Frequency of Rearrangements in Lynch Syndrome cases associated to MSH2. Characterization of a new deletion involving both EPCAM and the 5´part of MSH2.

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Running title. Deletions in MSH2-associated Lynch Syndrome cases.

Keywords: Lynch Syndrome, MSH2 gene, EpCAM (TASCTD1) gene, rearrangements, phenotype.

Financial Support: This work has been supported by the Regional Government of Castilla y León (Spain). L. Perez-Cabornero and A. Acedo were supported by a predoctoral FPI fellowship from the Government of Castilla y León (Spain).

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Conflicts of interest. The authors declare that they have no conflicts of interest.

Word count: 4226 words

Total number of figures and tables: 3 figures and 2 tables.
ABSTRACT

Lynch syndrome (LS) is caused by germline mutations in MSH2, MLH1, MSH6, and PMS2 mismatch-repair genes and leads to a high risk of colorectal and endometrial cancer. It was recently shown that constitutional 3’ end deletions of EPCAM can cause LS in tissues with MSH2 deficiency. We aim to establish the spectrum of mutations in MSH2 associated Lynch Syndrome cases and their clinical implications.

Probands from 159 families suspected of having LS were enrolled in the study. Immunohistochemistry (IHC) and microsatellite instability (MSI) analyses were used on the probands of all families. Eighteen cases with MSH2 loss were identified: 8 had point mutations in MSH2. In 10 Lynch syndrome families without MSH2 mutations, MSH2/EPCAM genomic rearrangement screening was performed with the use of multiplex ligation-dependent probe amplification (MLPA) and RT-PCR.

We report that large germline deletions, encompassing one or more exons of the MSH2 gene, cosegregate with the LS phenotype in 23% (8/35) of MSI families tested. A new combined deletion EPCAM-MSH2 was identified and characterized by breakpoint analysis, encompassing from the 3’end region of EPCAM to the 5´ initial sequences of the MSH2 (c.859-1860_MSH2:646-254del). EPCAM-MSH2 fusion transcript was isolated. The tumors of the carriers show high-level MSI and MSH2 protein loss.

The clinical correlation provided evidence that the type of mutation and the extension of the deletions involving the MSH2 gene could have different implications in cancer predisposition. Thus, the identification of MSH2/EPCAM rearrangements and their comprehensive characterization should be included in the routine mutation screening protocols for LS.
INTRODUCTION

Hereditary non-polyposis colorectal cancer (HNPCC) or Lynch Syndrome is the most frequent autosomal dominant colorectal cancer susceptibility syndrome caused by mutations inactivating one of the genes of the mismatch-repair system (MMR), most frequently MLH1 and MSH2, and less often in MSH6 and PMS2 (1) (2-4). The phenotype of tumors from these patients is characterized by widespread microsatellite instability (MSI) and loss of protein expression from the affected enzyme detected by immunohistochemical staining (IHQ). This syndrome is characterized by a high risk of early onset of colorectal cancer and several other extracolonic malignant tumors, especially endometrial cancer in women (5).

Mutations in two of these MMR genes, MSH2 and MLH1, account for the majority (about 40%) of the patients with HNPCC (6). Although the majority of the genetic defects in the human MMR genes responsible for HNPCC are a result of point mutations and small insertions and deletions, a substantial proportion result from gross genomic rearrangements. The role of genomic rearrangements in the etiology of HNPCC has been under-investigated because the screening for large deletions (e.g., by multiplex ligation-dependent probe amplification [MLPA] (7) or other techniques) is still not universal in diagnostic laboratories. One mechanism which could originate large genomic rearrangements is the unequal homologous recombination between repeat sequences with a high degree of homology SINE, including Alu repeats (8). In particular, there is a high incidence of genomic deletion in the MSH2 gene (4). This item has been reported as being due to the presence of a higher percentage of repetitive elements (Alu repeats) in the MSH2 gene. Most of them are present in the first half of the gene, including the 5´ upstream sequence of MSH2 (EPCAM/TACSTD1 and promoter region of MSH2).

