cancer; EC, endometrial cancer; CC, cervix cancer; Sar, undefined sarcoma; SC, skin ca sebaceous adenoma; Leu, leukaemia. GiC: gynaecological cancer; MSI, microsatellite instable; MSS, microsatellite stable; IHC+, conserv protein expression; MLH1–, loss of MLH1 expression.

$P = 0.007$, respectively) [25]. Monoallelic *MUTYH* mutations were found in eight of the 225 cases (3.6%) (Table A.2). No differences were observed in the frequency of monoallelic *MUTYH* mutations when compared with controls and unselected CRC ($P = 0.36$ and $P = 0.11$, respectively) [25].

Among the biallelic *MUTYH* carriers (Table 2 and Fig. 1), one case fulfilled Amsterdam criteria, another fulfilled both Muir–Torre and Bethesda criteria [26] and the remaining fulfilled Bethesda criteria. Two patients were homozygous for the p.Y179C, two for the p.G396D, three were compound heterozygous for p.Y179C/G396D, p.Y179C/p.R368Qfs*164 and p.G396D/exon 4–16 deletion. All biallelic carriers were diagnosed with CRC and the overlapping phenotype between Lynch and MAP syndromes was further evidenced by the presence of sebaceous adenomas and gastric, small bowel and endometrial tumours. Less than 10 adenomatous polyps were found at the time of CRC diagnosis, as reported by colonoscopy and/or pathological reports (Table 2). However, in most of the cases multiple polyps (adenomas and others) were diagnosed in the follow up colonoscopies.

Six out of nine (67%) analysed tumours (four CRC and two endometrial cancer) from six biallelic *MUTYH* carriers were *KRAS* mutant. All six mutated cases shared the somatic *KRAS* transversion c.34G>T (p.G12C). Six out of seven analysed tumours from four *MUTYH* monoallelic mutation carriers presented other *KRAS* mutations: c.34G>A (p.G12D), c.35G>T (p.G12V) and c.38G>A (p.G13D) (Table 2). A significant association between biallelic *MUTYH* germline mutation and *KRAS* p.G12C somatic transversion was found when compared to reported series of unselected CRC cohorts ($P < 0.0001$) [27,28].

No significant differences between biallelic, monoallelic and no mutation carrier groups were found regarding age ($P = 0.48$) or sex ($P = 0.36$) (Table 1). Considering the total number of polyps (adenomas and others), biallelic carriers had more polyps than wildtype patients ($P = 0.001$). No differences were detected between biallelic and monoallelic ($P = 0.06$) or monoallelic and wildtype patients ($P = 0.42$). When the total number of adenomatous polyps was considered, a higher number of adenomas were found in biallelic versus monoallelic ($P = 0.02$) and wild-type patients ($P < 0.001$).

4. Discussion

We have found a prevalence of biallelic *MUTYH* mutations of 3.9% in 225 patients fulfilling LS clinical criteria, revealing further overlapping phenotypes between Lynch and MAP syndromes in the largest study of *MUTYH* in LLS patients reported to date. This

Germanic-American cohort of 85 LLS cases (1
[21], and significantly higher than controls and unse
CRC from the same population [25].

Noteworthy, the prevalence of germline *MU*
mutations in these series might be even higher d
the mutation detection strategy utilised. *MUTYH*
initially analysed for the 3 Spanish hotspot mut
[13,23] and, only in heterozygous mutation ca
the study of the whole *MUTYH* was completed.
we focused in cases with LLS after exclusi
MLH1-methylated cases. Colebatch and collabo
reported a patient harbouring a *MUTYH* biallelic
tion diagnosed of a MMR-deficient CRC due to *M*
methylation [19], leaving open the door to the iden
tion of more cases if no tumour prescreening is m

LLS cases and their families have an intermedia
of cancer between LS and sporadic cancers [11].
ogeneity is likely to account for this intermediate p
type after confirming that part of this increased
associated with germline biallelic mutations in *MU*
Recently, double somatic mutations in MMR
have been reported in an important proportion o
tumours [29]. Moreover, two somatic G>T transv
mutations in *MSH2* have been identified in a M
deficient tumour from a biallelic *MUTYH* carrie
These findings suggest that *MUTYH* deficiency
eventually cause somatic MMR gene transversion
consequently, tumours with MSI phenotype mim
LS. Thus, biallelic somatic mutations in MMR
would not exclude the existence of germline mut
in genes other than MMR.

Until the identification in the present study of M
deficient tumours in seven biallelic *MUTYH* ca
only six cases had been reported [15,18–20]. In co
to those previously reported cases and the pro
clinical criteria for MAP syndrome diagnosis [12].
of the LLS cases with biallelic *MUTYH* mutation
less than ten adenomatous polyps at the time of
diagnosis. *MUTYH* biallelic mutations in the ab
of MAP-phenotype had been described in large po
tion-based CRC series [30–32]. These results su
that the scarcity of polyps or the presence of M
tumours should not exclude the *MUTYH* analysis
thermore, accumulation of polyps during follow-
the identified *MUTYH* biallelic cases strengthen
need of performing systematic reviews of surve
reports in patients with hereditary CRC suspicion

The frequency of *KRAS* transversion p.G1
unselected CRCs is about 3–4% [27,28] whil
reported frequency in MAP tumours is a
64%[28]. We found 67% (6/9) of tumours from bi
MUTYH carriers with the p.G12C mutation, confi
the potential role of KRAS analysis as a pre-scre
method that might help to select patients with
who are eligible for *MUTYH* testing. This wou

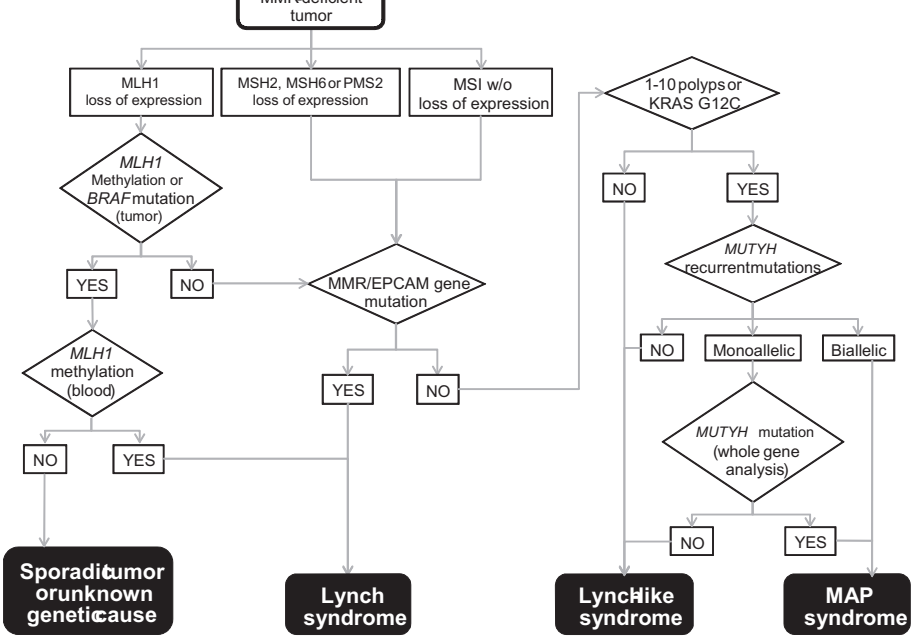

Fig. 2. Proposed decision support flow diagram for refining genetic diagnosis on cases with suspicion of LS with MMR deficient tum
without germline mutation in MMR genes. MMR, mismatch repair; MSI, microsatellite instable; MAP, *MUTYH* Associated Polypos

particularly useful in patients with early onset CRC in the absence of polyposis where screening of *MUTYH* mutations remains controversial [30].

It is generally assumed that patients with LLS tumours and their first-degree relatives are considered at high risk of LS and the need for special screening and surveillance strategies has been advocated. In those cases due to *MUTYH* mutations, specific recommendations for individuals and their at risk relatives affected by this autosomal recessive syndrome will be made.

The role of *MUTYH* monoallelic mutations in cancer risk has been debated. Whereas many researchers found that significant susceptibility to cancer risk was associated with monoallelic mutations [33–35] others have shown negative results in this regard [25]. Findings from a recent meta-analysis showed weak CRC susceptibility for monoallelic mutations versus wildtype [36]. The lack of significant differences in the number of polyps between monoallelic carriers and wildtype group is consistent with a weak susceptibility effect of these monoallelic mutations. Interestingly, two heterozygote carriers of *MUTYH* p.G396D were also carriers of the polymorphism p.Q338H. The role of p.Q338H is controversial. While it has been related to increased CRC risk [25,38] and deficient repair activity [39,40], no significant association has been found in large cohort studies [37].

Taking into consideration the prevalence of biallelic *MUTYH* mutations among LLS patients, we recommend the inclusion of *MUTYH* testing in the diagnostic strategy of LS-suspected patients (Fig. 2). Likewise, the obtained results reinforce the inclusion of *MUTYH* in the next-generation hereditary cancer panels that would help to decipher the phenotypic overlap betwe dromes. The refinement of the classification patients will allow a more precise and personali low-up of this heterogeneous set of patients.

Role of the funding source

The funders of the study had no role in study data collection, data analysis, data interpreta writing of the report.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejca.2014.05.022.

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Table A.1: Individual results and clinicopathological features.

M, Male; F, female; B, Bethesda (the number indicates the subcriteria fulfilled); AC, Amste
amplification; IHC, immunohistochemistry; SEQ, sequencing; VUS, variant of unknown significance
NV, not valuable; NM, non mutated; NSN, not specified number of polyps; NS, not specified
microsatellite stability; LR, large rearrangement; A, adenoma; H, hyperplastic. CRC, colorectal c
hepatic cancer; PC, pancreatic cancer; RC, renal cancer; RPC, renal pelvis cancer; UT, urinary tr
endometrial cancer; OC, ovarian cancer; SA, sebaceous adenoma; SEC, sebaceous carcinoma; A
scamous. Polyps from different colonoscopies are separated by ";" different polyps from the same c
* IHC performed upon a tumor metastasis.

Table A.1. Individual results and clinicopathological features

PATIENT INFORMATION			GERMLINE DATA									TUMOR								
			MMR MUTATIONAL ANALYSIS						MYH VARIANTS											
Patient ID	SEX	CLINICAL CRITERIA	TECHNIQUE UTILIZED FOR ANALYSIS	MLH1	MSH2	MSH6	PMS2	VUS Presence (Insight classification)	c.536 A>G (p.Y179C)	c.1187 G>A (G396D)	Other variants in MUTYH	TYPE OF TUMOR	AGE AT DIAGNOSIS	HISTOLOGICAL TYPE	LOCALIZATION	TNM STAGE	AJCC STAGE	HISTOLOGICAL GRADE OF DIFFERENCIATION	MLH1	MS
1	M	ACII	MLPA	NM	NM	NM	NM	-	AA	GG	NP	CRC	37	ADK	LEFT	T3N1M0	IIIB	GX	P	
												CRC	45	ADK	RIGHT	TisN0M0	0	GX	NP	
2	F	B1	MLPA	NM	NM	NP	NP	-	AA	GG	NP	CRC	38	ADK	RIGHT	T2N0M0	I	G1	N	
3	F	B4,5	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	CRC	60	ADK	RIGHT	T3N0M0	IIA	G2	P	
4	M	B5	MLPA; SEQ	NM	NM	NM	NP	-	AA	GC	NM	CRC	59	ADK	LEFT	T3N0M0	IIA	G2	N	
5	M	B1	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	26	ADK	RIGHT	T3N2M0	III	G2	N	
6	F	B1	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	36	ADK	RIGHT	T3N0M0	IIA	GX	N	
7	M	B1	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	39	ADK	RIGHT	T3N0M0	IIA	G3	N	
8	M	ACI	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	42	ADK	RIGHT	T3N0M0	IIA	G1	N	
9	F	AP	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	EC	52	ADK(EN)	NS	NS	NS	G1	N	
10	F	AP	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	OC	78	CA	RIGHT	NS	NS	G3	P	
11	F	B1,2,5	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	40	ADK	LEFT	T3N2M0	III	GX	N	
												EC	48	CA(S)	NS	T1bNxMx	NS	G3	NP	
12	F	B1	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	CRC	42	ADK	LEFT	T3N0M0	IIA	G1	P	
												EC	65	ADK(EN)	NS	T1bN0M0	IIB	G1	NP	
13	M	B2	MLPA; SEQ	NM	NM	NM	NP	-	AA	GG	NP	UT	63	CA	NS	T2N0M0	I	G2	P	
												UT	63	NS	NS	NS	NS	NS	NP	
												CRC	69	ADK(CC)	RIGHT	T3N0M0	III	G2	NP	
14	F	B5	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	64	ADK(CC)	RIGHT	T3N1M0	IIIB	G2	N	
												BC	46	NS	NS	NS	NS	NS	NP	
												BC	68	NS	NS	NS	NS	NS	NP	
15	F	B2	MLPA; SEQ	NM	NP	NP	NP	-	AG	GG	NM	EC	50	ADK(NS)	NOT LUS	T3N1M0	IIIC	G1	N	
												PC	69	ADK	NS	T4N0M0	III	G2	NP	
16	M	B2	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	CRC	75	ADK	RIGHT	T1N0M0	I	G2	P	
												CRC	75	ADK	RIGHT	T3N0M0	IIA	G1	NP	
17	F	AP	MLPA; SEQ	VUS	NP	NP	NP	c.1820T>A; p.L607H (Class 2)	AA	GG	NP	CRC	51	ADK (mucinous)	RIGHT	T4bN2Mx	NS	G2	N	
18	F	B1,4	MLPA; SEQ	NM	NM	NM	NP	-	AA	GG	NP	CRC	47	ADK(CC)	RIGHT	T3N0M0	IIA	G1	P	
												BC	NI	NS	NS	NS	NS	NS	NP	
19	M	AP	MLPA; SEQ	NP	NP	NM	NP	-	AA	GC	c.1014G>C ht; p.Q338H	CRC	59	ADK (mucinous)	RIGHT	T4aN1M1	IV	G1	P	
20	F	B5	MLPA; SEQ	NM	NM	NM	NP	-	AA	GG	NP	CRC	62	ADK	RIGHT	T2N0M0	I	G1	N	
21	F	AP	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	EC	57	ADK(EN)	NS	T1aN0M0	IA	G1	P	
22	F	B5	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	EC	58	ADK(EN)	NS	T1aN0M0	IA	G1	N	
23	M	ACI	MLPA; SEQ	NP	NP	NM	NP	-	AG	GG	NM	CRC	65	ADK	LEFT	T3N1M0	IIIB	G3	P	
												CRC	65	ADK	NS	NS	NS	G3	NP	
												RC	65	NS	NS	T1N0Mx	NS	G3	NP	
24	F	ACI	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	45	ADK	RIGHT	T3N0M0	IIA	G1	N	
25	F	B5	MLPA; SEQ	NP	NP	NM	NP	-	AA	GG	NP	EC	54	ADK(EN)	NS	T1aN0M0	IA	G1	P	
26	F	AP	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	EC	71	ADK(EN)	NS	T1bNxM0	NS	G3	P	
27	F	B4,5	MLPA; SEQ	NP	NP	NM	NP	-	AA	GG	NP	EC	40	ADK(EN)	NS	T1aN0M0	IA	G1	P	
28	F	B1	MLPA; SEQ	NM	NM	NM	NP	-	AG	GG	NM	CRC	34	ADK	RIGHT	T3N0M0	IIA	G2	P	
29	F	B1,5	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	EC	44	ADK(EN)	NS	T1aN0M0	IA	G3	N	
30	M	AP	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	54	ADK	RIGHT	T4N0M0	II	G1	N	
31	M	AP	MLPA; SEQ	NP	NP	NP	NM	-	AA	GG	NP	CRC	82	ADK	RIGHT	T3N1M0	IIIB	G1	P	
32	F	B1	MLPA; SEQ	NM	NP	NM	NP	-	AA	GG	NP	CRC	45	ADK	RIGHT	T3N1M0	IIIB	G3	N	
33	F	B5	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	GC	77	ADK	ANTRO	T3N2M1	IV	GX	N	
34	F	B1,5	MLPA; SEQ	NP	NP	VUS	NP	c.1450G>A; p.E484K (Class 3)	AA	GG	NP	CRC	42	ADK	RIGHT	T4N0M0	II	G1	P	
35	M	AP	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	CRC	64	ADK	RIGHT	T3N1M0	IIIB	G3	P	
36	F	AP	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	CRC	71	ADK	RIGHT	T3N0M0	IIA	G1	P	
												BC	71	NS	NS	NS	NS	NS	NP	
37	M	B5	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	CRC	69	ADK	RECTAL	T3N1M0	IIIB	GX	P	
												TEC	NI	NS	NS	NS	NS	NS	NP	
38	F	B2	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	EC	55	ADK(S)	NS	T3aN1M0	IIIC	G3	N	
												OC	55	NS	NS	NS	NS	NS	NP	
39	F	AP	MLPA; SEQ	NP	NP	NM	NP	-	AA	GG	NP	EC	72	ADK(EN)	NS	T3N0M0	III	G3	P	
40	F	AP	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	EC	66	ADK(EN)	NS	T1N0M0	I	G1	N	
41	F	AP	MLPA; SEQ	NP	NP	NM	NP	-	AA	GG	NP	EC	82	ADK(EN)	NS	T1N0M0	I	G1	P	
42	F	AP	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	EC	83	ADK(EN)	NS	T1aN0M0	IA	G3	P	
43	F	AP	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	EC	58	ADK(S)	NS	T1aN0M0	IA	G2	N	
44	F	AP	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	EC	81	ADK(EN)	NS	T1N0M0	I	G1	N	
45	F	AP	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	55	ADK	RIGHT	T3N0M0	IIA	G1	N	
46	F	AP	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	EC	52	ADK(EN)	NS	T1aN0M0	IA	G2	N	
47	F	ACII	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	EC	66	ADK(EN)	NS	T1aN0M0	IA	G1	N	
48	M	ACII	MLPA; SEQ	NM	NM	VUS	NP	c.2633T>C; p.V878A (Class 1)	AA	GG	NP	SBC	41	ADK	ILLEUM	T3N0M0	IIA	G1	P	
												SBC	47	ADK	ILLEUM	T3N0M0	IIA	G1	NP	
												CRC	47	ADK	RIGHT	T3N1M0	IIIB	G1	NP	
49	M	B2,5	MLPA; SEQ	NM	NM	NM	NP	-	GG	GG	NP	GC	52	ADK	NS	T2N1M0	IIA	G3	P	
												CRC	57	ADK	LEFT	T3N0M0	IIA	G2	NP	
												CRC	57	ADK	LEFT	T2N0M0	I	G1	P	
												CRC	57	ADK	RIGHT	T2N0M0	I	G1	NP	
												SA	63	AD	OTHER (FACE)	-	-	-	P	
												SBC	68	ADK	JEJUNUM	T2N0M0	I	G1	NP	
50	M	B1,4	MLPA; SEQ	NM	VUS	NM	NP	c.782G>A; p.R243Q (Class 3)	AA	GG	NP	CRC	44	ADK	RECTAL	NS	NS	GX	P	
51	F	B2,5	MLPA; SEQ	NM	NP	NP	NP	-	AA	CC	NP	CRC	36	ADK	LEFT	TxNxM0	NS	GX	P	
												EC	51	ADK(NS)	NS	T1bN1M0	IIIC	G2	N	
52	F	B1,5	MLPA; SEQ	NP	NP	NM	NP	-	AA	GG	NP	CRC	23	ADK	LEFT	T2N0M0	I	G1	P	
53	F	ACII	SEQ	NP	NP	NM	NP	-	AA	GG	NP	CRC	57	ADK	RIGHT	T3N0M0	IIA	G2	P	
54	M	B1,4,5	MLPA; SEQ	NM	NM	VUS	NP	c.1439T>A; p.V480E (Unclassified)	AA	GG	NP	CRC	61	ADK	RECTAL LEFT	T3N0M0	IIA	G3	NP	
55	M	B5	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	GC	71	ADK	NS	T3N1M0	IIB	G2	N	
56	M	B5	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	78	ADK	RIGHT	T4N0M0	II	G1	N	
57	M	B5	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	76	ADK	RIGHT	T3N1M0	IIIB	G2	N	
												CRC	76	ADK	LEFT	TisN0M0	0	GX	NP	
58	M	B1,2,4	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	77	ADK	RIGHT	T4N2M1	IV	G1	N	
												GC	77	ADK	NS	NS	NS	NS	NP	
59	M	ACII	MLPA	NP	NM	NM	NP	-	AA	GG	NP	CRC	48	ADK	RIGHT	T3N0M0	IIA	G3	P	
60	M	B4	MLPA; SEQ	NM	NM	NM	NM	-	AA	GG	NP	CRC	69	ADK	RIGHT	T2N0M0	I	G1	P	

Table A.1. Continued.

61	F	B1	MLPA; SEQ	NP	NP	NM	NP	-	AG	GG	1101dup; p.R368QfsX16	CRC	46	ADK	LEFT	T3N0M0	IIA	GX	P
												BC	67	NS	NS	NS	NS	NS	NP
62	M	ACI	MLPA; SEQ	NP	NP	VUS	NP	c.1450G>A; p.E484K (Class 3)	AA	GG	NP	CRC	61	ADK	LEEFT	T3N0M0	IIA	G1	P
												CRC	63	NS	RIGHT	NS	NS	NS	NP
63	M	ACI	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	CRC	47	ADK	RIGHT	T3N0M0	IIA	G1	P
64	M	B1,4,5	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	34	ADK	RIGHT	T3N0M0	IIA	G3	N
65	F	B2	MLPA; SEQ	NP	NP	NP	VUS	c.23+5G>A; p.? (Unclassified)	AA	GG	NP	OC	62	S	NS	T3N1M0	IIIC	G3	P
												CRC	67	ADK	RECTAL	T2N0M0	I	G2	NP
66	F	AP	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	SEC	79	CA	NS	NS	NS	G1	P
67	F	AP	MLPA; SEQ	NP	NP	VUS	NP	c.1109T>C; p.L370S (Class 3)	AA	GG	NP	EC	58	CA(S)	NS	T1N0M0	I	G3	P
68	F	B1,5	MLPA; SEQ	NP	NP	NM	NP	-	AA	GG	NP	EC	46	ADK(EN)	NS	T1N0M0	I	G1	P
69	F	ACII	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	40	ADK	RIGHT	T3N0M0	IIA	G2	N
70	F	B1,4	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	47	ADK	RIGHT	T4N2M0	IIIC	G2	N
71	F	AP	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	EC	59	ADK(EN)	NS	T1N0M0	I	G1	P
72	F	AP	MLPA; SEQ	NP	NP	NM	NP	-	AA	GG	NP	BC	45	CDI	NS	T3N2M0	IIIA	G2	P
												EC	59	ADK(EN)	NS	T1N0M0	I	G2	NP
73	M	B1	MLPA; SEQ	NM	NM	NM	NP	-	AG	GC	NP	CRC	45	ADK	LEFT	T4N2Mx	NS	G2	P
74	F	B1	MLPA; SEQ	NM	NM	NP	NP	-	AA	GG	NP	CRC	57	ADK	LEFT	T3N0Mx	NS	G2	NP
75	M	B1	MLPA; SEQ	NM	NM	NP	NP	-	AA	GG	NP	CRC	32	ADK	RIGHT	T3N0Mx	NS	G1	NP
												CRC	42	NS	NS	TisN0M0	0	NS	NP
76	F	B1	MLPA; SEQ	NM	NM	NP	NP	-	AA	GG	NP	CRC	49	ADK	RIGHT	T3N0Mx	NS	G1	NP
77	F	B2,5	MLPA; SEQ	NM	NM	NP	NP	-	AA	GG	NP	CRC	78	ADK	RIGHT	TisN0M0	0	G1,G2	N
												CRC	82	ADK	LEFT	NS	I	NS	NP
78	F	ACI	MLPA; SEQ	NM	NM	NM	NP	-	AA	GG	NP	CRC	40	ADK	RIGHT	T3N2Mx	NS	G3	NP
79	F	B1	SEQ	NM	NM	NM	NP	-	AA	GG	NP	CRC	40	ADK	LEFT	T3N0Mx	NS	G2	NP
80	F	B1,4	MLPA; SEQ	VUS	NM	NM	NP	c.1217G>A; p.S406N (Class 1)	AA	GG	NP	CRC	45	ADK	LEFT	NS	IV	G2	P
81	F	B1	SEQ	NM	NM	NM	NP	-	AA	GG	NP	CRC	34	ADK	LEFT	T3N0Mx	NS	G1	NP
82	M	AP	MLPA; SEQ	NM	NM	NP	NP	-	AA	GG	NP	CRC	56	ADK	RIGHT	T3N0Mx	NS	G1	N
												HC	NI	NS	NS	NS	NS	NS	NP
83	M	B1,4	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	57	ADK	LEFT	T3N0Mx	NS	G3	N
84	F	B1,4	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	CRC	46	ADK	LEFT	T3N0Mx	NS	GX	P
												CRC	52	ADK	RIGHT	T2N1Mx	NS	G1	NP
85	M	B2	SEQ	NP	NP	NM	NP	-	AA	GG	NP	CRC	65	ADK	RIGHT	T3N0Mx	NS	G2	P
												CRC	65	ADK	LEFT	T2N1Mx	NS	NS	NP
86	M	ACI	MLPA; SEQ	NM	NP	NP	NP	-	AG	GG	NM	CRC	74	ADK	RIGHT	T3N0Mx	NS	G2	N
87	M	B1	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	49	ADK	RIGHT	T2N1Mx	NS	G2	N
88	F	B1	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	49	ADK	RIGHT	T3N0Mx	NS	G3	N
89	M	B1	SEQ	NP	NP	NM	NP	-	AA	GG	NP	CRC	49	ADK	LEFT	T3N0Mx	NS	G2	P
90	M	B1	MLPA; SEQ	NP	NP	NM	NP	-	AA	GG	NP	CRC	46	ADK	RIGHT	T3N1Mx	NS	G1	P
91	F	AP	MLPA; SEQ	NP	NP	NM	NP	-	AA	GG	NP	CRC	52	ADK	LEFT	T3N0Mx	NS	G1	P
92	M	B1	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	CRC	36	ADK	RIGHT	T3N2Mx	NS	NS	P
93	F	B1	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	44	CC	RIGHT	T3N0Mx	NS	NS	N
94	F	B2	MLPA; SEQ	NP	NP	NM	NP	-	AA	GG	NP	CRC	71	ADK	LEFT	T2N0Mx	NS	G2	P
												RPC	71	NS	NS	NS	NS	NS	NP
95	F	B2	MLPA; SEQ	NM	NP	NP	NP	-	AA	GC	Deletion exons 4-16	EC	60	ADK(NS)	NS	T1N0M0	I	G1	N
												CRC	65	ADK	RIGHT	T3N0M0	IIA	G2	NP
96	M	B2	MLPA; SEQ	NP	NP	NP	NM	-	AA	GC	NM	CRC	56	ADK	RIGHT	T1N0M0	I	GX	P
												CRC	67	ADK	LEFT	T3N1Mx	NS	G2	NP
97	F	ACII	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	EC	53	ADK(EN)	NS	1B	IB	GX	P
												RPC	58	CA	NS	TaN0M0	0a	G2	NP
												UT	65	CA	NS	T1N0M0	I	GX	NP
												CRC	70	ADK	RIGHT	T3N0Mx	NS	G2	NP
98	M	B5	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	CRC	57	ADK	RIGHT	T4N2Mx	NS	G1	P
99	F	B1,2,4	MLPA; SEQ	NP	VUS	NM	NP	c.518T>G; p.L173R (Class 3)	AA	GG	NP	CRC	38	ADK	RIGHT	T3N0Mx	NS	G3	P
100	M	B1,4,5	MLPA; SEQ	NP	NP	NM	NP	-	AA	GG	NP	CRC	39	ADK	LEFT	T3N0Mx	NS	G3	P
101	F	B3	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	CRC	57	ADK	RIGHT	T3N0M0	II-A	G1	P
102	F	B3	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	CRC	55	ADK	LEFT	T2N1M0	III-A	G3	P
103	F	AP	MLPA; SEQ	NP	NM	NP	NP	-	AA	GG	NP	CRC	73	ADK	RIGHT	T3N0M1	IV	G1	P
104	F	B3	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	CRC	51	ADK	RIGHT	T3N0M0	II-A	G1	P
105	F	B1,3	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	CRC	49	ADK	RIGHT	T3N0M1	IV	G1	P
106	F	B1	MLPA; SEQ	NM	NM	NM	NP	-	AA	GG	NP	CRC	43	ADK	RIGHT	NS	NS	NS	P
107	F	B1	MLPA; SEQ	NM	NM	NM	NP	-	AA	GG	NP	CRC	39	ADK	RIGHT	T3N0M0	II-A	G3	P
108	F	B1,2	MLPA; SEQ	NM	NM	NP	NP	-	AA	GG	NP	CRC	32	ADK	RIGHT	T3N1M0	III-B	G2	P
												CRC	48	NS	NS	NS	NS	NS	NV
109	M	B1	MLPA; SEQ	NM	NM	NM	NP	-	AA	GG	NP	CRC	27	ADK	RIGHT	T4N1M1	IV	G4	P
110	M	B1	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	CRC*	43	ADK	LEFT	TxN2M1	IV	G3	P
111	F	B2	MLPA; SEQ	NP	NM	NM	NP	-	AA	GC	c.1014G>C ht; p.Q338H	CRC	51	ADK	RECTAL	T1N0M0	I	NS	NP
												CRC	51	ADK	RECTAL	T3N0M0	II-A	NS	NP
												EC	56	NS	NS	NS	NS	NS	P
112	M	B1	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	CRC	49	ADK	RIGHT	T3N0M0	II-A	G2	P
113	F	B1	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	CRC	49	ADK	RECTAL	T3N0M0	II-A	G2	P
114	M	B2,3,5	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	CRC	58	ADK	RIGHT	T2N0M0	I	G2	P
												CRC	58	ADK	RIGHT	T2N0M0	I	G3	NP
115	F	ACII	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	EC	77	ADK(CC)	CORPUS & 1/3 LUS	T2bN1; FIGO IIIC	II-B	G3	P
116	F	B1	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	CRC	48	ADK	RIGHT	T3N0M0	II-A	G1	P
117	F	B1,4	MLPA; SEQ	NP	VUS	NP	NP	c.518T>G; p.L173R (Class 3)	AA	GG	NP	CRC	44	CA	RIGHT	T4N2M0	III-C	GX	P
												CRC	49	ADK	RECTAL	TisN0M0	0	NS	NP
118	F	B1,2	MLPA; SEQ	NM	VUS	NM	NP	c.2069A>G; p.Q690R (Unclassified)	AA	GG	NP	CRC	31	ADK	RECTAL	T3N0M0	II-A	G3	NP
												CRC	35	ADK	RIGHT	T3N0M0	II-A	NS	P
												CRC	52	ADK	RIGHT	TisN0M0	0	NS	P
119	F	AP	MLPA; SEQ	NP	NM	VUS	NP	c.431G>T p.S144I (Class1)	AA	GG	NP	EC	45	ADK(EN)	NS	NS	NS	NS	P
120	M	ACII	MLPA; SEQ	NP	VUS	NP	NP	Duplicated exons 11-16; p.? (Unclassified)	AA	GG	NP	CRC	56	ADK	RIGHT	T4N1M0	III-B	G2	P
121	F	AP	MLPA; SEQ	NP	VUS	NM	NP	c.965G>A; p.G322D (Class 1)	AA	GG	NP	CRC	77	NS	NS	NS	NS	NS	P
122	F	B1	MLPA; SEQ	NP	VUS	NP	NP	c.518T>G; p.L173R (Class3)	AA	GG	NP	CRC	41	ADK	RIGHT	T3N1M0	III-B	G2	NV
123	M	B3	MLPA; SEQ	NP	NM	NP	NP	-	AA	GG	NP	CRC	61	ADK	RIGHT	T3N0M0	II-A	GX	P
124	F	AP	MLPA; SEQ	NP	VUS	NP	NP	c.1276G>A; p.G426R (Unclassified)	AA	GG	NP	OC	42	NS	NS	NS	NS	NS	NP
125	M	AP	MLPA; SEQ	NP	NP	NM	NP	-	AA	GG	NP	CRC	85	ADK	RIGHT	T4N0M0	II-B	G1	P
126	M	AP	MLPA; SEQ	NP	NP	NM	NP	-	AA	GG	NP	CRC	56	ADK	RECTAL	T2N0M0	I	GX	P
127	M	ACII	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	CRC	58	ADK	RECTAL	T3N0M0	II-A	NS	P
128	M	ACII	MLPA; SEQ	NM	NM	VUS	NP	c.1439T>A; p.V480E (Unclassified)	AA	GG	NP	CRC	61	ADK	RECTAL	T3N0M0	II-A	G3	P
129	F	B2,5	MLPA; SEQ	NP	NP	VUS	NP	c.1153_1155delAGG p.R385del (Unclassified)	AA	GG	NP	CRC	53	ADK	RIGHT	T3N0M0	II-A	G2	P
130	F	B1,4	MLPA; SEQ	NP	NP	VUS	NP	c.1618_1620delCTT; p.L540del (Unclassified)	AA	GG	NP	CRC	46	ADK	RIGHT	T3N0M0	II-A	G2	P

Table A.1. Continued.

