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A new strategy to screen MMR genes in Lynch Syndrome: HA-CAE, MLPA and RT-PCR ☆

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ABSTRACT

Aims: Hereditary non-polyposis colon cancer (HNPCC) is an autosomal dominant disorder that is genetically heterogeneous because of underlying mutations in mismatch repair (MMR) genes, primarily MLH1, MSH2 and MSH6. One challenge to correctly diagnose HNPCC is that the large size of the causative genes makes identification of mutations both labour intensive and expensive.

Methods: Our heteroduplex analysis by capillary array electrophoresis (HA-CAE) method, previously developed to increase the throughput and allow other multi-exon genes to be scanned, has been adapted for MMR genes. The altered peak patterns were then sequenced. Furthermore, the mutational scanning was completed using the Multiplex Ligation-Dependent Probe Amplification (MLPA) test in all negative HA-CAE cases, and these results were confirmed by RT-PCR.

Results: We studied 216 individuals belonging to 100 unrelated families that met the Amsterdam I/II criteria for HNPCC. We detected 40 different variants that are classified as follows: 8 (20%) deleterious mutation, 8 (20%) unknown pathogenic significance variants and 24 (60%) coding and intronic sequence variants.

Results: Pathogenic mutations were detected in 12% of the families and about 42% of these had a deletion variant. Unknown pathogenic significance variants (UVs) affected 13% of the families. We also found 12.5% of novel polymorphisms in the rest of the variants.

Concluding: In short, using a combined method that includes HA-CAE, MLPA and RT-PCR, it is possible to detect the entire mutational spectrum of MMR genes. Twenty percent of the mutations found in the three genes have not been reported before.

Concluding: Relatives at risk will be offered predictive molecular analysis with potential exclusion of non-carriers of mutations.

1. Introduction

Hereditary non-polyposis colorectal cancer (HNPCC) is a dominantly inherited cancer syndrome characterised by the familial accumulation of early onset colorectal, endometrial and other extracolonic tumours and accounts for around 3–5% of all colorectal cancer cases (CRC).[1,2]

HNPCC is caused by germline mutations in DNA mismatch repair (MMR) genes, human mutL homologue 1 (*MLH1*) on chromosome 3p22.3, human mutS homologue 2 (*MSH2*) on

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chromosome 2p21 and human mutS homologue 6 (*MSH6*) on chromosome 2p16.3, with 5–20% of mutations being large genomic rearrangements missed by conventional mutation screening techniques.[3–5]

The large size of these genes (19 exons for *MLH1*, 16 exons for *MSH2* and 10 exons for *MSH6*) encompassing 9241 nucleotides of coding sequence makes sequencing for germline mutations both labour intensive and expensive.[6] Carriers of germline MMR mutations have a >80% risk of developing cancer by the age of 75.[7–9]

Identification of HPNCC families is important because the diagnosis, treatment and follow-up of these individuals should be different from those with sporadic colorectal cancer.[10] However, the clinical diagnosis of HNPCC patients is very difficult due to a lack of a specific clinical phenotype. Though Amsterdam criteria I and II were established for HNPCC diagnosis,[11,12] many HNPCC families still do not meet the criteria.

Most reported pathogenic mutations are point mutations, comprising single base substitutions, small insertions and deletions.[13] The heteroduplex analysis by capillary array electrophoresis (HA-CAE) technique has already been properly validated by our laboratory in molecular analysis mutations in BRCA1 and BRCA2 genes in breast cancer.[14] In addition, genomic rearrangements such as large deletions and duplications, which cannot be detected by PCR and Sanger sequencing analysis, have been identified in a significant proportion of HNPCC families.[15–17] Yet, in most studies, only patients in whom no pathogenic point mutation had been found were screened for large genomic rearrangements in MMR genes.

Recently, the multiplex ligation-dependent probe amplification (MLPA) method has been introduced to assess DNA copy number changes semiquantitatively.[18]

A number of analytical methods to screen for mutations in the MMR genes have already been established. Several separation methods have been established for DNA mutation analysis.[19–21]

The majority of reported MMR mutations are nonsense, missense or frameshift mutations as well as changes affecting splice sites. However, more recently, the use of new techniques has led to the realisation that, in some populations, a relatively large proportion of pathogenic alterations are genomic rearrangements, in most cases single or multi-exonic deletions or duplications-inactivating MMR genes.[22]

Predictive genetic testing for germline mutations in MMR genes can allow the identification of HNPCC families, and are thus of great importance for the counselling, surveillance and management of at-risk patients.

A recent database catalogues over 2500 MMR gene variants [http://www.med.mun.ca/MMRvariants][23]; while the International Society for Gastrointestinal Hereditary Tumours (InSiGHT, http://www.insight-group.org/) lists over 400 pathogenic mutations that have been identified in the MMR genes.

In this communication, we report the identification of sequence variations in the *MLH1*, *MSH2* and *MSH6* genes using HA-CAE, Sequencing, MLPA and RT-PCR under optimised conditions, and the implementation and validation of this approach in a clinical laboratory setting.

