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EPCAM Germ Line Deletions as Causes of Lynch Syndrome in Spanish Patients

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The standard genetic test for Lynch syndrome (LS) frequently reveals an absence of pathogenic mutations in DNA mismatch repair genes known to be associated with LS. It was recently shown that germ line deletions in the last exons of *EPCAM* are involved in the etiology of LS. The aim of this study was to evaluate the prevalence of *EPCAM* deletions in a Spanish population and the clinical implications of deletion. Probands from 501 families suspected of having LS were enrolled in the study. Twenty-five cases with MSH2 loss were identified: 10 had mutations of *MSH2*, five had mutations of *MSH6*, and 10 did not show *MSH2/MSH6* mutations. These 25 cases were analyzed for *EPCAM* deletions using multiplex ligation-dependent probe amplification, and deletions were mapped using long-range PCR analysis. One subject with no *MSH2/MSH6* mutations had a large deletion in the *EPCAM* locus that extended for 8.7 kb and included exons 8 and 9. The tumor exhibited *MSH2* promoter hypermethylation. *EPCAM* deletion analysis followed by *MSH2* methylation testing of the tumor is a fast low-cost procedure that can be used to identify mutations that cause LS. We propose that this procedure be incorporated into clinical genetic analysis strategies and present a decision-support flow diagram for the diagnosis of LS. *(J Mol Diagn 2010, 12:765–770; DOI: 10.2353/jmoldx.2010.100039)*

Lynch syndrome (LS) is an autosomal dominant inherited cancer syndrome characterized by early-onset cancers of the colorectum and endometrium and tumors of the stomach, pancreas, small intestine, ovary, bladder, and bile duct.[1] In the Spanish population, about 2.5% of colorectal cancers are associated with LS.[2] The carcinogenetic etiology of this syndrome involves a DNA mismatch repair (MMR) inactivation caused by a germ line mutation of an MMR gene (*MLH1*, *MSH2*, *MSH6*, or *PMS2*) followed by somatic inactivation of the second allele.[1] As a consequence of MMR inactivation, these tumors exhibit microsatellite instability (MSI) and loss of expression of the mutated MMR gene.[1] It was recently shown that germ line deletions involving the last exon of the non-MMR gene, *EPCAM* (*OMIM#185535*), may silence its neighboring gene, *MSH2* (*OMIM#609309),* which is located 17 kb downstream of *EPCAM*, via promoter hypermethylation. This epigenetic inactivation seems to be effective only in tissues in which *EPCAM* is expressed.[3,4] The *EPCAM* gene codes for the epithelial cell adhesion molecule also known as CD326, which is expressed in all normal epithelial cells and in carcinoma tumors.[5] Thus, deletions of the last exon of *EPCAM* constitute a distinct class mutation associated with LS.

Currently, the standard genetic test for LS (point mutation and large-rearrangement analysis of *MLH1*, *MSH2*, *MSH6*, and *PMS2*) frequently fails to detect a pathogenic mutation. For this reason, we evaluated the association between *EPCAM* deletions and LS in a Spanish population and its clinical implications.

Materials and Methods

Patients

A total of 501 index subjects from Spanish families suspected of having LS were recruited from the Genetic Counseling in Cancer units of the La Fe and Elche University Hospitals between 2005 and 2009. All subjects fulfilled the Bethesda Guidelines (Amsterdam II criteria cases included).[6] The median age at diagnosis was 49 years (range 21–89 years). Clinical and molecular characteristics of this series are listed in Table 1. Patient selection was based on the results of MSI testing, immu-

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Table 1. Clinical and Molecular Characteristics of Index Subjects