131	M	B1,5	MLPA; SEQ	NM	NM	VUS	NP	c.1439T>A; p.V480E (Unclassified)	AA	GG	NP	CRC	39	ADK	LEFT	NS	III-B	G2	P	
132	F	B1,3	MLPA; SEQ	NM	NM	NP	NP	-	AA	GG	NP	CRC	44	ADK	RIGHT	T3N0M0	II-A	G2	N	
133	F	B1	MLPA; SEQ	NM	NM	NP	NP	-	AA	GG	NP	CRC	31	ADK	LEFT	T3N0M0	II-A	G2	N	
134	M	B1	MLPA; SEQ	NM	NM	NP	NP	-	AA	GG	NP	CRC	49	ADK	RIGHT	T3N0M0	II-A	NS	N	
135	F	B1	MLPA; SEQ	NM	NM	NP	NP	-	AA	GG	NP	CRC	48	ADK	RIGHT	T3N0M0	II-A	G3	N	
136	M	B1	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	40	ADK	RIGHT	T3N1M0	III-B	G2	N	
137	F	B1	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	30	ADK	RIGHT	NS	IV	G3	N	
138	F	B1	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	35	ADK	RIGHT	NS	NS	NS	N	
139	M	B1	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	48	CA	RIGHT	T3N0M0	II-A	G3	N	N
140	M	AP	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	66	ADK	NS	TXN0M0	NS	GX	N	
141	M	B4	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	70	ADK	RIGHT	T3N0M0	II-A	G1	N	
142	M	B3	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	59	ADK	RIGHT	T3N0M0	II-A	G1	N	
143	M	AP	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	63	ADK	RIGHT	T3N0M0	II-A	GX	N	
144	F	B4	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	71	ADK	LEFT	T2N0M0	I	G1	N	
145	F	AP	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	61	ADK	RIGHT	T3N0M0	II-A	GX	N	
146	F	B1	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	32	ADK	RIGHT	T3N0M0	II-A	G1	N	
147	M	AP	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	73	ADK	RIGHT	T3N1M0	III-B	G1	N	
148	M	B3	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	54	ADK	LEFT	T2N0M0	I	G1	N	
149	M	B3	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	55	ADK	RIGHT	T3N0M0	II-A	G1	N	
150	M	AP	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	82	ADK	RIGHT	T3N0M0	II-A	G4	N	
151	M	AP	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	66	ADK	RIGHT	T2N0M0	I	G1	N	
152	F	B1	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	41	ADK	RIGHT	T3N0M0	II-A	G2	N	
153	F	B1	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	42	NS	NS	NS	NS	NS	N	
154	F	B1	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	44	ADK	LEFT	T4N0M0	II-B	G1	N	
155	F	B1	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	25	ADK	LEFT	NS	NS	NS	N	
156	M	AP	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	52	ADK	RIGHT	T3N0M0	II-A	NS	N	
157	M	AP	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	57	ADK	LEFT	NS	NS	NS	N	N
158	M	B1	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	SBC	30	NS	DUODENUM	T4N1M0	III-B	NS	N	N
159	F	B3	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	55	ADK	RIGHT	T3N0M0	II-A	G1	N	
160	M	B1	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	41	ADK	RIGHT	NS	II-A	G2	N	
161	M	B3	MLPA; SEQ	VUS	NP	NP	NP	c.1852_1853delAA ins GC; p.K618A (Class1)	AA	GG	NP	CRC	39	ADK	RIGHT	T3N0M0	II-A	G1	N	
162	F	B1	MLPA; SEQ	VUS	NP	NP	NP	c.702G>A; p.= (Class2)	AA	GG	NP	CRC	52	ADK	RIGHT	T2N0M0	I	G1	N	
163	F	AP	MLPA; SEQ	VUS	NP	NP	NP	c.307-29C>A; p.= (Class 1)	AA	GG	NP	CRC	70	ADK	RIGHT	T4N1M0	III-B	G1	N	
164	M	B1	MLPA; SEQ	VUS	NP	NP	NP	c.2146G>A ; p.V716M (Class1)	AA	CC	-	CRC	48	ADK	RIGHT	T3N2M0	III-C	GX	N	
165	F	B1	MLPA; SEQ	NM	NM	NM	NP	-	AA	GG	NP	CRC	45	ADK	RIGHT	T3N0M0	II-A	G1	NV	
166	M	B1	MLPA; SEQ	NM	NM	NP	NM	-	AA	GG	NP	CRC	47	ADK	RECTAL	T3N0M0	II-A	G2	N	
167	F	AP	MLPA; SEQ	VUS	NP	NP	NP	c.2146G>A ; p.V716M (Class1)	AA	GG	NP	CRC	57	ADK	NS	T2N0M0	I	NS	NV	
168	M	B5	MLPA; SEQ	NP	NP	NP	VUS	c.59G>A, c.*17G>C ; p.R20Q (Class 1), p.= (Class 1)	AA	GG	NP	CRC	59	ADK	RIGHT	T3N1M0	III-B	G2	P	
169	M	B3	EXSEQ	NM	NM	NP	NP	-	AA	GG	NP	CRC	58	ADK	LEFT	T3N0M1	IV	G2	P	
170	M	AP	MLPA; SEQ	NM	NM	NP	NP	-	AA	GG	NP	CRC	72	ADK	RIGHT	T3N0M0	II-A	G2	NP	N
171	M	B4	EXSEQ	NM	NM	NP	NP	-	AA	GG	NP	GC	29	ADK	CORPUS	TisN0M0	I	G1	NP	N
172	F	B1,4	EXSEQ	NM	NM	NM	NP	-	AA	GG	NP	CRC	36	ADK	RIGHT	T4N1M0	III-B	NS	NV	
173	M	AP	MLPA; SEQ	NP	NM	NP	NP	-	AA	GG	NP	CRC	73	ADK	RIGHT	T4N0M0	II-B	G1	P	N
174	M	B5	MLPA; SEQ	VUS	NM	NP	NP	c.974G>A; p.R325Q (Class 2)	AA	GG	NP	CRC	65	ADK	RECTAL	TisN0M0	0	G1	P	
175	M	B3	MLPA; SEQ	NM	NM	NP	NP	-	AA	GG	NP	CRC	58	ADK	LEFT	T3N0M0	II-A	G1	P	
176	M	B5	MLPA; SEQ	NM	NM	NP	NP	-	AA	GG	NP	CRC	56	ADK	RECTAL	T1NXM0	I	G1	P	
177	F	B5	MLPA; SEQ	VUS	NP	NP	NP	c.307-29C>A; p.= (Class 1)	AA	GG	NP	CRC	78	ADK	RIGHT	T3N0M0	II-A	G2	N	
178	M	ACII	EXSEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	40	ADK	LEFT	T3N1M0	III-B	G2	N	N
179	M	B1	EXSEQ	NM	NM	NP	NP	-	AA	GG	NP	CRC	50	ADK	RIGHT	T3N0M0	II-A	G2	N	
180	F	ACII	MLPA; SEQ	NM	NM	NP	NP	-	AA	GG	NP	OC	47	ADK	LEFT OVARY	T1N0M0	I	G2	P	
181	F	ACII	MLPA; SEQ	NM	NM	NM	NP	-	AA	GG	NP	OC	25	NS	NS	NS	NS	NS	P	
182	M	B1	MLPA; SEQ	NM	VUS	NP	NP	c.1787A>G; p.N596S (Class 3)	AA	GG	NP	CRC	49	ADK	LEFT	T3N0MO	II-A	G3	N	
183	M	B2	MLPA; SEQ	NM	VUS	NM	NP	c.2045C>G; p.T682S (Unclassified)	AA	GG	NP	CRC	58	ADK	RIGHT	T4N1M0	III-B	G2	NP	N
												CRC	61	ADK	RIGHT	T3N0M0	II-A	G2	P	
184	F	B1	MLPA; SEQ	NM	VUS	NM	NP	c.965G>A; p.G322D (Class 1)	AA	GG	NP	CRC	36	NS	RIGHT	NS	NS	NS	P	
185	F	B1	MLPA; SEQ	NM	NP	NP	NM	-	AA	GG	NP	EC	50	ADK(NS)	LUS	NS	III-C	G3	N	N
186	M	ACII	MLPA; SEQ	NM	NP	NP	NP	-	GG	GG	-	CRC	45	ADK	RIGHT	T2N0M0	I	G2	N	N
187	M	ACII	MLPA; SEQ	NP	VUS	NM	NP	c.561_569delTGAGGCTCT; c.965G>A ; p.E188_L190del (Class3), p.G322D (Class 1)	AA	GG	NP	CRC	45	ADK	RIGHT	TisN0M0	0	G3	P	
188	F	B1	MLPA; SEQ	NM	NP	NP	NM	-	AA	GG	NP	CRC	42	ADK	RECTAL	T2N0M0	I	G2	N	
189	M	B1,4	MLPA; SEQ	NP	VUS	NM	NP	c.965G>A; p.G322D (Class 1)	AA	GG	NP	CRC	21	ADK	LEFT	T2N0M0	I	G2	P	
190	F	B1	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	49	ADK	LEFT	T3N1M0	III-B	G1	P	
191	M	B2	MLPA; SEQ	NP	VUS	NP	NP	c.1666T>C; p.= (Class 1)	AA	GG	NP	CRC*	58	ADK	LEFT	T4N0M0	II-B	G2	P	
192	F	B1	MLPA; SEQ	NM	NM	NP	NP	-	AA	GG	NP	CRC	48	ADK	LEFT	T3N1M0	III-B	G2	P	
193	F	B5	MLPA; SEQ	NP	NP	NM	NP	-	AA	GG	NP	CRC	65	ADK	RIGHT	T3N1M0	III-B	G3	P	
194	F	B5	MLPA; SEQ	NP	NP	NM	NP	-	AA	GG	NP	OC	55	ADK	LEFT	NS	NS	NS	P	
195	F	ACII	MLPA; SEQ	NM	NM	NM	NP	-	AA	GG	NP	CRC	46	ADK	RIGHT	T3N1bM0	III-B	G2	P	
196	M	B5	MLPA; SEQ	NM	NM	NP	NP	-	AA	GG	NP	CRC	55	ADK	LEFT	T3N0M0	II-A	G2	P	
197	M	B5	MLPA; SEQ	NM	NM	NP	NP	-	AA	GG	NP	CRC	54	ADK	LEFT	T3N1M0	III-B	G2	P	
198	M	ACII	MLPA; SEQ	NP	VUS	NM	NP	c.965G>A; p.322D(Class 1)	AA	GG	NP	CRC	44	ADK	RIGHT	T4N2M0	III-C	NS	P	
199	M	B2	MLPA; SEQ	NP	NP	VUS	NP	c.1450G>A; p.E487K (Unclassified)	AA	GG	NP	CRC	65	ADK	RIGHT	T3N0M0	II-A	G2	P	
200	F	B2	MLPA; SEQ	NP	NP	VUS	NP	c.3296T>A; p.I1099N (Unclassified)	AA	GG	NP	EC	52	ADK(EN)	NS	T1aN0M0/	I-A	NS	NP	N
												CRC	53	NS	RIGHT	T4N0M1	IV	G3	NP	N
201	F	ACII	EXSEQ	NM	NP	NP	NP	-	AA	GG	I	EC	60	ADK(EN)	NS	T1bN0M0	I	G2	N	
202	F	AP	EXSEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	66	ADK	RIGHT	T2N1M0	III-A	G2	N	
203	F	B5	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	51	ADK	RIGHT	T4N2M0	III-C	NS	N	N
204	M	B1	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	16	NS	NS	NS	NS	NS	N	
205	F	AP	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	69	ADK	RIGHT	T3N0M0	II-A	G3	N	
206	F	B1	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	42	ADK	NS	NS	NS	NS	N	
207	F	B2	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	69	ADK	LEFT	NS	NS	NS	N	
												EC	69	ADK(EN)	NS	NS	NS	NS	NP	N
208	F	B1	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	43	ADK	LEFT	T3N0M0	II-A	G2	N	
209	F	ACII	MLPA; SEQ	NP	NP	NM	NP	-	AA	GG	NP	CRC	56	ADK	RIGHT	TisN0M0	0	NS	P	
210	F	B2	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	64	ADK	RIGHT	NS	NS	NS	N	
												CRC	64	ADK	LEFT	NS	NS	NS	NP	N
211	M	B1,4	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	CRC	45	ADK	LEFT	NS	NS	NS	P	
212	M	B1	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	41	ADK	RIGHT	T3N0M0	II-A	G2	N	
213	F	B1	MLPA; SEQ	NP	VUS	NM	NP	c.1618_1620delCTT exon 4; p=? (Unclassified)	AA	GG	NP	CRC	45	ADK	RIGHT	T3N0M0	II-A	G2	P	
214	F	ACII	MLPA; SEQ	NP	VUS	NP	NP	LR exon 16; p=? (Unclassified)	AA	GG	NP	EC	40	ADK(EN)	NS	NS	NS	NS	P	
												CRC	47	ADK	NS	NS	NS	NS	NP	
215	F	AP	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	EC	56	ADK(EN)	NS	NS	NS	G2	N	
216	F	B2	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	EC	50	NS	NS	NS	NS	NS	N	
217	F	B1,4	MLPA; SEQ	NP	NP	NM	NP	-	AA	GG	NP	EC	48	ADK(EN)	NS	NS	NS	NS	P	
												BC	69	NS	NS	NS	NS	NS	NP	N
218	F	B1	MLPA; SEQ	NP	VUS	NP	NP	c.2702A>T; p.E901V (Unclassified)	AA	GG	NP	CRC	31	ADK	RIGHT	NS	NS	G3	P	
219	F	B1	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	CRC	50	NS	RIGHT	NS	NS	NS	P	
												CRC	63	NS	NS	NS	NS	NS	NP	N
												BC	78	NS	NS	NS	NS	NS	NP	N
220	M	B2	MLPA; SEQ	NP	NM	NP	NP	-	AA	GG	NP	CRC	51	ADK	RECTAL	T4N0M0	II-B	NS	N	
												CRC	81	NS	NS	NS	NS	NS	NP	N
												CRC	81	NS	NS	NS	NS	NS	NP	N
221	M	AP	MLPA; SEQ	NM	NP	NP	NM	-	AA	GG	NP	CRC	65	ADK	RIGHT	T4N2M0	III-C	G3	N	
222	F	B1,4	MLPA; SEQ	NP	VUS	NM	NP	c.518T>G; p.L173R (Class 3)	AA	GG	NP	CRC	48	ADK	RECTAL	NS	NS	NS	P	
223	M	B1,3	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	47	ADK	RIGHT	T4N0m0	II-B	NS	N	
224	M	B5	MLPA; SEQ	NP	NM	NM	NP	-	AA	GG	NP	CRC	74	ADK	RIGHT	T3N0M0	II-A	G3	P	
225	M	B1,5	MLPA; SEQ	NM	NP	NP	NP	-	AA	GG	NP	CRC	49	ADK	LEFT	T3N0M0	II-A	G2	N	

microsatellite stability; MMR, mismatch repair; IHC, immunohistochemistry; WT, wildtype; G, grad

is given by TNM Classification of malignant tumors.

Table A.2. Clinicopathological and molecular features of monoallelic *MUTYH* mutation carriers

Case	Sex	Clinical criteria	*MUTYH* mutation	*MUTYH* polymorphism	MMR VUS	Tumor location	Age at diagnosis	Stage	Degree of differentiation	Mucinous production	Infiltrating lymphocytes	MSI status	MMR IHC	*KRAS* status	Numbe Surgical re
4	M	Bethesda	G396D hetero	NI	NI	Left colon	59	T3N0M0	G2	ND	ND	MSI	Loss of MLH1 and PMS2	G13D	3 adeno
15	F	Bethesda	Y179C hetero	NI	NI	Endometrial	50	T3N1M0	G1	ND	ND	MSI	Loss of MLH1 and PMS2	WT	
						Pancreas	69	T4N0M0	G2	-	-	ND	ND	ND	
19	M	Anatomo-pathological	G396D hetero	Q338H hetero	NI	Right colon	59	T4N1M1	G1	YES	YES	MSI	Loss of MSH6	G13D	0
23	M	Amsterdam Criteria I	Y179C hetero	NI	NI	Left colon	65	T3N1M0	G3	ND	ND	MSI(1/5)	No loss	G12V	
						Colon	65	NS	ND	ND	ND	ND	ND	ND	0
						Renal	65	T1N0MX	G3	-	-	ND	ND	ND	
28	F	Bethesda	Y179C hetero	NI	NI	Right colon	34	T3N0M0	G2	ND	ND	MSI	Loss of MSH2 and MSH6	ND	0
86	M	Amsterdam Criteria I	Y179C hetero	NI	NI	Right colon	74	T3N0MX	G2	ND	ND	MSI	Loss of MLH1 and PMS2	G13D	0
96	M	Bethesda	G396D hetero	NI	NI	Right colon	56	T1N0M0	GX	ND	ND	MSI	Loss of PMS2	WT	ND
						Left colon	67	T3N1MX	G2	YES	ND	ND	ND	ND	3 adeno
111	F	Bethesda	G396D hetero	Q338H hetero	NI	Rectal	51	T1N0M0	ND	ND	ND	MSS	ND	ND	0
						Rectal	51	T3N0M0	ND	ND	ND	ND	ND	ND	
						Endometrial	56	T1N0M0	G1	NO	ND	ND	Loss of MSH2 and MSH6	G12D	

ARTICLE 3:

Identification of germline *FAN1* variants in MSH2-deficient Lynch-like syndrome patients.

Hypothesis: *FAN1* germline mutations, recently associated to familial CRC type X, could account for a proportion of LLS cases.

Aim: To determine the prevalence of germline *FAN1* variants in 30 MSH2-deficient LLS cases.

Summary of the obtained results: We identified 3 patients harboring rare or novel *FAN1* missense variants. One was classified as likely pathogenic by functional and computational analyses. The remaining two missense variants cosegregated with colorectal cancer-affected relatives. The obtained results suggest that germline *FAN1* variants may account for a significant proportion of LLS.

Contribution of the PhD candidate: Design of the probes for next generation sequencing (NGS). Target enrichment and library preparation. Variant calling, filtering and annotation of NGS results. *In silico* prediction studies of all *FAN1* variants. Analysis and interpretation of results, as well as preparing figures and tables. Writing the first draft of the article and preparing the final version.

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Short title: *FAN1* mutations in Lynch-like syndrome

ABSTRACT

In about 55% of individuals harboring mismatch repair (MMR) deficient tumors, germline mutations o
methylation in MMR genes are not identified, being referred as Lynch-like syndrome (LLS) patients. Rece
germline mutations have been associated to MMR proficient colorectal cancer (CRC) predisposition. The a
study was to determine whether germline *FAN1* play also a role in LLS. Germline analysis of *FAN1* was per
30 LLS individuals showing MSH2 deficiency in tumors. Three individuals harboring rare *FAN1* missense vari
identified. Two of the 3 identified variants, c.434G>A [p.(R145H)] and c.1129C>T [p.(R377W)], cosegreg
colorectal cancer-affected relatives. The remaining variant, c.1856T>A (p.M619K), was classified as likely p
by functional and computational analyses. The obtained results suggest the involvement of *FAN1* gene in LL

Keywords: Lynch syndrome, Lynch-like syndrome, *FAN1*, *MSH2*, DNA mismatch repair, interstrand crosslink repair, Fanconi anemia.

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Abbreviations

LS, Lynch syndrome; LLS, Lynch-like syndrome; CRC, col
cancer; MMR, mismatch repair; MSI, microsatellite inst
immunohistochemistry;

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Disclosures

The authors declare no conflict of interest.

Lynch syndrome (LS; MIM #120435) is the most common hereditary colorectal cancer syndrome, a for 2-5% of all CRC cases (Moreira et al. 2012). It is characterized by an increased risk of cancer, mainly colo endometrial tumors, and caused by heterozygous germline mutations (or epimutations) in mismatch repa genes (*MLH1, MSH2, MSH6* and *PMS2*) (Lynch et al. 2009). LS molecular diagnosis allows the ap management of patients and their families through clinical follow-up of carriers, mainly based on colo every 1-2 years starting at the age of 20 (Järvinen et al. 2000).

As a result of MMR deficiency, LS tumors exhibit microsatellite instability (MSI) and loss of MM expression by immunohistochemistry (IHC). These tissue markers are good pre-screening tools for LS iden which have been used both in the presence of familial/personal history of colorectal cancer (CRC) or other tumors, and in the universal screening of tumors. Although this feature is characteristic of the tumors dev LS individuals, the same deficiency is found in 10-15% of sporadic tumors, mainly due to somatic hyperme of *MLH1* (Yamamoto and Imai 2015). The germline mutational analysis of MMR genes is recommended wh deficient tumors are identified, in the absence of *MLH1* promoter methylation (Pineda et al. 2010).

Among the patients with MMR-deficient colorectal or endometrial cancers, about 55% do no pathogenic germline mutations in MMR genes, being thus grouped as Lynch-like syndrome (LLS) [rev (Buchanan et al. 2014). These patients, as well as their first-degree relatives, have an intermediate risk of d CRC (Rodriguez-Soler et al. 2013). However, appropriate clinical management and risk assessment for this patients has not been established yet (Buchanan et al. 2014).

LLS patients are a heterogeneous group. At the germline level we and others have identified mu other colorectal cancer (CRC) predisposing genes, such as *MUTYH* (Morak et al. 2014; Castillejo et al. 2014) (Elsayed et al. 2014), as responsible for a small proportion of LLS cases. Besides, double somatic events in genes have been found in 33-79% of MSH2-deficient and 25-89% of MLH1-deficient tumors from LLS (Haraldsdottir et al. 2014; Mensenkamp et al. 2014; Sourrouille et al. 2013).

Recently, our group identified *FAN1* (FANCD2/FANCI-associated nuclease 1; MIM #613534) as a r predisposing gene, finding it mutated in the germline in approximately 3% of Amsterdam-positive MMR- families (Seguí et al. 2015). *FAN1* is involved in maintenance of genome integrity, playing a role in the in crosslink repair as it belongs to the Fanconi Anemia pathway. In addition to its interaction with Fancon proteins, FAN1 also interacts with MMR proteins (Kratz et al. 2010; Liu et al. 2010; MacKay et Smogorzewska et al. 2010). Based on this, we hypothesized that germline mutations in the *FAN1* gene migh a proportion of LLS cases. To test this hypothesis we investigated the prevalence of germline mutations in series of 30 LLS patients with MSI tumors showing MSH2/MSH6 deficiency (Supplementary Table 2 and 3).

The *FAN1* c.1856T>A carrier (case 105, Figure 1) is a female who was diagnosed of CRC at 49 and cancer at 58, with no family history of cancer in her first-degree relatives. The CRC showed MSI in accord MSH2 and MSH6 loss of expression. Her mother died at 38 of a cardiac disease and her maternal grandfath otorhinolaryngological cancer. Cosegregation analysis was not possible in this family due to unavailability of *In silico* algorithms used to evaluate the effect of the identified variants on splicing predicted the creation acceptor splicing site two bases downstream of the c.1856T>A variant (Table 1). However, subsequen transcriptase-PCR analyses using patient cultured lymphocytes did not identify any changes (data not show protein level, the p.M619K (c.1856T>A) variant was predicted to be destabilizing of the protein struc deleterious for function (Table 1B). The variant p.M619K affected α-helix 15 in the TPR (tetratricopeptid domain, which is part of the dimerization interface formed by DNA-binding, and mediates inter-domain int (Supplementary Figure 2).

The *FAN1* c.434G>A carrier (case 114, Figure 1) was diagnosed with two synchronous CRC at age patient has a family history of LS-related tumors: his father had metachronic CRC at 65 and 75 years of age with no apparent MMR protein loss) and bladder cancer at 76, and his paternal aunt was diagnosed with years of age. Cosegregation analysis demonstrated the variant was present in the CRC affected proband's fa variant c.434G>A, p.(R145H), was predicted to be destabilizing of protein structure (Table 1). It is located i translated exon, which codes for the UBZ domain, essential for FAN1 localization to sites of damage (Smog et al. 2010). The putative pathogenic role of this variant is reinforced by the fact that c.418G>T p.(D140Y identified in an Amsterdam family and demonstrated to be pathogenic in functional analyses (Seguí et al. located nearby R145 (Supplementary Figure 2).

The *FAN1* c.1129C>T variant carrier (case 104, Figure 1) was diagnosed with CRC at 51 years of age. H who developed a prostate cancer at age 73 and a CRC at age 87, was also a carrier of the *FAN1* c.1129C> The variant c.1129C>T, p.(R377W), affects a highly conserved residue in α-helix 1 of the SAP' domain (Suppl Figure 2). Although this variant has no conclusive results through computational analyses on protein stability, the N-terminal region of α-helix 1 in SAP' is localized in the vicinity of the DNA-binding site and would impair the ligand-binding affinity by affecting electrostatic interactions.

Taken together, we have found 3 missense variants in *FAN1* gene among 30 LLS cases with MS deficient tumors. The variant c.1856T>A (p.M619K) was predicted probably pathogenic by *in silico* functional and structural levels) and c.434G>A [p.(R145H)] and c.1129C>T [p.(R377W)] demonstrated cose in CRC affected relatives. As FAN1 interacts with MMR proteins, the identification of germline *FAN1* v Lynch-like patients suggest that FAN1 deficiency could cause a impair MMR activity, leading to MMR tumors.

PATIENTS AND METHODS

Included patients

Mutational analysis of *FAN1* was performed in 30 probands (18 females and 12 males) diagnosed associated tumors showing loss of MSH2/MSH6 expression (Supplementary Tables 2 and 3). Previous ana not identify any germline MMR gene pathogenic mutation by Sanger sequencing and MLPA, although five were carriers of *MSH2* variants of unknown significance (class 3). The median age at diagnosis of the first tu 48 years (range 21-77). Concerning clinical criteria, 24 patients met Bethesda criteria, 4 fulfilled Amsterda and the remaining 2 were referred to the Genetic Counseling Unit for showing histological features suggesti and loss of MMR protein expression. Seven patients (23.3%) presented multiple LS-associated tumors. evident in all informative tumors analyzed (n=16).

Isolation of genomic DNA

DNA from peripheral blood lymphocytes (PBL) was extracted using FlexiGene DNA kit (Qiagen Germany) according to manufacturer's instructions. For available specimens of formalin-fixed paraffin e (FFPE) tissue from probands' relatives, 10-20 x 10-µm FFPE sections were cut and deparaffinized with Deparaffinization Solution (Qiagen, Hilden, Germany). Posterior DNA isolation was performed using e DNAeasy Tissue Kit or QIAmp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according to manu instructions.

Mutational analysis of *MSH2* and *MSH6* genes

Point mutation analysis of *MSH*2 (NM_000251.2; NG_007110.1) and *MSH6* (NM_000179.2; NG_007111.1) g performed by PCR amplification of exonic regions and exon-intron boundaries followed by Sanger se (primers and conditions available upon request). Genomic rearrangements in MMR genes were ana multiplex ligation dependent probe amplification using SALSA-MLH1/MSH2 P003-B1 and MSH6 P072 k Holland).

Mutational analysis of *FAN1* gene

FAN1 gene was analyzed by Next generation sequencing in 20 of the included individuals (see belo remaining 11 cases *FAN1* mutational analysis was performed by Sanger sequencing as previously described al. 2015).

Spectrophotometer (Thermo Fischer Scientific) and by electrophoresis in agarose gel.

Capture of the target regions was performed using HaloPlex Target Enrichment kit 1-500 kb Technologies, USA), according to the HaloPlex Target Enrichment System-Fast Protocol Version B. Br protocol consists of four steps: 1) digestion of genomic DNA using eight different restriction reac hybridization of restricted fragments to probes whose ends are complementary to the target fr circularization of fragments and incorporation of sequencing motifs including index sequences; 3) capture DNA using streptavidin beads and ligation of circularized fragments; 4) PCR amplification of captured target Quality control and dilution estimates of libraries were performed using High Sensitivity DNA chips in a Bioanalyzer. Library concentrations were normalized to 0.44 nM. Pooled libraries were sequenced on flowcell with paired 250 base reads plus an 8-base index read, using version 3 cartridges.

Agilent SureCall application was used to trim, align and call variants. *FAN1* exonic regions plus intronic boundaries as well as 650-bp upstream transcription start site were targeted. Filtering of called var performed depending on different features: base Phred quality >30, alternative allele ratio of variants ≥ alternative variant read depth ≥38x in PBL samples and ≥10x in FFPE samples. Variants with a MAF>1% acc 1000 Genomes or NHLBI Exome Sequencing Project (ESP) were filtered out, with exception of *MUTYH* varia rare variants identified were further confirmed by Sanger sequencing using independent DNA samples.

Pathogenicity assessment of identified *FAN1* variants

Cosegregation analysis. *FAN1* variants were screened in available DNA samples from proband's relatives b sequencing.