2. Materials and methods

2.1. Patients

This study involves 216 patients from 100 unrelated families suspected of HNPCC recruited through oncogenetic counselling at the General Yagüe Hospital (Burgos, Spain) through the Regional Hereditary Cancer Prevention Programme of the Regional Government of Castilla y León. Index patients were categorised as fulfilling the Amsterdam I/II criteria, and written informed consent was obtained from all patients included in the study.

2.2. Samples

Genomic DNA was isolated from peripherical blood using MagNA Pure Compact Nucleic Acid Isolation Kit I- Large Volume (ROCHE). RNA of all the samples was extracted following the instructions of the commercial QIAamp RNA blood Mini Kit (QIAGEN).

2.3. DNA amplification by multiplex-PCR

Fifty-one PCR fragments (19 for MLH1, 16 for MSH2 and 16 for MSH6) were amplified in 22 multiplex-PCR (9 for MLH1, 6 for MSH2, 6 for MSH6 and 1 mix MSH2-MSH6) groups in relation to their sizes and compatible amplification conditions, one primer of each pair was labelled with 6-carboxyfluorescein (FAM) for MLH1 and MSH6 and hexachloro-6-carboxyfluorescein (HEX) for MSH2; primer pairs, multiplex-PCR groups and annealing temperature conditions were newly designed (Table 1). All the primer sequences were designed to detect possible mutations that can affect splicing mechanisms.

Three different touchdown protocols were performed in a GeneAmp PCR System 9700 (Applied Biosystems) (Table 1): (I) Groups H1A, H1B, H1C, H1D, H1E, H2A, H2B, H2C, H2D, H2E, H6A, H6B, H6C, H6D and H6E: 35 cycles at 94 °C for 45 s, and touchdown annealing temperatures from 60 to 50 °C for 45 s and 72 °C for 60 s. (II) Groups H1F, H6F and H2-H6: 35 cycles at 94 °C for 45 s, and touchdown annealing temperatures from 60 to 55 °C for 45 s and 72 °C for 60 s. (III) Groups H2F: 35 cycles at 94 °C for 45 s, and touchdown annealing temperatures from 65 to 60 °C for 45 s and 72 °C for 60 s. An initial denaturation step at 95 °C for 2 min and a final extension step at 72 °C for 5 min were added to all thermocycling protocols. PCRs were carried out in a final volume of 25 μl containing 200 ng of genomic DNA, 1X buffer concentration was used in all groups except H2D (3.75X) and H2F (1.2X), and primer concentrations are specified in Fig. 1; 600 mM of each dNTP and 2.5U of Taq DNA polymerase (ROCHE).

To perform the heteroduplex formation, the multiplex-PCR fragments were heat denatured at 95 °C for 5 min and then gradually renatured by decreasing the temperature to 20 °C over a period of 90 min.

2.4. HA-CAE mutational analysis of MLH1, MSH2 and MSH6 genes

A detailed description of the HA-CAE method has previously been published by our group.[24] All PCR products were resolved

Table 1 – Primer pairs, multiplex-PCR groups and annealing temperature conditions for multiplex-PCR.

