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Germline variants in patients from the Iranian hereditary colorectal cancer registry

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Abstract

Background and aim Hereditary cancer syndromes account for 6–10% of all colorectal cancer (CRC) cases and 20% of early-onset CRC. Identifying novel pathogenic germline variants can impact genetic testing, counseling, and surveillance. This study aimed to determine the prevalence of germline variants associated with hereditary CRC in the Iranian population.

Methods Whole exome sequencing (WES) was conducted on DNA from 101 patients in the Iranian Hereditary Colorectal Cancer Registry (IHCCR). The cohort included 63 high-risk Lynch Syndrome (LS) patients and 38 colorectal polyposis patients. Germline variants and phenotype spectrum were assessed. Relatives of individuals with the mutations received counseling and cascade testing. Gene ontology and protein-protein interaction (PPI) analyses were conducted to elucidate gene roles on protein function.

Results Pathogenic/likely pathogenic (P/LP) variants were identified in Lynch-related genes in 36.51% of patients. P/LP variants in non-Lynch genes (*ATM*, *FH* (mono-allelic), *MSH3*, *PMS1*, and *TP53)* were identified in 26.98% of patients. Among polyposis patients, 50% had P/LP variants in the *APC* gene, and 15.79% had P/LP variants in the *MUTYH* gene. Additionally, 7.89% carried P/LP variants in non-FAP/MAP genes (*BLM*, *BRCA2*, and *PTEN)*. *MLH1* variants were most common in exons 10 and 18, *MSH2* in exon 12, and *APC* gene in exon 16. Cascade testing identified 50% of the tested relatives (40/80). Topology analysis of the protein-protein interaction networks in high-risk LS cases highlighted stronger connections among nodes for genes such as *TP53*, *ATM*, *POLD1*, *CDH1*, *MUTYH*, *WRN*, *NOTCH1*, *SMAD4*, *ERCC4*, *ERCC1*, and *MSH3*. These genes were associated with high penetrance in CRC. The protein-protein interaction analyses of polyposis patients indicated that genes like *POLE*, *MSH6*, *MSH2*, *BRCA2*, *BRCA1*, *MLH1*, *TOPBP1*, *BLM*, *RAD50*, *MUTYH*, *MSH3*, *MLH3*, *PTEN*, *BRIP1*, and *POLK* had a higher degree value and were also associated with high penetrance. Gene ontology and protein-protein interaction (PPI) analysis showed that some of the top-scoring non-Lynch genes were *TP53*, *ATM*, *POLD1*, *CDH1*, *MUTYH*, *WRN*, *NOTCH1*, *SMAD4*, *ERCC4*, *ERCC1*, and *MSH3*.

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Conclusions The study identified crucial germline variants for hereditary polyposis and non-polyposis CRC pathogenesis in the Iranian population. A selective strategy and cascade genetic testing are recommended for the diagnosis of hereditary colorectal cancer syndromes.

Keywords Hereditary colorectal Cancer, Germline variants, Whole exome sequencing (WES), Lynch syndrome, Polyposis syndrome

Introduction

Colorectal cancer (CRC) is among the five most common cancers worldwide [1]. It is the third most commonly diagnosed cancer and the second leading cause of cancer-related deaths [2]. Recent data from Iran's National Cancer Registry (INCR) shows that CRC is the third most common cancer in males and the second in females [3, 4]. The age-standardized incidence rate of CRC has increased in recent years [4]. While most CRC cases are sporadic, up to 35% of the inherited variability is due to genetic factors [5, 6], including a family history of CRC, hereditary cancer syndromes, and common low-penetrance genetic variations. Although some studies have provided more insights into the molecular mechanisms driving CRC, revealing its highly heterogeneous nature at the molecular level, many genetic factors remain unidentified [7, 8]. Recent studies have indicated the involvement of one or more moderate and high-penetrance cancer susceptibility gene(s) in 9.9–15.5% of patients with CRC. In early-onset CRC, this number shows an increased range of 16–25% [9]. Lynch syndrome (LS), the most common hereditary CRC syndrome, is caused by germline variants in the mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6*, *and PMS2*, and also in *EPCAM*, affecting one out of every 25 patients diagnosed with CRC [10]. Recent studies using next-generation sequencing (NGS) have revealed germline variants in cancer-predisposing genes other than Lynch-related genes, including *NTHL1*, *GERM1*, *GALNT12*, *RNF43*, *RPS20*, *MLH3*, and *MSH3* [11, 12, 13, 14].

Other hereditary CRC syndromes related to gastrointestinal (GI) polyposis include familial adenomatous polyposis (FAP; associated with *APC* pathogenic variants) and *MUTYH*-associated polyposis (MAP; caused by biallelic *MUTYH* pathogenic variants). Recently, more genes have been implicated in the development of other forms of hereditary colonic polyposis, such as polymerase proofreading–associated polyposis (PPAP) caused by the pathogenic variants of *POLE* or *POLD1*, *NTHL1*-associated polyposis, adenomatous polyposis related to the biallelic inactivation of MMR genes (such as *MSH3* and *MLH3*), *GREM1*-associated mixed polyposis, and *RNF43*-associated serrated polyposis. Other germline variants in genes such as *STK11*, *PTEN*, *BMPR1A*, and *SMAD4* can also be responsible for rarer cases of GI polyposis [15].

Identifying CRC susceptibility genes or familial polyposis syndromes can direct clinical management, leading to lifesaving interventions. In Iran, about 20–25% of CRC patients have a familial background, and 15% have a strong family history [16, 17], of whom almost 5% fulfil the Amsterdam II criteria, and 10% have loss of MMR protein expression [16, 18].

There is no study on the genetic landscape of hereditary CRC in the Iranian population, with most studies being case reports [19, 20, 21]. This study used whole exome sequencing (WES) to identify the genetic susceptibility to CRC and colorectal polyposis from 101 high-risk patients from three referral clinics [22]. We report common and novel germline variants that might be important in the pathogenesis and predisposition to hereditary CRC and polyposis. The updated phenotypic and genetic map of hereditary CRC could help develop national policies and guidelines for the screening and management of hereditary CRCs in Iran.

Methods

Settings, participants, and eligibility criteria

This study included participants from the Iranian Hereditary Colorectal Cancer Registry, a program for detecting and monitoring patients at high risk of hereditary CRC [16, 23]. The program involves Mashhad, Tehran, and Isfahan University of Medical Sciences. Between January 2019 and December 2021, 101 probands were selected from 2,500 registered patients based on clinical assessments indicating a high risk for hereditary CRC. The inclusion criteria for this study included patients who met the Amsterdam II or revised Bethesda criteria for LS [24, 25], and patients diagnosed with colorectal polyposis, defined as having more than ten polyps in the colon and rectum. This group comprised 63 CRC patients at high risk for LS and 38 with colorectal polyposis. These probands were invited and consented to participate in the study for genetic evaluation using WES. Colon and rectal cancer were defined based on ICD codes, and non-FAP-MAP cases were defined as cases with over ten colon polyps and a family history of cancer but with no pathogenic germline variants in *APC* and/or *MUTYH* genes. Non-Lynch cases were defined as cases that fulfilled the Amsterdam II or Revised Bethesda criteria but with no pathogenic variants in *MLH1*, *MSH2*, *MSH6*, and *PMS2* or *EPCAM* genes.