Computational analysis of functional and structural impact of FAN1 variants. DNA sequences conta identified *FAN1* variants were analyzed by bioinformatics tools addressed to evaluate its impact at the protein level using Alamut v.2.7.1. Evolutionary conservation of variants was evaluated using a multiple alig FAN1 sequences of evolutionary divergent species (e.g., Human, Mouse, Dog, *Platypus,* Chicken, Lizard, Zebrafish*, Pseudomona aeruginosa* on Align-GVGD (http://agvgd.iarc.fr/index.php)).

Human FAN1 (hFAN1; UniProt accession: Q9Y2M0) is a multi-domain protein with 1017 amino aci intact three-dimensional (3D) structure has not been determined yet. Only two crystal structures of hFAN1 4rec (2.20 Å resolution, a.a. 371-1009), 4ry3 (2.80 Å resolution, a.a. 371-1016)] have been determined (and Jiang 2015; Zhao et al. 2014). Protein domain annotations of FAN1 were retrieved from UniProt for a binding (UBZ) domain (a.a. 41-67) and a nuclease domain of the VRR_nuc family (a.a. 895-1007). The anno the canonical DNA-binding domain (SAP; a.a. 459-503) is according to crystal structures.

(3DM) Server. PyMOL Molecular Graphics System v1.5.0.4 (Schrodinger, LLC) was used to visualize structur
create Figure 2A. Input files were PDB file 3NA3 for MLH1 N-terminal domain and PDB file 3RBN for the C
domain.

mRNA splicing analysis and allele specific expression analysis. Human blood lymphocytes were incubated
without puromycin after one week of culture with Gibco® *PB-MAX*™ medium. Subsequently total RNA was
from cultured lymphocytes with *TRIzol*® Reagent. One μg of RNA was retro-transcribed using *iScript Sel*
synthesis kit. Amplification of *FAN1* coding region from exon 4 to 9 and from 5 to 9 containing c.1856T>A va
performed using specific primers (Supplementary Table 4; conditions available upon request). Sequences
transcripts were compared with transcripts from two control lymphocyte cultures.

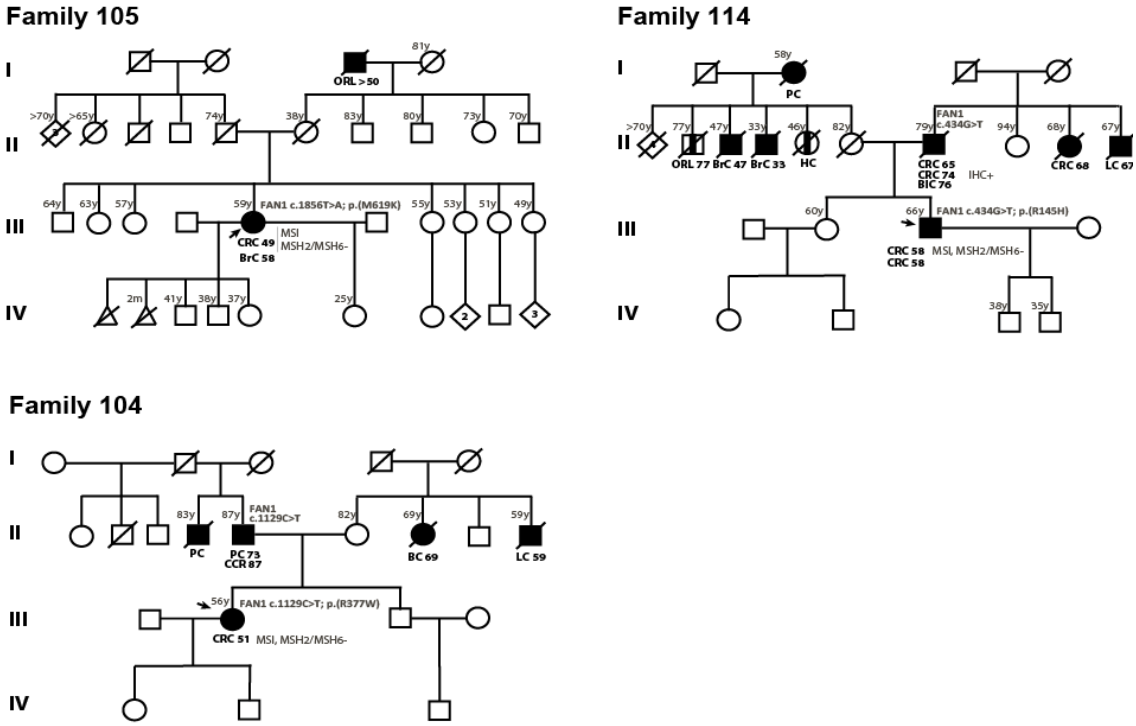

Figure 1. A) Pedigrees of the families with germline missense *FAN1* variants. Filled symbol, cancer; arrow, ir
Cosegregation results are indicated on the top-right corner of analyzed relatives. Current ages and ages
when available, are indicated on the top-left corner of each individual's symbol. CRC, colorectal cancer; PC,
cancer; LC, lung cancer; ORL, otorhinolaryngological cancer; BC, breast cancer; BrC, Brain cancer; HC, hepat
EC, endometrial cancer; SC, skin cancer; SA, sebaceous adenoma; MSI, microsatellite instable; IHC+, conser
protein expression.

Table 1. Rare germline *FAN1* missense variants identified in 30 MSH2-deficient Lynch-like syndrom predictions are shown. Evidence that supports pathogenicity of variants is highlighted in bold Supplementary Table 1.

Case	*FAN1* genetic variant	Splicing prediction	Structure prediction					
		Interpretation^	PoPMuSic	CUPSAT	ERIS	I-MUTANT 2.0	**Interpretation**	Pol
105	c.1856T>A; p.(M619K)	**New Acceptor Site**	**1.43 kcal/mol (D)**	**-0.15 kcal/mol (D)**	**>10 kcal/mol (D)**	**-3.43 kcal/mol (D)**	**Destabilizing**	**Possi**
114	c.434G>A; p.(R145H)	No effect	**0.46 kcal/mol (D)**	**-0.79 kcal/mol (D)**	**0.22 kcal/mol (D)**	**-1.42 kcal/mol (D)**	**Destabilizing**	
104	c.1129C>T; p.(R377W)	No effect	**1.30 kcal/mol (D)**	1.78 kcal/mol (S)	**6.19 kcal/mol (D)**	0.77 kcal/mol (S)	**Inconclusive**	

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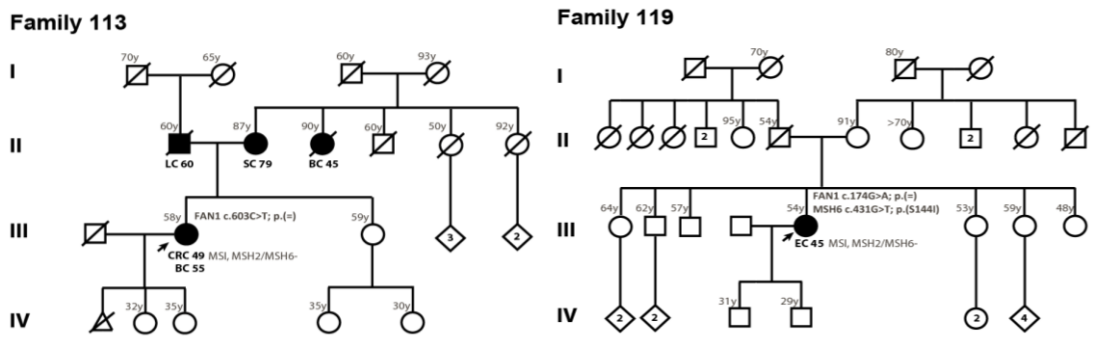
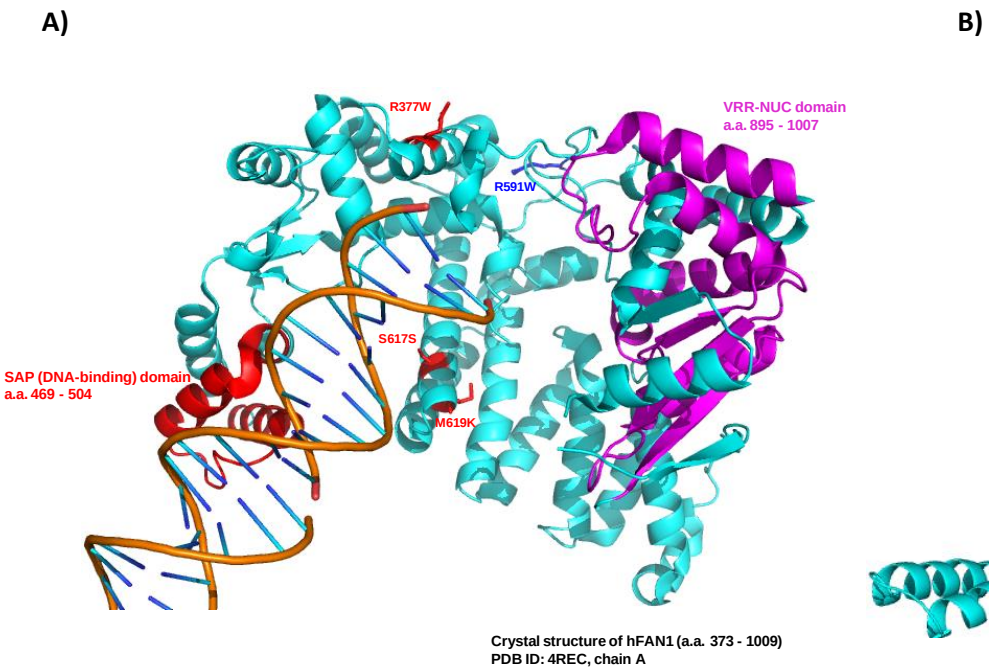


Supplementary Figure 1. A) Pedigrees of the families with germline silent *FAN1* variants. Filled symbol, cancer; arrow, index case. Current ages and ages at death, when available, are indicated on the top-left corner of each individual's symbol. CRC, colorectal cancer; PC, pancreas cancer; LC, lung cancer; ORL, otorhinolaryngological cancer; BC, breast cancer; BrC, Brain cancer; HC, hepatic cancer; EC, endometrial cancer; SC, skin cancer; SA, sebaceous adenoma; MSI, microsatellite instable; IHC+, conserved MMR protein expression; WT, wildtype



Supplementary Figure 2. Localization of FAN1 missense variants p.M619K and p.R377W in the crys and p.R377W in a 3D-model. Variants p.D140Y, R591W and P340S have been identified in the germ p.H324Q, identified in liver, cervix and kidney tumors, respectively.

Supplementary Table 1. Rare germline *FAN1* variants identified in 30 MSH2-deficient Lynch-like splicing *in silico* predictions are shown. Abbreviations: ESP, NHLBI GO Exome Sequencing Proje consensus splice site.

Patient ID	*FAN1* genetic variant	Reference SNP	Population MAF	Splicing		
			1000 genomes/ ESP EA	SSF [0-100]	MaxEnt [0-12]	NNSPLICE [0-1]
105	c.1856T>A; p.(M619K)	**NA**	**NA/NA**	**Acceptor: —>81.17 (c.1858); Donor: –->75.07 (c.1857)**	**Acceptor: –->4.47 (c.1858)**	**Acceptor: –->0.37 (**
114	c.434G>A; p.(R145H)	rs146408181	**0.0002/0.0018**	-	-	-
104	c.1129C>T; p.(R377W)	rs151322829	**0.0014/0.0081**	Acceptor: 70.02-->73.57 (c.1134)	-	-
119	c.174G>A; p.(=)	rs143965941	**NA/0.0010**	-	-	-
113	c.603C>T; p.(=)	rs142084532	**0.0010/0.0019**	-	-	-

Supplementary Table 2. Clinicopathological features of the included patients

Patient ID	CLINICAL DATA							TUMOR MOLECULAR DATA		MMR MUTATION	
	Gender	Clinical criteria	Cancer 1	Cancer 2	Cancer 3	Cancer 4	Cancer 5	IHC	MSI status	TECHNIQUES UTILIZED	
101	F	BC	CRC* (57)					MSH2/MSH6 loss	NP	MLPA; SEQ	
102	F	BC	CRC* (55)					MSH2/MSH6 loss	NP	MLPA; SEQ	
103	F	AP	CRC* (73)					MSH2 loss/MSH6 NP	NP	MLPA; SEQ	
104	F	BC	CRC* (51)					MSH2/MSH6 loss	NP	MLPA; SEQ	
105	F	BC	CRC* (49)					MSH2/MSH6 loss	NP	MLPA; SEQ	
107	F	BC	CRC* (39)					MSH2 loss/MSH6 NV	MSI	MLPA; SEQ	
108	F	BC	CRC* (32)	CRC (48)				MSH2 loss/MSH6 NV	MSI	MLPA; SEQ	
109	M	BC	CRC* (27)					MSH2/MSH6 loss	MSI	MLPA; SEQ	
110	M	BC	CRC* (43)					MSH2/MSH6 loss	NV	MLPA; SEQ	
111	F	BC	CRC (51)	CRC (51)	EC* (56)			MSH2/MSH6 loss	NP	MLPA; SEQ	
112	M	BC	CRC* (49)					MSH2/MSH6 loss	NV	MLPA; SEQ	
113	F	BC	CRC* (49)	BC (55)				MSH2 loss/MSH6 NV	MSI	MLPA; SEQ	
114	M	BC	CRC* (58)	CRC (58)				MSH2/MSH6 loss	MSI	MLPA; SEQ	
115	F	AC	BC (62)	BC (69)	EC* (77)			MSH2/MSH6 loss	NP	MLPA; SEQ	
116	F	BC	CRC* (48)					MSH2/MSH6 loss	MSI	MLPA; SEQ	
117	F	BC	CRC* (44)					MSH2/MSH6 loss	NP	MLPA; SEQ	MSH2 c.518T>G
118	F	BC	CRC (31)	CRC* (35)	CRC* (52)	CRC (58)	SC (37)	MSH2 loss/MSH6 NV and MSH2/MSH6 loss (respectively)	MSI	MLPA; SEQ	MSH2 c.2069A>G
119	F	BC	EC* (45)					MSH2/MSH6 loss	MSI	MLPA; SEQ	
121	F	AP	CRC* (77)					MSH2/MSH6 loss	NP	MLPA; SEQ	MSH2 c.965G>A
123	M	BC	CRC* (59)					MSH2/MSH6 loss	NP	MLPA; SEQ	
181	F	AC	OC* (25)					MSH2 loss/MSH6 NP	MSI	MLPA; SEQ	
183	M	BC	CRC (58)	CRC* (61)				MSH2/MSH6 loss	NP	MLPA; SEQ	MSH2 c.2045C>G
187	M	AC	CRC* (45)					MSH2/MSH6 loss	MSI	MLPA; SEQ	MSH2 c.561_569delTGAGG MSH2 c.965G>A
189	M	BC	CRC (21)					MSH2/MSH6 loss	MSI	MLPA; SEQ	MSH2 c.965G>A
191	M	BC	CRC* (58)					MSH2/MSH6 loss	NP	MLPA; SEQ	MSH2 c.1666
198	M	AC	CRC (44)					MSH2/MSH6 loss	MSI	MLPA; SEQ	MSH2 c.965G>
211	M	BC	CRC (45)					MSH2/MSH6 loss	MSI	MLPA; SEQ	
218	F	BC	CRC* (31)					MSH2/MSH6 loss	MSI	MLPA; SEQ	MSH2 c.2702A>T
219	F	BC	CRC* (50)	CRC (63)	BC (78)			MSH2/MSH6 loss	MSI	MLPA; SEQ	
224	M	BC	CRC* (74)					MSH2/MSH6 loss	MSI	MLPA; SEQ	

Note 1: Parenthesis after cancer type indicate years of age at diagnosis.

Note 2: Insight classification of *MSH2* and *MSH6* variants is indicated inside a parenthesis.

Abbreviatures: F: female, M: male, BC: Bethesda criteria, AC: Amsterdm criteria, CRC: colorectal cancer; EC: endometrial cancer; BC: breast cancer; SC: instability, MLPA: multiplex ligation-dependent probe amplification, SEQ: sequencing, NM: not mutated, NGS: next generation sequencing.

* Studied tumor for molecular data results.

^ Not reported variant; class 3 according to the Insight rules.

§ Sequenced only by Sanger.

Clinicopathological features	n (%)	No variant identified - n (%)	VUS Class 3 carrier n (%)
Total LLS	30 (100)	25 (83)	5 (17)
Sex			
Female	18 (60)	15 (60)	3 (60)
Male	12 (40)	10 (40)	2 (40)
Age at diagnosisº	48.3 (21-77)^	50 (21-77)^	44 (31-58)^
Clinical criteria			
Amsterdam	4 (13.3)	3 (12)	1 (20)
Bethesda	24 (80)	20 (80)	4 (80)
Anatomo-pathological	2 (6.7)	2 (8)	0 (0)
Patients with multiple primary tumors*	7 (23.3)	5 (71.4)	2 (28.6)
MSH2-deficient analyzed tumors			
Colorectal cancer	35 (89.7)	26 (86.7)	9 (100)
Endometrial cancer	3 (7.7)	3 (10)	0 (0)
Ovarian cancer	1 (2.6)	1 (3.3)	0 (0)

º First tumor diagnosis; ^ Age range; * LS spectrum (Bethesda)

Supplementary Table 4. Primers used for mRNA splicing analysis of *FAN1* c.1856T>A variant

Design 1				
Primers	Name	Sequence 5'→3'	Exon	Amplicon length
PCR	FAN1_Ex4_UP	TGAACTCCTTTCTGCTCCTGA	4	789bp
	FAN1_Ex9_DW	CCCTCTGTGATGCACTTGAT	9	
Sequencing	FAN1_Ex5_UP	GACAGCTTTCAACAGTCCTG	5	
	FAN1_Ex6_DW	AATCCCTTTTTGCACACTGA	6	

Design 2				
Primers	Name	Sequence 5'→3'	Exon	Amplicon length
PCR	FAN1_Ex5_UP	GACAGCTTTCAACAGTCCTG	5	490bp
	FAN1_Ex9_DW	CCCTCTGTGATGCACTTGAT	9	
Sequencing	FAN1_Ex6_DW	AATCCCTTTTTGCACACTGA	6	

ARTICLE 4:

Elucidating the molecular basis of MSH2-deficient tumors in suspected LS cases.

Hypothesis: A comprehensive analyses, both at the germline and somatic level, of genetic alterations in MMR and other CRC genes can be of help in elucidating the molecular basis of suspected LS.

Aim: To study the efficacy of the diagnostic algorithm of Lynch syndrome by means of comprehensive analysis of MMR genes and the implementation of a NGS panel for the analysis of germline and somatic mutations in colorectal cancer predisposition genes.

Summary of the obtained results: Only cases with MSH2/MSH6 deficient tumors were included. Twenty-seven germline pathogenic variants and 8 likely pathogenic variants were identified in *MSH2* gene. RNA splicing analysis identified aberrantly expressed transcripts in four of the seven evaluated *MSH2* variants. NGS panel testing in PBL DNA of Lynch-like syndrome cases revealed one previously unidentified germline *MSH2* mutation, two variants at the promoter region of MSH6 and predicted pathogenic variants in *MYH*, *SETD2*, *BUB1* and *FAN1*. In the six analyzed cases in which no germline alterations were found, the pattern of somatic alterations was as follows: double somatic hits in *MSH2* (n=1) and *MSH6* (n=1) were detected in 2 cases. In the remaining 4 cases compound heterozygous mutations in MMR genes *(MSH6, PMS2, MLH3*) and/or proof-reading polymerases (*POLD1* or *POLE*) were detected. Also, somatic mutations in other cancer genes (*APC, AXIN2, BMPR1A, PTEN* or *BUB1B*) coexisted with the above mentioned alterations. In LS suspected patients, pathogenicity assessment of MMR VUS and multigene panel testing is useful for the identification of double somatic hits and candidate germline mutations in CRC predisposing genes. This strategy could help to elucidate the molecular basis of LLS.

Contribution of the PhD candidate: Lymphocyte cultures, RNA extraction, retrotranscription and set up of long range PCR for *MSH2* splicing analysis. Analysis of germline mutations in *MSH2* promoter region. DNA isolation from formalin fixed paraffin-embeded (FFPE) tissues and sodium bisulfite treatment. Design and set up methylation studies of *MSH2* and *MSH6* promoter regions by MS-MCA. Design of the probes for next generation sequencing (NGS). Target enrichment and library preparation. Variant calling, filtering and annotation of NGS results. *In silico* prediction studies of missense variants found with NGS. Analysis and interpretation of results, as well as preparing figures and tables. Writing the first draft of the article and preparing the final version.

IN LYNCH SYNDROME SUSPECTED PATIENTS

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Short title: Molecular basis of MSH2-deficient tumors.

SUMMARY

Background and aim. Lynch syndrome (LS) is caused by germline mutations in mismatch repair (MMR) genes, r
MLH1 and *MSH2*. In a significant proportion of cases showing MMR-deficient tumors, no germline patho
mutations are identified in MMR genes thus hampering appropriate clinical management in these so-called Lyn
syndrome patients. Recently, mutations in *POLE* and *MUTYH* and double somatic events in MMR genes have
found in a significant proportion of these patients. The aim of this study was to evaluate the usefulness
diagnostic algorithm of Lynch syndrome of the implementation of a comprehensive analysis of MMR gene
germline and somatic mutations in other colorectal cancer predisposition genes in MSH2-deficient LS suspected ca
Patients and methods. Fifty-nine probands harboring MSH2-deficient tumors were included. *MSH2* and
mutational analysis was performed by Sanger sequencing of the coding region and MLPA, including probes at t
end of *EPCAM*. Pathogenicity assessment of *MSH2* variants was performed by means of *in vitro* RNA splicing an
and multifactorial likelihood calculations. Methylation at *MSH2* and *MSH6* promoter were evaluated by MS-M
customized next generation sequencing (NGS) panel for the analysis of CRC associated genes and potentially actio
targets in CRC was designed to support the analysis of PBL and matched FFPE DNA.
Results. Thirty-five individuals were carriers of pathogenic or probably pathogenic variants in *MSH2* and *EPCAM*,
were carriers of *MSH2* variants of unknown significance (VUS). RNA splicing studies identified aberrant transcrip
evaluated variants (c.211G>C, c.1276G>A and duplications of exon 11 and exons 11-16). Splicing and multifa
analyses allowed the reclassification as pathogenic mutations of 3 VUS and 6 probably pathogenic variants. NGS
testing in PBL DNA of Lynch-like syndrome cases revealed a germline *MSH2* mutation in one case, 2 *MSH6* pro
variants in another, and 5 cases harboring predicted pathogenic germline mutations in *BUB1*, *SETD2*, *FAN1* and *M*
In six analyzed cases in which no germline alterations were found, the pattern of somatic alterations was as fo
double somatic hits in MMR genes *MSH2* or *MSH6* were detected in 2 cases. In the remaining 4 cases c

with the above mentioned alterations.

Conclusions. The evaluation of germline and somatic mutational status of CRC-associated genes by means subexome panel and the pathogenicity assessment of identified variants is useful for the elucidation o molecular basis of up to 80% of MSH2-deficient suspected LS.

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Abbreviations
LS, Lynch syndrome
LLS, Lynch-like syndrome
CRC, colorectal cancer
MMR, mismatch repair
MSI, microsatellite instability
IHQ, immunohistochemistry

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Disclosures
The authors declare no conflict of interest.

INTRODUCTION

Lynch Syndrome (LS) is an inherited autosomal dominant cancer syndrome that confers an elevated r develop different types of cancer, mainly colorectal cancer (CRC) and endometrial tumors. It accounts for 2-4% newly diagnosed CRC and endometrial cancers[1–4]. It is caused by defective mismatch repair (MMR) activity c germline mutations in mismatch repair (MMR) genes (*MLH1, MSH2, MSH6* and *PMS2*). LS molecular diagnosis a the appropriate management of patients and their families[5].

Molecular diagnosis of LS is well established, based on the identification of microsatellite instability and/or loss of expression of MMR proteins by immunohistochemistry in tumors. Clinical suspicion of LS is trigger fulfillment of Amsterdam or Bethesda clinical criteria[6]. However, universal LS-screening of all newly diagnosed CR EC is also being implemented[7]. After identification of MMR deficiency (in the absence of *MLH1* promoter methy and/or *BRAF* p.V600E mutation), germline MMR testing is performed. Germline testing of MMR genes usually in analysis of point mutations in coding region as well as gross rearrangements. This strategy is being replac subexome gene panels' analysis using Next Generation Sequencing (NGS)[8–10].

in these so-called Lynch-like syndrome patients . These patients, as well as their first-degree relatives, ha intermediate risk of developing CRC[12]. However, appropriate clinical management and risk assessment in this gr patients has not been established[11].

Limitations in the molecular analysis techniques utilized could be responsible for the lack of detect germline MMR mutations, either due to false positives IHC/MSI results or false negatives in MMR mutational an due to complex or cryptic mutations[13–16] or lack of sensitivity (i.e. in mosaic cases)[17,18]. Moreover, MMR DNA varia unknown significance (VUS) are often identified, representing up to 30% of the identified DNA variants[19]. To fac their classification in terms of pathogenicity, a standardized classification scheme has been recently propos InSight, based on quantitative and qualitative algorithms[19]. Variants were classified according to the five class scheme as pathogenic (class 5), likely pathogenic (class 4), uncertain (class 3), likely non-pathogenic (class 2) and pathogenic (class 1). Therefore, further information on clinico-pathological, familial and functional data of a variant is highly valuable in order to finally establish the appropriate management of carrier individuals and families.

At somatic level, double hits have been reported in an important proportion of LLS tumors[17,20–22]. Mor since up to 60-70% of CRC showing loss of MLH1 protein expression harbor somatic *MLH1* hypermethylatio somatic methylation in other MMR gene promoters has been suggested as a cause of MMR-deficiecy. However, been poorly studied. To our knowledge only two series of MSH2 deficient tumors have been studied[25,26]. methylation was detected in tumor DNA of one case, not associated to germline *EPCAM* deletions[25].

In LLS patients overlapping phenotypes could mislead the screening of the underlying genetic cause. I regard, germline mutations in *MUTYH* (biallelic) and *POLE* have been reported in patients with MMR de tumors[22,27–30], strengthening the interest in the implementation of NGS gene panels in routine genetic diagnos date, several multiplex gene panels for the evaluation of hereditary colorectal cancer are commercially available the other hand, custom made panels allow more flexible designs and the inclusion of target regions of interest[32].

In the present work we aimed at elucidating the molecular basis underlying tumorigenesis in a cohort of suspected patients harboring MSH2 deficient tumors. We analyzed the presence of mutations and epimutati MMR genes and performed functional analysis of the identified MSH2 VUS. This was complemented by sequenc 26 CRC predisposing genes and actionable somatic targets with a high throughput technology designed to allow t of germline and FFPE samples.

Included patients

Mutational screening of *MSH2* gene was performed in a cohort of 59 probands with LS-associated tu showing loss of MSH2 protein expression by IHC. Twenty patients fulfilled Amsterdam criteria, 37 revised Bet criteria[33,34] and the remaining 2 were referred to the Genetic Counseling Unit for showing histological fe suggestive of MMR deficiency and loss of MSH2 expression[35]. Patients were assessed at Cancer Genetic Cour Units at the Catalan Institute of Oncology from 1998 to 2012. Clinical and pathological information of af individuals was recorded (Table 1). DNA samples from controls of a hospital based CRC case-control study were u analyze the frequency of the detected *MSH2* VUS[36]. Informed consent was obtained from all individuals enrolle internal Ethics Committees of participant hospitals approved this study. Of note, two patients initially classified were excluded from this cohort due to the detection of biallelic *MUTYH* mutations[29].

Isolation of genomic DNA

Peripheral blood lymphocyte (PBL) DNA was extracted using FlexiGene DNA kit (Qiagen, Hilden, Ger according to manufacturer's instructions. For each available specimen of formalin-fixed paraffin embedded tissue, 10-20 x 10-µm FFPE sections were cut from a single representative block per case, using macrodissection scalpel as needed to enrich for tumor cells. After deparaffinization with 480µl of Deparaffinization Solution (Q Hilden, Germany), DNA isolation was performed using either the DNAeasy Tissue Kit or QIAmp DNA FFPE Tiss (Qiagen, Hilden, Germany) according to manufacturer's instructions.

Mismatch repair genes mutational analysis

Mutational analysis of coding regions of MSH2 and MSH6 genes. Point mutation analysis of *MSH*2 (NM_000 NG_007110.1) and *MSH6* (NM_000179.2; NG_007111.1) genes was performed by PCR amplification of regions and exon-intron boundaries followed by Sanger sequencing (primers and conditions available upon rec Genomic rearrangements in MMR genes were analyzed by multiplex ligation dependent probe amplification SALSA-MLH1/MSH2 P003-B1 and MSH6 P072 kits (MRC-Holland), which include probes at the 3' end of EP Annotation of variants was done following the HGVS recommendations.

Direct sequencing of MSH2 and MSH6 promoter regions. A region encompassing 662 bases upstrea transcriptional start site (TSS) of *MSH2* gene and 915bp upstream the TSS of *MSH6* were amplified by PCR using D Megamix (Microzone Ltd., UK) and sequenced using the BigDye Terminator v.3.1 Sequencing Kit (Applied Biosys CA, USA) (Table S1; conditions available upon request). Sequences were analyzed on an ABI Prism 3100 G Analyzer (Applied Biosystems, CA, USA).

cultured lymphocytes with TRIzol® Reagent. One μg of RNA was retro-transcribed using iScript Select cDNA syn kit. The whole *MSH*2 transcript (2.8Kb) was amplified by LR-PCR (primers and conditions kindly provided by E. Ho Feder and M. Morak). Products were run in an electrophoresis gel and purified with Exonuclease 1 plus Shrimp Al Phosphatase (ExoSAP). Finally, 10 primers were used to analyze the whole coding region by Sanger sequencing.

Pathogenicity assessment of *MSH2* variants.

Variant frequency and cosegregation analysis. The identified *MSH2* variants were searched in the NHLBI E Sequencing Project (ESP) database (http://evs.gs.washington.edu/EVS) and screened by Sanger sequencing in a Sp population cohorts of 246 healthy controls[36]. *MSH2* variants were screened in DNA samples from family relativ Sanger sequencing.

In silico prediction of the functional impact. DNA sequences containing the identified *MSH2* variants were an using several bioinformatic tools addressed to evaluate its impact at the RNA and protein level, as prev reported[37,38].

Multifactorial likelihood analysis. Multifactorial likelihood analysis was based on estimated prior probabilit pathogenicity and likelihood ratios for segregation and tumor characteristics as described[39]. Variants were cla according to the 5 class IARC quantitative scheme[40], based on the calculated posterior probability.

mRNA splicing analysis and allele specific expression analysis. Human lymphocytes from variant carriers were cu as described above. Total RNA was extracted from cultured lymphocytes and cDNA was synthesized as descri Amplification of *MSH2* coding region containing the variants was performed using specific primers (Table S1; cond available upon request). Sequences of carrier transcripts were compared with transcripts from three c lymphocyte cultures. Allele specific expression (ASE) was analysed by SNuPE[37] (Table S1; conditions available request). ASE was calculated by dividing the proportion of variant/wildtype allele in cDNA by the proport variant/wildtype allele in gDNA. We used ≤0.5 as a threshold value for ASE definition. Experiments were perform quadruplicate.