Variables	n	%
Sex		
Male	233	46.5
Female	268	53.5
Criteria		
Bethesda	378	75.4
Amsterdam II	123	24.6
Tumor type		
CRC	463	92.4
Endometrial	24	4.8
Others	14	2.8
MSI analysis		
Positive	102	20.4
Negative	371	74.0
Not analyzed	28	5.6
IHQ analysis		
Normal expression	322	64.3
MLH1 loss	44	8.8
MSH2 loss	25	5.0
Not analyzed	110	21.9
BRAF V600E mutation and/or *MLH1* hypermethylation analysis*		
Positive	9	20.5
Negative	35	79.5

*Analysis performed to Bethesda cases with loss of MLH1.

nohistochemical (IHC) analysis of MMR proteins, and mutation analysis of *MLH1*, *MSH2*, and *MSH6*. Written consent for the diagnostic genetic tests was obtained from each patient.

Pregenetic Study of Tumor Tissue: Analysis of Expression of MMR Proteins, MSI, the Presence of the BRAF *V600E Mutation, and* MLH1 *Promoter Hypermethylation*

In all cases for which tumor tissues were available, we performed MSI analysis and IHC analysis of MMR proteins ($n = 483$). Tumors from non-Amsterdam II subjects with loss of *MLH1* expression were analyzed for the presence of the *BRAF* V600E mutation and for *MLH1* promoter hypermethylation. The presence of the *BRAF* V600E mutation or *MLH1* hypermethylation indicates that the tumor is of sporadic origin and is disregarded in genetic analysis.[7] Samples from subjects with loss of MSH2, MSH6, or MLH1 who did not have the *BRAF* V600E mutation or *MLH1* hypermethylation were analyzed for germ line mutations of the corresponding genes. Samples from subjects for whom tumor tissues were not available but who fulfilled the Amsterdam II criteria were also analyzed for germ line mutations ($n = 18$).

IHC analysis of MLH1, MSH2, MSH6, and PMS2 expression was performed as previously described.[2] To assess MSI, monomorphic markers (BAT26, BAT25, NR21, NR24, and NR27) were analyzed as reported by Buhard et al.[8] To detect the *BRAF* V600E mutation, which is located at exon 15, DNA was sequenced as described by Domingo et al.[9] Methylation analysis of the *MLH1* gene was conducted using methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA; kit ME011, MRC-Holland, Amsterdam, The Netherlands) according to manufacturer's protocol. The MS-MLPA technique for detecting *MLH1* hypermethylation has been validated.[10]

MLH1, MSH2, *and* MSH6 *Germ Line Mutation Analyses*

The selection of genes for analysis was based on IHC results. DNA from peripheral blood leukocytes was used for the analysis. Detection of point mutations was performed using PCR and direct sequencing of the whole coding sequence and the intron–exon boundaries of each gene.[11–13] Large rearrangements (deletions and insertions) were analyzed using MLPA [kits P003 (MLH1–MSH2), P248 (MLH1–MSH2 confirmation), and P008 (PMS2–MSH6); MRC-Holland] according to the manufacturer's recommended procedure.

EPCAM *Germ Line Deletion Analysis*

Samples from subjects with loss of MSH2 and MSI ($n = 25$) were analyzed for large deletions of the *EPCAM* locus using MLPA (kit P072-B1; MRC-Holland) according to the manufacturer's recommended protocol. This kit contains six probes for the region of interest: four are targeted at *EPCAM* exons 3, 8, and 9, and two are targeted at the intergenic region between the *EPCAM* and *MSH2* loci (Figure 1). Deletions were confirmed and mapped using multiple long-range PCR analysis of genomic DNA (Expand Long-range dNTP Pack; Roche, Mannheim, Germany) and various combinations of primer pairs that targeted the candidate chromosomal region (Figure 2). DNA sequencing was performed to characterize the deletion breakpoints.[14]