Demographic, clinical, and pathological characteristics, such as pathological grade, TNM staging system, tumor location, and molecular features, including MSI, MMR IHC, KRAS/NRAS, and BRAF variant analyses, were identified. Sex as a biological variable was analyzed. Family history up to the fourth generation was recorded, and pedigrees were drawn using the website familyhistory.invitae.com. Blood samples (10 ml) were collected for DNA extraction using the Blood DNA Isolation Kit (DENAzist Asia Co., Iran). Ethical approval was obtained, and participants provided written informed consent.

Immunohistochemistry

Expression of MMR proteins (MLH1, MSH2, MSH6, and PMS2) was assessed using IHC with primary monoclonal antibodies (Vitro SA, Master Diagnostica, Spain; RRID: AB_2140114). Tissue sections were prepared and visualized using Novolink Polymer Detection Systems (Leica Company, Wetzlar, Germany) and counterstained with hematoxylin and eosin. Two expert pathologists independently and blindly evaluated the results.

Whole-exome sequencing

Human whole exome enrichment was performed using the Agilent SureSelect V6 Target Enrichment Kit (RRID: SCR_01479), followed by NGS on the Illumina HiSeq4000 platform (RRID: SCR_016383) to yield an average coverage depth of approximately 100X. All exons and flanking 10 bp sequences were detected and analyzed. Quality control of sequencing data was performed on all samples before analysis using the fast QC software (RRID: SCR_014583) sequence pipeline (Illumina) to filter out low-quality reads. Sequenced reads were trimmed for adaptor sequences. The Burrows-Wheeler Aligner (BWA-MEM algorithm) was used for read mapping to the human reference genome (build hs37d5, based on NCBI GRCh37). PCR duplicates were discarded using the Mark Duplicates (Picard, Broad Institute, Cambridge, MA, USA) tool. We used the tools mentioned in the GATK (RRID: SCR_001876) Best Practices (The Haplotype Caller, MuTect2) pipeline for variant calling, and Strelka2 (Illumina) was used for SNV. Indel realignment and base quality score recalibration were performed (GATK, Broad Institute, Cambridge, MA, USA).

Finally, for variant annotation, we used the ANNOVAR tool (RRID: SCR_012821). Several databases were considered for variant annotation. Variants with a minor allele frequency (MAF) of ≥ 0.1% (for heterozygous variants) or ≥ 1% (for homozygous variants) were excluded using 1000 Genomes (Asian: RRID: SCR_008801), Iranom, and Gnomad. SIFT (prediction of damaging: RRID: SCR_012813), PolyPhen2 (HumVar prediction of probably damaging or possibly damaging), and CADD (Phred score ≥ 20) were used for pathogenicity prediction of missense variants. We also selected truncating (nonsense, splice site, and frameshift variants) or missense variants that fulfilled at least two of the three missense pathogenicity tools criteria. The clinical significance of these variants was classified for variant pathogenicity annotations according to Varsome and Gnomad per the Ambry five-tier variant classification protocol (pathogenic, likely pathogenic, variant of unknown significance, likely benign, and benign), which is based on guidelines published by the American College of Medical Genetics and Genomics, the Association for Molecular Pathology, and the International Agency for Research on Cancer. A pathogenic or likely pathogenic variant was defined as a variant that was predicted to result in a stop codon, a frameshift variant, a large duplication or deletion, or a missense variant in the coding region or splice site previously reported within the scientific literature and databases to be pathogenic or likely pathogenic. Particular attention was paid to genes known to be involved in predisposition to CRC and other neoplasms by reviewing data present on OMIM (Online Mendelian Inheritance in Man; http://www.omim.org/) and ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/; RRID: SCR_006169). The remaining rare non-synonymous variants were classified with reference from established databases such as ClinVar, Franklin, and Varsome. Variants classified as pathogenic or novel (not previously reported at the point of analysis) were assessed based on patients' clinical presentations, family history, and reference to the available literature.

Gene ontology (GO) and protein-protein interaction (PPI) network analyses

GO analysis was conducted on critical genes from WES of CRC patients. Candidate genes included 47 for LS and 22 for polyposis. To analyze cellular components (CC), molecular function (MF), and biological processes (BP), ClueGO (version 2.5.8, RRID: SCR_005748) and Cytoscape application (version 3.9.1, RRID: SCR_003032) were used [26]. A significance level of $p \leq 0.05$ was considered for gene ontology analyses.

Protein-protein interaction (PPI) networks were constructed using STRING (version 1.7.0; RRID: SCR_005223) and Network Analyzer (version 4.4.8) [27, 28] applications in Cytoscape networks as they play a significant role in interpreting biological processes [29] and protein functions. Additionally, interaction networks may reveal both the temporal and spatial aspects of forming functional cellular networks [30].

Cascade genetic testing and Sanger sequencing

Novel variants identified by WES were validated using Sanger sequencing (Codon Genetic Group, Iran). The sequencing results were analyzed using the SnapGene software (from Insightful Science; available at snapgene.

com, (RRID: SCR_015052). Family members of mutation carriers received genetic counseling and were offered mutation testing. PCR and sequencing techniques were used to assess whether first-degree relatives (FDR) and second-degree relatives (SDR) carried the mutation. DNA sequencing was conducted on 80 family members, comprising 52 family members related to 14 probands with high-risk LS and 28 family members related to 11 probands with hereditary polyposis.

Statistical analysis and presentation of data

The primary outcome was the detection of pathogenic, likely pathogenic, and variant of unknown significance (VUS) variants in cancer susceptibility genes. Age at CRC diagnosis was analyzed as a continuous variable; other characteristics were categorical. Circos plots (RRID: SCR_011798) were created using the shinyCircos package in R data (RRID: SCR_021252) visualization. To visually present the data distribution and trends, GraphPad Prism version 8.0.0 for Windows (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com) was used.

Results

Germline variants in patients at risk for Lynch syndrome

We categorized 63 CRC patients as high-risk for LS (Amsterdam II or revised Bethesda clinical criteria). WES showed that 36.51% ($n=23$) carried pathogenic/likely pathogenic (P/LP) variants in one or more of Lynch-related genes, while 26.98% ($n=17$) carried P/LP variants in non-Lynch genes (Fig. 1A). Among 23 patients with P/LP variants in Lynch-related genes, 11 had germline variants in *MLH1*, 10 in *MSH2*, 1 in *MSH6*, and 1 in *PMS2* (Table S1). We found P/LP variants in 36 non-Lynch genes, such as *ATM*, *ASXL1*, *FH*, *MSH3*, *PMS1*, and *TP53* (Table S1). No *EPCAM* gene deletion was found. Analysis of Lynch-related genes revealed that the most frequent P/LP variants in the *MLH1* gene are missense, while the *MSH6* gene showed a nonsense variant, and the *PMS2* gene showed a frameshift variant (Fig. 1B). Sanger sequencing was performed to confirm variants identified by WES (Figure S1). Among 52 family members of 14 probands with high-risk Lynch syndrome, half had variants.