Targeted Next Generation Sequencing.

Agilent SureDesign web-based application was used to design DNA capture probes of 509 target re including the coding exons plus 10 flanking bases of 26 genes associated to CRC, as well as their promoter re (comprising 650 bases upstream their TSS) (Table S2). Regions containing somatic hotspot mutations of 12 actio target genes and MSI CRC associated loci of 3 genes were also included (Table S2). Design was optimized for samples. Final design was composed of 11,012 amplicons covering 99.61% of the submitted target regions, in a sequenceable design size of 319,653kbp.

FFPE-derived DNA a PCR amplifying two *GAPDH* products (see Manual G9900-90050 from Agilent for more inform about primers and conditions) was performed and the products were visualized using High Sensitivity DNA chips Agilent Bioanalyzer. Capture of the target regions was performed using HaloPlex Target Enrichment kit 1-5 (Agilent Technologies, USA), according to the HaloPlex Target Enrichment System-Fast Protocol Version B. Briefl protocol consists of four steps: 1) digestion of genomic DNA using eight different restriction reactions; 2) hybridi of restricted fragments to probes whose ends are complementary to the target fragments, circularization of frag and incorporation of sequencing motifs including index sequences; 3) capture of target DNA using streptavidin and ligation of circularized fragments; 4) PCR amplification of captured target libraries. Quality control and d estimates of libraries were performed using High Sensitivity DNA chips in an Agilent Bioanalyzer. Library concentr were normalized to 0.44 nM. Pooled libraries were sequenced on aMiSeq flowcell with paired 250 base reads plus base index read, using version 3 cartridges.

Agilent SureCall application was used to trim, align and call variants. Variant filtering was performed bas Phred quality >30, alternative frequency ≥0.05, alternative read depth ≥38x in PBL samples and ≥10x in FFPE sar Germline rare variants and double somatic hits identified were further confirmed by Sanger sequencing independent DNA samples.

Methylation analysis of *MSH2* and *MSH6* genes

Methylation was evaluated by MS-MCA , consisting of a real-time PCR followed by temperature dissociat DNA previously treated with sodium bisulfite, using the EZ DNA Methylation-Gold Kit (Zymo Research, Orang USA). Each promoter region was preamplified using 2μl of external primers at 2μM, 1μl of bisulfite modified DN 5μl of Double MegaMix solution (Microzone Ltd., UK). Heminested PCRs of both promoter regions were carried ou LightCycler 480 II (Roche) using 1μl of a 1:10 dilution of preamplified fragments in 9μl of Light Cycler 480 SYBR G (Roche) containing 0.5μM of each internal primer. Sequences of primers are listed in Table S1. The amplified reg *MSH2* and *MSH6* promoters covered 13 and 18 CpGs, respectively. In vitro methylated DNA from CpG meth Jurkatt Genomic DNA (New England Biolabs) and a CRC sample from an *EPCAM* deletion carrier were us methylated controls in these experiments. Analytical sensitivity of the method to detect methylation was ass using serial dilutions of methylated Jurkatt DNA and lymphocyte DNA from a healthy patient (after bisulfite seque corroboration of unmehtylation). Analytical sensitivities of 10 and 25% were achieved in the analysis of MSH MSH6 promoters, respectively (Figure S1).

Clinical characteristics of patients with MSH2-deficient tumors

We identified 59 probands (22 males and 37 females) diagnosed with LS-associated tumors showing l MSH2 expression (Table 1). Accordingly, MSI was evident in all the informative tumors available (n=29). The media at diagnosis of the first tumor was 49.7 years (range 21-77). Mutational analysis of the *MSH2* gene and the 3'-e *EPCAM* identified a total of 27 patients harboring *bona fide* germline pathogenic (class 5) variants in *MSH2* and *E* (25 and 2, respectively), and 8 harboring likely pathogenic (class 4) *MSH2* variants (Table 1), according to the I classification rules (v 1.9). All these variants, identified in 59.3% of the patients analyzed (35/59), were conside responsible (or probably responsible) of Lynch syndrome. The remaining 24 patients were categorized as Lync syndrome (LLS): no *MSH2* variants were identified in 16 patients, one patient was carrier of a neutral (class 1) variant, and 5 were carriers of *MSH2* variants of unknown significance (VUS; class 3). Additional testing of *MSH6* detected only 1 neutral variant in the 17 LLS probands analyzed.

The age at first LS-associated-tumor diagnosis was of 45.8 (range from 21 to 59 years) in the identif patients, while it was of 50.7 years (range between 31 and 77) in LLS cases (Table S3). Concerning clinical c fulfillment, 52.4% of LS cases belonged to Amsterdam families, and 48.6% met Bethesda criteria. On the counte most of the LLS cases met Bethesda criteria (n=20; 83.3%), only 2 fulfilled Amsterdam criteria and the remaining 2 referred from the Pathology Department because of suspected MMR deficiency. Fifty-seven percent of LS case 25% of LLS patients (n=20 and n=6, respectively) presented multiple LS-associated tumors.

Pathogenicity assessment of *MSH2* variants

Thirteen probands were carriers of *MSH2* class 3 and 4 variants: 4 VUS (c.518T>G, c.2069A>G, ex duplication and exons 11-16 duplication) and 6 probably pathogenic variants (c.211G>C, c.989T>C, c.1276G>A, c 1G>A, c.2074G>C and c.[2635-3C>T;2635-5T>C]; Table 2 and Figure 2). None of them were described in the NHL Database nor identified in Spanish cohorts of control individuals (Table 2). cDNA splicing evaluation was perform the 7 variants (the 4 VUS, c.211G>C, c.989T>C and c.1276G>A) identified in carriers with available biological sa and predicted pathogenic by in silico algorithms (Table S4). The reverse transcriptase PCR analyses identified aber expressed transcripts in four of the seven *MSH2* variants (Table S4 and Figure S3). *MSH2* c.211G>C variant resulte partial deletion of exon 1 (r.195_211del), which is predicted to generate a truncated protein (p.Tyr66Serfs*10). c.1276G>A led to a partial deletion of exon 7 (r.1230_1277del48), which is predicted to generate an in-frame de of 16 amino acids (p.Ile411_Gly426del16) in the lever domain of MSH2. In these three cases, sequencing of the R products showed that variant alleles were absent from the whole-length wildtype transcript. The duplication of ex caused a tandem duplication of this exon (r.1662_1759dup), predicted to generate a truncated p (p.Gly587Alafs*3). According to the Insight classification rules, these variants were classified as pathogenic bas the generation of aberrant transcripts leading to premature stop codons or in frame-deletions disrupting func domains (Thompson 2014).

Table 1. Clinical and pathological information of MSH2 deficient cases

Patient ID	Gender	Clinical criteria	Technique utilized for analysis	MSH2/ EPCAM	MSH6	Identified variants (cDNA change)	Genetic variant (predicted protein change)	Initial classification (Insight v 1.9)	Cancer 1	Age at diagnosis (years)	Cancer 2	Age at diagnosis (years)	Can
229	M	BC	MLPA; EXSEQ	VI	NP	MSH2 c.1076+1G>A	p.?	Class 5	SC	49	SA	52	**CI**
230	M	AC	MLPA; EXSEQ	VI	NP	MSH2 c.689_691delinsTT	p.(Ala230Valfs*16)	Not reported (Class 5 according to the rules)	**BlC***	41	SC	40	S
231	F	BC	MLPA; EXSEQ	VI	NP	MSH2 c.897T>A	p.(Tyr299*)	Not reported (Class 5 according to the rules)	SA	50	BC	49	**E**
232	M	BC	MLPA; EXSEQ	VI	NP	MSH2 c.[2635-3C>T; 2635-5T>C]	p.?	Class 4	**CRC***	56			
233	M	BC	MLPA; EXSEQ	VI	NP	MSH2 c.528_529delTG	p.(Cys176*)	Class 5	**CRC***	40	PrC	51	
234	M	BC	MLPA; EXSEQ	VI	NP	MSH2 c.211G>C	p.(Gly71Arg)	Not reported (Class 4 according to the rules)	**CRC***	45			
235	M	AC	MLPA; EXSEQ	VI	NP	MSH2 c.[2635-3C>T; 2635-5T>C]	p.?	Class 4	**CRC***	21	PrC	50	
236	F	AC	MLPA; EXSEQ	VI	NP	MSH2 c.735_736insTGTT	p.(Lys246Cysfs*2)	Not reported (Class 5 according to the rules)	**OC***	43	CRC	44	
237	M	AC	MLPA; EXSEQ	VI	NP	MSH2 c.1387-?_1661+?del (del E9-10)	p.?	Class 5	CRC	32	CRC	34	**CI**
238	F	AC	MLPA; EXSEQ	VI	NP	MSH2 c.1216C>T	p.(Arg406*)	Class 5	CRC	33	**UC***	38	U
239	M	BC	MLPA; EXSEQ	VI	NP	MSH2 c.2074G>C	p.(Gly692Arg)	Class 4	**CRC***	36			
240	M	BC	MLPA; EXSEQ	VI	NP	MSH2 c.[2635-3C>T; 2635-5T>C]	p.?	Class 4	CRC	30	**CRC***	53	C
241	F	AC	MLPA; EXSEQ	VI	NP	MSH2 c.1705_1706delGA	p.(Glu569Ilefs*2)	Class 5	**CRC***	27			
242	F	AC	MLPA; EXSEQ	VI	NP	MSH2 c.(?_-68)_366+?del (del E1-2)	p.?	Class 5	CRC	28	CRC	33	**E**
243	F	AC	MLPA; EXSEQ	VI	NP	MSH2 c.2593dup	p.(Ile865Asnfs*17)	Not reported (Class 5 according to the rules)	OC	33	**CRC***	35	
228	F	AC	MLPA; EXSEQ	VI	NM	MSH2 c.989T>C	p.(Leu330Pro)	Class 4	**OC***	55			
245	M	AC	MLPA; EXSEQ	VI	NP	MSH2 c.942+3A>T	p.(Val265_Gln314del)	Class 5	CRC	39	**CRC***	45	
246	M	AC	MLPA; EXSEQ	VI	NM	EPCAM c.904-?_(*415_?)del (del E9)	p.?	Class 5	**CRC***	43	CRC	43	C
247	F	AC	MLPA; EXSEQ	VI	NP	MSH2 c.1345_1348delAAGT	p.(Lys449Phefs*4)	Class 5	CRC	51	**EC***	52	R
248	F	BC	MLPA; EXSEQ	VI	NM	MSH2 c.1511-1G>A	p.?	Not reported (Class 4 according to the rules)	**CRC***	56	BC	60	E
249	F	AC	MLPA; EXSEQ	VI	NP	MSH2 c.536dup	p.(Asp180*)	Not reported (Class 5 according to the rules)	CRC	43	**CRC***	44	S
250	F	AC	MLPA; EXSEQ	VI	NP	MSH2 c.602dup	p.(Leu201Phefs*31)	Not reported (Class 5 according to the rules)	**EC***	43	SA	36	S
251	F	AC	MLPA; EXSEQ	VI	NM	EPCAM c.904-?_(*415_?)del (del E9)	p.?	Class 5	**CRC***	28			
252	F	BC	MLPA; EXSEQ	VI	NP	MSH2 c.689_691delinsTT	p.(Ala230Valfs*16)	Not reported (Class 5 according to the rules)	**OC***	42			
253	M	BC	MLPA; EXSEQ	VI	NP	MSH2 c.536dup	p.(Asp180*)	Not reported (Class 5 according to the rules)	**CRC***	31			
254	F	BC	MLPA; EXSEQ	VI	NP	MSH2 c.1777C>T	p.(Gln593*)	Class 5	**CRC***	36			
255	F	BC	MLPA; EXSEQ	VI	NP	MSH2 c.970C>T	p.(Gln324*)	Class 5	**CRC***	37			
256	M	AC	MLPA; EXSEQ	VI	NP	MSH2 c.1077-?_(*272_?)del (del E7-12)	p.?	Class 5	**CRC***	59	CRC	59	
257	M	BC	MLPA; EXSEQ	VI	NP	MSH2 c.1035G>A	p.(Trp345*)	Class 5	**CRC***	41			
258	F	BC	MLPA; EXSEQ	VI	NM	MSH2 c.1276G>A	p.(Gly426Arg)	Not reported (Class 4 according to the rules)	**OC***	42			
259	F	AC	MLPA; EXSEQ	VI	NP	MSH2 c.1387-?_1661+?del (del E9-10)	p.(Val463Glnfs*7)	Class 5	CRC	37	**CRC***	48	C
260	M	BC	MLPA; EXSEQ	VI	NP	MSH2 c.942+3A>T	p.(Val265_Gln314del)	Class 5	**CRC***	42	CRC	42	
261	F	AC	MLPA; EXSEQ	VI	NP	MSH2 c.1277-?_1386+?del (del E8)	p.(Lys427Glyfs*4)	Class 5	EC	50	**CRC***	54	
262	M	BC	MLPA; EXSEQ	VI	NP	MSH2 c.942+3A>T	p.(Val265_Gln314del)	Class 5	**CRC***	34	SA	44	C
263	F	BC	MLPA; EXSEQ	VI	NP	MSH2 c.1165C>T	p.(Arg389*)	Class 5	EC	56	**CRC***	64	
122	F	BC	MLPA; EXSEQ	VI	NP	MSH2 c.518T>G	p.(Leu173Arg)	Class 3	**CRC***	41			
117	F	BC	MLPA; EXSEQ	VI	NP	MSH2 c.518T>G	p.(Leu173Arg)	Class 3	**CRC***	44			
264	F	BC	MLPA; EXSEQ	VI	NP	MSH2 c.1662-?_1759+?dup (duplication E11)	p.?	Class 3	**CRC***	29	**CRC***	51	
118	F	BC	MLPA; EXSEQ	VI	NM	MSH2 c.2069A>G	p.(Gln690Arg)	Not reported (Class 3)	CRC	31	**CRC***	35	**CI**
120	M	AC	MLPA; EXSEQ	VI	NP	MSH2 c.1662-?_(*272_?)dup (duplication E11-16)	p.?	Class 3	**CRC***	54	CRC	54	C
119	F	BC	MLPA; EXSEQ	NM	VI	MSH6 c.431G>T	p.(Ser144Ile)	Class 1	**EC***	45			
121	F	AP	MLPA; EXSEQ	VI	NM	MSH2 c.965G>A	p.(Gly322Asp)	Class 1	**CRC***	77			
101	F	BC	MLPA; EXSEQ	NM	NM				**CRC***	57			
102	F	BC	MLPA; EXSEQ	NM	NM				**CRC***	55			
103	F	AP	MLPA; EXSEQ	NM	NP				**CRC***	73			
104	F	BC	MLPA; EXSEQ	NM	NM				**CRC***	51			
105	F	BC	MLPA; EXSEQ	NM	NM				**CRC***	49			
123	M	BC	MLPA; EXSEQ	NM	NP				**CRC***	59			
106	F	BC	MLPA; EXSEQ	NM	NM				**CRC***	43			
107	F	BC	MLPA; EXSEQ	NM	NM				**CRC***	39			
108	F	BC	MLPA; EXSEQ	NM	NP				**CRC***	32	CRC	48	
109	M	BC	MLPA; EXSEQ	NM	NM				**CRC***	27			
110	M	BC	MLPA; EXSEQ	NM	NM				**CRC***	43			
111	F	BC	MLPA; EXSEQ	NM	NM				CRC	51	CRC	51	**E**
112	M	BC	MLPA; EXSEQ	NM	NM				**CRC***	49			
113	F	BC	MLPA; EXSEQ	NM	NM				**CRC***	49	BC	55	
114	M	BC	MLPA; EXSEQ	NM	NM				**CRC***	58	CRC	58	
115	F	AC	MLPA; EXSEQ	NM	NM				BC	62	BC	69	**E**
116	F	BC	MLPA; EXSEQ	NM	NM				**CRC***	48			

Abbreviatures: F: female, M: male, AC: Amsterdam criteria, BC: Bethesda criteria, MLPA: Multiplex Ligation-dependent Probe Amplification, EXSEQ: Exonic sequencing, VI: Variant identified, NM: not mutated, NP: not performed, C
BlC: bladder cancer, PrC: prostate cancer, PC: pancreas cancer, CNSC: central nervous system cancer, L: lymphoma.
Bold letter and (*) Indicate tumors in which MSI was studied

Table 2. *MSH2* class 3 and 4 variants identified in our series. Results of cDNA splicing and multifactorial likelihoo

MSH2 variant	RNA^; Protein	Frequency in controls (our cohort* /ESP database)	Initial classification (Insight v.1.9; September 2015)	Prior probability of pathogenicity	Prior used	Case ID	Reference	Ascertainment	Cancer	MSI/ IHC status	M
c.211G>C	**r.195_211del; p.Tyr66Serfs*10**	(0/188) / not reported	Probably pathogenic according to the rules (class 4, not reported)	NA (splicing aberation)	NA	234	This study	clinic	CRC	MSH2/MSH6 loss	
							This study	clinic	EC	MSH2/MSH6 loss	
c.518T>G	**r.518T>G; p.Leu173Arg**	(0/190) / not reported	Unknown significance (class 3)	0.953499658	0.9	122	This study	clinic	CRC	MSH2/MSH6 loss	
						117	This study	clinic	CRC	MSH2/MSH6 loss	
						A1	This study	clinic			
						A2	This study	clinic			
c.989T>C	**r.989T>C; p.Leu330Pro**	(0/236) / not reported	Probably pathogenic (class 4)	0.961065305	0.9	CTE-L0015	Liliana Varesco; LOVD	population		MSI-H	
						228	This study				
c.1276G>A	**r.1230_1277del48; p.Ile411_Gly426del16**	(0/246) / not reported	Probably pathogenic according to the rules (class 4, not reported)	NA (splicing aberation)	NA	258	This study	clinic	EC	MSH2/MSH6 loss	
c.1511-1G>A	**r.spl?; p.?**	NP/not reported	Probably pathogenic according to the rules (class 4, not reported)	NA	0.96	248	This study	clinic	CRC	MSI-H	
c.1662-?_1759+?dup (exon 11 duplication)	**r.1662_1759dup; p.Gly587Alafs*3**	NP / NA	Unknown significance (class 3)	NA (splicing aberation)	NA	264	This study	clinic	CRC1	MSH2/MSH6 loss	
							This study	clinic	CRC2	MSH2/MSH6 loss	
c.1662-?_(*272_?)dup (exon 11-16 duplication)	**r.1662_*23dup; p.?**	NP / NA	Unknown significance (class 3)	NA (splicing aberation)	NA	120	This study	clinic	CRC	MSI-H	
c.2069A>G	**r.2069A>G; p.Gln690Arg**	(0/190) / not reported	Unknown significance (class 3, not reported)	0.954182992	0.9	118	This study	clinic	CRC1	MSI-H	
							This study	clinic	CRC2	MSH2/MSH6 loss	
c.2074G>C	**r.?; p.(Gly692Arg)**	NP/not reported	Probably pathogenic (class 4)	0.961843012	0.9	B	Isidro 2000				
						239	This study	clinic	CRC	MSI-H	
c.[2635-3C>T;2635-5T>C]	**r.spl?; p.?**	NP/not reported	Probably pathogenic (class 4)	NA	0.26	232	This study	clinic	CRC	MSH2/MSH6 loss	
							This study	clinic	CRC	MSI-H	
						235	This study	clinic	CRC	MSI-H	
							This study	clinic	EC	MSI-H&MSH2/MSH6 loss	
						240	This study	clinic	CRC	MSI-H	

Abreviations: LR, likelihood ratio; NA, not available; NP, not performed; CRC, colorectal cancer; EC, endometrial cancer. (^)Result from this study.

stop codon the impact on the protein was uncertain. The remaining three *MSH2* variants included in this (c.518T>G, c.989T>C and c.2069A>G) had no apparent effect on mRNA splicing (data not shown). Evaluation variant allelic expression in cDNA samples from lymphocytes cultured in the presence or absence of puromycin d show allelic imbalances (Table S4).

Furthermore, clinico-pathological data from families carrying class 3 and 4 variants, for which probabilities were available (variants not affecting the RNA splicing), were used in multifactorial analyses (Table variant *MSH2* c.518T>G was further identified in two additional families from other centers (Figure S2C), they wer included in these calculations. Posterior probability of pathogenicity resulted >0.999 for the 6 analyzed variants.

In-depth germline analysis of LLS cases

In the mutational analysis of *MSH2* promoter region only two known polymorphisms (rs1863332 (c.-43 and rs2303425 (c.-118T>C)) were detected. Six out of 24 patients were carriers of rs1863332 (c.-433T>G) heterozygosis and 3 in homozygosis; ten cases were carriers of rs2303425 (c.-118T>C), 8 in heterozygosis an homozygosis (Table 3).

Table 3. Results from molecular characterization of LLS cases

Patient ID	VUS Presence	GERMLINE RESULTS			SOMATIC RESULTS	
		PROMOTER SEQUENCING		RNA ANALYSIS	METHYLATION	
		MSH2				
		rs1863332 c.-433T>G	rs2303425 c.-118T>C	MSH2	MSH2	MSH6
108		TT	TT	NP	NA	UM
109		TG	TT	2 aberrant transcripts associated to c.211G>C	NP	NP
110		TT	CC	NA	UM	UM
106		TT	TT	NP	UM	UM
112		GG	TT	WT	NP	NP
113		TT	TC	WT	NP	NP
114		GG	TT	NA	NP	NP
101		TG	TT	NA	NP	NP
102		TG	TT	NP	NP	NP
107		TT	TC	WT	NA	UM
116		TT	TT	NA	UM	UM
104		TT	TC	NA	NP	NP
105		GG	TT	WT	NP	NP
103		TT	TT	WT	NP	NP
111		TT	TC	WT	UM	UM
115		TT	TT	NA	UM	UM
123		TT	CC	WT	NP	NP
118	Not reported (Class 3)	TT	TC	WT	UM	UM
119	Class 1	TT	TT	WT	UM	UM
122	Class 3	TT	TC	NA	UM	UM
117	Class 3	TT	TC	NA	NP	NP
121	Class 1	TT	TC	NP	NA	UM
264	Class 3	TT	TT	NP	NP	NP
120	Class 3	TT	TT	NP	UM	UM

Abbreviations: WT, wildtype; NA, No amplification; NP, Not performed, UM, unmethylated.

Sequencing of the whole *MSH2* transcript was accomplished in 13 samples. Splicing alterations were de in case 109. In absence of puromycin, a deletion of almost all the first exon was identified (r.-16_211del; p.?) (1A). Moreover, in presence of puromycin, an inframe deletion of 16 bases (r.195_211del; p.Tyr66Serfs*10) wa present. Further NGS analysis (see below) revealed a mutation in the last nucleotide of the first *MSH2* exon (c.21 previously missed by Sanger due to primer design.

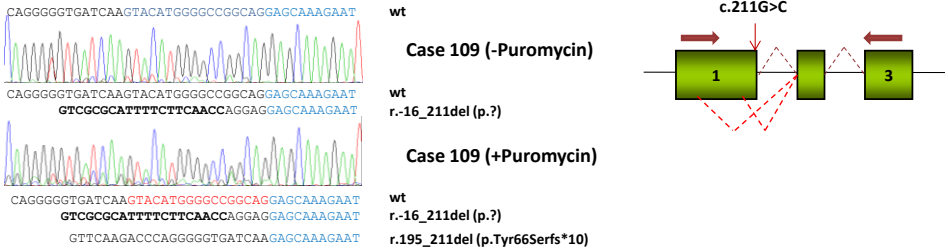

Figure 1. *MSH2* c.211G>C
A) RNA analysis from cul
lymphocytes in presence
absence of puromycin.
B) Schematic represent
of RNA alteration du
c.211G>C

Our NGS custom panel was used to analyze 17 PBL samples from LLS patients and 3 from the newly ider LS patients (Table 4). In addition to the 3 *MSH2* mutations identified in LS patients, the novel variant c.211G> detected that accounts for the splicing defect previously observed in the same case (Figure 1). Interestingly, on (ID: 102) harbored 2 variants at the promoter region of *MSH6* gene (c. -25C>T and c.-204C>G). The *MSH6* varia 25C>T is predicted to produce a premature out-of-frame start codon. Using PROMO c.-25C>T and c.-204C> predicted to affect FOXP3 and NF1/CTF binding, and binding of TFII-I, STAT4, NFkappaB1m c-Ets-1, RelA and respectively. As the patient was also carrier of heterozygous exonic SNPs in *MSH6* gene, ASE analysis cou performed upon RNA if it were available.

We also found missense variants predicted as pathogenic (by at least 3 *in silico* tools) in genes re associated to CRC: one in *BUB1,* three in the H3K36 trimethyltransferase *SETD2* and 1 in *FAN1* (previously submit Vargas et al.). Furthermore, we identified two heterozygous carriers of *MUTYH* recurrent variants c.1227_1228du c.1187G>A.

Molecular analysis of LLS tumors

No somatic methylation was evidenced in *MSH2* gene promoter (0/9 tumors) nor in *MSH6* (0/12 tu (Figure S4 and Table 3). Since previous studies have reported somatic methylation at the *MSH2* promoter in LS mutation carriers[41], 8 additional tumor samples from LS *MSH2* mutation carriers from our LS series were st resulting all unmethylated (data not shown).

Our NGS custom panel was used to analyze a total of seven tumors, 5 from 4 LLS individuals in whi germline predicted pathogenic alterations were found and 1 from a LS patient (Table 5). In LLS tumors, double so hits in *MSH2* (c.1600delC and c.1741delA) or *MSH6* (c.741delA and c.2765G>A) were detected in cases 114 and 1 two tumors from case 108 and one from case 121, double heterozygote mutations in MMR genes *(MLH1, MSH3, MLH3*) and/or proof-reading polymerases (*POLD1* and *POLE*) were detected. Also, somatic mutations in other c genes (*APC, AXIN2, BMPR1A, PTEN* or *BUB1B*) coexisted with the above mentioned alterations. Interestingly, th colorectal tumors from case 108 showed completely different profiles: the unstable tumor (cancer 1) harbored r deletions at homopolymeric sequences, whereas the stable tumor (cancer 2) harbored substitutions. No so

mutations in targeted exons from CRC actionable genes were identified in 5 of the 7 analyzed tumors. Three of harbored hotspot mutations in *KRAS*. Somatic hotspot mutations in *APC, PIK3CA, FBXW7, CTNNB1*, *TP53* and *PTEN* also detected.

Table 4. A) Germline variants found in LLS patients with Haloplex and results from in silico predictions

Patient ID	Variant calling			Position		Coverage						
	Gene	Transcript/cDNA change	Predicted protein change	chr	start	Allelic frequency	Read depth	SIFT (score)	Mutation Taster (p-value)	Polyphen2 / HumDiv (score)	Polyphen2 / HumVar (score)	Spl
105	**MUTYH**	**NM_001128425.1:c.1227_1228dup**	**p.Glu410GlyfsX43**	1	45797186	0.496	3690	-	-	-	-	
	FAN1	NM_014967.4:c.1856T>A	p.Met619Lys	15	31210411	0.558	5282	**D (0)**	**D (1)**	**PsD (0.937)**	**B (0.409)**	**Ga acce splici**
	SETD2	**NM_014159.6:c.1204C>T**	**p.Arg402Trp**	3	47164922	0.509	6441	**D (0)**	**D (0.99)**	**PrD (0.999)**	**PrD (0.923)**	Incon
104	FAN1	NM_014967.4:c.1129C>T	p.Arg377Trp	15	31197995	0.518	4623	D (0)	D (0.993)	B (0.398)	B (0.037)	No c
	APC	NM_000038.5:c.1959G>A	p.=	5	112173250	0.493	2504			-	-	Los acc splici
115	MLH3	NM_001040108.1:c.1870G>C	p.Glu624Gln	14	75514489	0.376	1024	B (0.05)	B (0.892)	PrD (0.990)	PsD (0.637)	Incon
	BUB1	**NM_004336.4:c.3005C>G**	**p.Thr1002Ser**	2	111397376	0.378	2652	B (0.63	B (0.639)	B (0.005)	B (0.018)	**Loss of splici**
111	**MUTYH**	**NM_001128425.1:c.1187G>A**	**p.Gly396Asp**	1	45797228	0.541	2944	**D (0)**	**D (1)**	**PrD (1.000)**	**PrD (0.999)**	
	BUB3	NM_004725.3:c.*1124G>A	p.?	10	124924475	0.456	580					
	MLH3	NM_001040108.1:c.*2058G>T	p.?	14	75481727	0.413	3036					
107	**SETD2**	**NM_014159.6:c.2798G>T**	**p.Gly933Val**	3	47163328	0.467	3621	D (0.01)	B (1)	B (0.000)	B (0.000)	**Loss o splici**
	ENG	NM_000118.3:c.1712G>A	p.Arg571His	9	130579457	0.483	10965	D (0.02)	B (1)	B (0.225)	B (0.028)	Incon
	EPCAM	NM_002354.2:c.-280G>C	p.?	2	47596365	0.408	3278					
	MLH3	NM_001040108.1:c.*2485G>C	p.?	14	75481300	0.402	1155					
110	**SETD2**	**NM_014159.6:c.2508T>G**	**p.Cys836Trp**	3	47163618	0.469	2135	**D (0)**	**D (1)**	**PsD (0.833)**	**B (0.176)**	No c
109	**MSH2**	**NM_000251.2:c.211G>C**	**p.Gly71Arg**	2	47630541	0.432	520	D (0.03)	D (1)	B (0.107)	B (0.076)	Loss o splici
	PMS1	NM_000534.4:c.2186A>G	p.Asn690Ser	2	190728798	0.482	2250	B (0.62)	B (1)	B (0.000)	B (0.000)	Incon
	TP53	NM_000546.5:c.*1175A>C	p.?	17	7571752	0.427	4674					
	APC	NM_000038.4:c.*1684A>G	p.?	5	112181507	0.321	594					
	ENG	NM_000118.3:c.*704delAGTT	p.?	9	130577491	0.995	6680					
112	MLH3	NM_001040108.1:c.2425A>G	p.Met809Val	14	75513934	0.508	1955	B (0.3)	B (1)	B (0.000)	B (0.000)	No c
	CDH1	NM_004360.3:c.2292C>T	p.=	16	68862204	0.408	1184			-	-	
	BUB3	NM_004725.3:c.*371A>G	p.?	10	124923722	0.358	1641					
	KLLN	NM_001126049.1:c.-1351G>A	p.?	10	89623595	0.489	1225					
	ENG	NM_000118.3:c.-186G>A	p.?	9	130616820	0.515	1932					
	ENG	NM_000118.3:c.-289A>T	p.?	9	130616923	0.524	2234					
119	AXIN2	NM_004655.3:c.1780G>A	p.Ala594Thr	17	63533114	0.492	3404	B (0.15)	B (1)	B (0.003)	B (0.003)	Incon
	FAN1	NM_014967.4:c.174G>A	p.=	15	31197040	0.489	3669	-	-	-	-	No c
	AXIN2	NM_004655.3:c.*884delT	p.?	17	63525208	0.485	3044					
	AXIN2	NM_004655.3:c.*476_*487delTGAGCTAGGAGT	p.?	17	63525606	0.463	3684					
	BMPR1A	NM_004329.2:c.*85G>A	p.?	10	88683561	0.538	817					
113	POLD1	NM_001256849.1:c.136G>A	p.Ala46Thr	19	50902244	0.467	4757	B (0.22)	D (0.988)	B (0.295)	B (0.037)	Incon
	FAN1	NM_014967.4:c.603C>T	p.=	15	31197469	0.544	1515			-	-	No c
114	FAN1	NM_014967.4:c.434G>T	p.Arg145His	15	31197300	0.484	2112	D (0.03)	B (1)	B (0.025)	B (0.007)	No c
	SMAD4	NM_005359.5:c.*2218G>T	p.?	18	48607055	0.582	212					
	ORMDL1	NM_001128150.1:c.-237C>G	p.?	2	190649224	0.515	2260					
103	CHEK2	NM_007194.3 c.1510G>C	p.Glu504Gln	22	29085155	0.304	2101	B (0.53)	B (1)	B (0.016)	B (0.005)	Incon
	EPCAM	NM_002354.2:c.831A>G	p.Ile277Met	2	47607081	0.192	2069	D (0.04)	B (0.956)	PsD (0.610)	B (0.125)	Incon
	AXIN2	NM_004655.3:c.623C>T	p.Ala208Val	17	63554116	0.166	482	B (0.06)	D (1)	B (0.228)	B (0.064)	No c
	ENG	NM_000118.3:c.1844C>T	p.Ser615Leu	9	130578230	0.287	3034	D (0)	D (0.745)	B (0.111)	B (0.011)	No c
	FBXW7	NM_033632.2:c.1200C>T	p.=	4	153250860	0.136	1400					
	POLD1	NM_001256849.1:c.-790T>C	p.?	19	50886861	0.198	4362					
	STK11	NM_000455.4:c.-325A>C	p.?	19	1206588	0.282	442					
102	MSH6	**NM_000179.2:c.-25C>T**	p.?	2	48010348	0.552	6303	-	-	-	-	Incon
	MSH6	**NM_000179.2:c.-204C>G**	p.?	2	48010169	0.459	2705	-	-	-	-	Incon
	SMAD4	NM_005359.5:c.*6293G>C	p.?	18	48611130	0.419	8047					
108	CDH1	NM_004360.3:c.2520C>T	p.=	16	68867273	0.468	5374	-	-	-	-	No c
	EPCAM	NM_002354.2:c.-485T>G	p.?	2	47596160	0.412	787					
	BUB3	NM_001007793.2:c.*173T>A	p.?	10	124924745	0.206	3229					
	ENG	NM_000118.3:c.*704delAGTT	p.?	9	130577492	0.491	4350					
116	TP53	NM_000546: c.-594insA	p.?	17	7591514	0.505	1692					
	MSH3	NM_002439.3:c.-457G>C	p.?	5	79950090	0.467	2088					
	TP53	NM_000546.5:c.*409C>A	p.?	17	7572518	0.51	937					
123	PMS2	NM_00535.5:c.-493insG	p.?	7	6049143	0.453	203					
101	MSH6	NM_000179.2:c.4002-10delT	p.?	2	48033891	0.693	913	-	-	-	-	No c

