

ORIGINAL ARTICLE

Co-occurrence of germline pathogenic variants for different hereditary cancer syndromes in patients with Lynch syndrome

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Abstract

Background: Lynch syndrome (LS) is a hereditary condition characterized by a high risk of colorectal cancer, endometrial cancer, and other neoplasia associated with germline alterations in DNA mismatch repair genes. The classical genetic diagnostic strategy for LS consists of the Sanger sequencing of genes associated with the suspected syndrome. Next-generation sequencing (NGS) enables the simultaneous sequencing of a large number of hereditary cancer genes. Here, we aimed to study whether other germline pathogenic variants of hereditary cancer genes are present in patients with LS.

Methods: A cohort of 84 probands with a previous genetic diagnosis of LS by Sanger sequencing was reanalyzed using NGS via a commercial panel of 94 hereditary cancer genes by hybrid capture. The American College of Medical Genetics and Genomics criteria were used to classify the clinical significance of the variants. The findings of NGS were confirmed by Sanger sequencing. When possible, genetic analyses of the new findings in the proband's relatives were also performed by Sanger sequencing.

Results: We identified five families (6%), out of 84, with at least two germline pathogenic variants conferring to high or moderate risk in different dominant cancer-predisposing genes: [*MLH1-BRCA2-NBN*], [*MLH1-BRCA1*],

Abbreviations: ACMG, American College of Medical Genetics and Genomics; CI, Confidence interval; CRC, Colorectal cancer; EC, Endometrial cancer; EUS, Endoscopic ultrasound; LS, Lynch syndrome; MINAS, Multilocus-Inherited-Neoplasia-Allele Syndrome; MMR, Mismatch repair; MRI, Magnetic resonance imaging; NCBI, National Center for Biotechnology Information; NGS, Next-generation sequencing; OR, Odds ratio; PSA, Prostate-specific antigen

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[*MSH2-ATM*], [*MSH6-NFI*], and [*MLH1-FANCA*]. Interestingly, only one out of these five families exhibited a clinical phenotype associated with the new pathogenic variants. The family with three pathogenic variants of the [*MLH1-BRCA2-NBN*] genes showed a high aggregation of tumors associated with LS and breast and ovarian cancer syndrome.

Conclusions: Our results showed that the co-occurrence of more than one pathogenic variant in cancer-predisposing genes was remarkable among cases of LS. In most cases, no clinical manifestations were associated with the secondary pathogenic variants. Further studies are needed to confirm these findings and elucidate their clinical impact. Reanalysis of LS families should be considered only in families with mixed clinical phenotypes.

KEYWORDS

cancer panel, hereditary cancer, lynch syndrome, moderate penetrance genes, multilocus inherited neoplasia alleles syndrome, next-generation sequencing, secondary findings

1 | BACKGROUND

Lynch syndrome (LS [MIM:120435]) is a hereditary condition characterized by a high risk of colorectal cancer (CRC), endometrial cancer (EC), and other neoplasia that are frequently diagnosed at an early age. LS is the most common hereditary CRC syndrome, accounting for approximately 1%–3% of all cases of CRC. In the majority of cases, germline variants in one of the DNA mismatch repair (MMR) genes [*MLH1* (MutL homolog 1), *MSH2* (MutS Homolog 2), *MSH6* (MutS homolog 6), and *PMS2* (PMS1 Homolog 2)] are responsible for this syndrome which exhibits autosomal dominant inheritance [1].

LS is characterized by incomplete penetrance and variable expressivity. There is high variability in cancer risk among the carriers of MMR gene pathogenic variants [2]. The causes of this variability are unknown, although they might be explained by age, sex, modifier genes, epigenetic changes, or environmental factors. The co-occurrence of other pathogenic variants may explain, in some cases, the absence of genotype–phenotype correlations [3]. Moreover, there is increasing evidence of the polygenic heritability of CRC risk, which might function as a penetrance-modifying factor in cases of LS [4].

The conventional model used for hereditary cancer risk assessment involves the identification of individuals whose histories fulfill the clinical criteria for a specific syndrome, followed by targeted germline testing of the gene(s) associated with that syndrome exclusively. The identification of a germline pathogenic variant in the probands and their relatives enables their inclusion in cancer surveillance programs. Such programs are effective in reducing cancer mortality in these families. Furthermore, family

members who do not carry the pathogenic variant can be treated safely as low-risk individuals, avoiding unnecessary screening and preventing anxiety in these individuals. Currently, multigene panel testing using next-generation sequencing (NGS) technologies tests multiple hereditary cancer genes in parallel, with lower economic burden and less time-consuming approaches [5].

At present, an increasing number of cases that carry two or more inherited cancer-predisposing alleles in the same individual are being described and are termed multilocus inherited neoplasia alleles syndrome (MINAS) [6]. This scenario has been reported in various disorders with both distinct and overlapping phenotypes. Although the frequency of MINAS is predicted to be low, such cases are probably underdiagnosed because of the standard diagnostic procedure used in clinical practice [6, 7].