Twenty patients (31.75%) carried VUS: four in *MLH1*, *MSH2*, and *MSH6* genes and 16 in non-Lynch genes (Fig. 1A), the most frequent in the *BCR* and *GXYLT1* genes (with an allele frequency of 0.019) (Table S1). Variants in the *MLH1* gene were mainly located in exons 10 and 18 and exon 12 of the *MSH2* gene (Figure S2).

We found 26.98% of high-risk LS probands with P/LP variants in non-Lynch genes such as *ATM*, *ASXL1*, *FH* (mono-allelic), *PMS1*, *MSH3* (mono-allelic), and *TP53* (Fig. 1, Table S1). Gene ontology and PPI analysis showed top-scoring non-Lynch genes were *TP53*, *ATM*, *POLD1*, *CDH1*, and others (Figures S3 and S4, Table S2).

Germline variants in patients with colorectal polyposis

Thirty-eight patients had more than ten colorectal polyps (polyposis). WES revealed that 19 patients (50%) carried P/LP variants in *APC* and six patients (15.79%) carried P/LP variants in *MUTYH* (Fig. 1C). Variants in the *APC* gene were most commonly found in the exon 16 (Figure S5). Three patients had biallelic variants in the *MUTYH* gene and three patients (7.89%) carried P/LP variants in non-FAP/MAP genes, including *BLM*, *BRCA2*, and *PTEN* (Fig. 1C, D; Table S2). Most P/LP variants of polyposis patients were heterozygous. Sanger sequencing was performed for some samples to confirm variants identified by WES. In the families of 11 of the polyposis probands, 29 at-risk family members were tested; of these, 15 were carriers (Figure S1).

Most P/LP variants in the *APC* and *MUTYH* genes were frameshift (Fig. 1D), whereas all VUS in the *APC* and *MUTYH* genes were missense (Fig. 1D). Eight patients (21.05%) carried VUS, one in *APC*, one in *MUTYH*, and six in non-FAP/MAP genes, such as *BRIP1*, *MSH3*, *MLH3*, and *TOPBP1* (Fig. 1C; Table S3).

Clinical characteristics of patients with pathogenic/likely pathogenic variants in Lynch-related and non-Lynch genes

Among patients with P/LP variants in Lynch-related genes, 78.26% (18/23) were identified with MSI high and/or dMMR (Table 1), whereas in patients with P/LP variants in non-Lynch genes, 47.05% (8/17) were MSI high and/or dMMR. Most (95.7%) of patients with P/LP variants in Lynch-related genes fulfilled the revised Bethesda criteria, while 69.56% fulfilled the Amsterdam II criteria. However, 82.35% and 47.05% of patients with P/LP variants in non-Lynch genes fulfilled the revised Bethesda and Amsterdam II criteria, respectively (Table 1). 65.22% and 58.83% of patients with P/LP variants in Lynch-related and non-Lynch genes were male, respectively (Table 1). The mean age of CRC diagnosis in patients with P/LP variants in Lynch-related genes was 44.7 years (ranging from 38 to 70 years), while for patients with non-Lynch genes was 45.8 years. While the majority of patients with P/LP variants in Lynch-related genes (73.91%) had early-onset (≤ 50 years) CRC, only 52.9% of patients with P/LP variants in non-Lynch genes were diagnosed with early-onset CRC (Table 1). The IHC loss of staining of both MLH1 and PMS2 was recorded in 16 out of 47 patients (Table 2).

Two patients had a previous diagnosis of endometrial cancer before CRC. The location of CRC tumors was mainly in the proximal colon (62.21% of patients in this study) and 26.08% were of mucinous histology. Of patients with P/LP variants in Lynch-related genes,