Table 4. B) Germline variants found in LS patients with Haloplex and results from in silico predictions

Patient ID	Variant calling			Position		Coverage						
	Gene	Transcript/cDNA change	Predicted protein change	chr	start	Allelic frequency	Read depth	SIFT (score)	Mutation Taster (p-value)	Polyphen2 / HumDiv (score)	Polyphen2 / HumVar (score)	Spl
117	**MSH2**	**NM_000251.2:c.518T>G**	**p.Leu173Arg**	**2**	47637384	0.38	24	**D (0)**	**D (1)**	**PrD (0.999)**	**PrD (0.992)**	**Incon**
	FAN1	NM_014967.4:c.1851C>T	p.=	15	31210406	0.546	2662	-	-	-	-	No c
	POLE	NM_006231.3:c.6072C>T	p.=	12	133209314	0.526	1415					
228	**MSH2**	**NM_000251.2:c.989T>C**	**p.Leu330Pro**	2	47643481	0.53	2033	**D (0)**	**D (1)**	**PrD (1.000)**	**PrD (1.000)**	
	STK11	NM_000455.4:c.945G>A	p.=	19	1223008	0.469	4487			-	-	Incon
	POLD1	NM_001256849.1:c.1138-8A>G	p.?	19	50906742	0.515	4834			-	-	Incon
118	**MSH2**	**NM_000251.2:c.2069A>G**	**p.Gln690Arg**	2	47703569	0.427	1931	**D (0)**	**D (1)**	**PrD (0.999)**	**PrD (0.992)**	Incon
	MLH1	NM_000249.3:c.*32_*34delCTT	p.?	3	37092170	0.501	1701					

Abbreviatures: B, benign; D, damaging; PrD, probably damaging; PsD, possibly damaging; N. D., not determined.

tested									(p-value)	(score)	(score)	
108_C2	**BUB1B**	NM_001211.5:c.1738G>T	p.Glu580*	15	40498388	0.0556	107	B (1)	-	-	-	Incon
	MLH1	NM_001167618.1:c.1253G>A	p.Arg418Gln	3	37090087	0.0976	204	B (0.07)	D (1)	PrD (1.000)	PrD (0.986)	Incon
	MSH6	NM_000179.2:c.2625G>T	p.Met875Ile	2	48027747	0.0731	423	B (0.17)	D (1)	B (0.001)	B (0.004)	No ch
	BMPR1A	NM_004329.2:c.878C>T	p.Ala293Val	10	88678938	0.272	440	D (0)	D (1)	PrD (1.000)	PrD (1.000)	No ch
	POLE	NM_006231.2:c.2284C>T	p.Arg762Trp	12	133244124	0.0511	704	D (0)	D (1)	PrD (1.000)	PrD (1.000)	No ch
	TP53	NM_000546.5:c.993+284C>T	p.?	17	7576569	0.131	106	-	-	-	-	Incon
108_C1	**SETD1B**	NM_015048.1:c.22del	p.His8Thrfs*27	12	122242656	0.309	6428	-	-	-	-	No ch
	MSH3	NM_002439.4:c.1114delAA	p.Lys383Argfs*32	5	79970914	0.158	796	-	-	-	-	Incon
	PMS2	NM_000535.5:c.1501G>A	p.Val501Met	7	6026895	0.114	6174	B (0.12)	B (1)	B (0.003)	B (0.002)	No ch
	MLH1	NM_001167618.1:c.697C>T	p.Arg233Trp	3	37070285	0.0758	131	D (0.02)	D (1)	PrD (0.990)	PsD (0.513)	No ch
	STK11	NM_000455.4:c.*787G>A	p.?	19	1228359	0.11	4842					No ch
	MSH2	NM_000251.2:c.-440delT	p.?	2	47629890	0.127	2152					Incon
	AXIN2	NM_004655.3:c.*631delT	p.?	17	63525462	0.277	2796					
	AXIN2	NM_004655.3:c.-330delA	p.?	17	63558069	0.167	3739					
	APC	NM_000038.5:c.*1884delT	p.?	5	112181707	0.087	137					
	STK11	NM_000455.4:c.-117del	p.?	19	1206796	0.229	667					Incon
114_C1	**MSH6**	NM_000179.2:c.741delA	p.K247fs*32	2	48025856	0.104	881	-	-	-	-	No ch
	AXIN2	NM_004655.3:c.1994delG	p.G665fs*24	17	63532584	0.121	1200	-	-	-	-	No ch
	MLH1	NM_001167617.1:c.713G>A	p.Gly238Asp	3	37061923	0.103	496	D (0.01)	D (1)	PsD (0.884)	PsD (0.596)	Incon
	MSH6	NM_000179.2:c.2765G>A	p.Arg922Gln	2	48027887	0.0724	607	D (0.04)	D (1)	PsD (0.680)	B (0.190)	No ch
	AXIN2	NM_004655.3:c.*631del	p.?	17	63525462	0.21	1109					
	AXIN2	NM_004655.3:c.957-3558_957-3559del	p.?	17	63558069	0.129	1673					
	SMAD4	NM_005359.3:c.*5757del	p.?	18	48610584	0.0693	722					
121_C1	**SETD1B**	NM_015048.1:c.22del	p.H8fs*27	12	122242655	0.466	10593	-	-	-	-	No ch
	PMS2	NM_000535.5:c.325del	p.Glu109Lysfs*3	7	6043348	0.205	420	-	-	-	-	No ch
	PTEN	NM_000314.4:c.968del	p.Asn323Metfs*21	10	89720811	0.0581	172	-	-	-	-	No ch
	SETD2	NM_014159.6:c.3165T>A	p.Asp1055Glu	3	47162961	0.185	3519	D (0)	D (0.992)	B (0.041)	B (0.044)	No ch
	MSH6	NM_000179.2:c.1082G>A	p.Arg361His	2	48026204	0.207	8083	B (0.21)	B (1)	PsD (0.837)	B (0.243)	No ch
	POLD1	NM_001256849.1:c.1330C>T	p.Arg444Trp	19	50909526	0.196	6980	D (0)	D (1)	PrD (1.000)	PrD (0.999)	Incon
	MLH3	NM_001040108.1: c.1755delA	p.E586fs*24	14	75514602	0.27	5221	-	-	-	-	No ch
	BUB3	NM_001007793.2:c.973T>C	p.Ser325Pro	10	124924564	0.0583	634	B (0.07)	D (0.999)	PsD (0.782)	PsD (0.838)	Loss of a splic
	STK11	NM_000455.4:c.-325A>C	p.?	19	1206588	0.214	3743					Incon
	AXIN2	NM_004655.3:c.*633del	p.?	17	63525459	0.241	11042					
	AXIN2	NM_004655.3:c.-618del	p.?	17	63558067	0.154	18797					
	STK11	NM_000455.4:c.-117del	p.?	19	1206796	0.236	6632					Incon
111_C3	**SETD1B**	NM_015048.1:c.22del	p.H8fs*27	12	122242656	0.83	1620	-	-	-	-	No ch
	MSH2	NM_000251.2:c.1600delC	p.Arg534Valfs*9	2	47693885	0.394	747	-	-	-	-	No ch
	MSH2	NM_000251.2:c.1741delA	p.Ile581Leufs*9	2	471698181	0.45	9	-	-	-	-	No ch
	MLH3	NM_001040108.1:c.1755del	p.Glu586Asnfs*24	14	75514603	0.39	136	-	-	-	-	No ch
	MSH3	NM_002439: c.1114delAA	p.Lys383Argfs*32	5	79970914	0.682	456	-	-	-	-	Incon
	BMPR1A	NM_004329.2:c.419del	p.Pro140Leufs*4	10	88659631	0.23	209	-	-	-	-	No ch
	CHEK2	NM_007194.3:c.880G>A	p.Ala294Thr	22	29099521	0.157	126	B (0.32)	D (0.993)	B (0.002)	B (0.001)	No ch
	MLH1	NM_001167618.1:c.443G>A	p.Arg148Gln	3	37067255	0.205	515	B (0.22)	D (1)	PsD (0.602)	B (0.100)	No ch
	MUTYH	NM_001128425.1:c.643G>A	p.Val187Met	1	45798293	0.346	1624	D (0)	D (1)	PrD (1.000)	PrD (0.999)	Incon
	POLE	NM_006231.3:c.2375A>G	p.Lys792Arg	12	133241981	0.47	2116	B (0.11)	D (1)	PrD (0.971)	PsD (0.887)	Gain of splic
	BUB3	NM_001007793.2:c.972-88G>A	p.?	10	124924475	0.603	67					
	SMAD4	NM_005359.5:c.*3760delT	p.?	18	48608588	0.331	181					
	AXIN2	NM_004655.3:c.*631delAA	p.?	17	63525462	0.441	1431					
	AXIN2	NM_004655.3:c.-619delT	p.?	17	63558069	0.129	1959					
	MLH3	NM_001040108.1:c.*2058G>T	p.?	14	75481727	0.551	496					
	MLH3	NM_001040108.1:c.-71G>A	p.?	14	75518090	0.421	1940					
	APC	NM_000038.5:c.*1884delT	p.?	5	112181707	0.299	147					
	STK11	NM_000455.4:c.-117del	p.?	19	1206796	0.479	572					Incon
115_C3												

B. Tumor from a Lynch syndrome patient

228_C1	**MSH3**	NM_002439.4:c.1141delA	p.Lys383Argfs*32	5	79970914	0.278	3154	-	-	-	-	Incon
	MUTYH	NM_001128425.1:c.1484G>A	p.Arg467His	1	45796222	0.242	14879	D (0.02)	B (0.901)	B (0.218)	B (0.049)	Incon
	MSH2	NM_000251.2:c.1601delG	p.Arg534Leufs*9	2	47693885	0.265	11983					Incon
	POLE	NM_006231.3:c.2865-4delT	p.?	12	133237747	0.506	24174					No ch
	MSH3	NM_002439.4:c.238-7G>A	p.?	5	79952223	0.238	21208	-	-	-	-	Incon
	BUB3	NM_004725.3:c.*1131delT	p.?	10	124924482	0.765	2396					
	SMAD4	NM_005359.5:c.*5835delT	p.?	18	48610584	0.28	12938					
	AXIN2	NM_004655.3:c.*636delAA	p.?	17	63525458	0.404	28588					
	PTEN	NM_000314.4:c.*655delT	p.?	10	89725884	0.193	4724					
	PTEN	NM_000314.4:c.*1631delT	p.?	10	89726860	0.196	1518					

The number of "C" in tumor tested corresponds to the Cancer number of Table 1.

Abbreviatures: B, benign; D, damaging; PrD, probably damaging; PsD, possibly damaging.

tested									(p-value)			
108_C2	APC	NM_001127511.2:c.2572C>T	p.Arg858*	5	112173917	0.0903	597	B (0.1)	-	-	-	Inco
	TP53	NM_000546.5:c.856G>A	p.Glu154Lys	17	7577082	0.348	1087	D (0)	D (1)	PrD (0.999)	PrD (0.982)	No
	KRAS	NM_004985.4:c.35G>A	p.Gly12Asp	12	25398284	0.248	104	D (0)	D (1)	B (0.385)	B (0.257)	No
108_C1	CTNNB1	NM_001098209.1:c.122C>T	p.Thr41Ile	3	41266125	0.114	454	D (0)	D (1)	PrD (0.996)	PrD (0.955)	No
	FBXW7	NM_001013415.1:c.1711C>T	p.Arg571Trp	4	153244092	0.0628	477	D (0)	D (1)	PrD (1.000)	PrD (1.000)	No
121_C1	APC	NM_001127511.2:c.4121C>A	p.Ser1374*	5	112175466	0.164	2068	-	-	-	-	Inco
	KRAS	NM_004985.4:c.38G>A	p.Gly13Asp	12	25398281	0.145	1164	D (0)	D (1)	B (0.215)	B (0.175)	Inco
	FBXW7	NM_001013415.1:c.1391C>T	p.Ser464Leu	4	153245446	0.352	4613	D (0.01)	D (1)	PrD (1.000)	PrD (0.988)	No
	PIK3CA	NM_006218.2:c.113G>A	p.Arg38His	3	178916726	0.227	2019	D (0.03)	D (1)	PrD (1.000)	PrD (0.992)	Inco
	GNAS	NM_001077489.2:c.429A>C	p.=	20	57480479	0.214	13390	-	-	-	-	Inco
111_C3	KRAS	NM_004985.4:c.35G>A	p.Gly12Asp	12	25398284	0.326	42	D (0)	D (1)	B (0.385)	B (0.257)	No
	PIK3CA	NM_006218.2:c.3145G>C	p.Gly1049Arg	3	178952090	0.401	226	D (0.01)	D (1)	B (0.300)	B (0.096)	Inco
114_C1												
115_C3												
B. Tumor from a Lynch syndrome patient												
228_C1	PTEN	NM_000314:c.636delT	p.Pro213Leufs*8	10	89717610	0.19	459	-	-	-	-	Inco

The number of "C" in tumor tested corresponds to the Cancer number of Table 1.

Abbreviatures: B, benign; D, damaging; PrD, probably damaging; PsD, possibly damaging.

DISCUSSION

In a series of LS suspected patients harboring MSH2-deficient tumors comprehensive somatic and ge mutational analysis allowed elucidating their molecular basis in a high proportion of cases. The reclassificat pathogenic of 3 *MSH2* VUS and the identification of a new *MSH2* splicing mutation yielded a 66% (40/59) mu detection rate. Furthermore, the identification of germline variants in *BUB1*, *SETD2*, *FAN1* and *MSH6* in 6 patie pathogenicity is confirmed) as well as the presence of double MMR or combined MMR/polymerase somatic l tumors from 4 LLS individuals, may increase this yield up to 81% (49/59). The obtained results further eviden great heterogeneity present in this subset of cases[20,22,27,29,30,42].

Eigth recurrent mutations (7 in *MSH2* and a deletion of EPCAM 3'-end) have been identified in two or unrelated families. Two of them (*MSH2* c.[2635-3C>T; 2635-5T>C] and *EPCAM* deletion) had been prev reported by our group as Spanish founder mutations[43], whereas *MSH2* c.942+3A>T was previously report recurrent in several populations[44].

RNA analyses allowed classifying three *MSH2* variants as pathogenic mutations affecting mRNA process the present work, splicing analysis in combination with multifactorial likelihood calculations offered a performance, allowing reclassification as pathogenic of 9 out of 10 variants (6 out of 6 class 4 variants and 3 ou class 3 variants). These results highlight the benefit of applying quantitative and qualitative analyses for v interpretation and classification, as well as the usefulness of collecting RNA samples and including RNA splicing an in the diagnostic routine. The variant *MSH2* c.211G>C, identified in two patients, illustrates the complexity classification process and the functional characterization. Splicing analysis of the whole transcript in patien identified two transcripts (r.-16_211del and r.195_211del). In contrast, in case 234 the splicing analysis perfo encompassed a smaller region containing the variant (from exon 1 -nucleotide c.85- to exon 4) identified on

Only the duplication of exons 11-16 could not be readily classified. Although it theoretically leads t generation of an aberrant transcript, as the duplicated region is inserted after the stop codon, its pathogenic effe not demonstrated so far. Of note, multifactorial analysis was not performed for variants causing splicing aberr (and located outside the consensus splice sites). In these given cases, the use of prior probabilities of missense va that assume no changes at RNA level would lead to wrong classifications. Current multifactorial models will be improved when information concerning IHC patterns and MSI/IHC results of extracolonic tumors, frequently noted suspected families, will be included. Also, their informativeness will certainly improve if the impact on RNA spli tested before multifactorial analysis.

The contribution of promoter variation of MMR genes to LS is likely marginal. Sanger sequencing and Ha analysis of the *MSH2* promoter indicates that variants at this region are not relevant in our series. Previously, c 77del, c.-190C>T and c.-80insA variants have been shown to reduce promoter activity [45,46]. Intriguingly, varia 225G>C was found to increase the transcriptional efficiency [46]. In our hands, two germline variants at the promoter, c.-25C>T and c.-204C>G have been reported whose functional impact is still unknown.

The lack of detection of *MSH2* methylation in LLS MSH2-deficient tumors is in agreement with th proportion of methylated tumors in MSH2 deficient LLS patients (1 of 46) reported in previous series[25,41]. MS (Methylation specific- Melting Curve Analysis) is a robust technique that simultaneously analyzes 24 CpG. The u methylation-independent primers further increases the dependability validated by the inclusion of adequate po and negative controls in each run. Moreover, none of the 8 available tumors from *MSH2* mutated LS cases methylated. While Nagasaka's approach analyzed a region not included in our amplicon[41], they did not co methylation by other techniques.

The germline and somatic mutational analysis of selected CRC-associated genes has yielded promising r in this set of MSH2 deficient cases. Germline biallelic *MUTYH* mutations[29] were detected and excluded prior t analysis. The identification of 3 putative pathogenic alterations in *FAN1* was previously reported (Vargas submitted). To the best of our knowledge, this is the first report of a germline predicted pathogenic *BUB1* varian patient with breast and endometrial cancers. Recently, germline heterozygous mutations in this gene, a compon the spindle assembly checkpoint (SAC) responsible for correct chromosome segregation[47], have been identif patients with an early onset and familial CRC[48,49]. In our series, the identified variant in *BUB1* (c.3005C>G, p.T1 case 115), affected the protein kinase catalytic domain and is predicted to destabilize the protein (data not sh Studies in mice have shown that mutations affecting the kinase catalytic domain appear to have a dominant ne function by competing for kinetochore binding or preventing interactions with other SAC components[47]. Since mutations can be responsible for variegated aneuploidy, cytogenetic analysis as well as cosegregation analysis family should be of help in clarifying the pathogenicity of the identified variant[49].

MSH2/MSH6 to chromatin . The identification in our series of 3 LLS patients harboring germline *SETD2* pre
pathogenic variants may be consistent with a putative causal role in LLS. The 3 identified probands were diagno
CRC before age 50. Besides cosegregation and functional analysis, epigenetic analysis in biological samples of
patients should be of help in their characterization. So far, other indirect evidences point to a role for SETD2 i
tumors. Somatic SETD2 mutations have been detected in a subset of gastric tumors displaying MSI without k
MMR gene mutations[51]. However, in renal cell carcinoma SETD2 mutations have been associated with demethylat
non-promoter regions[52]. Finally, it is noteworthy that we were not able to confirm a role for germline *POLE* and *
mutations in this subset of MMR-deficient tumors [53].

Somatic subexome analysis at a high coverage has provided interesting results in this preliminary an
Somatic double hits in MMR genes were evidenced in two tumors confirming previous reports [17,20–22]. The rem
three, double heterozygote mutations in MMR genes and/or proof-reading polymerases were identified. The li
number of cases analyzed precludes drawing conclusions on these findings although it must be beared in mind
pediatric tumors arising in CMMRD cases strongly associate with mutations in the exonuclease domain of proof-re
polymerases. Finally, our observations reinforce the notion that variations in *MSH2* or *MSH6* may be a frequent ev
these cases in line with previous reports while somatic hypermethylation does not play a significant role.

The yield of subexome testing is directly related to the selection of genes, the sample analyzed as well
quality and depth of the analysis. While mean coverage is high (1200x) is similar for PBL and FFPE DNA in FFPE is
variable depending upon the amplicon chosen. Using this coverage we have ruled out germline mosaicisms with
cut-off value in PBLs. Regarding somatic testing, all reported mutations have been detected in amplicons with a
coverage (1400x) making our findings dependable. However, variability may have lead to the loss of other re
findings.

In all, comprehensive germline and somatic analysis has proved useful in the elucidation of the unde
molecular basis of suspected LS in MSH2 deficient cases. Subexome analysis opens the scope of the genes invol
this set of cases. Further studies of larger series and more in-depth functional characterization of variants detect
mandatory in order to establish the true clinical validity of the proposed strategy.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing
report.

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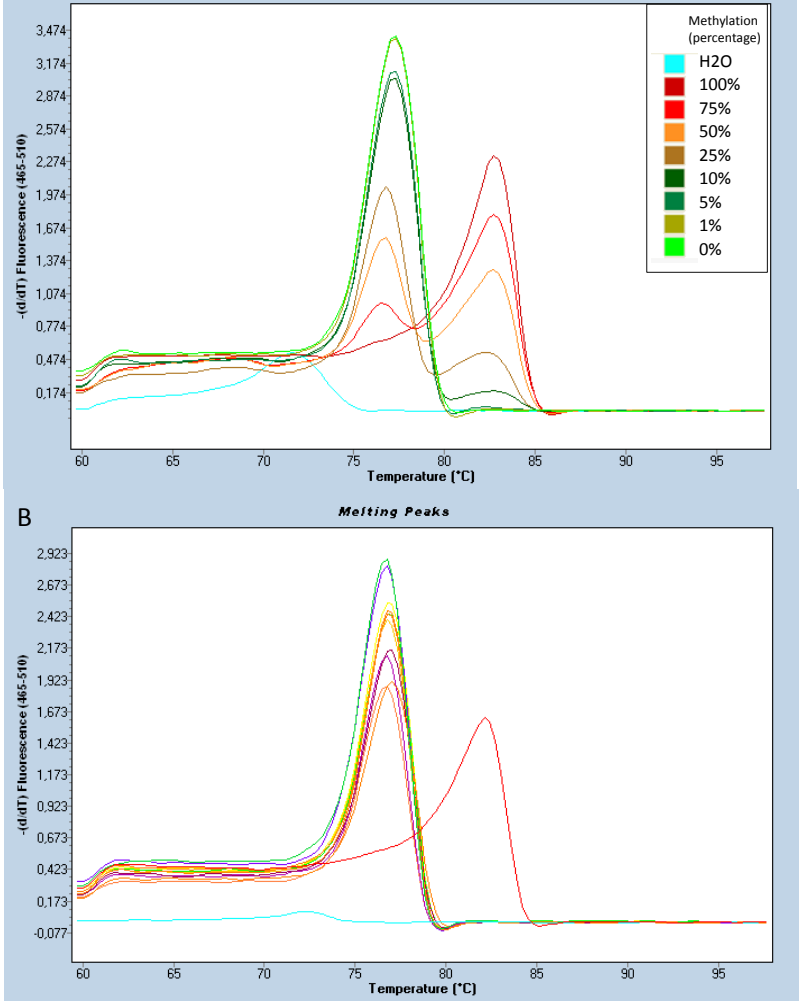
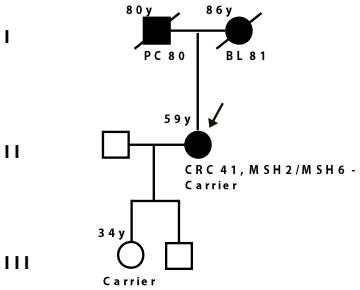
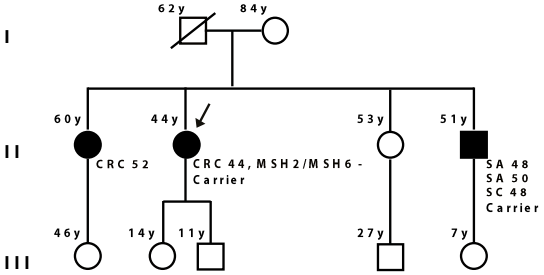


Figure S1. MS-MCA (analytical sensitivity). A) *MSH2* promoter methylation sensitivity gradient (in percentage) by Meth Specific - Melting Curve Analysis. The sensitivity is of 5% methylation. 100% methylated peak corresponds to CpG Methylated Genomic DNA from New England Biolabs. Mehtylated peak is at 82.6°C and unmethylated control at 77.2°C. B) Example of promoter results by MS-MCA, the positive control (in red) has a melting temperature of 82.6°C and the rest of the sample methylated) of 76.8°C.

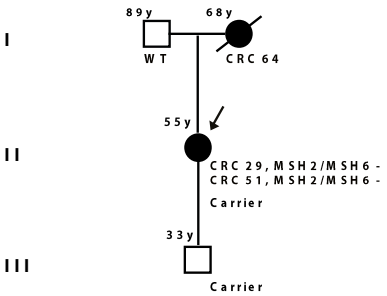
Family 122: c.518T>G



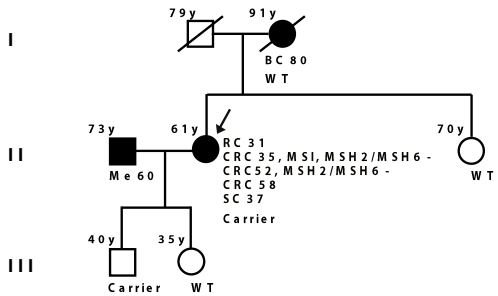
Family 117: c.518T>G



Family 264: Duplication E11



Family 118: c.2069A>G



Family 120: Duplication E11-16

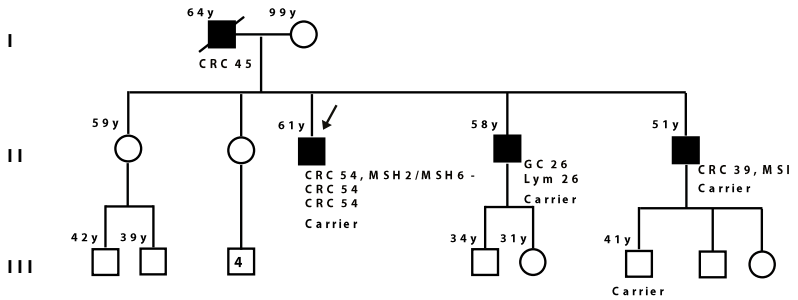
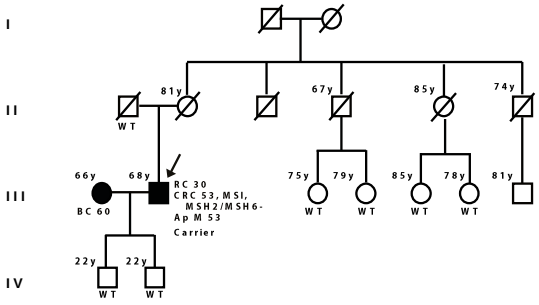
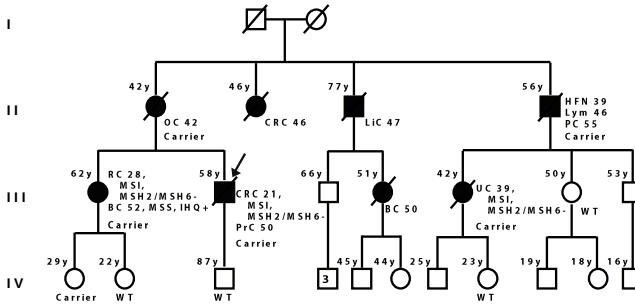


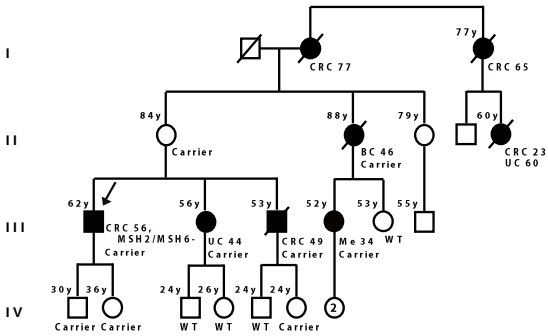
Figure S2. Family pedigrees from carriers of *MSH2* class 3 variants (A), class 4 variants (B) and further identified c.518T>G varia
Filled symbol, cancer; arrow, index case. Cosegregation results are indicated below individual's symbols as "carrier" or "WT". C
ages and ages at death, when available, are indicated on the top-left corner of each individual's symbol. CRC, colorectal canc
pancreas cancer; BC, breast cancer; SC, skin cancer; SA, sebaceous adenoma; BL, Bladder cancer; RC, Rectum cancer; GC,
cancer; Lym, Lymphoma; UC, Uterine cancer; Me, melanome; Ap M, appendix malignant; OC, Ovarian Cancer; LiC, Liver cance
head/face/neck cancer; PrC, prostate cancer; MSI, microsatellite instable; MSS, microsatellite stable; IHC+, conserved MMR p
expression; the pattern of expression of MSH2 and MSH6 proteins is indicated (-, loss; NV, non-valuable).