This present study aimed to estimate the prevalence of the co-occurrence of pathogenic variants in cancer-predisposing genes in a cohort of LS probands with pathogenic variants in MMR genes. The underlying objective was to deepen the understanding and achieve a more precise assessment of the cancer risk in these families. Herein, we report five cases with multiple heterozygous pathogenic germline variants in different cancer syndrome genes.

2 | PATIENTS AND METHODS

2.1 | Design

A descriptive retrospective and analytical study was performed using a comprehensive genetic analysis of

hereditary cancer genes to identify additional pathogenic variants in patients with LS.

2.2 | Patients

The cohort of patients included in this study corresponded to probands with a suspicion of LS based on their personal or family history of cancer. All families included here fulfilled the revised Bethesda criteria and were referred to and attended a Genetic Counseling in Cancer consultancy from the Hereditary Cancer Program of the Valencian Region (Spain) between May 2005 and December 2016. The total number of patients with suspected LS with pathogenic/likely pathogenic variant in MMR genes was 189. All participants signed proper informed consent for genetic diagnosis. About 85% of them also signed the informed consent for the Valencian Biobanks Network ($n = 161$). Patients included in this group were considered for the current study. These individuals also consented to participate in any future research project related to hereditary cancer. Participation implies the use of biological samples and associated clinicopathological information. The patient's autonomy and desire to know or to ignore secondary findings were respected according to the Spanish laws. This study was approved by the corresponding Ethical and Scientific Committees. Eleven DNA samples were excluded because of insufficient DNA quantity ($n = 150$). Random selection of the 84 patients was carried out among the 150 individuals with enough DNA quality who signed the Valencian Biobank Network informed consent, due to funding limitations (Supplementary Table S1).

2.3 | Genetic diagnosis procedures

For all participants, a genetic diagnosis of LS was established previously by Sanger sequencing after the application of standard procedures of clinical selection criteria, MMR immunohistochemical screening, and other complementary analyses (i.e., assessment of *MLH1* methylation and *BRAF-V600E* mutation in tumors) [1].

2.3.1 | NGS library preparation and bioinformatics analysis

Ninety-four genes associated with hereditary cancer syndromes were sequenced via NGS in DNA extracted from blood cells (TruSight Cancer Illumina kit; Illumina, San Diego, CA, USA) (Supplementary Table S2). Libraries

were generated using TruSight-RapidCapture (Illumina), according to the manufacturer's protocol. Sequencing was performed on an Illumina-MiSeq apparatus and sequence data were analyzed using the on-instrument MiSeq-Reporter (Illumina) software. BAM files were created after the alignment of FASTQ files to the human reference genome (hg19, NCBI_build GRCh37) using the Burrows-Wheeler aligner. The Genome Analysis Toolkit was used to perform variant calling (VCF_files), and the VariantStudio software (Illumina) was used to manage the data after the application of a custom pipeline for variant filtering. Briefly, we considered a minimal coverage of 20 \times ; a Q score of 30; a region of interest corresponding to the coding sequence ± 10 nt, and; variants with an allelic population frequency $\leq 5\%$, according to the 1000 Genomes Project and gnomAD. The threshold for the variant allele frequency was >0.30 . The Integrative Genomics Viewer was used for the manual review of the variants of interest.

2.3.2 | Pathogenic assessment of genetic variants

The American College of Medical Genetics and Genomics (ACMG) criteria were used to classify the clinical significance of the variants [8]. The genomic variant search engine VarSome [9] and the public archive of the relationships among human sequence variations and phenotypes with supporting evidence, ClinVar [10], were used to facilitate the variant classification process. Briefly, after the application of population-frequency filters, we filtered variants with the following molecular consequences: frameshift, nonsense, consensus splice sites, start loss and stop gain, in-frame in/del, and missense variations. We screened for these variants in the VarSome and ClinVar databases and reviewed the classifications offered considering the criteria provided. The variants that were classified in ClinVar as being pathogenic or likely pathogenic by an expert panel or with multisource consistency were considered as pathogenic or likely pathogenic in the current study. Variants with conflicting interpretations among submitters were investigated in-depth, considering the VarSome classification and the evidence of pathogenicity found in all the integrated resources.

All pathogenic variants detected here were confirmed by Sanger sequencing. When possible, genetic analyses of the new findings in the proband's relatives were also performed by Sanger sequencing.

In this present study, we did not analyze variants in genes with high homology to pseudogenes, such as *PMS2*. We also excluded the analysis of copy number variations.

2.4 | Patient follow-up

Within the operation of the Hereditary Cancer Program of the Valencian region, the annual update of the pedigrees and the monitoring of adherence to the follow-up recommendations were contemplated by phone and reviewing medical records.

2.5 | Statistical analysis

Descriptive statistics of categorical variables are presented as frequencies and proportions. The association between qualitative variables was analyzed using the chi-squared test, followed by Fisher's exact test. Statistical analysis was performed using R (The R Project for Statistical Computing: <https://www.r-project.org/>), and a confidence level $P < 0.05$ was used.