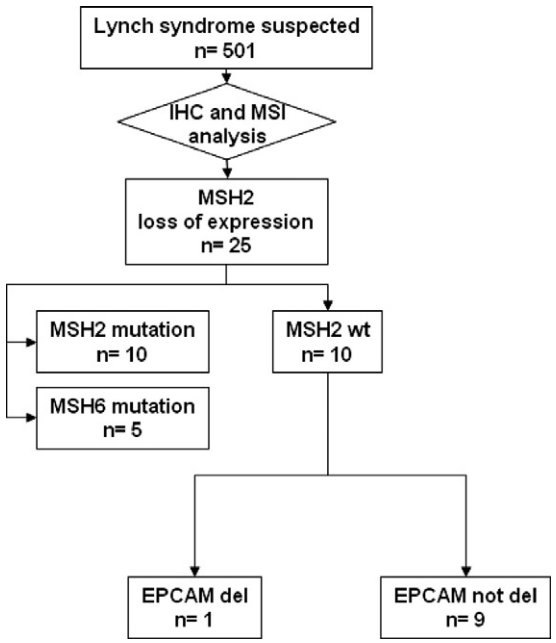

Figure 1. Schematic overview of the MMR and *EPCAM* genetic analysis. IHC, immunohistochemical; MSI, microsatellite instability.