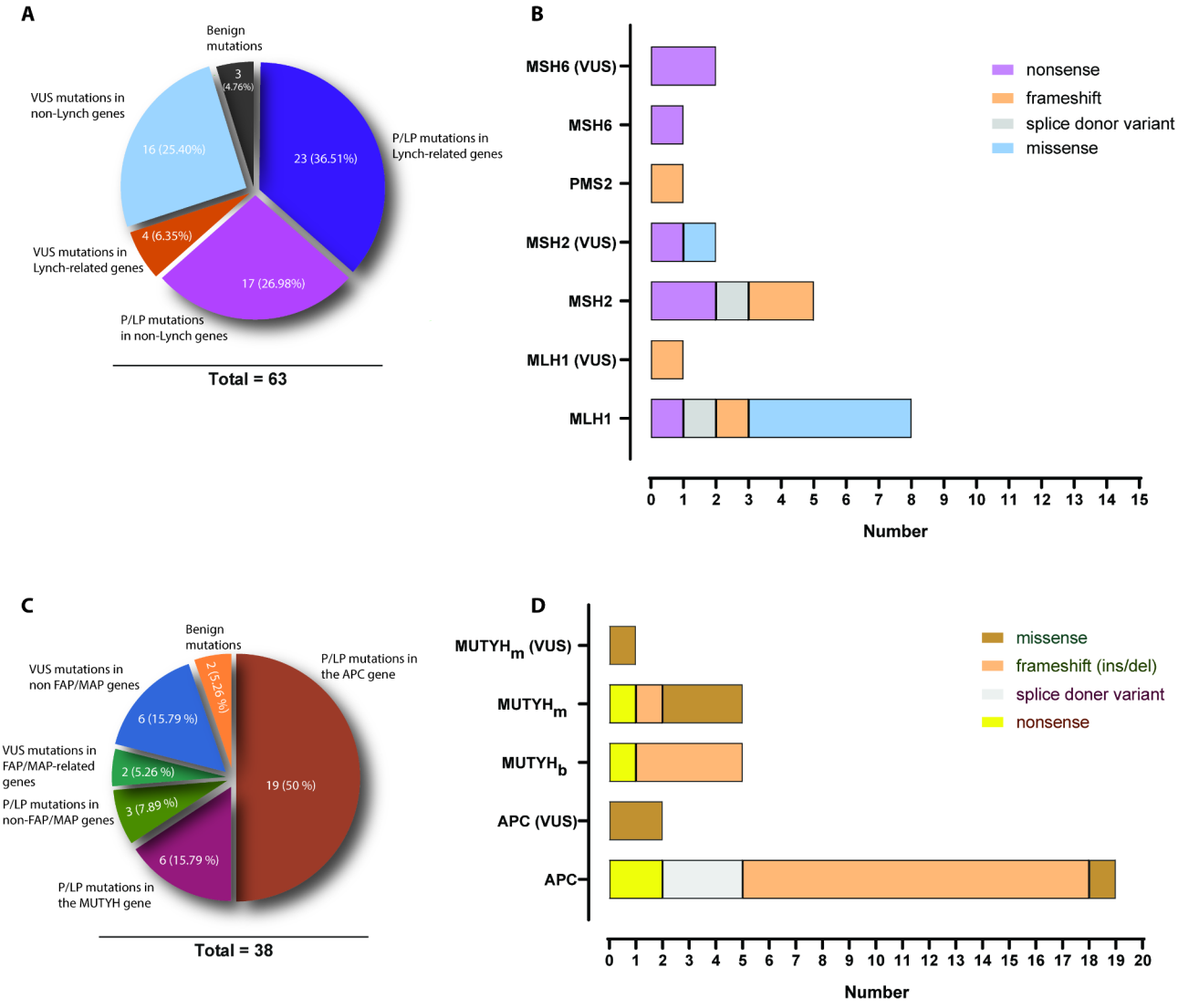


Fig. 1 Germline variants in patients at risk for Lynch syndrome and colorectal polyposis. (**A**) Percentage of pathogenic/likely pathogenic (P/LP) variants and VUS in Lynch-related and non-Lynch genes of patients at risk for Lynch syndrome. (**B**) Number of different variants in four Lynch-related *MLH1*, *PMS2*, *MSH2*, and *MSH6* genes. For example, in the *MLH1* gene, from the P/LP variants, there is one nonsense, one frameshift, one splice donor variant, and five missense variants, while there is only one VUS frameshift variant. (**C**) Percentage of P/LP and VUS variants in patients with colorectal polyposis. (**D**) Number of different types of variants in two FAP/MAP genes of *APC* and *MUTYH*. $MUTYH_m$: mono-allelic variants in the *MUTYH* gene; $MUTYH_b$: bi-allelic variants in the *MUTYH* gene

34.78% had CRC at clinical stage II, whereas 35.29% of patients with P/LP variants in non-Lynch genes had stage III CRC (Table 1).

In this cohort, the most common cancers diagnosed in the relatives of LS patients were colorectal, stomach, breast, prostate, and endometrial (Fig. 2A). Of 11 probands with P/LP variants in the *MLH1* gene, the mean age of CRC diagnosis was 40 years, and 9 (81.81%) fulfilled the Amsterdam II criteria (Fig. 2A), CRC was mainly in the proximal colon and mucinous type. One patient with a VUS in *MLH1* (c.c380G p.s127w) was a 42-year-old male with distal CRC, dMMR (with loss of expression of MLH1/PMS2) who fulfilled the Amsterdam II and the revised Bethesda criteria and developed metachronous colorectal cancer in the rectum and transverse colon. He had a family history of gastric cancer and CRC in FDR.

P/LP variants in the *MSH2* gene were identified in 10 probands. The mean age of these patients for CRC diagnosis was 40 years. Among these patients, 7 (70%) fulfilled the Amsterdam II criteria, and 80% had dMMR CRC, mostly (60%) in the proximal colon (Fig. 3A; Table 1). Their family members had mostly colorectal, gastric, prostate, and breast cancers (Fig. 2A). All the known criteria of a typical case of LS (Amsterdam II criteria, dMMR proximal CRC) were identified in only four

Table 1 The clinic-pathological characteristics of CRC patients with dMMR

		Patients with P/LP mutations in Lynch-related genes	Patients with P/LP mutations in non-Lynch genes	Patients at risk for Lynch syndrome
Number of patients		23	17	63
Mean age (year)		44.7	45.8	47.5
Age at diagnosis (year)	≤ 50	73.91% (n = 17)	52.95% (n = 9)	57.15% (n = 36)
	≥ 50	26.09% (n = 6)	47.05% (n = 8)	42.85% (n = 27)
Sex	Female	34.78% (n = 8)	41.17% (n = 7)	44.44% (n = 28)
	Male	65.22% (n = 15)	58.83% (n = 10)	55.56% (n = 35)
Location of the colorectal tumor	Proximal	62.21% (n = 15)	35.29% (n = 6)	57.14% (n = 36)
	Distal	30.43% (n = 7)	35.29% (n = 6)	26.98% (n = 17)
	Rectum	4.34% (n = 1)	17.64% (n = 3)	11.11% (n = 7)
CRC staging *	TIS	4.34% (n = 1)	0	4.76% (n = 3)
	I	26% (n = 6)	11.76% (n = 2)	15.58% (n = 10)
	II	34.78% (n = 8)	11.76% (n = 2)	31.74% (n = 20)
	III	26% (n = 6)	35.29% (n = 6)	22.22% (n = 14)
	IV	4.34% (n = 1)	11.76% (n = 2)	20.63% (n = 13)
Synchronous cancer		n = 2	n = 0	n = 3
Metachronous cancer		n = 1	n = 0	n = 2
Mucinous type CRC		n = 6	n = 1	n = 7
Amsterdam II		69.56% (n = 16)	47.05% (n = 8)	55.55% (n = 35)
Revised Bethesda		95.65% (n = 22)	82.35% (n = 14)	88.88% (n = 56)
MSI high or dMMR		78.26% (n = 18)	47.05% (n = 8)	74.60% (n = 47)

* Based on the eighth edition of AJCC Cancer Staging Manual [Amin et al. 2017]

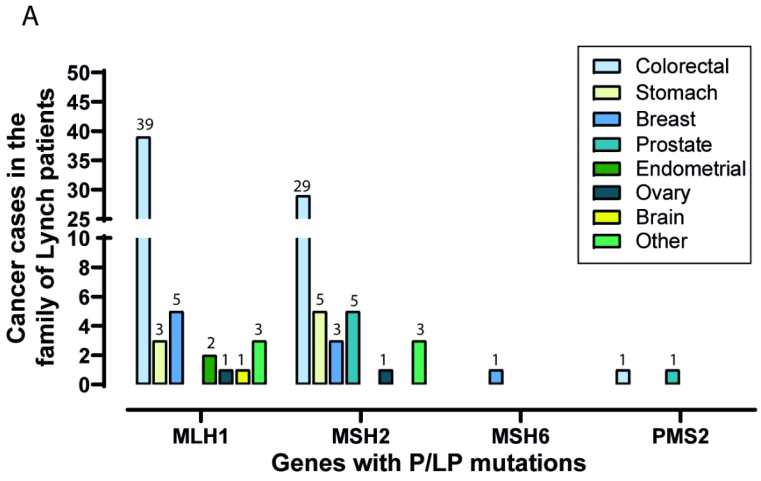
Table 2 Comparison of germline mutations (WES) and immunohistochemical (IHC) staining of tumor tissues for MMR genes