3 | RESULTS

A cohort of 84 LS probands with previously characterized pathogenic germline variants in the *MLH1* ($n = 36$), *MSH2* ($n = 31$), and *MSH6* ($n = 17$) genes was studied.

3.1 | General results in the NGS analysis

The average coverage at 20× of the 255 Kb target sequence was 96.2%. A total of 23,664 variants passed the initial qualifying criteria. After the application of our custom pipeline, we compiled 1020 variants for classification based on their clinical significance, which revealed that 84% ($n = 852$) of them were benign or likely benign variants, 8% ($n = 79$) had uncertain significance, and 9% ($n = 87$) were pathogenic or likely pathogenic variants (including MMR pathogenic variants) (Supplementary Table S3). The raw data have been uploaded to the NCBI Sequence Read Archive, which is a public repository of NGS data, coded as PRJNA594841.

Genes containing homology with pseudogenes, such as *NFI* (Neurofibromin 1) or *SDHA* (Succinate Dehydrogenase Complex Flavoprotein Subunit A), were not excluded from further analysis because of the good quality of the results. We obtained good coverage of specific reads that were mapped to these genes. Only *PMS2* was disregarded because the majority of its coding sequence had low mapping quality, except for exons 6–8, where no pathogenic or likely pathogenic variants were detected.

All pathogenic variants in the MMR genes previously detected by Sanger sequencing were confirmed

by NGS, except for two *MSH2* splicing variants. These variants (c.942+2T>A and c.942+3A>T) were missed in the four probands who were heterozygous for any of them (LS18, LS56, LS60, and LS70; Supplementary Table S1). Both variants were located in the intron 5 of *MSH2*, near a difficult-to-analyze homopolymer stretch of 26 adenine residues, and are frequently missed [11].

3.2 | Identification of additional variants in other cancer predisposition genes

In addition to the known pathogenic variants in MMR genes, we found additional five heterozygous pathogenic variants in five patients. These variants were associated with different hereditary cancer syndromes. The new pathogenic variants detected in these patients with LS were classified according to their nature, frequency, and reported clinical significance. The evidence used for the classification of the new pathogenic and likely pathogenic variants is presented in Table 1. Briefly, four of them generated premature stop codons: three frameshift variants in *ATM* (ATM Serine/Threonine Kinase) [c.6711_6715delGGAAA], *FANCA* (FA Complement Group A) [c.1115_1118delTTGG], and *NFI* (Neurofibromin 1) [c.5129_5141del], and one nonsense variant in *NBN* (Nibrin) [c.2140C>T]. Moreover, one splice variant in the *BRCA1* (BRCA1 DNA Repair Associated) gene [c.5152+5G>A] was detected. Three of these variants (*NBN*, *BRCA1*, and *FANCA*) were classified as pathogenic/likely pathogenic by VarSome and ClinVar, two (*NFI* and *ATM*) were not reported by ClinVar but were classified as pathogenic/likely pathogenic by VarSome.

All of the pathogenic and likely pathogenic variants found are rare or extremely rare in the normal population. None of these variants have been reported in our control population database, which includes 1041 healthy individuals from the Collaborative Spanish Variant Server database. The only variant described in the gnomAD database (filters used: gnomAD v2.1.1-non-cancer and European non-Finnish population) was *NBN* [c.2140C>T], with very low frequency: five alleles were detected among the 102,568 alleles sequenced. Significant frequency differences were observed between our LS cohort and gnomAD (Table 1).

We were able to test 26 relatives of these probands (range, 1–11 relatives per family) for pathogenic variants in MMR and secondary genes. We found 10 (38%) positive individuals for the newly detected pathogenic variants (Figures 1 and 2).

TABLE 1 Description of patients and families in whom new pathogenic/likely pathogenic variants were detected