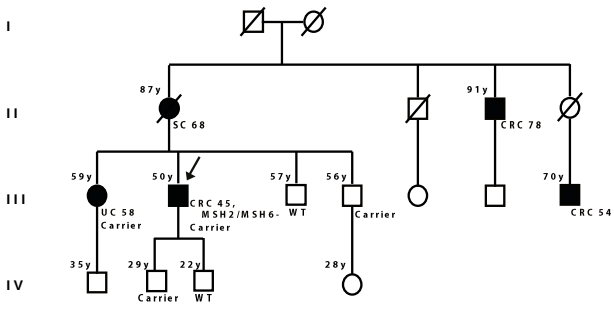
Family 232: c.[2635-3C>T;2635-5T>C]



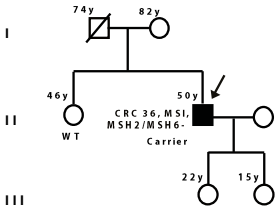
Family 234: c.211G>C



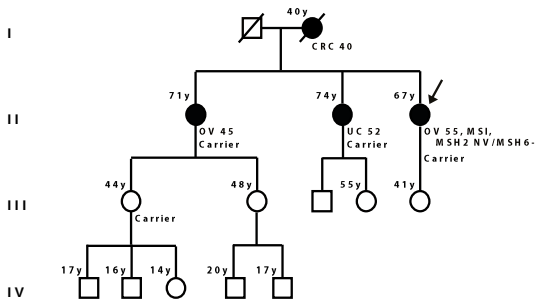
Family 239: c.2074G>C



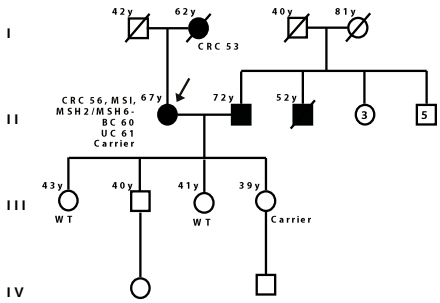
Family 228: c.989T>C



Family 248: c.1511-1G>A



Family 258: c.1276G>A



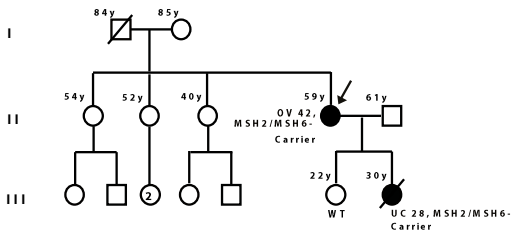


Figure S2 cont. Family pedigrees from carriers of *MSH2* class 3 variants (A), class 4 variants (B) and further identified c.5 variant (C).

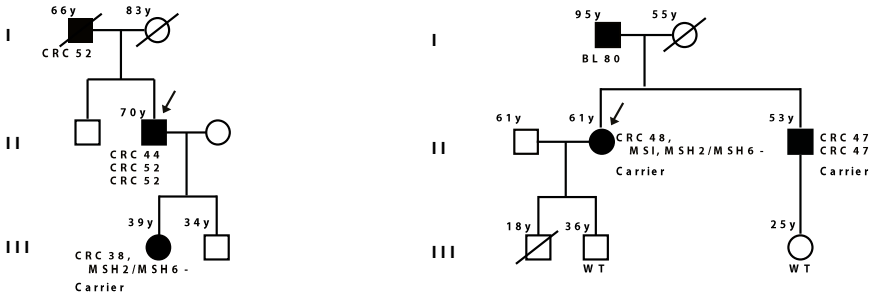


Figure S2 cont. Family pedigrees from carriers of *MSH2* class 3 variants (A), class 4 variants (B) and further identified c.5 variant (C).

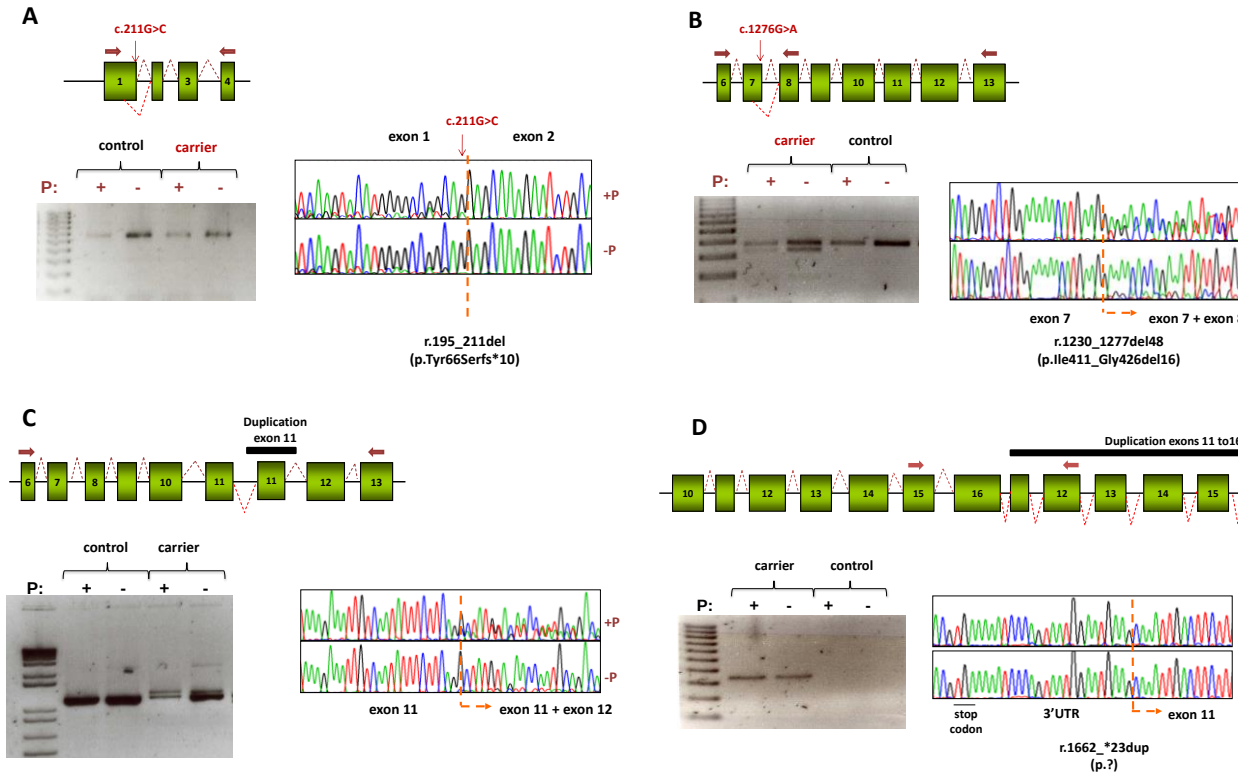


Figure S3. cDNA characterization of the MSH2 c.211G>C (A), c.1276G>A (B), duplication of exon 11 (C), duplication of exons 1 (D). In green boxes a schematic representation of the normal and aberrant transcripts caused by the mutations. On the botto the gels showing RT-PCR products from controls and carriers in absence and prescence of puromycin. On bottom right, sequencing of the RT-PCR products from variant carriers.

Gene	Analysis	Primer name	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon length	CpGs interrogated
MSH2	MS-MCA	MS-MCA MSH2_PCR	TTTTTTTAATTAGGAGGTGAGGAG	CACCCCCTAAATCTTAAACACCT	221bp	24
		MS-MCA MSH2_Heminested	TTTTTTAGGGTATGTYGGAGAAG	CACCCCCTAAATCTTAAACACCT	125bp	13
	Sanger sequencing (gDNA)	MSH2Pr-2_PCR&SEQ	GCCAAGAAGAGTCTGGGACA	ACGCGCATCCTTAGTAGAGC	404bp	
		MSH2Pr-2_SEQ	TTCAAGTTTCCTTCTGATG	GCCTTCCTCCTCCTCCCAG		
		MSH2Pr-1_PCR&SEQ	TCAAGCCTTGCAGCTGAGTA	CCATGTCGAAACCTCCTCAC	315bp	
		MSH2Ex1_PCR&SEQ	TCGCGCATTTTCTTCAACCA	GTCCCTCCCCAGCACG	285bp	
	Long range_PCR	MSH2_E1up_EX/ MSH2_c16R_neu	TCGCGCATTTTCTTCAACCA	TACCTTCATTCCATTACTGGG	2.8 Kb	
	Sanger sequencing (cDNA)	MSH2_E1up_EX	TCGCGCATTTTCTTCAACCA			
		MSH2_E2/3dw_EX		GCCAGGAGAAGCCTTATATG		
		MSH2_E4up_EX	AGGAATTCTGATCACAGAAAG			
		MSH2_E5dw_EX		TGAAAAAGGTTAAGGGCTCTG		
		MSH2_E7up_EX	CTAATGTTATACAGGCTCTGG			
		MSH2_E8dw_EX		TTCCTGAAACTTGGAGAAGTCA		
		MSH2_E12up_EX	GCTATGTAGAACCAATGCAGACAC			
		MSH2_E12dw_EX		AGTGTCTGCATTGGTTCTACATAG		
		MSH2_E14up_EX	GGGAAGAGGAACTTCTACCTACG			
		MSH2_E14dw_EX		CTCTTCAGTGGTGAGTGCTGT		
		MSH2_c16R_neu		TACCTTCATTCCATTACTGGG		

Table S2. Genes and exons covered by NGS subexome panel

Gene	Transcript	Exons	Promoter
APC	NM_000038	All	Yes
BUB3	NM_004725	All	Yes
MUTYH	NM_001128425	All	Yes
STK11	NM_000455	All	Yes
POLE	NM_006231	All	Yes
POLD1	NM_002691	All	Yes
BMPR1A	NM_004329	All	Yes
SMAD4	NM_005359	All	Yes
PTEN	NM_000314	All	Yes
ENG	NM_000118	All	Yes
FAN1	NM_014967	All	Yes
TP53	NM_000546	All	Yes
CDH1	NM_004360	All	Yes
CHEK2	NM_001005735	All	Yes
BUB1B	NM_001211	All	Yes
BUB1	NM_004336	All	Yes
EXO1	NM_130398	All	Yes
AXIN2	NM_004655	All	Yes
EPCAM	NM_002354	All	Yes
MLH1	NM_000249	All	Yes
MLH3	NM_001040108	All	Yes
MSH2	NM_000251	All	Yes
MSH3	NM_002439	All	Yes
MSH6	NM_000179	All	Yes
PMS1	NM_000534	All	Yes
PMS2	NM_000535	All	Yes
AKT1	NM_005163	3	No
BRAF	NM_004333	11 and 15	No
CTNNB1	NM_001904	3	No
EGFR	NM_005228	3, 7, 15 and 18 to 21	No
FBXW7	NM_033632	8 to 12	No
GNAS	NM_000516	6 and 8	No
KRAS	NM_004985	2 to 4	No
MAP2K1 (MEK1)	NM_002755	2	No
MET	NM_000245	2, 5, 14, 16 to 19, and 21	No
NRAS	NM_002524	2, 3, 4 and 5	No
PIK3CA	NM_006218	2, 3, 8, 10, 14 and 21	No
SRC	NM_005417	14	No
SETD2	NM_014159	3	No
SETD1B	NM_015048	1	No
SETDB2	NM_031915	13	No

- Targeted regions of exons were designed including -/+10bp of the intron-exon boundaries.
- Promoter region comprise 650bp upstream the TSS.

Total number of cases	59 (100)	35 (59,3)	24 (40,7)	19 (32,2)	5 (8,5)
Sex					
Female	37 (62,7)	19 (54,3)	18 (75)	14 (73,7)	4 (80)
Male	22 (37,3)	16 (45,7)	6 (75)	5 (26,3)	1 (20)
Age at diagnosisº	49.7 (21-77)^	45.8 (21-59)^	50,7 (31-77)^	51.6 (32-77)^	42.5 (31-54)^
Clinical criteria					
Amsterdam	20 (33,9)	18 (51,4)	2 (8,3)	1 (5,3)	1 (20)
Bethesda	37 (62,7)	17 (48,6)	20 (83,3)	16 (84,2)	4 (80)
Anatomo-pathological	2 (3,4)	0 (0)	2 (8,3)	2 (10,6)	0 (0)
Patients with multiple primary tumors*	26 (44,1)	20 (57,1)	6 (25)	3 (15,8)	3 (60)
MSH2-deficient analyzed tumors					
Colorectal cancer	48 (78,7)	25 (71,4)	23 (87,5)	16 (84,2)	7 (100)
Endometrial cancer	7 (11,5)	4 (11,4)	3 (12,5)	3 (15,8)	0 (0)
Ovarian cancer	4 (6,6)	4 (11,4)	0 (0)	0 (0)	0 (0)
Ureter cancer	1 (1,6)	1 (2,9)	0 (0)	0 (0)	0 (0)
Bladder /other non-LS associated	1 (1,6)	1 (2,9)	0 (0)	0 (0)	0 (0)

º First tumor diagnosis; ^ age range; * LS spectrum (Bethesda)

Table S4. In silico predictions and result of the splicing analysis of MSH2 variants functionally evaluated in

			Splice Site Prediction									Enhancer site prediction					
			NNSplice		Spliceport		NetGene2		Softberry		Interpretation	Rescue ESE	ESE finder	Interpretation	Functional domain	PolyPhen-2 (score)	SIF
VUS	Exon	SS	wildtype	variant	wildtype	variant	wildtype	variant	wildtype	variant							
c.211G>C	E1	A	—	—	—	—	—	—	—	—	**Inconclusive**	No change	1 site destroyed / 3 created	**Inconclusive**	DNA binding domain	Benign (0.107)	Toler
p.Gly71Arg		D	0.95	0.59	NR	NR	0.00	0.00	11.56	NR							
c.518T>G	E3	A	0.98	0.98	NR	NR	0.00	0.00	4.5	4.5	**No effect**	1 created	1 created	**Aberrant ESE**	Connector domain	Probably Damaging (0,986)	Dan
p.Leu173Arg		D	1	1	NR	NR	0.00	0.00	14.64	14.64							
c.989T>C	E6	A	0.98	0.98	1.76	1.93	0.00	0.00	9.3	9.3	**No effect**	No change	1 destroyed	**Inconclusive**	Lever domain	Probably Damaging (1,000)	Dan
p.Leu330Pro		D	0.98	0.98	NR	NR	0.00	0.00	11.14	11.14							
c.1276G>A	E7	A	0.91	0.91	1.22	1.22	0.36	0.36	NR	NR	**Inconclusive**	No change	No change	**No change**	Lever domain	Benign (0.063)	Toler
p.Gly426Arg		D	0.91	NR	NR	NR	0.00	NR	11.98	NR							
c.2069A>G	E13	A	0.95	0.95	1.2	1.44	0.77	0.77	7.88	7.88	**No effect**	No change	2 created	**Inconclusive**	ATPase domain	Probably Damaging (0,999)	Dan
p.Gln690Arg		D	1.00	1.00	1.64	1.64	0.00	0.00	15.06	15.06							
dup exon 11	E11		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	Lever domain	**NA**	
dup exons 11-16	E11-16		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	Lever/ATPase/ Helix-turn-helix	NA	

Predictions are interpreted as inconclusive when the same results are not obtained by all the programs used.

Abbreviations: SS, splice site; A, acceptor consensus splice site; D, donor consensus splice site; NR, consensus splice site not recognized.

DISCUSSION

This thesis aimed at gaining insight into the molecular basis of LS suspected patients, in particular in LLS cases. The comprehensive analysis, both at the genetic and epigenetic level of MMR genes, including coding and promoter sequences together with the use NGS multigene panel testing (germline and somatic) has been useful for the identification of constitutional *MLH1* methylation and novel germline variants in other CRC-associated genes (*FAN1*, *BUB1*, *SETD2* and *MUTYH*). Also, double or compound somatic events in MMR and polymerase genes account for a proportion of these cases. This strategy has proved useful for the refinement of the molecular basis of LLS and provides with a complex picture of this subset of cases. In this joint discussion we aimed at providing an overview of all results obtained and it should complement the discussion of the papers enclosed herein.

1. THE ROLE OF METHYLATION IN MMR GENES IN SUSPECTED LYNCH SYNDROME

1.1. MSH2 and MSH6 promoter methylation does not appear to play an important role in LLS

The distinction between LS-associated tumors and sporadic ones is of great clinical importance since the altered molecular pathways and management strategies largely differ between them. It is well established the effectiveness of using either *MLH1* methylation or *BRAF* mutation analysis in CRC as a filter to select cases that will not continue with the diagnostic algorithm of LS (Mireia Gausachs et al. 2012; Leticia Moreira et al. 2012; Newton et al. 2014; Perez-Carbonell, Cristina Alenda, et al. 2010). Although *MSH2* promoter methylation has been associated to *EPCAM* deletions at germline level being acknowledged as an epigenetic heritable defect present in all cases reported so far, somatic *MSH2* methylation as a sporadic cause of MSI tumors has been poorly studied.

We did not detect *MSH2* promoter methylation in the 13 samples from LLS patients harboring tumors with MSH2 deficient expression or in the 3 from MSH6 negative tumors. This

is in agreement with the low proportion of methylated tumors in MSH2 deficient LLS patients (1 of 46) reported in previous series (Nagasaka et al. 2010; Rumilla et al. 2011) (Table 14).

Table 14. Previous and present results on *MSH2* and *MSH6* methylation studies in LS-associated tumors

Cases	Affected gene	Ours	Nagasaka 2010	Rumilla 2011
Methylation assay		MS-MCA	COBRA	MSP
Lynch syndrome	*MSH2* mutated	0 out of 8	24% (11 out of 26)	-
	EPCAM mutated/ MSH2 deficiency	1 out of 1	100% (3 out of 3)	100% (10 out of 10)
VUS carriers	*MSH2* variant	0 out of 5	0% (0 out of 2)	-
	MSH6 variant	0 out of 3	-	-
Lynch-like syndrome	*MSH2* deficiency	0 out of 8	0% (0 out of 6)	2% (1 out of 40)
	MSH6 deficiency	0 out of 1	-	-

Methodological issues are relevant to the robust identification of hypermethylation in the clinical setting. Rumilla et al, used the MSP technique comprising the region from c.-105 to c.27, equivalent to the MSP1 region utilized by Ligtenberg for the analysis of *MSH2* promoter methylation in cases with *EPCAM* deletions (Ligtenberg et al. 2009). In the present work we set up MS-MCA (Methylation specific- Melting Curve Analysis) for the study of methylation in the promoter region of *MSH2*. Since our DNA samples were isolated from FFPE tissues, our amplicon was constrained to less than 150bp. We chose a region that comprised 24 CpGs from c.-32 to c.189, including probe +126 of MS-MLPA ME011-B1 kit, that is 100bp downstream the region analyzed by Rumilla. The inclusion of several CpG residues as well as the use on methylation-independent primers increases the dependability of our results that was validated in each run by the inclusion of adequate positive and negative controls. We used a commercially available Jurkat methylated cell line DNA that previously sequenced (after bisulfite conversion) in order to assure 100% methylation levels. This sample was used in combination with DNA from control lymphocytes at different proportions, in the analysis of the analytical sensitivity of the MS-MCA assays. As a positive control a colonic samples from an *EPCAM* deletion carrier was used (Table 14). False positive results were ruled out when validation by MS-MLPA and bisulfite sequencing was performed.

Prior studies revealed a prevalence of *MSH2* promoter methylation of 24% in cases harboring germline pathogenic mutations in *MSH2* gene (Nagasaka et al. 2010). None of the 8 available tumors from *MSH2* mutated LS cases were positive (Table 14). It must be emphasized that Nagasaka's approach analyzed an upstream region (c.-196 to c.-38) not included in our

amplicon. Thus, we could be missing methylation at those specific CpG sites. However, it must be emphasized that Nagasaka did not confirmed methylation by other techniques.

Also, we found no evidence of methylation at the *MSH6* promoter in the 9 cases analyzed, representing the first attempt to assess MMR-deficient LS suspected patients. Previously, MSH6 methylation was studied in 99 sporadic tumors from LS spectrum with the same outcome (Lima 2008; Vymetalkova et al. 2014). Notwithstanding, hypermethylation at the *MSH6* promoter is frequent in breast tumor and normal DNA samples (Kornegoor et al. 2012; Moelans, Verschuur-Maes, and van Diest 2011). While a robust technique, MS-MLPA ME-002-B1 methylation kit (MRC-Holland) using a 15% cut-off was used, the fact that they did not study non cancer individuals limits the relevance of their findings. Noteworthy, a small proportion of the cases harboring MLH1-deficient tumors being tested for LS show a modest amount of germline *MSH6* methylation ranging between 0-20% when using MS-MLPA ME011-B1 kit (M Pineda, unpublished observations).

Of note, we have not performed methylation studies of *PMS2* promoter region yet in our series of 5 cases harboring tumors with loss of PMS2 protein alone. To date, only one study evaluated *PMS2* methylation status in 100 MLH1/PMS2 and PMS2 deficient CRC samples, finding no methylation (Truninger et al. 2005).

1.2. Identification of constitutional epimutations and their characterization among LS suspected patients

Constitutional epimutation carriers, as referred to patients that harbor epigenetic abnormalities that are widely distributed within normal somatic tissues, originate in the parental germline or early embryo, and are potentially meiotically heritable (Hitchins 2015). They have been documented in 2 of the 4 MMR genes involved in LS pathogenesis, *MLH1* and *MSH2*, the latter secondary to germline EPCAM deletions.

Two *MLH1* epimutation carriers were found in a series of 34 suspected LS cases harboring *MLH1*-methylated tumors and no identified *MLH1* germline mutation in its coding

region (Castillejo et al. 2015; van Roon et al. 2010). This corresponds to a prevalence of 5.9% (2 out of 34) and to 14.2% (2 out of 14) of patients with CRC diagnosis before 50 years of age. This is in line with the prevalence reported in series of patients with *MLH1*-methylated tumors, enriched for cases with an early age of onset (Castillejo et al. 2015; van Roon et al. 2010). In our LS series, *MLH1* epimutations account for up to 2% of the LS cases similar to other reported series (Crepin et al. 2012; Niessen et al. 2009).

Epimutant cases had developed multiple LS tumors at an early age, a feature that has been previously observed in about half of the reported epimutant cases (Hitchins and Ward 2009). Epimutation carriers usually exhibit an earlier age of onset (~39 years), that is approximately 5 years younger than in patients with germline *MLH1* mutations (Hitchins 2013; Wagner et al. 2001). Although, this may not only reflect the phenotype associated with the epimutation but also the selection criteria used so far in most studies.

For the purpose of the present thesis characterization of case 34 (one of the 2 *MLH1* epimutants) is further discussed. Clinically, case 34 was a patient diagnosed with her first colorectal tumor at 29 years of age, a second CRC at 44 and an endometrial cancer at 49. The last two tumors were analyzed for MSI and both resulted positive. *BRAF* p.V600E mutation was also absent. However, the presence of somatic *BRAF* mutation has been previously reported in tumors from three epimutation carriers (Crepin et al. 2012; Goel and Boland 2012; van Roon et al. 2010), representing 15.8% (3 out of 22) of the reported cases, suggesting that tumors from epimutant patients can mimic MSI sporadic CRCs. As expected, somatic *MLH1* hypermethylation was present in the two tumors analyzed.

It is relevant to characterize in detail the epimutants in order to be able to provide with a robust counseling. In case 34 only 20% of the alleles were methylated which is lower than the 50% previously reported for most of the cases (Crepin et al. 2012; Gazzoli et al. 2002; Hitchins et al. 2007; Megan P Hitchins et al. 2011; Morak et al. 2008; Suter et al. 2004). The functional impact of promoter methylation was analyzed by SNuPe at the heterozygous *EPM2AIP1* c.*2570G>T (rs9311149) (Supp. Fig. S2b of Article 1) showing partial silencing of the G allele, suggesting the presence of mosaicism. While LOH is the most frequent mechanism of inactivation of the wildtype allele in tumors from epimutation carriers (Goel et al. 2011), it could not be assessed for this case.

So far, in all cases identified but one, the methylated allele was of maternal origin (Crepin et al. 2012; Goel and Boland 2012; Hitchins and Ward 2007; M P Hitchins, Owens, C.-T. Kwok, et al. 2011; Morak et al. 2008). Further cosegregation studies were made in case 34 family (Fig. 25) after the publication of Article 1. Unfortunately, no samples were available from her parents, making it impossible to know if methylation was inherited or *de novo* acquired. However, the absence of identified genetic alterations *in cis* and the absence of methylation in 4 sisters PBL suggest it is probably a primary epimutation.

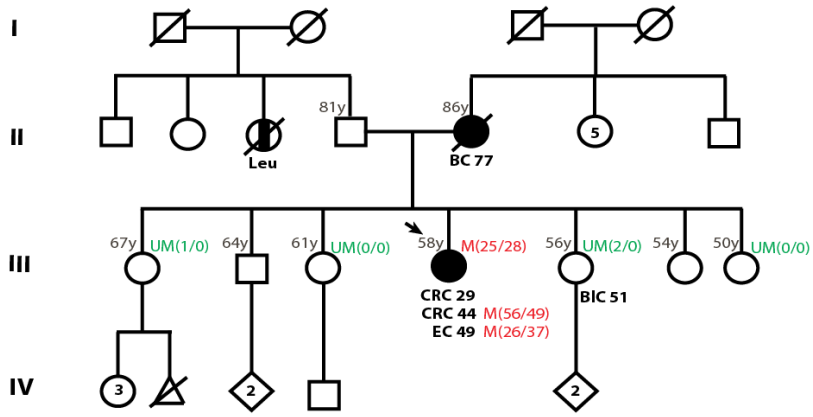


Figure 25. Family pedigree of case 34.

Circles, females; squares, males; filled, cancer affected; vertical bar at center, non-confirmed cancer affected. Cancer localization (CRC, colorectal cancer; EC, endometrial cancer; BC, breast cancer; Leu, leukemia) and age at diagnosis are indicated. The epimutation carrier is indicated by an arrow. Result of the MLH1 methylation analysis in parenthesis (C region/D region); for PBLs is indicated at the top right corner of the individual and for tumors at the right: UM, unmethylated; M, methylated.

As mentioned, despite having made a thorough search by means of Sanger sequencing of the *MLH1* promoter and coding regions, no genetic alteration underlying the epimutated alleles was observed. Previously, genetic aberrations *in cis*, gross rearrangements in two cases (one deletion of *MLH1* exons 1 and 2, one duplication involving the whole gene), and in a third one the haplotype c.-27C>A/c.85G>T within the promoter region have been identified as responsible for *MLH1* epimutations (Gylling et al. 2009; Megan P Hitchins et al. 2011; Morak et al. 2011). In contrast to primary epimutants who have non-Mendelian inheritance due to reversible methylation, dominant transmission pattern is observed in these cases.

Conclusive evidence of the etiological role of *MLH1* and *MSH2* epimutations in LS and the increasing detection of epimutations has led to the suggestion that the molecular diagnosis of these defects should be implemented on a routine screening basis to enable carriers to be early diagnosed, and that genetic counseling and clinical management are conceived on time (Hitchins 2013). So far, the detection of somatic *MLH1* hypermethylation is often used to exclude patients from further MMR mutation analysis, based on cost effectiveness considerations (M Gausachs et al. 2012; Perez-Carbonell, C Alenda, et al. 2010). *MLH1* methylation analysis showed higher specificity than *BRAF* V600E analysis in four previous studies (Mireia Gausachs et al. 2012; Leticia Moreira et al. 2012; Newton et al. 2014; Perez-Carbonell, Cristina Alenda, et al. 2010). In contrast, sensitivity of *BRAF* and *MLH1* methylation is similar.

Patients with somatic *MLH1* hypermethylation could now be considered as candidates to screen for constitutional *MLH1* epimutations. Based on the clinical presentation of the reported cases (Hitchins and Ward 2009) and our experience, this screening could be restricted to those diagnosed earlier than 50 years or with multiple tumors the first one before the age of 60. If this was the case, MS-MLPA could be a good methodological approach. The robustness and informativeness already shown for paraffin-embedded tissues (Mireia Gausachs et al. 2012) has been confirmed when being used in the germline.

Identification of *MLH1* constitutional epimutation carriers will lead to LS diagnosis in that patient. For this reason we consider of great importance its confirmation by other techniques. In this work we have used bisulfite sequencing, MS-MCA, pyrosequencing and MS-MLPA. Specific traits of the techniques that have been used in this thesis to analyze the methylation status are summarized in Table 15. Besides, functional impact should be evaluated by transcription assays.

Table 15. Summary of the techniques used for DNA methylation analysis in this thesis.

Technique	DNA treatment	Assay details	Considerations for assay interpretation	Quantitative output
Bisulfite-sequencing	Bisulfite	Comparison of reference genome and bisulfite-treated DNA provides single nucleotide resolution information about methylation patterns.	- Require micrograms of DNA input. - Chemical DNA treatment can lead to its damage. - Primers must be designed in non CpG regions or be degenerated. - Incomplete bisulfite conversion is a risk, therefore careful analysis of C's (not CpG's) within the amplified fragment must be performed.	No
MS-MCA		Comparison of annealing temperatures between methylated and unmethylated sequences.		Semi
Pyrosequencing		Quantitative analysis of individual CpG sites with real time monitoring.		Yes
MS-MLPA	None	Peaks from digested (unmethylated) and undigested (methylated) DNA are normalized to control within each experiment (at least 8 samples per batch).	Requires low amounts of DNA input.Determination of methylation status is limited by the enzyme recognition site. Necessity of working in batch.	Semi

As commented in the introduction of this thesis, *MSH2* epimutations are associated to deletions in the neighboring *EPCAM* gene, probably after generation of a fusion transcript between *EPCAM* and *MSH2*, thus promoting hypermethylation of the *MSH2* promoter (Kovacs et al. 2009; Ligtenberg et al. 2009). In our series of LS suspected patients with MSH2 deficient tumors, long rearrangement analyses had been performed by MLPA (kit P008, MRC Holland), that includes probes for the analysis of both *MSH2* and *EPCAM*, allowing the identification of the exon 9 deletion of *EPCAM* gene in 3 families (Mur et al. 2013). Since EPCAM deletions were already discarded by previous screening, no further studies were performed.

2. THE SEARCH FOR UNIDENTIFIED GERMLINE MMR GENE MUTATIONS

2.1. Mutational analysis of MMR promoters identify variants of putative relevance in LLS

With the aim of identifying mutations affecting MMR transcriptional activity, we sequenced the promoter region of the silenced genes in tumors from LLS patients. We also analyzed promoters of heterodimer partners due to the implications of pathogenic mutations within MutL and MutS complexes (Halvarsson et al. 2006; de Jong et al. 2004; Loconte et al. 2014; Niessen et al. 2009).