It has recently been shown that constitutional 3´ end deletions of the EPCAM gene (OMIM#185535) (non-MMR gene) can cause Lynch syndrome through the epigenetic...
silencing of \textit{MSH2} in \textit{EPCAM}-expressing tissues, resulting in tissue-specific \textit{MSH2} deficiency (9). Thus, deletions of the last exon of \textit{EPCAM} constitute a distinct class mutation associated with LS. Several investigators have reported families with \textit{EPCAM} deletions (9),(10),(11),(12),(13).

In a recent study, Kempers et al, 2011 (13), established different cancer risks associated with \textit{EPCAM} deletions depending on whether a deletion affects only the \textit{EPCAM} gene or both the \textit{EPCAM} and its neighbouring gene \textit{MSH2} (\textit{EPCAM–MSH2}). These risks are then compared with those for Lynch syndrome carriers of a mutation in \textit{MMR genes}. This is the first study that describes the cumulative cancer risks and cancer profile of \textit{EPCAM} deletion carriers. They show a profound difference in the frequency of cases of endometrial cancer in this group compared with other Lynch syndrome families with mismatch-repair gene mutations.

Strikingly, endometrial cancer was observed only in carriers with large \textit{EPCAM} deletions that extended close to the \textit{MSH2} gene. The authors described the cumulative risk of endometrial cancer at 70 years of age in \textit{EPCAM} deletion carriers as being 12%. This risk is much lower than that for \textit{MSH2} mutation carriers (51%) or combined \textit{MSH2–EPCAM} deletion carriers (55%). These data suggest that the risk for endometrial cancer in carriers of \textit{EPCAM} deletions is dependent on the size and location of the deletion. The exact criteria of deletions, conferring a low risk of endometrial cancer, remain to be defined by further assessments of endometrial cancer incidence in carriers of different \textit{EPCAM} deletions and analyses of the \textit{EPCAM–MSH2} intergenic region for transcription mediating capacity.

These results highlight, on the one hand, the importance of performing strategies for defining the exact extent of rearrangements and, on the other hand, that the determination of the tumor spectrum and age-specific cancer risk in families carrying
different mutations associated with Lynch syndrome will help to generate optimal recognition and surveillance strategies.

This study was designed to confirm the prevalence of large genomic rearrangements in MSH2 and EPCAM genes in Spanish families. To characterize them, a study was proposed at the molecular level to determine their extent, identify their breakpoints and characterize the impact of the genomic alteration on the correct splicing of the gene. We also evaluated whether different types of MSH2 gene changes (point mutations or deletion extensions affecting MSH2 or both EPCAM-MSH2) were associated with distinct clinical characteristics within the present study series.
PATIENTS AND METHODS

Patients

Samples from 159 independent families were referred for MMR mutation analysis under the Junta de Castilla y Leon Cancer Genetic Counselling Program (Spain). The criteria for entry into the mutational study were defined in accordance with Amsterdam or Bethesda guidelines. Informed consent was obtained from each patient.

DNA was extracted from blood samples from all of our patients using the MagNa Pure Systems (Roche).

Immunohistochemical and tumor microsatellite instability testing.

Immunohistochemical (IHC) staining of tumors for MLH1, MSH2 and MSH6 genes was analyzed by a pathologist in the General Yagüe Hospital, Burgos (Spain).

Microsatellite instability (MSI) analysis was performed on matched normal and tumor DNA pairs using the National Cancer Institute/International Collaborative Group on HNPCC reference marker panel (including two mononucleotide repeats: Bat25, Bat26; and three dinucleotide repeats: D2S123, D5S346, D17S250). DNA was extracted using the DNAeasy Tissue kit (Qiagen). Fluorescently labelled PCR products were detected using the ABI 3130 Genetic Analyzer and the GeneScan software. We classified tumors as MSI-positive only if two or more markers showed instability (14).

Mutation analysis

Samples from subjects with MSI were analyzed for the detection of point mutation using heteroduplex analysis by capillary array electrophoresis (HA-CAE). This method was developed in our laboratory (15) and the validation for MMR genes has been recently published (16).