Family & Proband-ID	P/LP variant in MMR GeneVariant-Penetrance	Second / ThirdP/LP variant GeneVariant-Penetrance	Evidence for classification of the new P/LP variant	Associated cancer/syndrome to new genetic findings	Proband clinical phenotype and age at dx	First-degree relatives clinical phenotype and age at dx(affected/total)
F-LS9 / LS9	<i>MLH1</i> c.1717_1718delGT p.(Val573SerfsTer11) High	<i>BRCA2</i> † c.3492dupT p.(His1165SerfsTer19) High	ClinVar: P (3) CSVS: NR gnomAD: NR VarSome: P ACMG: PVS1, PM2, PP5	HBOC	EC 46y; BC 49y	ThC BC 60y; OC 64y CRC 49y CRC 43y; OC 53y (4/8)
		<i>NBN</i> c.2140C>T p.(Arg714Ter) Moderate	ClinVar: P/LP (2) CSVS: NR gnomAD: 5/102568 VarSome: P ACMG: PVS1, PM2, PP3, PP5	BC		
F-LS15 / LS15	<i>MLH1</i> c.701delA p.(Glu234GlyfsTer5) High	<i>BRCA1</i> c.5152+5G>A p.? High	ClinVar: LP (1) CSVS: NR gnomAD: NR VarSome: LP ACMG: PS3, PM2, PP3, PP5	HBOC	CRC 59y; EC 60y	CRC LC 64y (2/8)
F-LS30 / LS30	<i>MSH2</i> c.2633_2634delAG p.(Glu878AlafsTer3) High	<i>ATM</i> c.6711_6715delGGAAA p.(Lys2237AsnfsTer10) Moderate	ClinVar: NR CSVS: NR gnomAD: NR VarSome: P ACMG: PVS1, PM1, PM2	BC	CRC 39y; EC 52y	CRC 43y BrC (2/6)
F-LS44 / LS44	<i>MSH6</i> c.762dupT p.(Glu255Ter) High	<i>NF1</i> c.5129_5141del p.(Cys1711TrpfsTer5) High (NF1) Moderate (BC)	ClinVar: NR CSVS: NR gnomAD: NR VarSome: LP ACMG: PVS1, PM2	NF1 BC	CRC 47y	CRC 69y PC 70y (2/8)
F-LS49 / LS49	<i>MLH1</i> c.1731G>A p.(Ser577 =) High	<i>FANCA</i> c.1115_1118delTTGG p.(Val372AlafsTer42) Moderate	ClinVar: P (2) CSVS: NR gnomAD: NR VarSome: P ACMG: PVS1, PP5, PM2, PP3	BC	CRC 40y	CRC 40y (1/6)

Symbols: (†) indicates that LS9 is not a carrier of this *BRCA2* pathogenic variant. The ClinVar stars represent the aggregate review status on web pages: (3) reviewed by an expert panel; (2) two or more submitters with assertion criteria and evidence (or a public contact) provided the same interpretation; (1) multiple submitters provided assertion criteria and evidence (or a public contact), but there are conflicting interpretations, or one submitter provided an interpretation with assertion criteria and evidence (or a public contact).

Databases used: ClinVar: Clinical Variation (<https://www.ncbi.nlm.nih.gov/clinvar/>); CSVS: Collaborative Spanish Variant Server (<http://csvs.babelomics.org/>); gnomAD: The Genome Aggregation Database: (<http://gnomAD.broadinstitute.org/>); VarSome: the Human Genomics Community (<https://varsome.com/>)

Abbreviations: BC, breast cancer; BrC, brain cancer; CRC, colorectal cancer; EC, endometrial cancer; HBOC, hereditary breast, and ovarian cancer syndrome; LC, lung cancer; LP, likely pathogenic; NF1, neurofibromatosis type 1; NR, not reported; OC, ovarian cancer; P, pathogenic; PC, pancreatic cancer; PM1, PM2, PM4, moderate evidence of pathogenicity; PP2, PP3, PP5, supporting evidence of pathogenicity; PS3, strong evidence of pathogenicity; PVS1, very strong evidence of pathogenicity; ThC, throat cancer; VUS, variant of uncertain significance.

3.3 | Clinical phenotype in families with co-occurrent pathogenic variants

Family (F-LS9) fulfilled the criteria for LS (Amsterdam-II) and hereditary breast and ovarian cancer. Pathogenic

variants that are responsible for these syndromes were previously detected in this family. *MLH1*: c.1717_1718delGT; p.(Val573SerfsTer19) was initially identified in the LS9 proband, who had diagnoses of endometrial and breast cancers at the ages of 46 and 49 years, respectively.

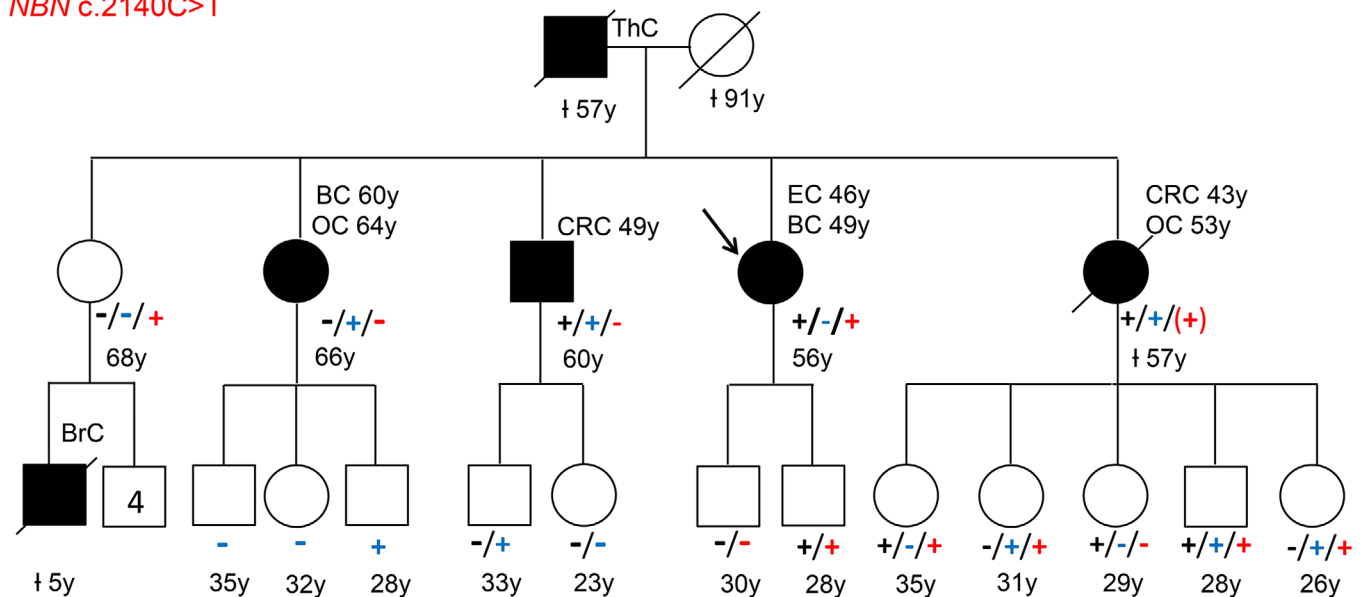
F-LS9*MLH1* c.1717_1718delGT*BRCA2* c.3492insT*NBN* c.2140C>T