Gen	Annealing (°C)	Group	Exon	Sequence primer forward	Sequence primer reverse	Primer (μl)	Length (pb)
MLH1	60–50	H1A	12	cttcttattctgagtctct	acagaataaaggaggtag*	2.0–2.0	490
			1	gaggtgattggctgaaggc*	tcgtagcccttaagtgagcc	2.0–2.0	162
		H1B	17	ttgttccctgtcctttttc*	cctttccctccagcacaca	1.0–1.0	226
			4	tcagataacctttcccttg*	actcaagatctctgccaaaaa	1.0–1.0	276
			16	cttgctccttcatgttcttg*	agaagtataagaatggctgtc	1.0–1.0	182
		H1C	9	agtttatgggaaggaaccttg*	ccctgtgggtgtttcctg	0.5–0.5	264
			8	ccttgtgtcttctgctgtttg*	taatgtgatggaatgataaacc	0.75–0.75	186
		H1D	13	caagaataataatgatctgcac*	gacaacatgactgctttctc	0.25–0.25	273
			14	ctattacttacctgtttttgg*	gctctgcttgttcacacac	0.75–0.75	183
		H1E	15	ttacttctcccattttgtcc*	gtggagagctactattttcagaa	1.5–1.5	214
			10	cacccctcaggacagttt	tggttgaggagtttggtg*	1.0–1.0	169
MLH1	60–55	H1F	18	agtagtctgtgatctccgttt	ctgtcctagtcctggggt*	1.0–1.0	352
			5	ttgttgatatgattttctcttt	gcttcaacaatttactctcc*	1.0–1.0	207
MLH1	60–50	H1G	19	cagggaggcttatgacatct	gagaaagaagaacacatccc*	0.5–0.5	243
			7	ctagtgtgtgtttttggc*	tccaccagcaaactatta	2.0–2.0	172
		H1H	2	acattagagtagttgcagac*	acaaacatcctgctactt	0.5–0.5	174
			6	cttttgccaggacatcttg*	agcactagaacacattactttga	2.0–2.0	231
		H1I	3	ttaaatcaagaaaatgggaa	tcacaggaggatattttacac*	0.5–0.5	224
			11	ctccccctcccactatctaa	caaaggccccagagaagtag*	2.0–2.0	245
MSH2	60–50	H2A	14	ttgtttttgtatgtgtatgtta	aagtttcccattaccaag*	2.0–2.0	365
			10	tggtagtaggtatttatggaa*	tgttagagcatttagggaa	2.0–2.0	258
			16	gtgtgatatgtttagatggaa	tagcttatcaatattaccttca*	1.5–1.5	312
		H2B	4	ttttcttattccttttctca*	ttgagataaatatgacagaaat	2.0–2.0	214
			15	cataaattgctgtctcttc	aactatgaaaacaaactgac*	0.25–0.25	257
			5	tggatccagtggtatagaaat	ctgaaaaaggttaagggctc*	1.5–1.5	198
		H2C	3	gttcaagagtttgttaaatt*	tggaatctcctctatcact	2.0–2.0	358
			2	gaagtccagctaatacagtg	cttcacatttttattttttctactc*	1.5–1.5	286
			11	cacattgcttctagtacacattt*	caaaagccaggtgacatt	0.25–0.25	204
		H2D	7	tgcttagttgataaattttaatt	tattgtatgagttgaaggaaaa*	2.0–2.0	313
			13	cttgctttctgatataatttg*	caagggactaggagatgcac	0.5–0.5	258
		H2E	1	gggacgtgggaggggag	actctctgaggcgggaaag*	1.5–1.5	370
			9	ggattttgtcactttgttctgtt*	ggggacagggaacttataaaa	1.5–1.5	249
MSH2	65–60	H2F	12	atttttacggcttatatctgttta	gttacccccacaaagccc*	1.0–1.0	352
			8	tgagatctttttatttgtttgtttt*	agtggcctttgctttttaa	1.0–1.0	207
MSH6	60–50	H6A	9	ttttttgagagggcacttt	ccccttttactgtttctttg*	1.0–1.0	328
			8	ccttttttgttttaattcct*	cctctcaaaaaaccgaa	1.0–1.0	236
		H6B	5	aaaaccccccaaacgatgaag*	tggaaaatgatcacctaagtatgtt	1.0–1.0	391
			4D	gttcattgtcctgttctcttc*	aaaaggagtatggcaagtatc	1.0–1.0	476
		H6C	1	tccgtccgacagaacggttg*	gcaggcgctaccgatctc	1.0–1.0	287
			4C1	ggatgaggaagtggtggcagat*	tatgcacgagtatggccagaaga	1.0–1.0	441
		H6D	3	gggattacaggcgtgagc	ccccatcaccctaacataaa*	0.5–0.5	280
			4B	cgttagtggaggtggtgatg*	tttcatgaataccagcccc	1.5–1.5	307
			4F	gaaaaggctcgaaagactgg*	gggataatatacagctggcaaa	0.5–0.5	487
		H6E	6	tcataagaaagacaaaagtt*	ctcatatacaagaagcaaat	0.5–0.5	268
			4C2	ggtgatccctctgagaac*	tccctaaaatattcttcctc	1.5–1.5	329
			4E	gtgacattaaacaacttggagat	agcctgctttgggagtaa*	1.0–1.0	586
MSH6	60–55	H6F	10	tttaattttaagggaagt	ccattatgtagaaggtagat*	2.0–2.0	257
			2	gaaacttgaccaaatattaact	cctgtctgtctgtttctctc*	1.0–1.0	317
			4A	aaagtcaaaaaatcataagt	aagccattctaaagtttc*	0.5–0.5	580
MSH2	60–55	H2-H6	H2-6	gcttgccattctttctattt*	agtggtataatcatgtgggtaa	2.0–2.0	238
MSH6			H6-7	ggtgaaagtacatttttgttg*	tgcgtgctctaaaaacattc	1.0–1.0	212

*Labelled primer.

on an ABI 3100 DNA sequencer (16 capillaries; Applied Biosystems). Fragment analysis was carried out using GeneScan software v3.7 (Applied Biosystems). Heteroduplexes were identified by the presence of altered peak patterns as compared to a control sample.

2.5. *DNA sequencing*

Fragments showing an HA-CAE-altered pattern were sequenced with the BigDye Terminator Sequencing Kit v3.1 (Applied Biosystems) with unlabelled forward and reverse primers on an ABI 3100 DNA sequencer (4 capillaries; Applied Biosystems).

2.6. *Screening for genomic rearrangements and confirmation by RT-PCR analysis*

This analysis was performed using the SALSA P003-MSH2/MLH1 MLPA kit (MRC Holland, Amsterdam, The Netherlands; www.mrc-holland.com) according to the manufacturer's

Table 2 – Mutations and variants detected by HA-CAE and MLPA techniques.