The selection of genes for analysis was based on IHC results. DNA from peripheral blood leukocytes was used for the analysis. Fragments showing an HA-CAE-altered pattern were sequenced with the BigDye Terminator Sequencing Kit v3.1 (Applied...
Biosystems, Forster City, CA, USA) with unlabelled forward and reverse primers on an ABI 3100 DNA sequencer (4 capillaries; Applied Biosystems).

**MSH2 and EPCAM Genomic rearrangement Identification.**

To detect genomic deletions affecting the *EPCAM* and *MSH2* gene loci, the MLPA multiplex ligation-dependent probe assay (MRC-Holland, Amsterdam; The Netherlands) was used. The test kits used were SALSA MLPA kits P003 and P008 (MRC Holland, Amsterdam, the Netherlands) following the manufacturer’s instructions. The P003 MLH1/MSH2 kit contains oligonucleotide probes targeting all exons of *MSH2* and an additional probe to test exon 1. The P008 MSH6/PMS2 kit contains probes targeting *EPCAM/TACSTD1* exons 3 and 8: one 27 kb upstream and the other 15 kb upstream from the *MSH2* gene. PCR products were analyzed on an ABI 3130 capillary sequencer using GeneMapper software (Applied Biosystems, Forster City, CA, USA).

**Fusion transcript EPCAM-MSH2. RNA isolation and Reverse Transcription PCR (RT-PCR).**

The synthesis of complementary DNA (cDNA) was performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Forster City, CA, USA) using DNase-treated RNA in the presence of random primers. The cDNA amplification was performed with specific primers that encompassed the predicted rearrangement designed for the coding sequences flanking the putative mutation. Short amplicons from RT-PCR were sequenced with the same primers. TASCTD1-Ex7-FW: 5´-ggttggtgtgatagcagttg-3´ MSH2-Ex4-Rev: 5´-ggttgaggtcctgataaatg-3´.

**Breakpoint characterization and Long Range PCR.**

A CGH array strategy was done to confirm the extension of deletions identified by MLPA. A specific Human Array CGH 44K was designed by Nimgenetics for coverage of chromosome 2: 47419322-47580004 (NCBI 36) with a high resolution (500bp).
Based on the information obtained from the CGH array, the interspersed repeats in regions around the breakpoints were examined using the Repeat Masker Program (www.repeatmasker.org).

In order to characterize the rearrangements and determine the exact site of their breakpoints, we performed Long-range PCR of gDNA using primers designed to span the putative breakpoints: EPCAM-In6Fw: 5´-TCCCATTTT TAGACCCCCAAA-3´ and MSH2-In3Rv: 5´ GTG GCTCATGCGCTGTACA-3´. The Expand Long Template PCR System (Roche Diagnostics GmbH, Mannheim, Germany) was used according to the manufacturer’s protocol. PCR products were separated on a 0.8% agarose gel and visualized by ethidium bromide staining. PCR products containing the expected rearrangement were cut from the gel and purified using GFX PCR DNA and the Gel Band Purification Kit (Illustria, GE Healthcare UK limited, Buckinghamshire, United Kingdom). Isolated PCR fragments were sequenced by walking on both strands using the Big Dye V3.1 Terminator Kit (Applied Biosystems, Forster City, CA, USA) and an automated sequencer.

**EPCAM-MSH2 deletion detection**

As a diagnostic tool, we designed a multiplex-PCR strategy based on three primer sequences (one forward and two reverse) to screen these deletions in patients’ first degree relatives. This analysis of genomic DNA produces a unique band sized 705bp when primers EPCAM-In6Fw and MSH2-In3Rv are used. This situation happens in a wild-type case, and produces an additional band when the third primer, EPCAM-In6´Rev: 5´-CAATGTGCAGACACTGATGAT-3´, is used in deletion carrier samples (Figure 3). The experimental conditions are supplied as supplementary material (Supplementary Material 1).
RESULTS.

MSH2 point Mutation Analyses.