FIGURE 1 Pedigree of family LS9. The pathogenic variants found in the family are shown at the top. Filled symbols indicate patients affected by cancer (each color refers to a specific type). Age when the pedigree was drawn, age of death (symbol “†”), and age at diagnosis, when available, are also detailed. The proband is marked by an arrow. Carrier status was studied in the available relatives, and those carrying or not carrying the pathogenic variants are marked with + and -, respectively: in black for MMR gene variant, blue for the second, and red for the third pathogenic/likely pathogenic variant, if any. BC, breast cancer; BrC, brain cancer; CRC, colorectal cancer; EC, endometrial cancer; MMR, mismatch repair; OC, ovarian cancer; ThC, throat cancer

BRCA2: c.3942dupT; p.(His1165SerfsTer19) was originally detected in her elder sister, who was diagnosed with breast and ovarian cancers at the ages of 60 and 64 years, respectively. The LS9 individual did not carry the *BRCA2* variant detected in the family, despite a diagnosis of breast cancer at the age of 49 years. In this study, we identified a pathogenic variant with moderate penetrance in *NBN* [c.2140C>T; p.(Arg714Ter)] that may be the underlying cause of the breast tumor. Another six relatives (including four females) carried this *NBN* pathogenic variant and, consequently, have an increased genetic risk for breast cancer (Table 1). Details on the genotypes and clinical phenotypes of the remaining relatives are shown in Figure 1.

In contrast, patient LS44 who was diagnosed with CRC at the age of 47 is the only *NF1*-variant carrier in her family. A recent physical examination looking for clinical characteristics of neurofibromatosis type 1 had been performed in patient LS44, and no signs of the disease were detected. We reviewed again the medical history, and no clinical manifestation of neurofibromatosis was observed. Besides, no clinical records related to associated phenotypes were found in the other three families.

In summary, only one out of the five families in whom a second pathogenic variant was detected showed a clinical

phenotype that was associated with the second pathogenic variant (Table 1, Figure 1, and Figure 2).

4 | DISCUSSION

To the best of our knowledge, this was the first study to analyze a comprehensive panel of cancer-predisposing genes by NGS among LS probands who had already been diagnosed by Sanger sequencing. In this present study, we found that ~6% of LS families with co-occurrent pathogenic variants in non-MMR hereditary cancer genes. A better knowledge of the architecture of pathogenic genetic variants for cancer predisposition is essential for more precise risk estimation and personalized counseling for the patients and their families, including adequate cancer prevention and surveillance strategies.

Here, the number of LS probands with a second pathogenic variant for cancer predisposition was unexpectedly high. Five families out of 84 (6%) had at least two germline pathogenic/likely pathogenic variants: [*MLH1-BRCA2-NBN*], [*MLH1-BRCA1*], [*MLH1-FANCA*], [*MSH2-ATM*], and [*MSH6/NF1*].

Two of the non-MMR pathogenic variants identified in this study (2/84; 2.46%) were located in genes that are