Family code	Gen	Location	Mutation	*Type of mutation	Clinically	Detection method	Previously reported
Deleterious mutations							
VA44	MLH1	EX3	c.306+5G>A	S	Deleterious	HA-CAE	YES
VA67							
VA104							
VA2	MLH1	EX19	c.2284-2287delACCT; 2284-2313ins30	F	Deleterious	HA-CAE	NO
VA117	MSH2	EX2	C.227_228delAG, STOP80	F	Deleterious	HA-CAE	YES
VA6	MSH2	EX10	c.1661G>A	S	Deleterious	HA-CAE	NO
VA8	MSH2	EX16	c.2651T>G, p.I884S	M	Deleterious	HA-CAE	YES
VA25	MSH2	EX1–3	c.1-?_306+?del, p.Met1_Glu102del	Deletion of Exon 1-3	Deleterious	MLPA	YES
VA4	MSH2	EX7	c.1077-¿_1276+?del; p.Leu360_Gly426>LysfsX16	Deletion of Exon 7	Deleterious	MLAP	YES
VA17	MSH2	EX4–8	p.Ile216_Gln462del	Deletion of Exon 4-8	Deleterious	MLPA	NO
VA20							
VA32							
Unknown variants							
VA111	MLH1	EX5	c.394G>C, p.D132H	M	Unknown	HA-CAE	YES
VA30	MLH1	EX13	c.1574G>A, p.S505N	M	Unknown	HA-CAE	NO
VA47	MLH1	EX16	c.1852_1853AA>GC, p.K618A	M	Unknown	HA-CAE	YES
VA55							
VA82							
VA108							
VA121							
VA70	MLH1	EX19	c.2146G>A, p.V716M	M	Unknown	HA-CAE	YES
VA43	MSH2	EX2	c.212-5delT	UV	Unknown	HA-CAE	YES
VA4	MSH6	EX2	c.431G>T, p.S144I	M	Unknown	HA-CAE	YES
VA94							
VA22	MSH6	EX4	c.2633T>C, p.V878A	M	Unknown	HA-CAE	YES
VA85	MSH6	EX5	c.3425C>T, p.T1142M	M	Unknown	HA-CAE	NO
Sequence changes							
	MLH1	IN3	c.307–29C>A	UV	Sequence change	HA-CAE	NO
	MLH1	EX8	c.655A>G, p.I219V	M	Sequence change	HA-CAE	YES
	MLH1	IN13	c.1558+14G>A	P	Sequence change	HA-CAE	YES
	MLH1	IN14	c.1668–19A>G	P	Sequence change	HA-CAE	YES
	MLH1	EX17	c.1959G>T, p.L653L	P	Sequence change	HA-CAE	YES
	MSH2	IN1	c.211+9C>G	P	Sequence change	HA-CAE	YES
	MSH2	EX2	c.339G>A, p.K113K	P	Sequence change	HA-CAE	YES
	MSH2	IN9	c.1511–9T>A	P	Sequence change	HA-CAE	YES
	MSH2	IN10	c.1661+12G>A	P	Sequence change	HA-CAE	YES
	MSH2	IN12	c.2006–6T>C	P	Sequence change	HA-CAE	YES
	MSH6	EX1	c.116C>A, p.G39E	SNP	Sequence change	HA-CAE	YES
	MSH6	EX1	c.186G>A, p.A62A	P	Sequence change	HA-CAE	YES
	MSH6	EX2	c.276A>G, p.P92P	P	Sequence change	HA-CAE	YES
	MSH6	IN2	c.458–52G>T	SNP	Sequence change	HA-CAE	YES
	MSH6	EX3	c.540T>C, p.D180D	P	Sequence change	HA-CAE	YES
	MSH6	EX4	c.642C>T, p.Y214Y	P	Sequence change	HA-CAE	YES
	MSH6	EX4	c.1164C>T, p.H388H	P	Sequence change	HA-CAE	YES
	MSH6	EX4	c.1186C>G, p.L396V	P	Sequence change	HA-CAE	YES
	MSH6	EX4	c.2400T>C, p.V800V	P	Sequence change	HA-CAE	NO
	MSH6	EX5	c.3246G>T, p.P1082P	P	Sequence change	HA-CAE	YES
	MSH6	IN5	c.3438+14A>T	P	Sequence change	HA-CAE	YES
	MSH6	IN5	c.3439–16C>T	P	Sequence change	HA-CAE	YES
	MSH6	IN7	c.3646+29delCTAT	P	Sequence change	HA-CAE	YES
	MSH6	EX10-3́UTR	c.4083+85T>A	UV	Sequence change	HA-CAE	7NO

*Type of mutation: S: splicing, F: frameshift, M: missense, UV: unspecified variant, P: polymorphisms, SNPs: single-nucleotide polymorphisms.

protocol using 180 ng of genomic DNA (gDNA) from each patient. Reactions were analysed using an ABI 3130 automated sequencer (Applied Biosystems, Applied Biosystems, Foster City, CA; www.appliedbiosystems.com), and data analysis

was performed by GeneMapper Analysis software (Applied Biosystems). All the alterations detected were confirmed by RT-PCR. Three microlitres of total RNA were used to synthesise complementary DNA (cDNA) by High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) using random primers. RT-PCR was performed with specific primers designed for the coding sequences flanking the putative mutation. To confirm deletions, short amplicons from RT-PCR were sequenced.