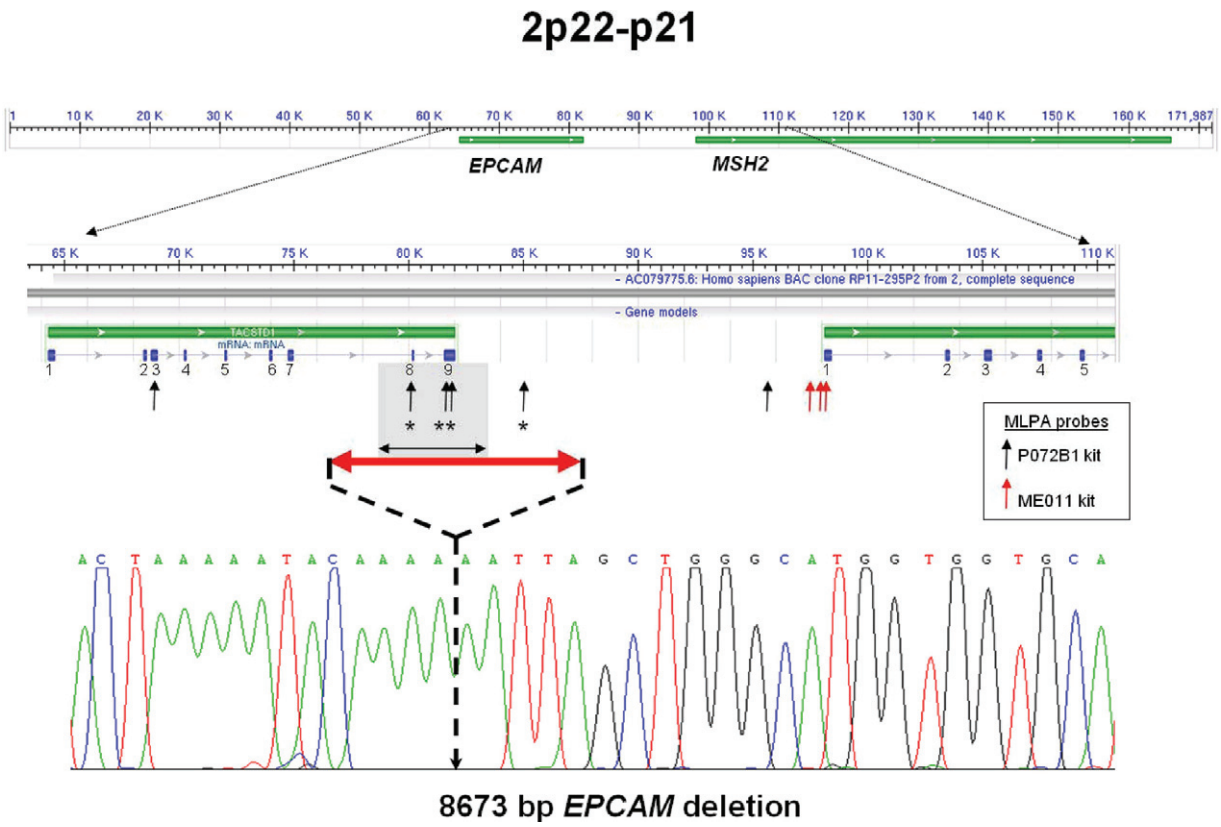

Figure 2. Structural organization of the *EPCAM-MSH2* locus. The *EPCAM* and *MSH2* genes are indicated in green, with an expanded region showing positions of relevant exons. Positions of the MLPA probes are indicated by **black** and **red vertical arrows**. **Arrows** with **asterisks** indicate probes that revealed the *EPCAM* deletion discovered in this study. The minimal deleted region (5 kb) is depicted as a gray rectangle. The **red double arrowhead** illustrates the length of the deletion, and a sequencing trace that includes the deletion breakpoint of the 8673-bp deletion is shown.

MSH2 *Promoter Hypermethylation Analysis*

Subjects with a germ line *EPCAM* deletion were tested for tumor and peripheral blood leukocyte hypermethylation of the promoter region of the *MSH2* gene using MS-MLPA (kit ME011; MRC-Holland). This kit contains three probes for that region. The threshold of methylated versus unmethylated samples was 15% based on a previous study.[10]

Results

Of the 501 index subjects analyzed, 126 belonged to families that fulfilled the Amsterdam II criteria and 375 subjects fulfilled the Bethesda guidelines. After screening using MSI testing, IHC analysis of MMR proteins, testing for the presence of the *BRAF* V600 mutation, and *MLH1* methylation analysis, samples from 155 subjects were analyzed for point mutations and large rearrangements of *MLH1*, *MSH2*, and *MSH6* (Figure 1). Mutations of these genes were detected in 51 subjects (32.9%) (details of these mutations will be published elsewhere). Twenty-five subjects had MSH2 loss and MSI. Of these, 10 had a mutation of the *MSH2* gene and five had a mutation of the *MSH6* gene. The remaining 10 cases did not have alterations of the *MSH2* or *MSH6* genes. None of the mutated *MSH2* or *MSH6* genes showed *EPCAM* deletions, and only one patient with no *MSH2–MSH6* mutation harbored a large deletion at this locus (1/10; 10%). This deletion was confirmed by MLPA analysis. We then analyzed samples from the proband's brother, who had colorectal cancer (CRC) that was diagnosed at the age of 36 years, and detected the same *EPCAM* deletion. This subject also had MSH2 loss and MSI. A subsequent test using the three MS-MLPA probes revealed a clear hypermethylated pattern in the tumors of both subjects. Moreover, no *MSH2* methylation was found when peripheral blood leukocytes from these two subjects was analyzed. A mosaic silencing of *MSH2* occurs only in tissues that express *EPCAM*, which is characteristic of these deletions.

Deletion mapping using long-range PCR showed that this deletion extended for 8.7 kb (g.77525_86198del8674 from AC079775.6), including the 5-kb minimal deleted region (*EPCAM* exons 8 and 9).[4]

The pedigree of the family is shown in Figure 3. This family fulfilled the Amsterdam I criteria for LS. There were five CRCs in three consecutive generations, four of which were diagnosed before the age of 50. Individuals IV-1 and IV-2 were positive for the *EPCAM* deletion. Family members are currently undergoing analysis for this mutation to predict their risk of developing LS.