We have found uncommon variants in the *MLH1* promoter in 2 out 57 cases harboring MLH1-deficient tumors. One case (ID: 206) resulted heterozygous for *MLH1* c.-1018G>A (rs190305737). She had a MSI CRC with loss of MLH1 and PMS2 expression by IHC. Unfortunately no RNA sample from this patient was available. Should samples from carriers become available ASE analysis could then be performed. The second case (ID: 141) met Bethesda 4 criteria having a CRC affected daughter at the age of 36 years. Her daughter's tumor did not lack MLH1 expression and was stable. Index case was heterozygous for the novel variant, *MLH1* c.-574T>A. RNA from the index patient was not available. His tumor was MSI and had loss of *MLH1* expression with confirmed unmethylation at the *MLH1* promoter region by MS-MLPA. Both variants are predicted to affect different transcription factor binding sites (TFBS); *MLH1* c.-1018 affects the activating enhancer binding protein 2 alpha (AP-2α) and the glucocorticoid receptor (GR), and *MLH1* c.-574 affects NF1, NF1/CTF and C/EBPβ transcription factors (PROMO).

Further studies could be carried out to determine the pathogenicity of these variants localized at promoter regions. For example, transcriptional activity could be studied by luciferase reporter assays or *MLH1* allelic specific expression analysis (Paul, Soranzo, and Beck 2014). These assays were used for evidencing reduced *MLH1* transcriptional activity of the *MLH1* c.-411_-413del, c.-42C>T, c.-11C>T and c.-27C>A variants (Robyn L. Ward et al. 2013). Analyses of the functional impact of promoter *MLH1* variants c.-28A>G and c.-7C>T have been recently made by Hesson and colleagues, whom demonstrate a partial loss of constitutional *MLH1* expression to ~50% in the two identified carriers (Hesson et al. 2015). Other *MLH1* promoter variants reported in LS suspected cases are c.-432_-435del, c.-64G>T, c.-53G>T and c.-28A>T (Green et al. 2003; C. T. Kwok et al. 2014; B. A. Thompson et al. 2014).

In a series of 36 LLS cases with MSH2/MSH6 deficient tumors and 13 with MSH6 deficiency alone, Sanger sequencing and/or HaloPlex analysis of the MSH2 promoter indicates that variants at *MSH2* promoter region are not likely involved in the pathogenesis of these cases in our series. In contrast, other variants have been previously identified at this region. *MSH2* c.-78_-77del found in a LLS case harboring 3 MSI CRCs was demonstrated to reduce promoter activity and impair DNA binding of nuclear protein (Yan et al. 2007). Transcriptional downregulation secondary to *MSH2* c.-190C>T and c.-80insA has also been reported (Shin et al.

2002). Intriguingly, variant c.-225G>C, reported in 3 LS-suspected cases harboring MSI tumors, was found to increase the transcriptional efficiency (Shin et al. 2002).

MSH6 promoter region was sequenced by Sanger in the same series of LLS patients, only known high prevalent polymorphisms were found: *MSH6* c.-557T>G (rs3136228) and c.-448G>A (rs3136229). Despite its population frequency, Gazzoli et al evidenced that these SNPs altered different Sp1 binding sites affecting *MSH6* transcription at mRNA and protein level (Gazzoli and Kolodner 2003). This suggestion lays on the fact that promoter activity in genes lacking a TATA box, as is the case of *MSH6*, is regulated by Sp1 transcription factors (Liu et al. 2005), which activate mRNA synthesis by RNA polymerase II (Dynan and Tjian 1985). On the other side, a third SNP associated with the polymorphic haplotype (c.-557T>G, c.-448G>A, c.-159C>T) was evaluated in a large cohort of CRC patients, finding no association with the disease (Mrkonjic et al. 2007), besides their high frequency among Caucasian population difficult a pathogenic association. Interestingly, HaloPlex analysis of MSH2/MSH6- LLS cases (Article 4) identified two germline variants at the *MSH6* promoter, c.-25C>T and c.-204C>G, within the uncovered region by Sanger sequencing. The *MSH6* variant c.-25C>T is predicted to produce a premature out-of-frame start codon and to affect FOXP3 and NF1/CTF binding. Moreover, *MSH2* c.-204C>G is predicted to affect the binding of TFII-I, STAT4, NFkappaB1m c-Ets-1, RelA and Elk-1 by PROMO. As the patient was also carrier of heterozygous exonic SNPs in *MSH6* gene, ASE analysis could be performed upon RNA if it were available.

At the time of writing this dissertation the study of *PMS2* promoter has not been completed. The promoter is included in the custom panel design for the analysis of CRC predisposing genes but this study will unlikely provide meaningful results due to the co-amplification of pseudogenes. For the mutational analysis of *PMS2* coding region, the study by long range PCR (Clendenning et al. 2006) in combination with a modified MLPA panel have allowed the analyses of *PMS2* point mutations and long rearrangements at the 3'UTR, avoiding pseudogene amplification with success (Borràs et al. 2013; Vaughn et al. 2013).

2.2. Mutational analysis at RNA level allows the identification of splicing mutations

One reported cause of missed germline MMR mutations is the presence of deep intronic mutations not readily identified in the DNA analysis of coding regions (Chen 2008; Clendenning et al. 2011; Rhees, Arnold, and Boland 2014; Wagner et al. 2002). In one of thirteen LLS cases where complete sequencing of *MSH2* cDNA was performed (Article 4) an aberration at the RNA level was detected. In the absence of puromycin a deletion of almost all the first exon of *MSH2* r.-16_211del (p.?) was evident. Moreover in the presence of puromycin an inframe deletion of 16 bases: r.195_211del (p.Tyr66Serfs*10) was observed. Later, NGS analysis revealed a mutation in the last nucleotide of the first *MSH2* exon, that was previously undetected by Sanger due to the location of the primer used. Interestingly, the same DNA variant c.211G>C had been previously detected in another LS suspected case, initially classified as VUS and now catalogued as pathogenic. The reclassification is based on the generation of aberrant transcripts, according to the Insight classification rules (Thompson 2014).

Bona fide cryptic mutations in the *MSH2* gene have been previously identified. Clendenning and collaborators identified an intronic mutation 478bp upstream of *MSH2* exon 2 (c.212-553_c.212-479), causing the creation of a novel splice donor site. The subsequent insertion of 75 nucleotides contained a stop codon at the 3'end, which is predicted to result in a truncated protein (Clendenning et al. 2011). Furthermore, Liu et al identified a cryptic paracentric inversion of *MSH2* from exon 2 to 6 in 2 LLS cases harboring MSH2-deficient tumors. The aberrant transcript produced an imbalance of 18Kb at DNA level, resulting in the deletion of the 4 implicated exons (Liu et al. 2015). In all, no cryptic mutations have been found in this subset of cases. However, our findings reinforce the utility of collecting RNA samples for the analysis of splicing and/or cryptic aberrations.

1.2.5. Pathogenicity assessment of MSH2 variants

RNA analyses allowed classifying three *MSH2* variants as pathogenic mutations affecting mRNA processing. In the present work, splicing analysis in combination with multifactorial likelihood calculations offered a good performance, allowing reclassification as pathogenic of 9 out of 10 variants (6 out of 6 class 4 variants and 3 out of 4 class 3 variants). These results highlight the benefit of applying quantitative and qualitative analyses for variant interpretation and classification as well as the usefulness of collecting RNA samples and including RNA splicing analyses in the diagnostic routine. The variant *MSH2* c.211G>C, identified in two patients, illustrates the complexity of the classification process and the functional characterization. Splicing analysis of the whole transcript in patient 109 identified two transcripts (r.-16_211del and r.195_211del). In contrast, in case 234 the splicing analysis performed encompassed a smaller region containing the variant (from exon 1 -nucleotide c.85- to exon 4) identified only the r.195_211del transcript. The variant was finally classified as pathogenic based on the generation of aberrant transcripts, according to the Insight classification rules (Thompson 2014).

Only the duplication of exons 11-16 could not be readily classified. Although it theoretically leads to the generation of an aberrant transcript, as the duplicated region is inserted after the stop codon, its pathogenic effect was not demonstrated so far. Of note, multifactorial analysis was not performed for variants causing splicing aberrations (and located outside the consensus splice sites). In these given cases, the use of prior probabilities of missense variants that assume no changes at RNA level would lead to wrong classifications. Current multifactorial models will be likely improved when information concerning IHC patterns and MSI/IHC results of extracolonic tumors, frequently noted in LS suspected families, will be included. Also, their informativeness will certainly improve if the impact on RNA splicing is tested before multifactorial analysis.

3. THE ROLE OF OTHER CRC-ASSOCIATED GENES

3.1. MUTYH is a bona fide LLS cancer gene

Multiple and redundant mechanisms of DNA repair coexist within the cells. It is well known that DNA repair is the result of the coordinated action of many components that organized in mutimeric complexes. Components of the MMR repair may cooperate with proteins involved in other DNA repair mechanisms such as Base Excision Repair. We have found a prevalence of biallelic *MUTYH* mutations of 3.1% in LLS for the whole series. The prevalence was similar (3.9%) when only cases fulfilling LS clinical criteria (Amsterdam or Bethesda) were considered. Thus, our study supports the existence of overlapping phenotypes between Lynch and MAP syndromes in the largest study of *MUTYH* in LLS patients reported to date (August, 2015). This prevalence is higher than the reported in a Germanic-American cohort of 85 LLS cases (1.18%) (Morak et al. 2014). Moreover, it is also significantly higher than the frequency observed in controls and unselected CRC from the Spanish population (F Balaguer et al. 2007).

Noteworthy, the prevalence of germline *MUTYH* mutations in our series could be even higher due to the limitations of the mutation detection strategy utilized. The 3 Spanish hotspot mutations (Gomez-Fernandez et al. 2009; Guarinos et al. 2014; Nielsen et al. 2011; Win et al. 2011) were initially analyzed and, only in heterozygous mutation carriers, sequencing of coding region and MLPA was completed (Article 2). In fact this was made evident in a further study from our group (Seguí, Navarro, et al. 2015), in which one of our LLS cases (case 106, Supp. Table A1 of Article 2) reported as wildtype for *MUTYH* hotspots, was found to be a compound heterozygous for *MUTYH* c.1147delC (p.Ala385Profs*23) and c.43A>G (p.Met15Val) by exome NGS. In contrast, the analysis of the whole coding region of *MUTYH* by NGS in the subset of LLS patients with MSH2-deficient tumors did not identify any additional MAP patient (Article 4).

Until the identification of these seven biallelic *MUTYH* carriers in our work, only seven additional cases had been previously reported in patients with MMR deficient tumors (Cleary et al. 2009; Colebatch et al. 2006; Gu et al. 2002; Lefevre et al. 2010; Morak et al. 2014). To the best of our knowledge, so far only 1 more Lynch-like case (previously mentioned) has been reported (Seguí, Navarro, et al. 2015). While, Yurgelun and collaborators identified 3 biallelic

MUTYH carriers in a series of 1260 of CRC patients, neither clinical information nor tumor MSI status was available for the positive cases (Yurgelun et al. 2015). Of note, LS suspected patients harboring *MLH1* methylated tumors were not included in our studies. We may have been missed some additional cases as a biallelic *MUTYH* mutations have been reported in *MLH1* methylated tumors (Colebatch et al. 2006).

In contrast to those previously reported cases and the proposed clinical criteria for MAP syndrome suspicion (Brand 2013), most of the LLS cases with biallelic *MUTYH* mutations reported in our series had less than 10 adenomatous polyps at the time of CRC diagnosis (Article 2). *MUTYH* biallelic mutations in the absence of MAP-phenotype had been described in large population-based CRC series (Giráldez et al. 2009; Knopperts et al. 2013; Wang et al. 2004). In fact, two of the 5 *MUTYH* biallelic cases, developed more than 10 adenomatous polyps after CRC was evidenced by follow-up colonoscopies. Recently, Guarinos and collaborators reported that an important proportion of MAP patients (40.8%) can debut with serrated polyps (Guarinos et al. 2014). Thus, the scarcity of adenomatous polyps, the presence of serrated polyps or the presence of MSI in tumors should not exclude the *MUTYH* analysis. Furthermore, our findings reinforce the need to perform systematic reviews of surveillance reports in patients with hereditary CRC suspicion.

Double somatic MMR mutations have been reported in a subset of LLS tumors (range 10-52%) (Sourrouille et al. 2013; Mensenkamp et al. 2014; Geurts-Giele et al. 2014; Article 4). Interestingly, the LLS case with germline *MUTYH* biallelic mutations found in the Germanic-American series cohort from Morak et al., harbored double somatic *MSH2* transversions. This finding suggests that MUTYH deficiency could eventually cause somatic mutations in MMR genes, phenotypically mimicking LS. Thus, it is important to bear in mind that biallelic somatic mutations in MMR genes do not necessarily exclude the existence of germline mutations in genes other than MMR.

It has been previously reported that defective excision of A/8-oxoG mismatches in tumors from MAP patients induce an overrepresentation of G:C>T:A somatic transversions in genes such as *APC* and *KRAS* with an incidence of up to 40 and 63%, respectively (Lipton et al. 2003; van Puijenbroek et al. 2008). G>T transversions appear to have a preference for G bases in GAA sequences in *APC,* whereas in *KRAS* a preferential GGT>TGT (c.34G>T, p.G12C) is found

(Al-Tassan et al. 2002; Jones et al. 2004). Conversely, the frequency of *KRAS* transversion p.G12C in unselected CRCs is about 3-4% (Andreyev et al. 2001; van Puijenbroek et al. 2007). Accordingly, sixty-seven percent of the analyzed tumors (6 out of 9) from biallelic *MUTYH* patients had *KRAS* c.34G>T mutation. Consequently, the *KRAS* mutation analysis could be useful as a pre-screening method to select patients with CRC who are eligible for *MUTYH*. This might be particularly relevant in patients with early onset CRC in the absence of polyposis, (Knopperts et al. 2013). Noteworthy, the analysis of the series of patients with MSH2/MSH6-negative tumors by means of our NGS custom panel identified in case 106 (a germline biallelic *MUTYH* carrier), double somatic G>T transversions in *APC*, but a transition c.35G>A (p.G12D) in *KRAS* (data not shown).

So far, the role of germline *MUTYH* monoallelic mutations in cancer risk is a matter of controversy. Many researchers have found a modest increased susceptibility to cancer risk associated to monoallelic mutations (Croitoru et al. 2004; Jones et al. 2009; Khalaf et al. 2013; Win et al. 2014), especially when codon 396 is affected (Khalaf et al. 2013). However, larger studies have failed to replicate these findings (Francesc Balaguer et al. 2007; Lubbe et al. 2009; Ma, Zhang, and Zheng 2014; Theodoratou et al. 2010). The lack of differences in the number of polyps between monoallelic carriers and wildtype group observed in our study is consistent with a weak susceptibility effect of these monoallelic mutations. It may well be that monoallelic carriers are predisposed to somatic mutations in *MUTYH* gene. In fact, (from Article 4), the endometrial tumor of a carrier of the germline heterozygous *MUTYH* p.G396D had acquired a somatic *MUTYH* heterozygous missense variant (c.643G>A; p.V215M). This variant is predicted deleterious by all *in silico* analyses, probably constituting a *bona fide* second hit in this tumor. While the patient had a personal history of 3 LS-associated tumors (2 colorectal and 1 endometrial) only the endometrial one was available. Further analyses are needed to elucidate the role of somatic second hits in *MUTYH* gene.

3.2. The role of the DNA repair FAN1 gene in LLS

We found three missense variants in the *FAN1* gene among 30 LLS cases with MSH2/MSH6 deficient tumors. The c.1856T>A (p.M619K) was predicted probably pathogenic

by *in silico* tools (at functional and structure levels) and c.434G>A (p.R145H) and c.1129C>T (p.R377W) demonstrated cosegregation in CRC affected relatives. As FAN1 interacts with MMR proteins MLH1, PMS2 and PMS1 (Cannavo et al. 2007) and has been related to maintenance of genome stability (Kinch et al. 2005; MacKay et al. 2010; O'Donnell and Durocher 2010), the identification of germline *FAN1* variants in Lynch-like patients suggest that FAN1 deficiency might impair MMR activity to a certain degree, leading to MMR deficient tumors.

FAN1 biallelic mutations are associated to karyomegalic interstitial nephritis (KMIN) (Zhou et al. 2012), FAN1 copy number variants have been associated to neurological conditions (Ionita-Laza et al. 2014) and FAN1 monoallelic mutations recently associated to hereditary MSS CRC (Seguí, Mina, et al. 2015). Biallelic mutations in KMIN patients are usually localized towards the C-terminus of *FAN1*, in contrast CRC associated mutations do not appear to have a preferential location (Seguí, Mina, et al. 2015; Article 3).

Our work is the first study linking *FAN1* to Lynch-like syndrome. The obtained results, together with the recently reported association between *FAN1* and fCRC type X suggest that *FAN1* may be included in the next-generation hereditary cancer panels that would help to decipher at the genotype level the phenotypic overlap between distinct colorectal cancer syndromes. While suggestive, these results must be taken with caution. Further studies in larger series and functional analysis of identified variants are mandatory to refining the role of *FAN1* mutations in LLS. In this regard, immortalization of lymphocytes B of carriers of *FAN1* missense variants is ongoing to enable studying their sensitivity to mytomicin C as a surrogate of DNA interstrand crosslink ability.

3.3. Germline and somatic mutations in other CRC-associated genes

To the best of our knowledge, this is the first report of a germline predicted pathogenic *BUB1* variant in a patient with breast and endometrial cancers. Recently, germline heterozygous mutations in this gene, a component of the spindle assembly checkpoint (SAC) responsible for correct chromosome segregation (Leland et al. 2009), have been identified in patients with in early onset and familial CRC (Hanks et al. 2004; de Voer et al. 2013). In our

series, the identified variant in *BUB1* (c.3005C>G, p.T1002S; case 115), affected the protein kinase catalytic domain and is predicted to destabilize the protein (data not shown). Studies in mice have shown that mutations affecting the kinase catalytic domain appear to have a dominant negative function by competing for kinetochore binding or preventing interactions with other SAC components (Leland et al. 2009). Since *BUB1* mutations can be responsible for variegated aneuploidy, cytogenetic analysis as well as cosegregation analysis in the family should be of help in clarifying the pathogenicity of the identified variant (de Voer et al. 2013).

SETD2, a gene encoding for a H3K36 trimethyltransferase, was included in our customized NGS gene based on the observation that depletion of SETD2 resulted in MSI and elevated mutation rates *in vivo* as H3K36me3 is necessary for recruiting MSH2/MSH6 to chromatin (Li et al. 2013). The identification in our series of 3 LLS patients harboring germline *SETD2* predicted pathogenic variants may be consistent with a putative causal role in LLS. The 3 identified probands were diagnosed of CRC before age 50. Besides cosegregation and functional analysis, epigenetic analysis in biological samples of these patients should be of help in their characterization. So far, other indirect evidences point to a role for *SETD2* in MSI tumors. Somatic *SETD2* mutations have been detected in a subset of gastric tumors displaying MSI without known MMR gene mutations (Boussioutas et al. 2006). However, in renal cell carcinoma *SETD2* mutations have been associated with demethylation at non-promoter regions (Creighton et al. 2013). Finally, it is noteworthy that we were not able to confirm a role for germline *POLE* and *POLD1* mutations in this subset of MMR-deficient tumors (Church et al. 2013).

Somatic subexome analysis at a high coverage has provided interesting results in this preliminary analysis. Somatic double hits in MMR genes were evidenced in two tumors confirming previous reports. The remaining three, double heterozygote mutations in MMR genes and/or proof-reading polymerases were identified. The limited number of cases analyzed precludes drawing conclusions on these findings although it must be beard in mind that pediatric tumors arising in CMMR-D cases strongly associate with mutations in the exonuclease domain of proof-reading polymerases. Finally, our observations reinforce the notion that variation MSH2 or MSH6 may be a frequent event in these cases in line with previous reports (Geurts-Giele et al. 2014; Haraldsdottir et al. 2014; Mensenkamp et al. 2014; Sourrouille et al. 2013) while somatic hypermethylation does not play a significant role.

The yield of subexome testing is directly related to the selection of genes, the sample analyzed as well as the quality and depth of the analysis. While mean coverage is high (1200x) is similar for PBL and FFPE DNA in FFPE is highly variable depending upon the amplicon chosen. Using this coverage we have ruled out germline mosaicisms with a 5% cut-off value in PBLs. Regarding somatic testing all reported mutations have been detected in amplicons with a good coverage (1400x) making our findings dependable. However, variability may have lead to the loss of other relevant findings.

4. FINAL REMARKS

In a series of 160 LS suspected patients 15 cases have been definitively reclassified as LS, MAP or sporadic (double hits or somatic methylation cases). Furthermore, we have provided with suggestive evidence that germline variants in other relevant CRC genes may account for a minority of these cases. Altogether, the results obtained further evidence the great heterogeneity present in this subset of cases (Castillejo et al. 2014; Elsayed et al. 2014; Haraldsdottir et al. 2014; Mensenkamp et al. 2014; Palles et al. 2012; Seguí, Navarro, et al. 2015).

We propose an alternative strategy (Fig. 26), starting with the analysis of constitutional *MLH1* methylation in cases with multiple primary CRCs or in patients younger than 50 years when somatic MLH1 methylation is present. Furthermore, our results point to the use of high-throughput mutational analysis both at germline and somatic level for the analysis of multiple susceptibility genes. These analyses will be eventually complemented by functional analysis of the variants observed aiming at determining the clinical relevance of variants. Further studies of larger series and more in-depth functional characterization of variants detected are mandatory in order to establish the true clinical validity of the proposed strategy.

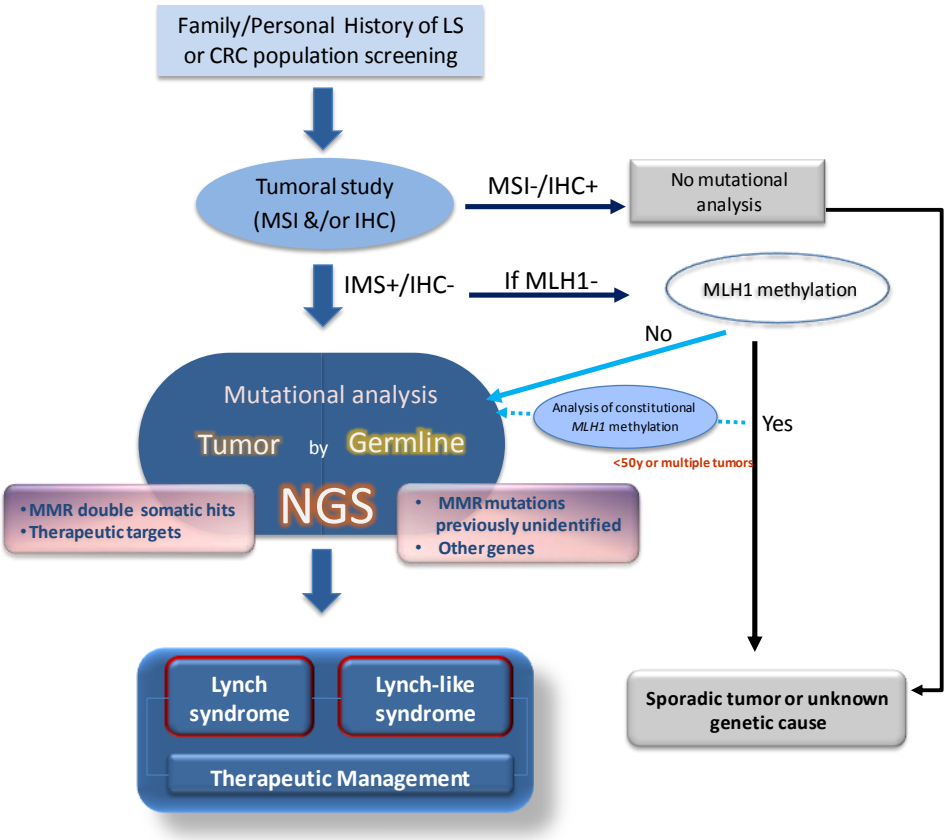


Figure 26. Proposed algorithm for Lynch syndrome screening.

CONCLUSIONS

1. Somatic methylation in *MSH2* and *MSH6* tumors is not of help in ruling out LS in contrast with somatic *MLH1* methylation.

2. Constitutional epimutations in *MLH1* gene represent a minor fraction (1-2%) of suspected Lynch syndrome cases. A refined molecular characterization of these cases is essential for genetic counseling of probands and relatives.

3. A small number of germline variants have been identified in the promoter regions of MMR genes in LLS cases. Its significance remains unclear until further functional characterization is performed.

4. Pathogenicity assessment of *MSH2* variants by means of cDNA study and multifactorial analysis allows the identification of LS in a significant number of MSH2/MSH6 negative cases.

5. Germline biallelic *MUTYH* mutations are responsible for up to 3% of Lynch-like syndrome.

6. Germline mutations in the DNA repair *FAN1* gene may account for a relevant proportion of Lynch-like syndrome.

7. The combined germline and somatic assessment of the mutational status of CRC-associated genes by means of a subexome panel is useful for the elucidation of the molecular basis of a relevant number of suspected LS.

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ANNEXES

ANNEX I: Additional publication

New insights into POLE and POLD1 germline mutations in familial colorectal cancer and polyp

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Germline mutations in DNA polymerase ε (*POLE*) and δ (*POLD1*) have been recently identified in fam multiple colorectal adenomas and colorectal cancer (CRC). All reported cases carried *POLE* c.1 (p.Leu424Val) or *POLD1* c.1433G>A (p.Ser478Asn) mutations. Due to the scarcity of cases reported s accurate clinical phenotype has not been defined. We aimed to assess the prevalence of these recurre tions in unexplained familial and early-onset CRC and polyposis, and to add additional information to d clinical characteristics of mutated cases. A total of 858 familial/early onset CRC and polyposis patie studied: 581 familial and early-onset CRC cases without mismatch repair (MMR) deficiency, 86 ca MMR deficiency and 191 polyposis cases. Mutation screening was performed by KASPar genotypin and/or Sanger sequencing of the involved exons. *POLE* p.L424V was identified in a 28-year-old polyp CRC patient, as a *de novo* mutation. None of the 858 cases studied carried *POLD1* p.S478N. A new m *POLD1* c.1421T>C (p.Leu474Pro), was identified in a mismatch repair proficient Amsterdam II family. I genicity was supported by cosegregation in the family, *in silico* predictions, and previously publish assays. *POLE* and *POLD1* mutations explain a fraction of familial CRC and polyposis. Sequencing the pr ing domains of *POLE* and *POLD1* should be considered in routine genetic diagnostics. Until additional is gathered, *POLE* and *POLD1* genetic testing should not be restricted to polyposis cases, and the pre *de novo* mutations, considered.

INTRODUCTION

Estimates indicate that familial colorectal cancer (CRC) defined by the presence of two or more first-degree relatives affected with CRC involves over 20% of all cases (1–3). Nevertheless, CRC syndromes caused by known high-penetrance G collectively account for only 2–6% of all CRC cases mutations and epimutations in the DNA mismatch repa genes *MLH1*, *MSH2*, *MSH6* and *PMS2* cause Lynch explaining a proportion of hereditary non-polyposis C

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mutations in *APC* and *MUTYH* primarily predispose to multiple colonic adenomas, a benign precursor of CRC; a 40 kb upstream duplication in *GREM1* cause hereditary mixed polyposis; and several types of hamartomatous polyposes are explained by mutations in *SMAD4*, *BMPR1A*, *STK11* and *PTEN* (4–9). Nevertheless, there are still a number of CRC families suggestive of carrying a mutation in a high-penetrance predisposition gene, but without mutations in the known genes. Among these, a number of familial adenomatous polyposis cases are not explained by germline mutations in *APC* or *MUTYH*.

Recently, using a combination of whole-exome sequencing and linkage analysis in probands with >10 adenomas by age 60 but no germline mutations in *APC*, *MUTYH* or the *MMR* genes, Palles *et al*. identified DNA polymerase ε (*POLE;* MIM #174762) and δ (*POLD1;* MIM #174761) mutations in individuals/families with multiple colorectal adenomas and CRC (10). In all, two pathogenic variants, *POLE* c.1270C>G (p.Leu424Val) (NM_006231) and *POLD1* c.1433G>A (p.Ser478Asn) (NM_002691), and an additional variant whose pathogenicity has not yet been determined, *POLD1* c.981C>G (p.Pro327Leu), were identified. All three genetic changes affect the proofreading (exonuclease) domain of the respective polymerase, suggesting deficient proofreading repair during DNA replication (10–13).

After a comprehensive screening of the identified pathogenic mutations in over 3800 CRC patients of European ancestry enriched for a familial CRC history, multiple adenomas and early-onset disease, a total of 13 families with *POLE* p.L424V and 3 with *POLD1* p.S478N were identified (10). To date, no additional *POLE*/*POLD1* mutated families have been reported in the literature. Clinical data from the reported families indicate that the two pathogenic mutations show dominant inheritance and confer high risk to multiple colorectal adenomas, large adenomas, early-onset CRC or multiple CRCs. *POLD1* p.S478N also confers increased risk to endometrial cancer in female carriers. Nevertheless, the phenotype varies among carriers, and until additional cases are identified, an accurate description of the clinical characteristics of this syndrome cannot be provided (13).

In this study, we aimed to assess the prevalence of *POLE* p.L424V and *POLD1* p.S478N in polyposis and non-polyposis familial and early-onset CRC cases, and to add additional information to help define the phenotypic/clinical characteristics of mutated cases.

RESULTS

Neither *POLE* p.L424V nor *POLD1* p.S478N was identified in genetically uncharacterized familial non-polyposis CRC cases, including 581 MMR-proficient and 86 MMR-deficient cases. Likewise, *POLD1* p.S478N was not detected in 191 polyposis cases.

POLE p.L424V was identified in a polyposis family (Series no. 1) (Fig. 1A). The index case was a female patient diagnosed with CRC (pT2pN0pM0) and >35 colonic polyps at age 28. From a total of 33 polyps analyzed, 31 were adenomas, 1 a hyperplastic polyp and 1 a mixed polyp. At 30 years old, 2 years after the surgery, she had developed 8 additional adenomas. No genetic alterations in *APC* and absence of the common *MUTYH* variants had been identified. No loss of heterozygosity (LOH) of the *POLE* chromosomal region, analyzed with two informative microsatellites 1.13 Mb apart, and studying the alle of the mutated and wild-type alleles by SNaPshot, w tumor DNA extracted from the colon tumor dev mutation carrier (Supplementary Material, Fig. S1)

Based on the clinical findings of the proband, h subjected to a colonoscopy at age 56, whic pT2pN0pM0 tumor (adenocarcinoma arising fro lous adenoma) at the proximal colon and one polyp. Her mother was diagnosed with breast ca and died at 41. No information on gastrointestinal cl in the mother had been reported, and no colon cance measures were followed in the maternal family bra of cosegregation revealed that the father did not carr mutation, suggesting a different etiology for his C was confirmed by microsatellite analysis (data not wise, p.L424V was not identified in the DNA extr archived cytology sample obtained from an affected tasis) of the mother's breast cancer. Therefore, these cated that *POLE* p.L424V occurred as a *de n* mutation in the index case.