Primers pair for RT-PCR: *c.306+5G>A*: forward in exon 2: 5′-agacaatggcaccgggat -3′ and Reverse in exon 4: 5′-cacatggcttatgctggc -3′. *Exon 7 deletion*: Forward in exon 6: 5′-tgctgaataagtgtaaaacccc -3′ and Reverse in exon 8: 5′-ggagaagtcagaacgaagatcag -3′. *Exon 4-8 deletion*: Forward in exon 3: 5′-gttggagttgggtatgtggatt -3′ and Reverse in exon 9: 5′-tgttgactgcatcttcttttcc -3′.

3. Results

The results of screening MLH1, MSH2 and MSH6 gene mutations through the multi-method HA-CAE plus MLPA in our population are described in Table 2.

The new strategy revealed a total of 40 different DNA sequence variants: 11 in MLH1, 12 in MSH2 and 17 in MSH6, and these were categorised as 8 definitely pathogenic mutations, 8 unspecified variants and 24 polymorphisms. About 20% of the mutations (8 of 40) had not been reported before in databases [http://www.insight-group.org/; http://www.med.mun.ca/MMRvariants/].

Thirty-seven different alterations were detected by the HA-CAE method in 100 unrelated families (SM1, SM2, SM3, SM4 and SM5 in Supplementary material). MLPA was performed on those families in whom no germline mutation was found, and we report here three different genomic rearrangements, one of which is novel.

Twelve families were the carriers of a deleterious mutation: two in MLH1 (4 families) and six in MSH2 (8 families), one and two of which, respectively, were novel. Fig. 1 shows the electropherograms obtained by HA-CAE of these variants. No deleterious mutations were found in the MSH6 gene.

Two splicing mutations were detected and then confirmed by RT-PCR (Fig. 2), one of which was in exon 3 of MLH1: c.306+5G>A (this was found in three families); and the other one in exon 10 of MSH2: c.1661G>A (this is a novel mutation and we found it in only one family).

Five families revealed a genomic deletion, all in the MSH2 gene (Table 2). The MLPA assay, RT-PCR products and Sequencing pattern are all shown in Fig. 3. We have identified a novel deletion more than once: three different families (VA17, VA20 and VA32) have the exons 4–8 deletion.