IHC finding	WES-IHC agreement		WES-IHC no agreement			Total
	All	Mutations in Lynch-related genes	All	Mutations in Lynch-related genes	Mutations in non-Lynch genes	
Loss of MLH1 and PMS2	10.63% (5)	MLH1 (1)	23.40% (11)	-	SMAD4 (1), SUFU (1), ATM (1), CDH1 (1), CTSA (1), PTCH2 (1), DIS3L2 (1), COL6A3 (1), BCR (1), No Mutation (2)	34.04% (16)
Loss of MSH6 and PMS2	-	-	4.25% (2)	MLH1 (1)	RNF4 (1)	4.25% (2)
Loss of MSH2 and PMS2	-	-	2.12% (1)	-	POLD1 (1)	2.12% (1)
Loss of MSH2 and MSH6	19.14% (9)	MSH2 (8), MSH6 (1)	6.38% (3)	-	RB1 (1), GALN12 (1), WRN (1)	25.53% (12)
Loss of PMS2	2.12% (1)	-	10.63% (5)	MLH1 (2)	PMS1 (1), MLH3 (1), No Mutation (1)	12.76% (6)
Loss of MSH2	-	-	2.12% (1)	-	MSH3 (1)	2.12% (1)
Loss of MLH1	2.12% (1)	-	4.25% (2)	-	EPHA3 (1), DICER1 (1)	6.38% (3)
Loss of MSH6	-	-	4.25% (2)	MSH2 (1)	FH (1)	4.25% (2)
Normal	-	-	8.51% (4)	MSH6 (1)	PDGFRL (1), TTN (1), TENM3 (1)	8.51% (4)
Total	34.04% (16)		65.95% (31)			47

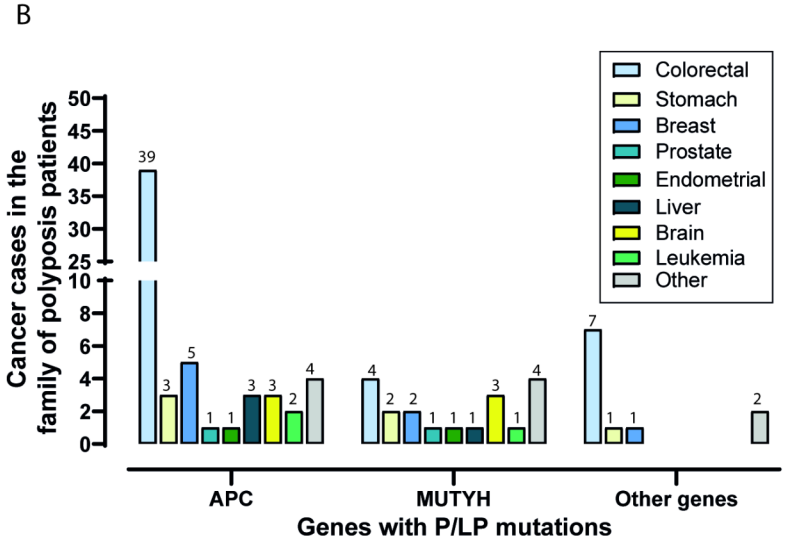
Numbers in parentheses indicate number of patients with the mutated gene

patients (out of 10) with P/LP variants in the *MSH2* gene. One patient had a VUS in *MSH2*, a 48-year-old male with with loss of expression of MSH2/MSH6 in a synchronous CRC in the proximal and distal colon (Fig. 3A). He had a family history of gastric cancer in an SDR.

One patient with P/LP variants in *MSH6* was a 69-year-old male with loss of expression of MSH6/MSH2 in a distal CRC. He had a family history of breast cancer in an SDR. A 54-year-old male patient with a VUS in *MSH6* met the Amsterdam II clinical criteria and had a proximal CRC. Interestingly, the IHC findings revealed MMR proficiency and a family history of gastric cancer in an FDR and 4 CRCs in SDR. We found one case with a pathogenic variant in *PMS2*, a 53-year-old male with loss of expression of PMS2 in a distal CRC, and a family history of prostate cancer in his father at age 76. Pathogenic variants in MSH6 and PMS2 are the most common in LS cases [31].



	P/LP mutation in MLH1 (11)	P/LP mutation in MSH2 (10)	P/LP mutations in Lynch-related genes (23)	P/LP mutations in non-Lynch genes (17)
FDR with CRC	81.80 % (9)	70 % (7)	73.91 % (17)	23.52 % (4)
SDR with CRC	54.54 % (6)	50 % (5)	47.82 % (11)	41.17 % (7)
FDR with stomach cancer	0 %	20 % (2)	8.69 % (2)	0 %
SDR with stomach cancer	18.18 % (2)	30 % (3)	21.73 % (5)	17.64 % (3)



	P/LP mutation in APC (No.19)	P/LP mutations in MUTYH(3)	P/LP mutations in non-FAP/MAP genes(3)
One or more FDR with CRC	31.57% (6)	100% (3)	66.66%(2)
One or more SDR with CRC	36.84% (7)	33.33% (1)	66.66%(2)
One or more FDR with stomach cancer	0	33.33% (1)	33.33%(1)
One or more SDR with stomach cancer	10.52% (2)	33.33% (1)	0
One or more FDR with breast cancer	5.26% (1)	0	0
One or more SDR with breast cancer	21.05% (4)	33.33% (1)	66.66%(2)

Fig. 2 (See legend on next page.)

(See figure on previous page.)

Fig. 2 The number of cancer cases observed in the family of patients at risk for Lynch syndrome and polyposis. (**A**) The number of cancer cases observed in the family of patients at risk for Lynch syndrome is categorized based on P/LP variants in *MLH1*, *MSH2*, *MSH6*, and *PMS6* genes. The bottom panel shows the percentage of CRC and stomach cancers in FDR (first-degree relatives) and SDR (second-degree relatives) of patients with P/LP variants in *MLH1*, *MSH2*, Lynch-related genes, and non-Lynch genes. (**B**) The number of cancer cases observed in the family of patients with colon polyposis, categorized based on P/LP variants in *APC*, *MUTYH*, and non-FAP/MAP genes. The bottom panel shows the number of CRC, stomach, and breast cancers in FDR (first-degree relatives) and SDR (second-degree relatives) of patients with P/LP variants in *APC*, *MUTYH*, and non-FAP/MAP genes

Clinical characteristics of polyposis patients with P/LP variants in FAP/MAP genes

In 38 patients with polyposis criteria and germline P/LP variants in FAP/MAP genes, 52.63% ($n=20$) had over 100 polyps (Table 3), of whom 78.94% (15/19) had pathogenic *APC* germline variants (Fig. 3B). Of the ten patients who had CRC at the time of diagnosis, seven had P/LP variants in the *APC* gene, and two in the *MUTYH* gene (one monoallelic, and one biallelic). The mean age at the time of diagnosis of polyposis patients was 46.26 years (ranging from 21 to 73 years; 63.15% of them ≤ 50 years). The mean age at the time of diagnosis of polyposis patients with pathogenic variants of *APC* and *MUTYH* was 43.9 and 42.3 years, respectively. Most patients (11/19) with *APC* and *MUTYH* pathogenic variants had frameshift variants (Fig. 1D). Six probands were carriers of the *MUTYH* variants, four as biallelic and two as monoallelic (Fig. 3B). Most of the CRC tumors in polyposis patients were at stages III and IV (Table 3).