A total of 35 MSI families were tested for point mutation in MMR genes by combining HA_CAE and sequencing analysis. Eighteen of these families showed a deficiency in the expression of MSH2/MSH6 proteins in tumors and the screening began for the MSH2 gene. A total of 8 families with a pathogenic germline mutation were detected in the MSH2 gene (Figure 1). Clinicopathological features, molecular findings of the index patients and sample numbers are listed in Supplementary material 2.

MSH2/EPCAM Genomic rearrangement Analyses.

The ten families tested negative for point mutations in MSH2 were screened for the presence of large genomic rearrangements in EPCAM/MSH2 using MLPA (Figure 1). MLPA detected two different rearrangements in MSH2, involving the deletion of exon 7 and exons 4-8, in three and four unrelated families, respectively, using the SALSA MLPA kit P003 MSH2/MLH1 (Table 1). Also, a new deletion encompassing EPCAM-MSH2 in two members from an additional family was detected using both the SALSA MLPA kit P003 and kit P008 (Table 1). No cases involving EPCAM deletion alone (without MSH2 5’ involvement) were detected.

Two different rearrangements in MSH2, involving the deletion of exon 7 and exons 4-8, were previously confirmed through an RT-PCR analysis and sequencing (16). The exact breakpoints were determined in the article with the reference number CAPR-11-0227. Both of them are positioned within Alu elements. (Table 1)

In order to confirm the extension of multi-exonic MSH2 deletion involving exons 1_3 in the two carriers (cases C43 and C132), we used the SALSA MLPA kit P008 PMS2/MSH6. A reduction of the peak area at the probe was observed for the MSH2 exon, which also exhibited an aberrant hybridization signal for one or two EPCAM probes (the one located in exon 8), which was confined to the deletion beginning in the...
3’ region of the EPCAM gene, located upstream of MSH2 (Fig. 2A). RT-PCR on RNA from index subject C43, in which exon 7 of EPCAM is fused to exon 4 of MSH2, was detected (Fig. 2C). A CGH-Array analysis was used to determine the extension of the deletion. The results obtained indicate a deletion extension in chromosome 2: 47464677-47492513 (NCBI 36).

Primer pairs were designed to obtain a patient-specific junction fragment, which produced a length of approximately 550 bp (Fig. 3A). Sequence analysis of the junction fragment confirmed a 28.9 Kb deletion (c. 859-1860_MSH2:646-254del) (Fig. 3B). The breakpoints are located within two interspersed elements, one AluY and an AluSx in inverse orientation (Fig. 3A). Interestingly, the crossover site lies within a 3-bp sequence of perfect identity. This deletion has not been reported in previous studies, but many deletions involving exons 1-3 of MSH2 have been described in the Leiden Open Variation Database (http://chromium.liacs.nl/LOVD2/colon_cancer/home.php?action=switch_db) and their breakpoints have still not been investigated.

We designed a PCR test based on the discrimination of deletion or wild-type alleles in order to screen this complex deletion in first degree relatives of the VA-25 family (Figure 3C). This procedure is faster, cheaper and easier than MLPA.

Combined EPCAM-MSH2 deletion was detected in a 34-year-old man diagnosed with a primary CCR at the same age (C43 case). An additional asymptomatic 43-year-old woman carrier was also detected (C132 case). The pedigree of the family is shown in Figure 2B. This family fulfilled the Amsterdam I criteria for LS. There were six cancers associated to the Lynch Syndrome spectrum in two consecutive generations, two of which were diagnosed before the age of 50. Individuals C43 and C132 were carriers of the combined EPCAM–MSH2 deletion. Family members are currently undergoing analysis for this mutation to predict their risk of developing LS.
Clinical correlations.

The present patients with deletions in MSH2 were divided into groups (with vs. without) involving the EPCAM 3’ region gene, and they were compared to index individuals from our regional registry known to carry MSH2 point mutations (Table 2).