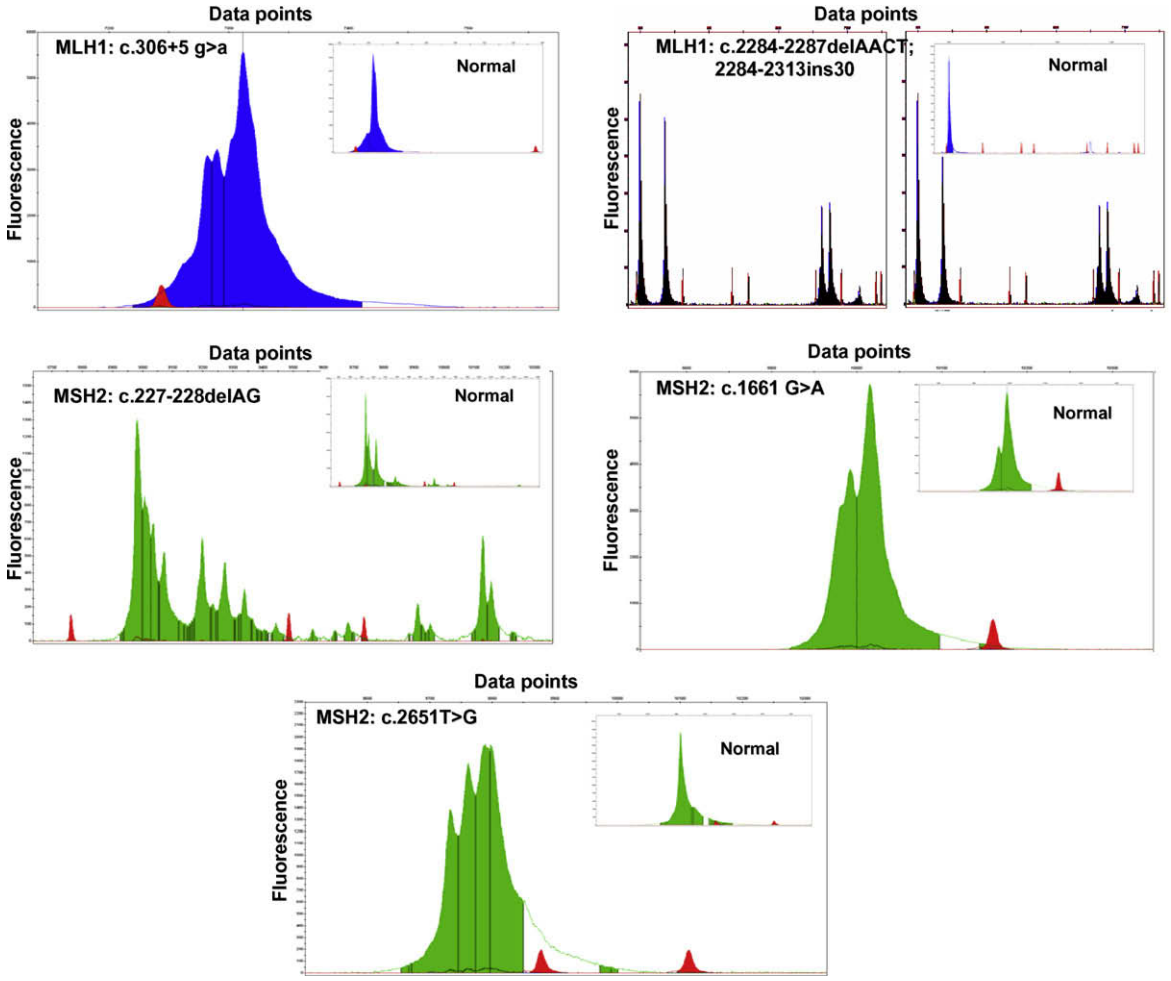

Fig. 1 – Electropherograms of deleterious mutation obtained by HA-CAE technique.

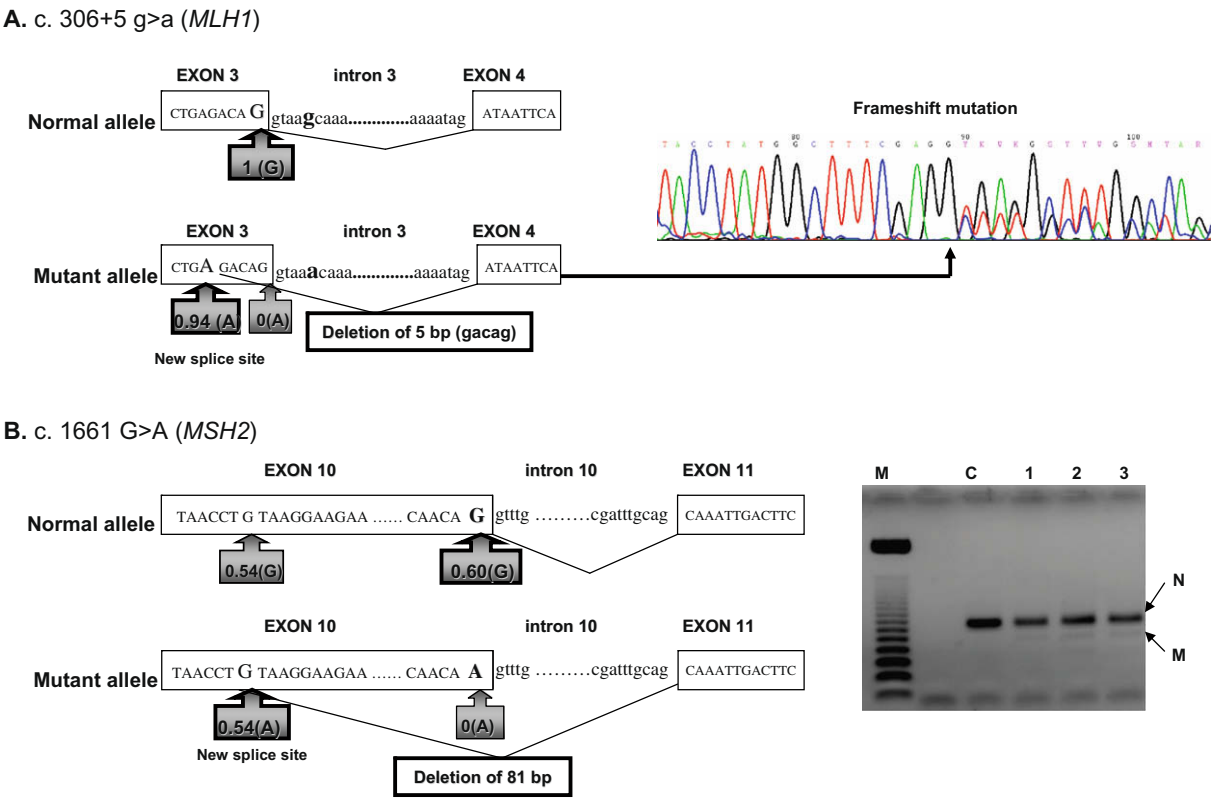

Fig. 2 – Graphical representation of splicing mutation. Characterisation of c.306+5G>A mutation by sequencing (A), RT-PCR results of c.1661G>A mutation (B).

We characterised seven different missense variants and one unclassified variant in 13 families, two of which were novel: p.S505N in hMLH1 and T1142M in MSH6. (Peak patrons in SM1 and SM2 in Supplementary material) (Unknown Variants in Table 2).