Discussion

The most accepted strategy for LS screening comprises the analysis of samples from patients who fulfill the Be-

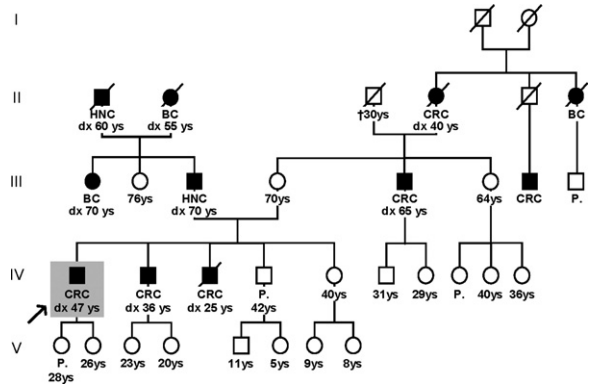

Figure 3. Pedigree of a family with an *EPCAM* mutation. Family members indicated by solid symbols were diagnosed with CRC at the ages indicated. Index subject IV-1 is indicated by a **gray rectangle** and an **arrow**. HNC, Head and neck cancer; BC, Breast cancer; CRC, Colorectal cancer; and P, polyps.

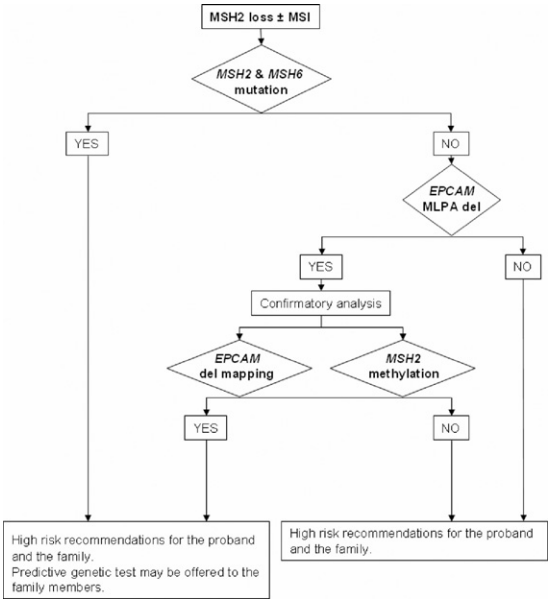

Figure 4. Proposed decision-support flow diagram for incorporating *EPCAM* deletion testing into the LS genetic diagnosis strategy.

thesda guidelines for DNA MMR status, followed by genetic analysis of MMR genes, if indicated. Markers such as the *BRAF* V600E mutation and *MLH1* promoter hypermethylation may help to distinguish sporadic from familial tumors when *MLH1* loss is present in Bethesda-positive cases.[7,10] Our laboratory used this strategy to analyze 501 probands recruited over four years from two genetic counseling units of the Comunidad Valenciana (Spain). The efficacy of detection of mutations associated with LS was about 10% for the 501 consultant patients and 33% for the 155 who underwent genetic testing, which is similar to previous results.[15] Cost-effective screening methods that are more sensitive and specific than current methods are required.[16] Mutations in genes that regulate expression of MMR genes may account for the low rate of detection of mutations associated with LS. Germ line hypermethylation of the *MLH1* or *MSH2* genes is an atypical alteration.[17,18] *MSH2* germ line hypermethylation might be caused by the positional effect of large deletions that affect the last exon of the *EPCAM* gene located 17 kb upstream of *MSH2*.[3] A variety of mechanistically different phenomena classed as negative chromosomal position effects may induce gene silencing through changes in the chromosomal environment rather than by direct targeting of the gene.[19] The presence of *cis*-acting elements would be involved in somatic epigenetic events as reported for *MGMT* and *MLH1* genes.[20,21] The *EPCAM* gene, which lacks a normal polyadenylation signal, may cause mosaic patterns of epigenetic inactivation of its neighboring gene, *MSH2*, depending on its tissue-specific expression pattern.[3] Deletions that remove the transcriptional termination sequences of an upstream gene result in multiple aberrant *EPCAM/MSH2* fusion transcripts and consequent inactivation of these two genes. The altered allele may place the *MSH2* gene under the control of the *EPCAM* promoter in *cis*-.[4]