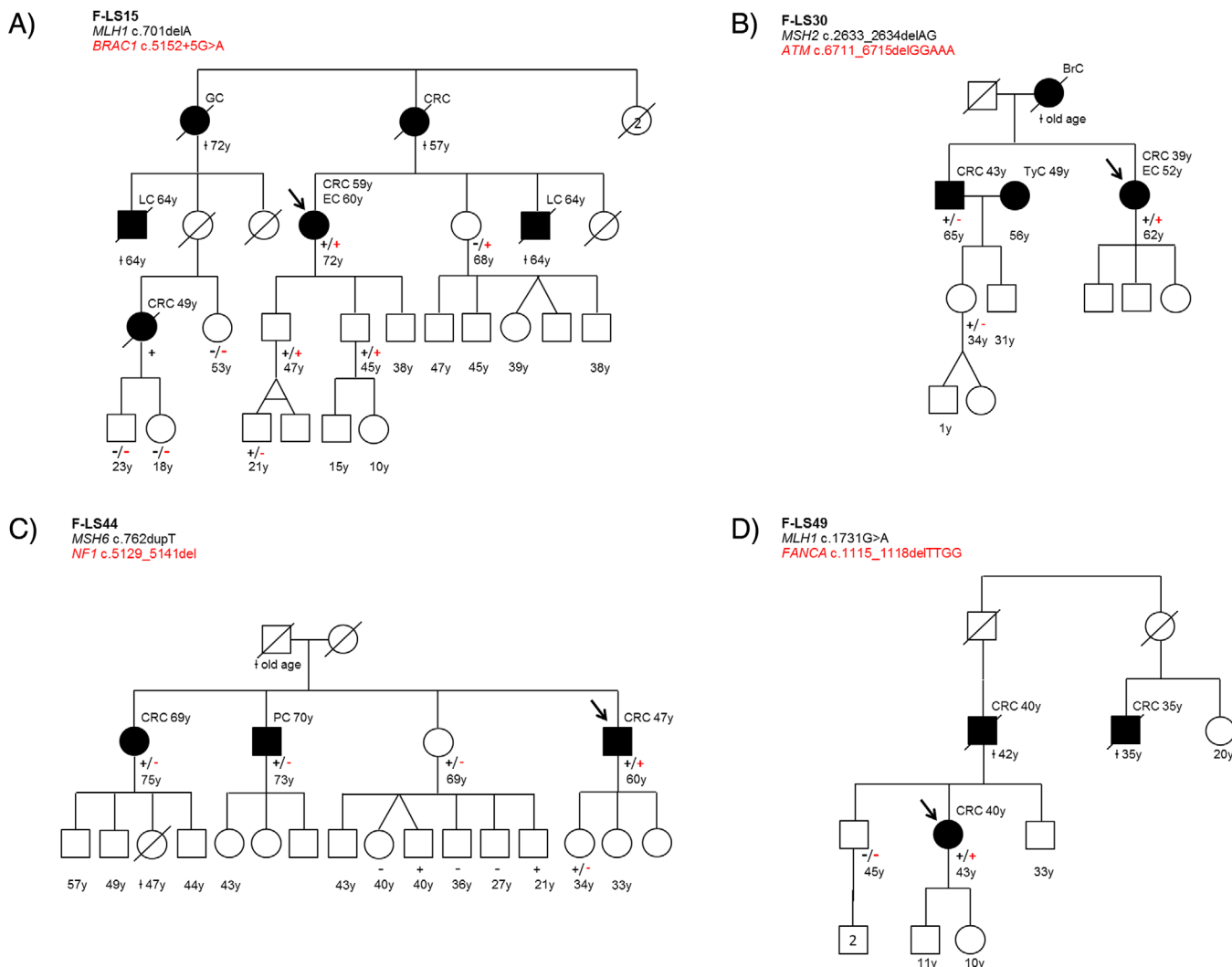


FIGURE 2 Pedigrees of the Lynch syndrome families with a second pathogenic variant in other hereditary cancer genes: A) Pedigree of family F-LS15; B) Pedigree of family F-LS30; C) Pedigree of family F-LS44; D) Pedigree of family F-LS49. The pathogenic variants found in the family are shown at the top. Filled symbols indicate patients affected by cancer (each color refers to a specific type). Age when the pedigree was drawn, age of death, and age at diagnosis, when available, are also detailed. The proband is marked by an arrow. Carrier status was studied in the available relatives, and those carrying or not carrying the pathogenic variants are marked with + and -, respectively: in black for the MMR gene pathogenic variant, and in red for the second pathogenic variant. BoC, bone cancer; BrC, brain cancer; CRC, colorectal cancer; EC, endometrial cancer; GC, gastric cancer; LC, lung cancer; MMR, mismatch repair; PC, pancreatic cancer; TyC, thyroid cancer

considered as having high penetrance for hereditary breast and ovarian cancer (two variants in *BRCA1*) and neurofibromatosis type 1 (one variant in *NF1*). Pathogenic variants in *NF1* are also considered a moderate risk for breast cancer [12, 13]. Moreover, an additional three pathogenic variants were found in genes of moderate penetrance for breast cancer (*ATM*, *NBN*, *FANCA*) [12–15] (Table 1).

Furthermore, the heterozygous recurrent pathogenic variant c.1187G>A; p.Gly396Asp in the *MUTYH* gene was detected in case LS41, a female patient who was diagnosed with endometrial cancer at age of 51 years and belongs to a family fulfilling the Bethesda criteria (Supplementary Table S3). This gene is responsible for attenuated

polyposis and CRC with a recessive mode of inheritance (*MUTYH*-associated polyposis syndrome). A low cancer risk effect of *MUTYH* monoallelic pathogenic variants has been described [16]. Moreover, it has been suggested that a monoallelic pathogenic variant at the *MUTYH* gene could confer additional risk of CRC for carriers of an MMR gene pathogenic variant alone. The evidence supporting this is still insufficient [17]. In any case, the few studies in this regard suggest a digenic inheritance of concomitant heterozygous pathogenic variants in *MSH6* and *MUTYH* that confer an increased risk of colorectal cancer. In this regard, there is no data available on endometrial cancer. Consequently, this heterozygous case was not considered

important in our study. Thus, our results showed that 6% (5/84) of the LS probands with pathogenic variants in MMR genes carried pathogenic variants in other genes associated with a moderate/high risk of different hereditary cancers.