On account of the mutation-screening method use no.2, consisting of sequencing exons 13 and and *POLD1*, respectively, a novel genetic cha c.1421T>C (p.Leu474Pro), was detected in an MMR-proficient family. The index case was a f diagnosed with a well-differentiated left c (pT2pN0pM0) and a synchronous gastrointes tumor (GIST) in the large bowel at age 36. No pol found during surgical removal or follow-up. Her mo nosed with endometrial cancer at age 52. A mate diagnosed with metachronous CRC (pT3pN0pM0) rial cancer (Stage IB) at ages 33 and 56, respect polyps were found in the intestinal tract during surg tion and follow-up. A maternal uncle was diagnosed cancer at age 72, and his daughter died of a brain tu The maternal grandmother died from a bladder ca (Fig. 1B). Cosegregation analysis performed in aunt, diagnosed with CRC (33 years) and endon (56 years), confirmed her status of heterozygous c fore, the mother of the index case was an obligate mu

The variant *POLD1* p.L474P is localized conserved residue located within the proofrea of DNA polymerase δ. *In silico* analysis usi PolyPhen-2, Condel and SIFT algorithms predi functional effects with scores of −3.36 (deleteri ably damaging), 1 (deleterious) and 0 (damaging), Human *POLD1* p.L474 is the homologous residue *Saccharomyces cerevisiae.* The mutation p.L479 organism has been shown to cause a mutator ph Moreover, human *POLD1* p.L474 is the paralog the human *POLE* p.L424, the residue where *POLE* p.L424V mutation occurs (10). In summa from cosegregation, *in silico* predictions of the v tionality and yeast functional assays strongly sug genic nature for *POLD1* p.L474P.

Mutation screening of the driver genes *KRAS* (c 13, and exons 3 and 4), *NRAS* (exons 2–4), and *BR* in the colorectal tumor developed by the index ca endometrial tumors developed by her maternal a no somatic mutations.

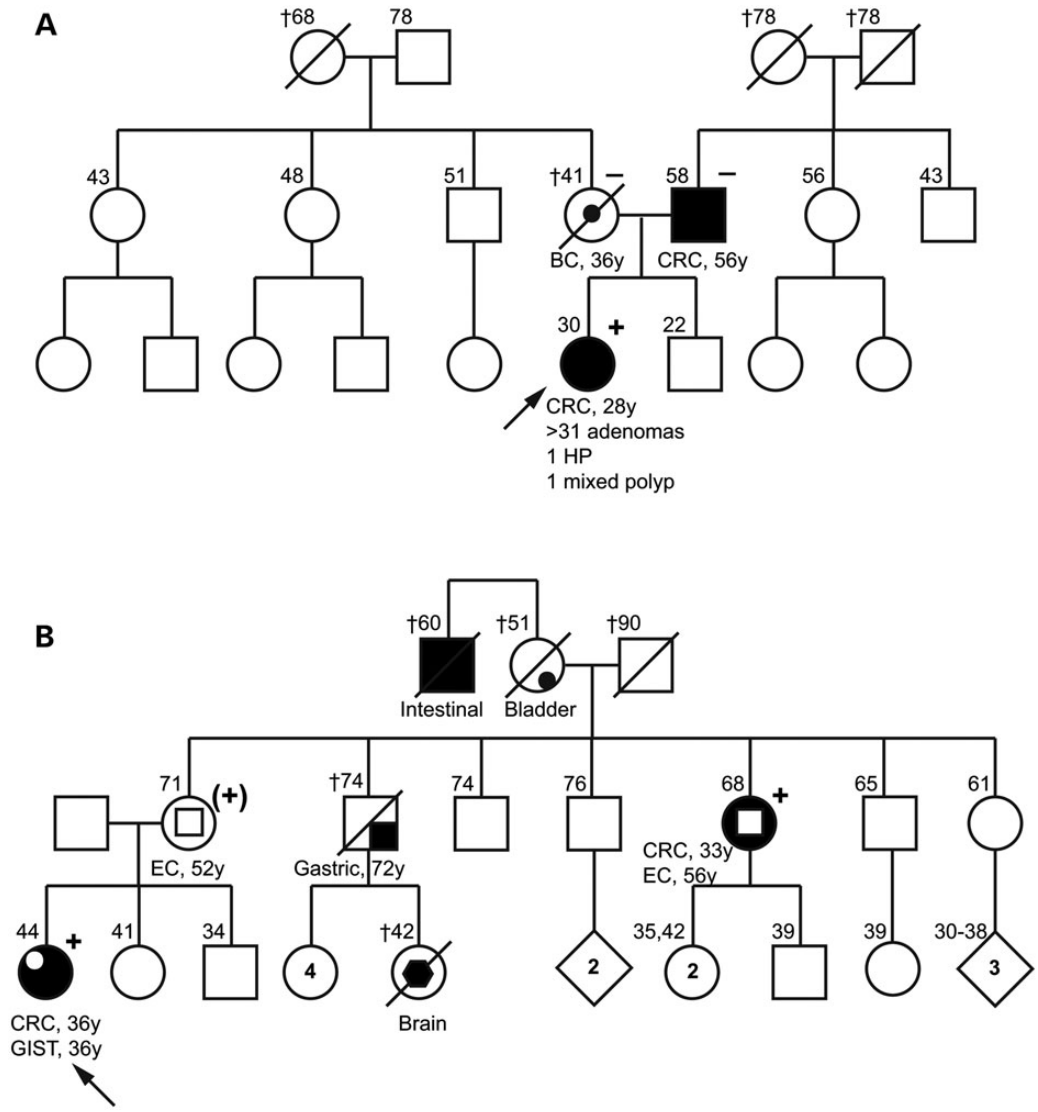

Figure 1. Pedigrees of the families with *POLE* p.L424V (**A**) and *POLD1* p.L474P (**B**) mutations. Filled symbol, CRC; centered filled circle, breast ca right filled circle, bladder cancer; centered unfilled square, endometrial cancer; top-left unfilled circle, GIST; bottom-right filled square, gastric cancer; fi tumor of the central nervous system; +, mutation carrier; (+) obliged mutation carrier; -, wild-type; arrow, index case. Ages at information gathering or a available, are indicated on the top-right corner of each individual's symbol. CRC, colorectal cancer; BC, breast cancer; EC, endometrial cancer; GIST, ga stromal tumor; HP, hyperplastic polyp; y, years.

DISCUSSION

POLE p.L424V and *POLD1* p.S478N mutation screening in 858 Caucasian (Spanish) patients with CRC and/or colonic polyposis, enriched for a family history of colorectal tumors, multiple colonic polyps and/or early-onset disease, identified one carrier of *POLE* p.L424V. This accounts for 0.12% (1/858) of the total, 0.52% (1/191) of the polyposis cases, and 0.86% (1/116) of the adenomatous polyposes studied. Despite its infrequency and based on the simplicity of the test, our findings provide further evidence to advice that at least *POLE* p.L424V, as a recurrent mutation, should be tested in adenomatous polyposis cases without mutations in *APC* and *MUTYH*.

Together with the family identified in our series, a total of 14 families carrying the *POLE* p.L424V mutation have been reported and described in the literature (10). Eleve were CRC-only and/or polyposis families. Of the othe ilies previously described, an astrocytoma and tum ureter, ovary and breast were reported in mutation c probable mutation carriers, who had also been diagn at least two additional colorectal tumors (10). Re additional carrier of a *POLE* mutation, the deletio 5622delGT, has been identified in a patient diagn CRC at 26 years of age, with no further informatio about family history of cancer or polyposis (15). In c the p.L424V mutation occurred *de novo* and caused e CRC (28 years) and adenomatous polyposis. To date first *de novo* case reported for *POLE*/*POLD1* germ tions. Nevertheless, as occurs in 20% APC mutatio

with an apparent *de novo* mutation (16), it could also be a consequence of a somatic mosaicism in one parent. Being *POLE* p.L424V a recurrently found mutation, it can be hypothesized that its recurrent nature may be the result of a founder effect. Even though this could still be true for certain instances, the existence of *de novo* p.L424 V mutations supports the idea of a mutation hotspot, which may also originate non-founder recurrent mutated cases.

In this study, we also report a novel mutation, *POLD1* p.L474P, identified in an Amsterdam II family without defects in the MMR system. This finding suggests that polymerase proofreading mutations in *POLD1* explain a proportion of the uncharacterized hereditary non-polyposis CRC cases. This finding supports the screening of the genes, at least of *POLD1*, in non-polyposis CRC cases. In this regard, the term 'polymerase proofreading-associated polyposis' may be misleading and should be carefully used, at least until more POLE/POLD1 families are described and the full phenotypic spectrum of this syndrome is refined.

In addition to the family herein identified, carrying *POLD1* p.L474P, three additional families with germline *POLD1* mutations have been previously described, all of them carrier of p.S478N (10). In three of the four *POLD1* families reported, including the one described here, two or more endometrial tumors have been diagnosed, indicating the importance of cancer surveillance of this type of tumor in *POLD1* mutation carriers (10,17).

Tumors developed in the context of polymerase proofreading mutations, both germline and somatic, show an ultramutated, apparently microsatellite-stable phenotype, sometimes leading to over a million base substitutions per tumor. In these tumors, the mutation spectrum is changed, with a particular increase in the proportion of G : C to T : A and A : T to C : G transversions. Therefore, it would be expected to find numerous mutations in common CRC genes. However, except for rare mutations in APC, the frequency of mutations in other driver genes is low (13). Here, the screening of common *KRAS*, *NRAS* and *BRAF* mutations in two tumors (1 colorectal and 1 endometrial cancer) from two *POLD1* p.L474P carriers, which also showed MMR proficiency, revealed no mutations, even when some of the most common mutations found in those genes in colorectal cancer, such as *KRAS* c.34G>T (p.G12C) or *BRAF* c. 1799T>A (p.V600E), are transversions. This agrees with the results obtained by Palles *et al*. (10), where no mutations in driver genes were identified in 4 of 6 tumors from five *POLD1* mutation carriers, and in 5 of 10 tumors from three *POLE* mutation carriers. Additional studies analyzing the mutation burden and the presence of mutations in additional known driver genes in the tumors developed by *POLE* and *POLD1* mutation carriers, both CRC and endometrial, will provide a clearer picture of the somatic molecular landscape of this syndrome.

Our results, together with the information gathered so far, support the recommendation of sequencing the exons encoding the proofreading domains of *POLE* and *POLD1* in all familial CRC and polyposis cases without mutations in the known predisposing genes. Based on our findings, *POLE* and *POLD1* mutation screening should not be restricted to polyposis cases, and the presence of *de novo* mutations should be taken into account. Also, whenever endometrial cancer cases are reported in the family, genetic testing of *POLD1* should be prioritized.

MATERIALS AND METHODS

Patients

A total of 858 familial/early-onset CRC and poly
from 840 families were included in the anal
informed consent was obtained from all subjects
received the approval of the Ethics Committees o
institutions.

Series no.1

A total of 612 familial and/or early-onset colorect
or polyposis patients from 594 families witho
mutations in the known CRC or polyposis genes
in the study. All had been referred to the Geneti
Units of the Catalan Institute of Oncology in
region of Catalonia between 1999 and 2012.
based on familial history of colorectal cancer or pol
of early-onset colorectal cancer and/or person
polyposis at early age.

All non-polyposis cases ($n = 524$) had been pre
for MMR deficiency, either by immunohistoche
MMR proteins MLH1, MSH2, MSH6 and PMS
PCR-based microsatellite instability analysis. M
tumors ($n = 86$) did not present somatic *MLH1* pro
lation and/or the *BRAF* V600E mutation, thus sug
editary component. Nevertheless, no germline m
identified in the candidate MMR genes, according
protein expression pattern in the tumor. Clinical fe
polyposis cases are shown in Table 1.

All polyposis patients ($n = 88$) had underg
genetic testing of the three most recurrent genet
Spanish population, i.e. p.Tyr179Cys, p.Gly
p.Glu410Glyfs*43 (NM 001128425.1) (18). If
three was detected, all the coding regions of *MUT*
sequently sequenced. When the number of adenor
the *APC* gene, including exons and flanking regi
sequenced. Clinical features of polyposis cases
Table 2.

Series no.2

Series no.2 consisted of 246 uncharacterized he
and/or polyposis index patients. The 143 non-po
patients included in the study were recruited
Cancer Genetic Counseling Units of the Spani
Valencia between 2005 and 2013. Of them, 63
the Amsterdam criteria (I or II) and were MMR-p
remaining 80 cases were diagnosed with MMR-p
polyposis CRC and had two or more first or s
relatives diagnosed with a Lynch syndrome-relat
gardless of age (Bethesda criterion no. 5) (Table
of MMR deficiency was assessed either by immunc
try of the MMR proteins and/or by PCR-based
instability analysis. Biological samples and clinic
information were obtained from the Valenc
Network and from the Hereditary Cancer Pro
Valencia Region, both in Spain.

A total of 103 polyposis cases were recruited thr
POLIP project, which comprises a multicentric S
(19). All cases were diagnosed with attenuated p
>10 polyps and at least one first-degree relative

Table 1. Characteristics of the non-polyposis CRC cases analyzed

	N (fam.)	Criteria *n* (%) Ams. I	Ams. II	Beth.	n.a.	Age at can Mean (±
Series no.1 (506 families)[a]						
MMR-proficient[b]	438 (423)	31 (7.1%)	11 (2.5%)	390 (89.0%)	6 (1.4%)	49.0 (± 12
MMR-deficient[c]	86 (86)	1 (1.2%)	4 (4.7%)	63 (73.3%)	18 (20.9%)[d]	51.4 (± 13
Series no.2 (143 families)						
MMR-proficient[b]	143 (143)	17 (11.9%)	46 (32.2%)	80 (55.9%)	0	49.4 (± 11
Total	667 (649)	49 (7.4%)	61 (9.1%)	533 (79.9%)	24 (3.6%)	–

MMR, mismatch repair; *N*, number of individuals; fam., number of families; Ams., Amsterdam criteria (I or II); Beth., Bethesda criteria; n.a., not availa
standard deviation.
[a]Three families shared MMR-proficient and MMR-deficient cases.
[b]Non-polyposis cases whose tumors showed microsatellite stability and intact expression of the MMR proteins MLH1, MSH2, MSH6 and PMS2.
[c]Non-polyposis cases whose tumors showed microsatellite instability and/or loss of expression of at least one MMR protein.
[d]Cases referred from the Department of Pathology (CSUB, IDIBELL) to the Hereditary Cancer Program (ICO, IDIBELL) based on tumor histopatholo
suggestive of MMR deficiency, which was subsequently confirmed. Somatic promoter *MLH1* methylation was discarded and/or the presence of *BRA*
confirmed. No information on familial cancer history was available.

Table 2. Characteristics of the polyposis cases analyzed

	Adenomatous polyposis[a]	Attenuated adenomatous polyposis[b]	Non-adenomatous polyposis[c]
Series no.1 (n = 88)			
N (%)	15 (17.0%)	42 (47.7%)	14 (15.9%)
Mean age at polyposis diagnosis (± SD)	43.0 (± 11.1)	53.3 (± 13.3)	51.8 (± 8.5)
CRC; *n* (%)	9 (60.0%)	31 (73.8%)	8 (57.1%)
Polyposis family history; *n* (%)	4 (26.7%)	14 (33.3%)	3 (21.4%)
CRC family history; *n* (%)	2 (13.3%)	11 (26.2%)	4 (28.6%)
Series no.2 (n = 103)			
N (%)	0	58 (56.3%)	42 (40.8%)
Mean age at polyposis diagnosis (± SD)	0	60.6 (± 10.5)	50.9 (± 9.7)
CRC *n* (%)	0	21 (36.2%)	9 (21.4%)
Polyposis family history; *n* (%)	0	16 (27.6%)	40 (95.2%)
CRC family history; *n* (%)	0	54 (93.1%)	13 (31.0%)
TOTAL (*n* = 191)	15 (7.8%)	100 (52.4%)	56 (29.3%)

N, number of individuals; SD, standard deviation; CRC, colorectal cancer; n.a., not available data.
[a]Adenomatous polyposis: >100 adenomatous polyps.
[b]Attenuated adenomatous polyposis: 10–100 adenomatous polyps.
[c]Non-adenomatous polyposis includes hyperplastic, serrated and mixed polyposis.
[d]Referred to the corresponding unit of genetic diagnosis as 'polyposis' but with no specific clinical information available.

CRC. All cases had previously undergone genetic testing of the three most frequent *MUTYH* genetic variants in Spanish population, by sequencing exons 7 and 13 of the gene. If one of these three was detected, all the coding regions of *MUTYH* were subsequently sequenced. The APC gene was analyzed by Sanger sequencing in all individuals with >10 adenomas.

POLE p.L424V and *POLD1* p.S478N screening

In Series no.1, KASPar assays (KASP-By-Design genotyping assays, LGC group, Teddington, UK) were used to genotype the two mutations. Reactions were carried out in the LightCycler 480 real-time PCR detection system (Roche Diagnostics GmbH, Germany), including a corresponding positive control in each run. Positive controls for *POLE* p.L424V and *POLD1* p.S478N were kindly provided by Professor Ian Tomlinson (The Wellcome Trust Center for Human Genetics, Oxford, UK). Genotype calling was performed automatically by the LightCycler 480 II software. Validation of genotyping results deviated from the wild-type cluster, analysis of samples that had failed (no amplification) the genotyping experiment, and cosegregation studies, were carried out by direct auto
quencing. Primers and PCR conditions are shown in Su
tary Material, Table S1. Sequencing was performed
Sequencer 3730 and data analyzed using Mutation
v.3.10.

In Series no.2, Sanger sequencing was used to
mutations in exon 13 of *POLE*, where *POLE* p.
located, and in exon 11 of *POLD1*, where *POLD1*
is located. Primers and PCR conditions are shown
mentary Material, Table S1. Sequencing was perfo
3130 Genetic Analyzer (Applied Biosystems, Fo
CA, USA), and data were analyzed using Sequencin
v.5.1 and Variant Reporter v.1.1 (Applied Biosyste
City, CA, USA).

Loss of heterozygosity

Microsatellites mapping close to *POLE* and expand
Mb, D12S1723, D12S1628, D12S357 and D12S1638
lyzed to assess LOH in DNA extracted from form
paraffin-embedded tissue (10). Also, SNaPshot tar

mutation p.L424V was used to assess LOH and to discriminate wild-type and mutated alleles. Primers and conditions are shown in Supplementary Material, Table S1. LOH was scored if the intensity of any allele was reduced by $\geq$50% relative to the other allele after taking account of the relative allelic intensities in paired constitutional DNA.

KRAS, NRAS and *BRAF* mutation screening

Analysis of *KRAS* mutations at codons 12 and 13 was performed using KRAS StripAssay (VienaLab Diagnostics GmbH, Vienna, Austria), following manufacturer's instructions. Exons 3 and 4 of *KRAS*, exons 2, 3 and 4 of *NRAS,* and *BRAF* V600E were assessed by direct automated (Sanger) sequencing. Primers, and PCR and sequencing conditions are available upon request.

In silico prediction analysis

In silico studies to assess the impact of amino acid substitutions (missense variants) on protein structure, function and evolutionary conservation were performed with SNPs3D, PolyPhen-2, SIFT and CONDEL algorithms (20–23).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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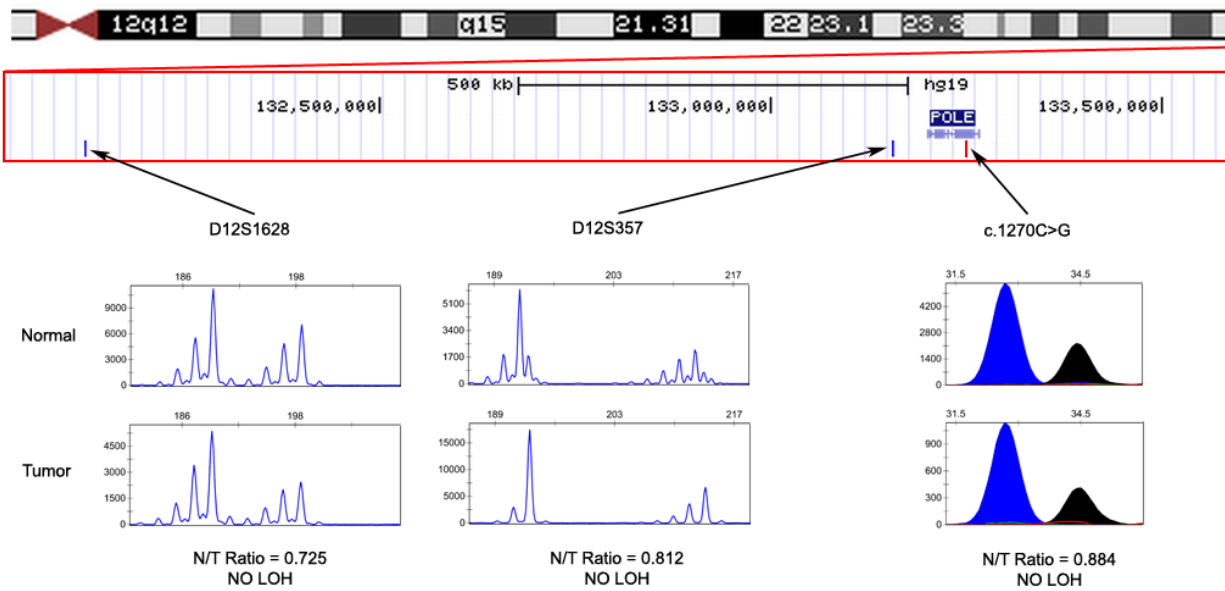
Suppl. Table 1. Primers and PCR conditions.

	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplicon size	Annealing temperature	Series
*POLE*_exon 13	CATCCTGGCTTCTGTTCTCA	GTGGCCATCTGGATGTGTG	223	60ºC	No.2
*POLD1*_exon 11	GTGTGTCCCTGTCCTTGGAA	GTCAGAGGTTGGGGTGAGAG	217	60ºC	No.2
*POLE*_L424V	GGTGCCTGTTAGGAACTTGC	CCGCACACACAGTAAGGAGA	449	57ºC	No.1
*POLD1*_S478N	GGAGTACAAGCTCCGCTCCT	GAAAAAGTGGGCGTCAGGTA	250	57ºC	No.1
SNaPshot					
*POLE*_L424V_LOH	TTACCTTCCTGTGGGCAGTC	TAGCTCCACGGGATCATAGC	73	54ºC	
SNaPshot extension	TTCCTGTGGGCAGTCATAAT	-	-	-	

Suppl. Figure 1. Absence of LOH at *POLE* in the colon tumor developed by the *POLE* L424V mutation carrier. LOH results using two informative microsatelites, D12S1628 and D12S357, an the mutation, assessed by SNaPshot, are shown.



ANNEX II: Directors' Report

As directors of the doctoral thesis of Gardenia María Vargas Parra, titled "**Elucidating the molecular basis of Lynch-like syndrome",** we certify that the doctoral candidate has actively participated in designing and conducting experimental work included in this thesis, analysis of results, discussion and drawing conclusions, and in preparing the final article. The specific contributions in each work are listed below, together with their impact factors at the date of publication.

PUBLISHED ARTICLES

ARTICLE 1:

***MLH1* methylation screening is effective in identifying epimutation carriers.**

Marta Pineda*, Pilar Mur*, María Dolores Iniesta, Ester Borrás, Olga Campos, **Gardenia Vargas**, Silvia Iglesias, Anna Fernández, Stephen B Gruber, Conxi Lázaro, Joan Brunet, Matilde Navarro, Ignacio Blanco and Gabriel Capellá.

(*) Authors contributed equally to this work.

European Journal of Human Genetics (2012) 20, 1256–1264; doi:10.1038/ejhg.2012.136.

Impact factor (2012 JCR Science Edition): 4.319

Contribution of the PhD candidate: Molecular characterization of one (case 34) of the two epimutants reported in this work. Direct sequencing of *EPM2A1P1* gene and *MLH1* gene promoter. *EPM2AIP1* allele-specific expression analysis by single-nucleotide primer extension (SNuPe). Bisulfite conversion of PBL DNA and bisulfite sequencing of the promoter region of *MLH1* gene. Methylation-specific melting curve analysis (MS-MCA) of regions C and D of *MLH1* promoter. Refinement of the pyrosequencing design for the methylation analysis of *MLH1* C and D promoter regions. Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) of PBL and tumoral DNA of case 34. Participation in the writing of the article and preparation of all figures and sections of tables related to the characterization of case 34.

Prevalence of germline *MUTYH* mutations among Lynch-like syndrome patients

Adela Castillejo*, **Gardenia Vargas***, María Isabel Castillejo, Matilde Navarro, Víctor Manuel Barberá, Sara González, Eva Hernández-Illán, Joan Brunet, Teresa Ramón y Cajal, Judith Balmaña, Silvestre Oltra, Sílvia Iglesias, Àngela Velasco, Ares Solanes, Olga Campos, Ana Beatriz Sánchez Heras, Javier Gallego, Estela Carrasco, Dolors González Juan, Ángel Segura, Isabel Chirivella, María José Juan, Isabel Tena, Conxi Lázaro, Ignacio Blanco, Marta Pineda, Gabriel Capellá and José Luis Soto.

(*) Authors contributed equally to this work.

European Journal of Cancer (2014) 50, 2241–2250; doi.org/10.1016/j.ejca.2014.05.022.

Impact factor (2014 JCR Science Edition): 5.417

Contribution of the PhD candidate: Selection of cases for analysis and collection of the samples from five different Catalonian hospitals. Collection of clinico-pathological information from the set of all Catalonian patients. Analysis of *MUTYH* mutations c.536A>G (p.Y179C) and c.1187G>A (p.G396D) by real-time PCR allelic discrimination assay. Analysis of the *MUTYH* c.1227_1228dup (p.E410Gfs*43) by Sanger in this series. Analysis and interpretation of the results, preparation of all figures and tables contained in the article, writing the first draft and preparing the final version of the manuscript. Dr. Adela Castillejo, who shares first co-authorship of this article, was responsible of the study of *MUTYH* variants in the Valencian series of cases.

ARTICLE 3:

Identification of germline *FAN1* variants in MSH2-deficient Lynch-like syndrome patients.

Gardenia Vargas, Estela Dámaso, Matilde Navarro, Tirso Pons, Anna Fernández, Lídia Feliubadaló, Ares Solanes, Silvia Iglesias, Àngela Velasco, Alfonso Valencia, Joan Brunet, Conxi Lázaro, Laura Valle, *Marta Pineda, *Gabriel Capellá.

Submitted for publication.

Contribution of the PhD candidate: Design of the probes for next generation sequencing (NGS). Target enrichment and library preparation. DNA quantification using Qubit and/or Agilent bioanalyzer as well as quality controls of FFPE samples for NGS. Variant calling, filtering and annotation of NGS results. In-silico prediction studies of all FAN1 variants found with NGS, with exception of protein structure predictions. Analysis and interpretation of results, as well as preparing figures and tables. Writing the first draft of the article and preparing the final version.

ARTICLE 4:

Elucidating the responsible cause of MSH2-deficient tumors with no germline mutation detected in suspected LS cases.

Gardenia Vargas, Maribel González, Bryony A. Thompson, Carolina Gómez, Anna Fernández, Jesús del Valle, Silvia Iglesias, Àngela Velasco, Ares Solanes, Joan Brunet, Lídia Feliubadaló, Conxi Lázaro, Marta Pineda, Gabriel Capellá.

Submitted for publication.

Contribution of the PhD candidate: Lymphocyte cultures with and without puromycin, RNA extraction, retrotranscription and set up of long range PCR for *MSH2* splicing analysis. Methylation studies of *MSH2* and *MSH6* promoter regions: design and set up of *MSH2* and *MSH6* genes' promoters sequencing by Sanger. DNA isolation from formalin fixed paraffin-embeded (FFPE) tissues. Sodium bisulfite treatment of FFPE tumor DNA. Design and set up methylation studies of *MSH2* and *MSH6* promoter regions by MS-MCA. Study of promoter hypermethylation by MS-MCA in available tumor samples. Bisulfite sequencing of *MSH2* and *MSH6* promoter regions of Jurkatt cell line. Exhaustive search for the best methodologies for high-throughput parallel study of germinal and tumoral FFPE DNA. Design of the probes for next generation sequencing (NGS). Target enrichment and library preparation. DNA quantification using

NGS, with exception of protein structure predictions. Analysis and interpretation of results, preparing figures and tables. Writing the first draft of the article and preparing the final version.

ANNEXED ARTICLE (PUBLISHED)

ARTICLE 5:

New insights into *POLE* and *POLD1* germline mutations in familial colorectal cancer and polyposis.

Laura Valle, Eva Hernández-Illán, Fernando Bellido, Gemma Aiza, Adela Castillejo, Maria-Isabel Castillejo, Matilde Navarro, Nuria Seguí, **Gardenia Vargas**, Carla Guarinos, Miriam Juarez, Xavier Sanjuán, Silvia Iglesias, Cristina Alenda, Cecilia Egoavil, Angel Segura, María-José Juan, María Rodriguez-Soler, Joan Brunet, Sara González, Rodrigo Jover, Conxi Lázaro, Gabriel Capellá, Marta Pineda, José Luís Soto, Ignacio Blanco.

Human Molecular Genetics, 2014, 23: 13, 3506–3512; doi:10.1093/hmg/ddu058.

Impact factor (2014 JCR Science Edition): 6.677

Contribution of the PhD candidate: Selection of MSI cases and preparation of those samples for analysis. Clinico-pathological data collection of MSI cases. Critical revision of the article before submission.

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As directors of the doctoral thesis of Gardenia María Vargas Parra, titled "**Elucidating the molecular basis of Lynch-like syndrome",** we certify that Adela Castillejo, PhD., co-author of the article "*Prevalence of germline MUTYH mutations among Lynch-like syndrome patients*" exposed on this thesis, had not used these results for the completion of her doctoral thesis. Therefore, we confirm the participation of the PhD candidate in this work.

Prevalence of germline *MUTYH* mutations among Lynch-like syndrome patients.

Adela Castillejo*, **Gardenia Vargas***, María Isabel Castillejo, Matilde Navarro, Víctor Manuel Barberá, Sara González, Eva Hernández-Illán, Joan Brunet, Teresa Ramón y Cajal, Judith Balmaña, Silvestre Oltra, Sílvia Iglesias, Àngela Velasco, Ares Solanes, Olga Campos, Ana Beatriz Sánchez Heras, Javier Gallego, Estela Carrasco, Dolors González Juan, Ángel Segura, Isabel Chirivella, María José Juan, Isabel Tena, Conxi Lázaro, Ignacio Blanco, Marta Pineda, Gabriel Capellá and José Luis Soto. (*) Authors contributed equally to this work.

Contribution of the PhD candidate: Coordination of the study of the Catalonian series. Gardenia Vargas participated in the selection of cases for analysis and collection of the samples in collaboration with five different Catalonian hospitals, as well as active contact with Valencian collaborators. She collected clinico-pathological information from the set of all Catalonian patients. She tuned the analysis of *MUTYH* mutations c.536A>G (p.Y179C) and c.1187G>A (p.G396D) by real-time PCR allelic discrimination assay. She studied all 125 Catalan cases for the three *MUTYH* most prevalent variants in Spain. Gardenia Vargas conducted the analysis and interpretation of the results, she prepared all figures and tables contained in the article, writing the first draft and preparing the final version of the manuscript. Dr. Adela Castillejo, who shares first co-authorship of this article, coordinated the study of the Valencian series and performed the study of *MUTYH* variants in that set of 100 patients.

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