Furthermore, we have detected 24 different sequence variants, predicted to be polymorphisms, in several families (Sequence Changes in Table 2). A large number of polymorphisms were found in both coding and intronic sequences, and 12.5% of the polymorphisms were novel (1 of 5 in MLH1 and 2 of 14 in MSH6). Some polymorphisms have been detected once in our 100 families: c.655A>G and c.1959G>T in MLH1; c.339G>A in MSH2 and c.116G>A, c.642C>T, c.1164C>T and c.1186C>G in MSH6. Sometimes, two of these polymorphisms appear together and this produced another different peak patron in HA-CAE (P5 of SM1, P16 of SM3, P26 of SM5 and P21 of SM4 in Supplementary material).

4. Discussion

Detecting germline mutations of the responsible genes for cancer predisposition is a recognised need in cancer genetics. To date, hundreds of mutations, distributed in all the exons of the MMR genes, have been identified in a large number of families.

The rapid detection of disease-causing mutations allows the identification of asymptomatic carriers who may benefit from tailored screening and prevention protocols. Therefore, it is increasingly important to develop simple, low-cost, sensitive, high-throughput methods to identify sequence variations in such complex genes, HA-CAE fulfills these criteria, and allows the mutational analysis of MMR genes in a short period of time. Consequently, this technique may be of interest for genetic testing laboratories that routinely screen these complex genes in a large number of samples.

The overall mutation detection rate for pathogenic mutations in this series was 20%. These pathogenic mutations were detected in 12% of the families, mutations in MLH1 and MSH2 account for about 33% and 67% of cases, respectively. Thus, 37% of deleterious mutations are novel.

In the MLH1 gene, we found two different pathogenic mutations: the variant *c.306+5G>A*, a G to A transversion affecting position +5 of the splice donor site of MLH1/exon 3; the sequencing of this revealed a deletion of five nucleotides (Fig. 2A) which gave rise to the aberrant splicing previously identified.[25] We describe a rare novel mutation in the MLH1 gene in a family, whose frameshift mutation was found by our group and published previously.[26] This complex mutation (*c.2284-2287delACCT; 2284-2313ins30*) that consists of a deletion of 4 nucleotides (ACCT) and the insertion of 30 nucleotides led to an extension of 46 new amino acids on the extreme COOH-terminus (Fig. 1). An important functional role for this region of the protein has been suggested.[27] Seven members were studied, and the mutation was also seen to segregate. The affected members showed the same mutation and one asymptomatic carrier was detected.