The most common cancers in the relatives were CRC, breast, stomach, and brain cancers (Fig. 2B). Three cases with P/LP germline variants in the *APC* gene developed desmoid tumor, one patient with c.3921_3925del(p.I1307fs), one patient with the c.3389 del G(p.c1130fs), and another patient with the c.3180_3184del (p.I1060fs) (Fig. 3B). In the FDR of patients with P/LP variants in the *APC* and *MUTYH* genes, 7 and 3 cases of CRC were identified, respectively (Table 3).

Remarkably, a *PTEN* variant was found in a 40-year-old woman with rectosigmoid polyposis (20–50 polyps). Following screening, she was diagnosed with early-stage breast cancer. Additionally, we observed intriguing *MSH3* monoallelic variants (c.214 C > T p.P72S) in a 37-year-old male with more than 50 colon polyps and a family history of cancer in a second-degree relative.

Two patients with VUS in *APC* were suggested to be classified as pathogenic: a 51-year-old male with more than 100 colon polyps and rectal cancer with a family history of CRC in his father; and a 72-year-old male with more than 100 colon polyps and a family history of endometrial cancer in FDR and CRC in SDR (Figure S8). We also found two patients with two different monoallelic variants (compound heterozygote) in *MUTYH*, a 35-year-old female with 50–100 colon polyps and CRC in his brother, and a 44-year-old female with more than 100 colon polyps and distal CRC (Figure S9). For polyposis patients, the PPI network analysis identified 41 nodes and 120 edges (Figure S10). Genes like *POLE*, *MSH6*, *MSH2*, *BRCA2*, *BRCA1*, *MLH1*, *TOPBP1*, *BLM*, *RAD50*, *MUTYH*, *MSH3*, *MLH3*, *PTEN*, and *BRIP1* genes had a higher degree value indicating high penetrance in colorectal cancer [11, 32] (Figure S10, Table S4). Analysis showed 62.5% of terms related to colon carcinoma, 25% to familial breast cancer, and 12.5% to endometrial carcinoma (Figure S10). Proposed biomarkers included *PTEN*, *POLE*, *BRCA1/2*, *MSH2*, *MSH6*, *MLH3*, and *MSH3*, as potential candidates associated with polyposis and colorectal cancer. However, further investigation is necessary to validate these findings and establish their clinical significance.

Discussion

In this study, we conducted WES germline analysis from 63 high-risk LS patients based on clinical criteria and 38 polyposis patients. We identified P/LP variants in Lynch-related and non-Lynch genes in 63.49% of high-risk LS proband (Fig. 1). 36.51% (23/63) had variants in Lynch-related genes, and 26.98% had variants in non-Lynch genes, including *ATM*, *ASXL1*, *FH* (mono-allelic), *MSH3* (mono-allelic), *PMS1*, *MUTYH* (mono-allelic), *WRN*, and *TP53*. These findings align with other studies and may suggest that the known Lynch-related genes do not explain all inherited susceptibility to CRC in high-risk cases that fulfill clinical criteria [33, 34, 35, 36, 37]. These findings also indicate that a broader number of genes should be tested in affected patients and their relatives. In the polyposis cohort, 71% had P/LP germline variants in FAP/MAP- and non-FAP/MAP genes. Most of these variants were in *APC* and *MUTYH* (mono- and bi-allelic) genes, with three patients having *PTEN*, *BRCA2*, and *BLM* P/LP germline variants. These findings highlight the need for a comprehensive hereditary cancer risk assessment in CRC and polyposis with appropriate pre and post-test genetic counseling.

Previous studies have identified various germline variants predisposing individuals to cancer. The National Comprehensive Cancer Network (NCCN) recommends universal MMR deficiency screening for all colorectal and endometrial tumors, regardless of age [36]. However, in low-resource settings like Iran, selective strategies using clinical criteria such as Bethesda or Amsterdam II are more practical due to the limited access to genetic testing and financial constraints in identifying high-risk patients [32].