We selected subjects with MSH2 loss and MSI who lacked an *MSH2* or *MSH6* mutation for *EPCAM* deletion analysis. Heterodimeric proteins containing MSH2 and MSH6 monomers are recognized by the monoclonal antibodies used in the IHC analysis. Mutations in *MSH2* or *MSH6* may result in IHC loss of both genes.[22] Our results showed that 40% (10/25) of subjects with MSH2 loss and MSI lacked a pathogenic mutation. Of these, only one subject had a *EPCAM* deletion (10%). Likewise, Ligtenberg et al[3] detected four mutated cases of 10 unexplained Dutch putative LS families with *MSH2* loss and MSI. Niessen et al[23] detected three probands with *EPCAM* deletions of 11 patients from the northern part of the Netherlands who were suspected of having LS. These authors only tested for the presence of the Dutch founder deletion.[3] Kovacs et al[4] detected four different *EPCAM* deletions in five of 27 probands selected from clinically well-defined Hun-

Table 2. List of Published *EPCAM* Mutations Related to Lynch Syndrome

Reference	*EPCAM* mutation	Frequency*	Ethnicity
Ligtenberg et al[3]	c.859-1462_*1999del	4/10 (40%)	Dutch
	c.555 + 894_*14194del	2/?	Chinese
Niessen et al[21]	c.859-1462_*1999del	3/11 (27%)	Dutch
Kovacs et al[4]	g.77631_92364del14734	5/27 (19%)	Hungarian
	g.77436_86109del8674		
	g.79459_85516del6058		
	g.72468_82822del10355		
Present study	g.77525_86198del8674	1/10 (10%)	Spanish

*Frequency: number of index subjects with *EPCAM* deletions/number of index subjects analyzed with loss of MSH2 expression and no *MSH2*/*MSH6* mutation.

garian LS families. The outcome of our literature review was that only 14 unrelated subjects have been shown to carry one of the six deletions reported to date (Table 2).[3,4,21] The alteration reported here is a new deletion and was detected in a family with a very high incidence of early-onset CRC and no extracolonic tumors. The number of potential deletion carriers (ie, unexplained cases of *MSH2* loss and MSI) included in our study is small but sufficient to conclude that these types of alterations are present in Spanish LS patients. Taking our results into consideration, the expected proportion of such alterations in cases with *MSH2* loss is 10–40%.[3] Detection of *EPCAM* deletions using MLPA followed by MS-MLPA analysis of *MSH2* methylation in tumors is a fast low-cost procedure that should be incorporated into clinical LS genetic analysis strategies. We propose a decision-support flow diagram to facilitate genetic analysis, which should include a related cancer patient, if available, to minimize false-positive results. If the two related patients have the same MLPA deletion pattern and the same tumor behavior (eg, MSH2 loss, hypermethylation, and MSI), the alteration can be considered the cause of the LS and therefore genetic counseling should be carried out (Figure 4). Deletion mapping should be performed to characterize and define deletion breakpoints. Although deletion mapping is definitive, it is time-consuming and may delay clinical decisions. For patients in whom *MSH2* methylation testing is not possible (eg, inaccessible tumor tissue, low quality or quantity of tumor DNA, etc) deletion mapping is mandatory.

None of the mutated *MSH2* or *MSH6* genes showed *EPCAM* deletions, indicating the high specificity of this decision tree for identifying *EPCAM* deletions. The majority of *MSH2* loss subjects who lack pathogenic mutations do not exhibit *EPCAM* deletions. The underlying causes of these cases are as yet unknown.

In summary, the combination of MLPA analysis for detection of *EPCAM* germ line deletions and MS-MLPA analysis for *MSH2* promoter hypermethylation in tumors can facilitate identification of mutations responsible for LS. These analyses should be incorporated into routine genetic diagnosis protocols for LS.

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