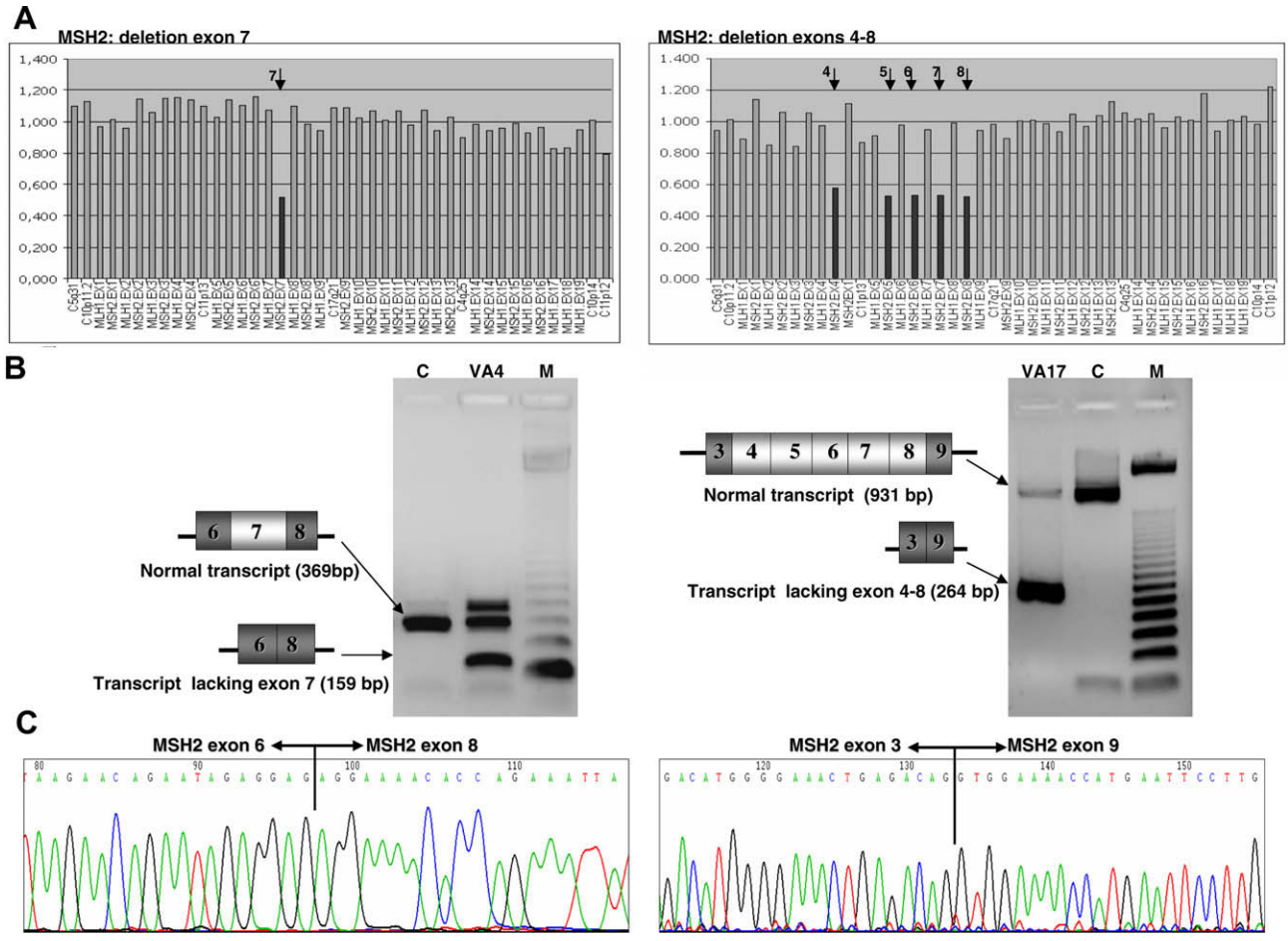

Fig. 3 – MLPA imaging (A), RT-PCR results (B) and sequencing (C) of 7 exon and 4–8 exon deletions found in MSH2.

In the MSH2 gene, we found six different deleterious mutations: the frameshift mutation *c.227_228delAG* in exon 2 created a premature stop in codon 80 that led to premature protein truncation (Fig. 1). We detected a novel transversion (*c.1661G>A*) affecting the last nucleotide of exon 10. The consensus value for guanine at this position of the splice donor sites of primate genes is 0.78.[28] In this case, it has been observed that the change from guanine to cytosine makes the natural splicing site 5′ (value 0.60) disappear, using a new donor cryptic site in position c.1580 (value 0.56), thus producing the deletion of 81 nucleotides in the mRNA. This point was confirmed by means of RT-PCR analysis, so that the normal fragment would have a size of 357 bp and the aberrant fragment 246 bp (Fig. 2B). The variant c.2651T>G (*p.I884S*), classified as a missense mutation (Fig. 1), probably alters the protein function, but not its translation, and would consequently not be detected by immunohistochemistry, which evaluates protein expression. This variant only appears once in the MMRvariants Database. Besides, we found and confirm three deletion rearrangements: in family VA25, the exons 1–3 *deletion* also included the hMSH2 promoter,[29] CRC occurred in the proband at the age of 34, his healthy sister is a carrier; an exon *7 deletion* in another family, this rearrangement was described previously[30]; and we have identified one novel deletion more than once, three different families (VA17, VA20 and VA32) have exons *4–8 deletion* (Fig. 3), twenty six members of these families were checked and we found 17 carriers, exons 4–8 deletion has not been detected in any studies. This mutation can be shown to represent a founder mutation, and their pathogenicity is supported by the cosegregation of the mutation with the disease in three unrelated families.

Our results are not comparable to those observed in another Spanish study, which found a majority of MLH1 mutations.[31]

Another finding presented here is that 50% of pathogenic mutations in MSH2 are large genomic deletions, about 42% of the families with deleterious mutation have a deletion. These results in our Spanish population show higher percentages of rearrangements than those previously published by other authors,[32] but then again, they are similar to others.[33]

In this study, we found 20% of variants that we classified as of unknown pathogenic significance (UVs). Twenty percent of UV variants were novel variants: *p.S505N* in exon 13 at MLH1, this missense mutation has not been described previously but in the database, another variant, V506A, appears in the next amino acid that displayed a 25–65% reduction in binding to hPMS2.[34] This variant was found in a 52-year-old female and three synchronimous CRC and other cancers through the maternal line; the proband, her brother and mother all have CRC.

At MSH6 in exon 5, we found *p.T1142M* at the index case of VA85 family with onset 29. In the database, another missense mutation has been described with three codons upstream, and this variant is found in the P-loop of the ATP-binding domain required for the hydrolysis of ATP.[35]

On the other hand, 60% of the variants found are polymorphisms. Single-nucleotide polymorphisms (SNPs) represent an important class of genetic variation, and SNPs within and outside coding sequences are under intense examination for possible associations or mechanistic links to disease.[36] Preliminary studies have demonstrated increased throughput of HA-CAE in polymorphism analysis[37], and here we demonstrate its successful application for MMR genes.

In short, the present work demonstrates the successful application of the HA-CAE method to detect mutations in the MLH1, MSH2 and MSH6 genes. We believe that, with these screening methods, the risk of missing a family carrying a germline mutation is low, and that with the HA-CAE and MLPA combined method, a high mutation detection yield is guaranteed.

In this study, relatives at risk from patients will be offered predictive molecular analysis with potential exclusion of non-carriers of mutations from the surveillance programme.

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 doi:10.1016/j.ejca.2009.01.030.

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