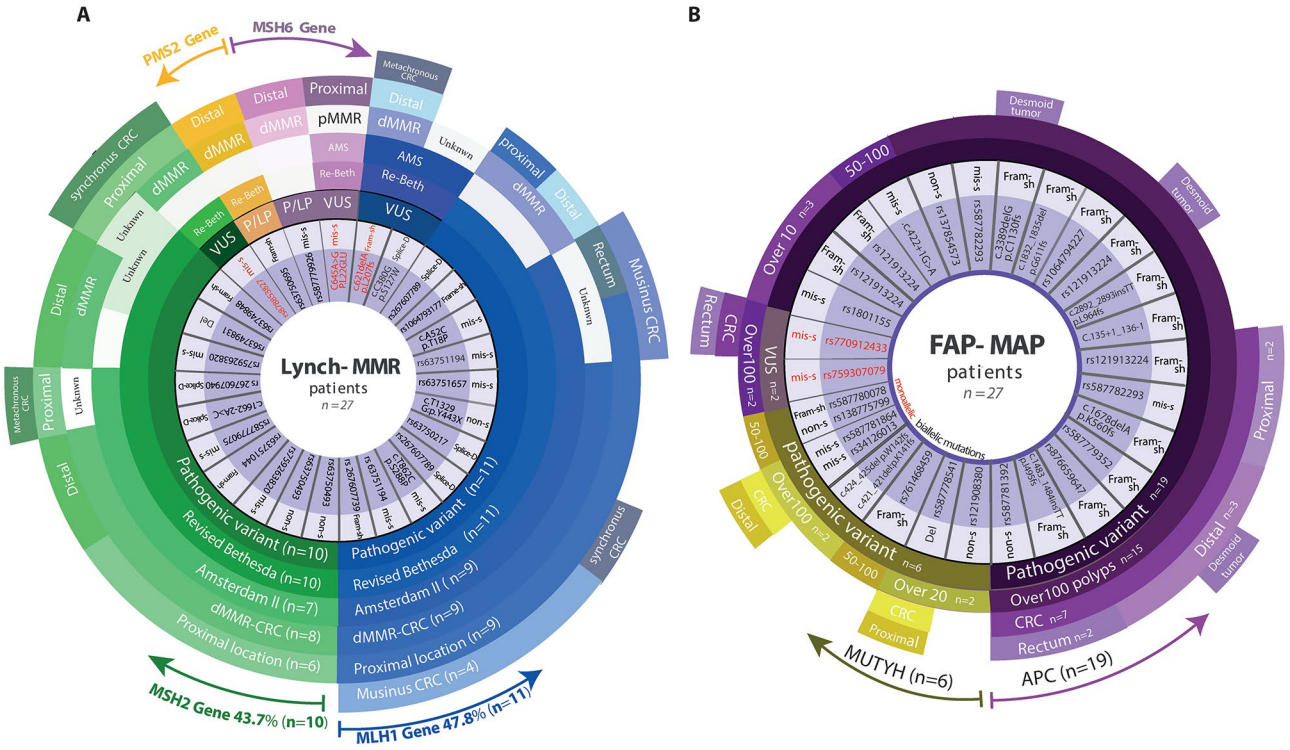


Fig. 3 Circular visualization of clinical characteristics of patients with variants in Lynch-related and FAMP/MAP genes. (**A**) 27 patients had P (pathogenic)/LP (Likely Pathogenic) and VUS (variants of uncertain significance) variants in Lynch-related genes. Rectangles with green gradients represent the clinical characteristics of patients with the MSH2 variants. Rectangles with blue gradients represent the clinical characteristics of patients with the MLH1 variants. Rectangles with orange gradients represent the clinical characteristics of patients with the PMS2 variants. Rectangles with purple gradients represent the clinical characteristics of patients with the MSH6 variants. The white rectangles show unknown characteristics. The white rectangle in the center of the circle shows the characteristics of the rsID number for dbSNP, Mis-s: missense, Fram-sh: frameshift, Del: deletion, Ins: Insertion, Splice-D: splice donor, and Non-s: nonsense. The red words correspond to the novel variants. **(B)** 25 patients had P/LP/VUS variants in FAP/MAP genes in polyposis patients. Rectangles with purple gradients represent the clinical characteristics of patients with the APC variants, and rectangles with gold gradients represent the clinical characteristics of patients with the MUTYH variants

We identified P/LP variants in Lynch-related genes in 23 (36.51%) patients, of whom 18 were confirmed to be MMR-deficient by IHC. (Fig. 3A). Studies using universal screening for MMR deficiency in all CRC cases found lower germline variant rates. In Australia, screening of 813 CRC cases identified 11.1% with MMR deficiency and 5.2% with germline variants [33]. In China, screening of 4802 patients found 5.37% with MMR loss, with only 20% confirmed as Lynch families [38].

Using a selective strategy focusing on high-risk LS patients, this study showed higher rates of germline variants, similar to other low-resource countries, such as Brazil, where 41% of high-risk LS patients had MMR-deficient tumors and about half carried pathogenic germline variants [37]. Another study using WES on familial colorectal cancer type X identified 32 patients with cancer-associated variants. Sanger sequencing had previously confirmed the absence of germline MMR mutations in these patients, classifying them as FCCX cases. Interestingly, 4 patients were found to have MMR gene variants, suggesting a potential role of somatic or other mechanisms [39]. Our study, which focused on clinically high-risk individuals, showed higher germline variant rates than studies with broader CRC screenings.

An ATM mutation in a 52-year-old male with proximal adenocarcinoma emphasized the importance of including ATM in genetic testing for high-risk CRC patients [40]. Other variants found were: *ASXL1* (c.2728 C > T; p.Q910X), *FH* (c.557G > A; p.S186N), pathogenic stop-gain variants of *PMS1* (c.301 C > T; p.R101X), and a VUS (c.473 C > T; p.T158M). *ASXL1* is associated with CRC progression (45) and *FH* gene variants are linked to hereditary leiomyomatosis and renal cell cancer (HLRCC) [41]. While *PMS1* variants are rare in LS, some studies have identified germline *PMS1* mutations in families with hereditary non-polyposis colorectal cancer (HNPCC), suggesting a potential, though limited, role in the disease [42].

MSH3 variants were identified in three families meeting Amsterdam criteria for LS and one with colon polyposis (Figure S6). While MSH3 deficiency is linked to genetic instability [43, 44, 45] and tumorigenesis, its exact role in CRC remains uncertain.

Table 3 Clinic-pathological characteristics of polyposis patients

		Patients with P/LP mutations in APC	Patients with P/LP mutations in MUTYH	Patients with P mutations in non-FAP/MAP genes	Patients with colorectal polyposis
Number of patients		19	6	3	38
Mean age (year)		43.9	42.3	46	46.26
Age at diagnosis (year)	≥ 50	26.3% (n = 5)	16.7% (n = 1)	n = 1	36.8% (n = 14)
	≤ 50	73.6% (n = 14)	83.3% (n = 5)	n = 2	63.15% (n = 24)
Sex	Female	42.1% (n = 8)	66.7% (n = 4)	n = 2	39.47% (n = 15)
	Male	57.89% (n = 11)	33.3% (n = 2)	n = 1	60.5% (n = 23)
Number of colon polyps	10–49	15.78% (n = 3)	33.3% (n = 2)	n = 2	13.15% (n = 5)
	50–100	5.2% (n = 1)	33.3% (n = 2)		15.78% (n = 6)
	≥ 100	78.9% (n = 15)	33.3 (n = 2)	n = 1	52.63% (n = 20)
CRC	Yes	(n = 7)	(n = 2)	n = 1	26.31% (n = 10)
Location of colorectal tumor	Distal	(n = 3)	(n = 1)		10.52% (n = 4)
	Proximal	(n = 2)	(n = 1)	n = 1	13.15% (n = 5)
	Rectum	(n = 2)	0		10.52% (n = 4)
Mean age at CRC diagnosis (year)		45.8	37.5	57	47.3
CRC in first-degree relatives		(n = 7)	(n = 3)	(n = 2)	(n = 16)
FAP extra colonic manifestations		Thyroid nodule (2)	0	Thyroid nodule (1)	Thyroid nodule (6)
		Desmoid tumor (3)			Desmoid tumor (3)
		Duodenal polyp (4)			Duodenal polyp (4)
CRC staging *	TIS	0	0	0	(n = 1)
	I	0	(n = 1)	0	(n = 1)
	II	(n = 1)	0	0	(n = 2)
	III	(n = 1)	(n = 1)	0	(n = 2)
	IV	(n = 2)	0	0	(n = 4)

* Based on the eighth edition of AJCC Cancer Staging Manual [Amin et al. 2017]

A germline variant (c.614G > A:p.R205H) in the *TP53* gene was found in a patient with early-onset CRC, suggesting that early-onset CRC may be the first presentation of Li-Fraumeni Syndrome (LFS), warranting the inclusion of *TP53* in CRC NGS panels. VUS found in MMR genes in families with a strong history of cancer are potential candidates to be reclassified as pathogenic or likely pathogenic. In this study, VUS variants were identified in *MLH1* (c.621delA p.L207fs), *MSH2* (c.2116G > A p.Val706Met), and *MSH6* (c.64 A > G; p.K22E) (Table S1, Figure S7).

We also found VUS variants in non-Lynch genes such as in the breakpoint cluster region (*BCR*) gene, and *GXYLT1* with the potential to be involved in the pathogenesis of LS (Table S1). BCR alterations are present in several cancers (56) and *GXYLT1* variants promote CRC metastasis via the MAPK pathway (57). These findings warrant the need for further studies due to the complexity of genetic factors in CRC tumorigenesis and reinforce the importance of comprehensive genetic testing for high-risk populations.