Five of the second pathogenic variants are considered to have moderate penetrance for breast cancer (*ATM*, *FANCA*, *NBN*, and *NFI* variants) [12–15]. This could confer an increase in breast cancer risk in carriers. Cumulative evidence suggests that monoallelic mutations in *FANCA* may predispose to breast cancer. A recent study performed by del Valle et al. [15] reports an odds ratio of 3.14 with a 95% confidence interval of 1.40–6.17 ($P = 0.003$) for breast cancer risk. The associated cancer risk for these variants should also be considered in the surveillance protocols [13].

None of the 84 probands carried a second pathogenic variant in MMR genes, confirming the low prevalence of carriers with pathogenic variants in two different MMR genes among classical LS probands, especially when the sequencing was performed by Sanger and guided by the immunohistochemistry results of MMR protein expression in tumor tissues [18].

The known prevalence of MINAS is low, although it has probably been underdiagnosed because the standard clinical practice consists of testing candidate inherited cancer genes sequentially until a pathogenic variant is detected. In a recent review, the authors reviewed 82 cases with co-occurrence of pathogenic variants in different cancer-predisposing genes in the literature and reported another five new cases with mixed clinical phenotypes [6]. Moreover, they created a specific public database to compile this kind of case. To date, the database contains 103 entries [19]. More recently, Whitworth et al. [20] reported that up to 3% (2/67) of patients with multiple primary tumors had evidence of MINAS.

Conversely, Stradella et al. [21] performed an important prospective study in which they found 13 new MINAS cases out of 1023 unrelated cases with suspicion of hereditary cancer using an extended NGS gene panel. Interestingly, 10 of these MINAS cases presented with clinical manifestations associated with only one of the pathogenic variants identified at the time of diagnosis. The authors concluded that patients with two pathogenic variants usually do not exhibit more severe clinical manifestations, and recommended further analysis and follow-up of those patients to obtain an improved knowledge of the clinical impact of MINAS [21]. In the study reported by Stradella et al. [21], 63 probands carried a pathogenic/likely pathogenic variant in MMR genes. In two of them (3.2%), a second pathogenic/likely pathogenic variant was detected in another hereditary cancer gene. That study [21] and ours have some methodological dissimilarities that could explain the differences in the results obtained: in the study

of Stradella et al. [21], the cohort was prospective, whereas in ours it was retrospective. Moreover, the sample size of our study was slightly larger (63 vs. 84 LS probands), the NGS gene panels were different (135 vs. 94 genes), and the pipelines used for result interpretation were distinct.

In our work, the finding of a second pathogenic variant in cancer-predisposing genes was also unexpected, considering the patients' medical histories. There was no clinical suspicion of a second cancer syndrome in any of the families included here, except for F-LS9. This family is a paradigm of the complexity of genetic risk management in cancer. Two hereditary cancer syndromes coexist in the same family with previously described causal genetic alterations, i.e., LS (*MLHI*) and hereditary breast and ovarian cancer (*BRCA2*). Individual LS9 was diagnosed with endometrial and breast cancers (at the ages of 46 and 49 years, respectively), and she did not carry the *BRCA2* pathogenic variant of the family. Interestingly, in this present study, we were able to identify a new pathogenic variant in the *NBN* gene associated with breast cancer in this patient, which could help explain this event.

The genotypic scenario presented by family LS9, with the combination of three pathogenic alterations in three different hereditary cancer genes, is extremely complex. None of the 10 analyzed relatives were carriers for any of the three pathogenic variants identified in this family, which implies that all of them have a moderate-to-high risk of developing different types of tumors. In such cases, personalized cancer surveillance programs are mandatory to reduce the impact of the disease.

Genotype-phenotype correlation is essential for diagnosing genetic disease in a patient known to carry a disease-associated genetic variant [22]. We expected to find a stronger genotype-phenotype correlation for the secondary pathogenic alleles found using an agnostic approach. In some cases, the lack of a clinical phenotype associated with the risk conferred by the secondary genetic alterations might be caused by the young age of the proband, incomplete penetrance, the small family size, insufficient available information about carrier status in the relatives, or the absence of a comprehensive clinical examination of probands and relatives, to detect expected clinical manifestations. In unusual clinical circumstances, such as MINAS, the possibility of *de novo* or mosaicism mutations cannot be ruled out. In this regard, our data, which were obtained from the relatives tested in the five families with more than one pathogenic variant in different hereditary cancer genes, showed that, at least in two of these families, the new pathogenic variants were not *de novo* mutations (LS9 and LS30). At this time, we do not possess information to determine the *de novo* or inherited

status of the new pathogenic variants in the remainder of the families (Figure 2).