We compared clinical information of individuals in the study in whom a point mutation in MSH2 (N = 19) was identified with those in whom such a deletion in the same gene was identified, depending on the extension (only MSH2 gene N = 15 or EPCAM-MSH2 gene N = 1) (Table 2). The average age of CCR diagnosis for those cases with a point mutation was ~47 years, compared to ~38 and ~34 years for those in the study with deletion abnormalities in males. In females, the average age of CCR diagnosis for patients with a point mutation was 50 years, as compared to 33 and ~38 years for MSH2 or EPCAM-MSH2 deletion positive cases, respectively. The sex distribution (male/female) was approximately 6:5 (MSH2 point mutation) and 1:1 for both cases MSH2 or EPCAM-MSH2 deletion. The average age of endometrial diagnosis for those cases with a point mutation was 43 years, compared to ~51 years for those in the study with an MSH2 deletion.

When compared to patients with point mutations, MSH2 deletion patients had a two and a half times higher chance of developing endometrial cancer. EPCAM-MSH2 deletion patients did not present any endometrial cancer in women in the family described.
DISCUSSION

In this study, we carried out MSH2/EPCAM MLPA analyses of (10) high risk Lynch Syndrome cancer patients with loss of MSH2 protein expression in the tumor. We have found copy number alterations in about 23% of high risk families for HNPCC with microsatellite instability (8/35) who were previously screened negative for point mutations in MLH1, MSH2 and MSH6 genes. MLPA detected two different rearrangements in MSH2 in seven unrelated families and a new deletion encompassing EPCAM-MSH2 was identified in an additional family. Therefore, all of them were found to involve the MSH2 gene. Particularly, two new rearrangements encompassing exon 7 and exons 4-8 deletion in MSH2 were detected in three and four non-related families respectively, and an additional deletion affecting both EPCAM and its neighbouring gene MSH2 (EPCAM–MSH2). However, the two rearrangements involving the MSH2 gene occurred in multiple kindred and, in a paper to be published in parallel, we will show evidence of a common origin in the two deletion of MSH2 (data published on CAPR-11-0227 manuscript).

Our results provide the first evidence that, as in many other studied populations, large genomic changes involving the MSH2 gene is an important event in our HNPCC family series (almost 50% of pathogenic mutations). The frequency of large rearrangements in MSH2, as compared to MLH1, depends on the studied population. Several studies have demonstrated that these rearrangements correspond to between 15% and 55% of the mutations in MMR genes (17). In a study of the Spanish population, an exceptionally low frequency of rearrangements in MLH1/MSH2 genes (<1.5%) was reported (18); though a higher frequency of rearrangements was found in a Basque Country population (~25%) (19). Our data imply that the high frequency of deletions in this study is caused by strong founder effects in our population.
On the other hand, deletions in the EPCAM gene have been reported in several populations with a different frequency, from 19% (10) to 40% (9) in Hungarian and Dutch populations respectively. In the Spanish cohort, one family carrier of this kind of mutation has been identified (~10% incidence) (11). All the subjects of EPCAM deletion carriers were selected from patients with tumors with MSH2 loss and MSI who lacked an MSH2 or MSH6 point mutation. No deletion confined to the EPCAM gene alone was identified in our patient series, but we have identified and characterized a new combined deletion EPCAM-MSH2 by breakpoint analysis, encompassing from the 3’end region of EPCAM (TACSTD1) (exons 8 and 9) to the 5’ initial sequences of the MSH2 (exons 1-3). From this, one expressed EPCAM-MSH2 fusion transcript was identified and it was predicted to be in frame. The tumors of the carriers show high levels of MSI and MSH2 protein loss.

Knowing the frequency of deletion in the population can significantly influence the screening algorithms for patients at risk of HNPCC. Considering these results, as well as the rapid and easy of perform techniques for the characterization of these mutations (such as MLPA, RT-PCR, CGH-Array and sequencing), we proposed that the mutation screening algorithm should begin with MLPA and not with conventional screening/scanning methods, especially in cases where the protein expression pattern of the tumor shows a loss of MSH2 protein or is unknown. Also, our PCR-based assay could be useful for rapid cost-effective HNPCC screening of EPCAM-MSH2 deletion first-degree relatives.