Other studies have reported variants in non-Lynch genes, including *BRCA1/2*, *APC*, and biallelic *MUTYH*, *CHEK2*, *TP53*, *PTEN*, *CDH1*, and *SMAD4* in individuals with clinical LS features [46]. In this study, we also found variants in the majority of these non-Lynch genes, including *TP53* (:c.1010 C > T p.Arg337Trp), *PTEN* (:c.323 C > T p.Arg108Cys), *CDH1* (c.1557T > G p.Val519Gly), and *SMAD4* (c. T977T > C p. I326T).

Most LS variant carriers (32 patients) and non-LS variant carriers (26 patients) fulfilled the National Comprehensive Cancer Network (NCCN) criteria for LS. Thus, limited panel gene testing for MMR variants may miss the non-LS variants. Additionally, this study observed discordance between IHC results and genomic findings in 17.39% (4/23) of LS patients, consistent with recent studies that showed 15.2% of MSI and or IHC results for patients with *MSH6* or *PMS2* mutations were discordant, potentially leading to missed mutations without the use of multi-gene panel testing (MGPT) (64–67). This highlights the limitations of single gene testing based on IHC results and reinforces the need for comprehensive genetic testing to accurately identify pathogenic variants.

Germline variants in the *APC* and *MUTYH* genes were identified in 65% (25/38) of patients with over ten colorectal polyps and 68% (17/26) of patients with more than 100 colon polyps. These results aligned with previous studies showing *APC* variants in 33.5% and the *MUTYH* variant in 7.8% of adenoma cohorts [47]. The higher prevalence of APC and biallelic MUTYH variants in patients with over 100 polyps compared to previous studies [47, 48] might be due to differences in inclusion criteria and study

population. Findings of WES revealed that 7% (3/38) of patients with 10–50 colon polyps had *APC* variants, and 5% (2/38) had *MUTYH* variants. These findings are consistent with previous findings showing a prevalence of 2.3-9% for variants in these genes in patients with a similar polyp burden [47, 48]. These results further emphasize the significance of genetic testing for patients with ten or more polyps and highlight the need for analyzing both polyposis and non-polyposis colorectal cancer genes in this population, as we have identified P/LP variants in the *PTEN*, *BLM*, *BRCA2*, and *MSH3* genes. Consistent with a previous study [49], our findings suggest that *MSH3* variants may represent a recessive subtype of colorectal adenomatous polyposis, providing further insights into the potential role of *MSH3* in colon polyposis.

LS individuals should undergo regular colonoscopies and endometrial screening. A study of 1745 LS individuals showed that 16% had malignancies beyond colorectal and endometrial cancers, including breast, urinary bladder, and small bowel carcinoma [50]. We found gastric cancer as the second most common cancer and breast cancer as the third most common among LS probands. In another Iranian study, gastric cancer was the most common cancer in men and breast cancer in women, which should guide surveillance strategies [51].

The higher prevalence of gastric cancer in both Iranian and Japanese populations than in Western countries, suggests that environmental factors might also contribute, so endoscopic surveillance and H. pylori eradication are recommended in these populations (46, 47).

Evidence suggests breast cancer with medullary features as LS-related tumors [25], particularly for *PMS2* variant carriers. A Canadian study highlighted 41 breast cancer cases in LS families, emphasizing the importance of intensified breast cancer surveillance in LS women [52]. Our findings support the potential association between LS and breast cancer, necessitating further investigation as well as long-term registry and follow-up of carrier cases.

This study had limitations, including a small size population, limited genomic coverage of WES, and reliance on Clinvar and Gnomad for variant classification due to the scarcity of data on subpopulations, such as Iranian. On the other hand, its strength lies in using a clinically selected cohort of high-risk LS cases to better correlate phenotype with genotype. We emphasize the limitations of target gene testing and the preference of using a broader gene panel for testing high-risk groups, to further incorporate them in genetic testing protocols for the Iranian population.

Finally, these results are consistent with other studies related to cascade testing [53]. Hampel et al. reported that cascade testing could find three carriers out of six family members tested [54]. This highlights the importance of cascade testing to identify carriers in probands.

Conclusion

Here we report the first assessment of the prevalence and type of germline variants associated with hereditary CRC and polyposis in the Iranian population. Since clinical criteria for LS analysis appear to identify many probands with unexpected P/LP variants in highly penetrant non-LS cancer susceptibility genes, and because of the growing reduction of WES costs, we suggest that comprehensive panel testing or even WES could eventually replace targeted genetic testing. A selective strategy followed by cascade genetic testing in FDR and SDR may potentially identify carriers with low cost, and help healthcare professionals indicate the most appropriate gene panels for testing. Finally, establishing a national registry for hereditary cancer syndromes, as the IHCCR, is the utmost need for under-resourced populations. This study might help develop national policies and guidelines for the screening and management of hereditary CRCs in Iran.

Abbreviations

INCR	Iranian National Cancer Registry
CRC	Colorectal cancer
GWASs	Genome-wide association studies
LS	Lynch syndrome
MMR	Mismatch repair
FAP	Familial adenomatous polyposis
MAP	MUTYH-associated polyposis
NGS	Next-generation sequencing
WES	Whole exome sequencing
PPAP	Polymerase proofreading–associated polyposis
IHCCR	Iranian Hereditary Colorectal Cancer Registry
HNPCC	Hereditary non-polyposis colorectal cancer
NCCN	National Comprehensive Cancer Network
IHC	Immunohistochemistry
MAF	Minor allele frequency
GO	Gene ontology
PPI	Protein-protein interaction
CC	Cellular component
MF	Molecular function
BP	Biological process
VUS	Variations of uncertain significance
P	Pathogenic variants
LP	Likely pathogenic
SDR	Second-degree relative
FDR	First-degree relative
NCCN	National Comprehensive Cancer Network
HRC	High-risk colorectal cancer
MSI	Microsatellite instability
EMAST	Elevated microsatellite alterations at selected tetranucleotide repeats
LOH	Loss of heterozygosity
EC	Endometrial cancer
ED	Exonuclease domain
NER	Nucleotide excision repair

Supplementary Information

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Supplementary Material 1

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Author contributions
HD, LaG, and HanV designed the study. LeG, MRA, NED, JSK, MHE, AJ, FA, MS, FM, SH, AB, MehZ, MRK, MB, MA, FR, and HV were involved in the data gathering and interpreting the results. LaG, MA, MorZ, and LeG performed analyses. LeG, LaG, AT, BH, and HD wrote the first draft of the manuscript. HD, HanV, JSK, LaG, MRA, FR, and LeG edited the final version of the manuscript. All authors read and approved the final version of the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
This study was approved by the Ethics Committee of Mashhad University of Medical Sciences (ethics code: IR.MUMS.REC.1396.164) and conformed to the ethical principles in the Declaration of Helsinki. The participants signed an informed consent form before the study for experiments involving human participants (including tissue samples).

Competing interests
The authors declare no competing interests.

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