Pathogenic germline alterations in actionable genes should motivate specific clinical surveillance for carriers. According to the Spanish laws, the patient's autonomy and desire to know or to ignore the genetic results from research projects were respected. Those who manifested their right to know about the actionable results from a research project were contacted and received adequate genetic counseling. According to the Spanish Society of Medical Oncology, for *BRCA1* and *BRCA2* women carriers, annual breast contrast magnetic resonance imaging (MRI) at the age of 30-70 years is recommended. Semi-annual transvaginal ultrasound and CA125 tumor marker assessment surveillance may be considered from the age of 30 as ovarian cancer screening. For men, consider mammography in the case of gynecomastia. Other cancer screenings include annual screening with prostate-specific antigen (PSA) for prostate cancer from the age of 40 years is recommended in *BRCA2*, and also offered to male *BRCA1* carriers. For pancreatic cancer, cancer surveillance with endoscopic ultrasounds and MRI in carriers with a first-degree relative with pancreatic cancer from the age of 50 or 10 years before the youngest diagnosis in the family is recommended. Skin and eye examination for melanoma screening are also recommended according to personal/familial risk factors [23].

In general, breast screening MRI is recommended for women with a >20% lifetime breast cancer risk, including those with mutations in highly penetrant genes and the majority of moderately penetrant genes, like *ATM*, *NBN*, and *NFI*. No data are available on the benefit of risk-reducing mastectomy. This procedure may be considered based on family history. Screening for other cancers (ovarian, pancreatic, or prostate cancers) is not recommended in the absence of familial antecedents [13]. For *NFI* unaffected mutation carriers, annual physical and eye examinations for every age is recommended [12]. A significant association with the risk of breast and ovarian cancer have been found in *FANCA* mutation carriers [15]. Nevertheless, *FANCA* is not widely considered as a moderate risk gene, and no surveillance recommendations have been established. In any case, women should be encouraged to participate in breast cancer screening.

Most hereditary cancer-predisposing syndromes are Mendelian disorders in which the phenotypes are complex traits because of the effects of additional independently inherited genetic variations and/or environmental effects. These factors might be responsible for the absence of correlations between the genotypes and clinical phenotypes [24]. The concept of polygenic risk score is being introduced in the field of hereditary cancer. This concept tries to integrate the cumulative effects of moderate- and high-

penetrance germline variants for a better understanding of the architecture of the genetic risk in patients [25].

Our knowledge regarding hereditary cancer syndromes is mainly based on high-risk populations and shows that most of these syndromes have incomplete penetrance and variable expressivity [1]. General population data about the prevalence of high-penetrance pathogenic alleles for hereditary diseases suggest an underestimation of pathogenic allele prevalence and consequently, an overestimation of their real penetrance [26]. Moreover, an increasing overlap in the clinical phenotype among different hereditary cancer syndromes is being reported [7, 27, 28]. The information generated by massive genetic analyses is revealing a new dimension and a more complex view of the genetics reality, thus changing the classical paradigms in this field of study [26–28].

An important limitation of this study was that CNV alterations and a gene containing pseudogenes (*PMS2*) were not analyzed. A more comprehensive study will likely offer a higher diagnostic yield regarding the detection of various pathogenic variants in hereditary cancer genes within a family.

5 | CONCLUSIONS

In summary, our results showed that the prevalence of secondary pathogenic variants in other non-MMR hereditary cancer genes among LS cases is remarkable (about 6%). Further studies are needed to confirm this finding and elucidate its clinical impact. According to our results, we suggest the reanalysis of LS families only if the pedigree suggests a cancer predisposition beyond that explained by LS.

DECLARATIONS

AUTHORSHIP

JLS and AC conceived of the study and participated in its design and coordination. RFA and MIC conducted the experiments, acquired, and analyzed the data. NG, TM, ACA, and ED offered technical support. ABSH and AS collected clinical data, recruited and re-contacted patients for the current study. VDO and ED offered bioinformatics support. RFA, JLS, and AC drafted this manuscript. All authors have read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Informed consent was obtained from all participants. The study fulfilled the Declaration of Helsinki ethical principles for medical research involving human

subjects (64th-WMA-General-Assembly 2013) and received the approval of the Ethics Committee of the Valencian Region. Biological samples and clinicopathological information were obtained from the Valencian Biobank Network and the Hereditary Cancer Program of the Valencian Region (Spain).

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST STATEMENT

The authors of this article certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements) or nonfinancial interest (such as personal or professional relationships, affiliations, knowledge, or beliefs) in the subject matter or materials discussed in this manuscript.

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The raw data have been uploaded to the NCBI Sequence Read Archive, which is a public repository of NGS data, coded as PRJNA594841: Co-occurrence of pathogenic variants for different hereditary cancer syndromes in patients with Lynch syndrome.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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