The possibility that cancer risks may vary, depending on the type of mismatch repair gene mutation, may have significant implications for cancer screening recommendations. The proportion of pathogenic point mutations versus rearrangements in MSH2 versus EPCAM-MSH2 deletions identified in this set of samples is of 50% versus 43.8% versus 6.3%. To the best of our knowledge, the
clinical features of families carrying the detected rearrangements were not different from those of families exhibiting other types of mutations, despite results published by other groups like (13), which showed that endometrial cancer was observed only in carriers with large EPCAM deletions that extended close to or into the MSH2 gene.

In conclusion, our data show that large genomic rearrangements occur in MSH2 with a high frequency and genetic evidence has been provided that a certain proportion of these deletions involve the EPCAM gene. The need to incorporate techniques to routinely detect large genomic rearrangements and confirm the extension of the deletions involving the MSH2 gene is emphasized, as it could be involved in the predisposing to Lynch syndrome.

ACKNOWLEDGMENTS

Thanks are due to the National DNA Bank of Salamanca and the Science Foundation AECC. We would also like to thank Noemy Martínez and Lara Hernández for their excellent technical support and Alan Hynds for his critical reading of this manuscript.

GRANT SUPPORT

This work was supported by the Regional Government of Castilla y León (Spain). L. Perez-Cabornero and A. Acedo were supported by a predoctoral FPI fellowship from the Government of Castilla y León (Spain).
REFERENCES


FIGURE LEGENDS

Figure 1. Schematic overview of the MSH2 and EPCAM genetic analysis. HA_CAE (Heteroduplex Analysis by Capillary Array Electrophoresis)

Figure 2. (A) Representation of MLPA results of index subject from families VA-25. Exon 1 from the MSH2 gene is analyzed in duplicate with two synthetic probe mixes. This probe was also present in the SALSA P008 kit used for routine EPCAM diagnostics. The bar diagram is also shown. A peak reduction of MSH2 exon 1 and EPCAM exon 7 probes was recognized. (B) Pedigree of family VA-25 carrier of a combined mutation EPCAM-MSH2 with Lynch-associated tumors. The index subject is indicated by an arrow (C-43) and family member carrying the deletion is indicated by “C” (Carrier) (C132). The sequencing chromatogram of fusion transcript EPCAM-MSH2 is also shown. RT-PCR on RNA from index subject C-43, in which exon 7 of EPCAM is fused to exon 4 of MSH2, was previously performed.

Figure 3. (A) Structural organization of the EPCAM-MSH2 locus. Exons from both EPCAM and MSH2 genes are depicted as blue or green boxes, respectively. The positions of the long-range PCR primers are indicated by horizontal red arrows. An additional reverse primer used in diagnosed PCR is indicated by a horizontal blue arrow. (B) Long range PCR, using primers flanking the deletion, detects a wild type (wt) fragment (555bp) in two carrier VA-25 family members (C-43 and C-132). The sequence alignment of the region encompassing the breakpoint is shown. Sequence identities are indicated by vertical bars. The breakpoint is indicated in grey. Alu
sequences involved in the deletion are highlighted in pink (Alu Y in intron 7 of EPCAM gene) and blue (Alu Sx in intron 3 of MSH2 gene). (C) Diagnostic PCR tool in which the wt band 705bp appears in both control and deletion carrier DNA, while deleted fragment 555bp is only amplified in the deletion carrier DNA. The additional bands in the PCR are non-specific products.

TABLES LEGENDS

Table 1. Characteristics of the EPCAM/MSH2 Deletions.

Table 2. Mean age at diagnosis of colorectal and endometrial cancers in carriers of a mutation in MSH2- associated Lynch syndrome cases.

* Included the carrier mutation probands and their relatives. SD (Standard Deviation)
Lynch Syndrome suspected index cases
N=159

Microsatellite instability
N=35

MSH2/MSH6 loss of expression
N=18

HA-CAE
Point mutations analysis

MSH2 point mutations
N=8

MSH2 Wt
N=10

MLPA
MSH2/EPCAM Large rearrangements analysis

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Table 2. Mean age at diagnosis of colorectal and endometrial cancers in carriers of a mutation in MSH2- associated Lynch syndrome cases.

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* Included the carrier mutation probands and their relatives. SD (Standard Deviation)