Research Article
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Cancer Prevention Research

Frequency of Rearrangements in Lynch Syndrome Cases Associated With *MSH2*: Characterization of a New Deletion Involving both *EPCAM* and the 5′ Part of *MSH2*

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Abstract

Lynch syndrome 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* could cause Lynch syndrome 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 Lynch syndrome were enrolled in the study. Immunohistochemistry and microsatellite instability (MSI) analyses were used on the probands of all families. Eighteen cases with *MSH2* loss were identified: eight had point mutations in *MSH2*. In 10 Lynch syndrome families without *MSH2* mutations, *EPCAM-MSH2* genomic rearrangement screening was carried out with the use of multiplex ligation–dependent probe amplification and reverse transcriptase PCR. We report that large germline deletions, encompassing one or more exons of the *MSH2* gene, cosegregate with the Lynch syndrome phenotype in 23% (8 of 35) of MSI families tested. A new combined deletion *EPCAM-MSH2* was identified and characterized by break point 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 *EPCAM-MSH2* rearrangements and their comprehensive characterization should be included in the routine mutation screening protocols for Lynch syndrome. *Cancer Prev Res; 4(10); 1556–62. ©2011 AACR.*

Introduction

Hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch syndrome is the most frequent autosomal-dominant colorectal cancer (CRC) susceptibility syndrome caused by mutations inactivating one of the genes of the mismatch repair (MMR) system, most frequently in *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 (IHC) staining. This syndrome is characterized by a high risk of early-onset CRC and several other extracolonic malignant tumors, especially endometrial cancer in women (5).

Mutations in 2 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 results from gross genomic rearrangements. The role of genomic rearrangements in the etiology of HNPCC has been under investigation because the screening for large deletions [e.g., by multiplex ligation–dependent probe amplification (MLPA; ref. 7) or other techniques] is still not universal in diagnostic laboratories. One mechanism that could originate large genomic rearrangements is the unequal homologous recombination between repeat sequences with a high degree of homology of short interspersed nuclear elements (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

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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 *MSH2* in *EPCAM*-expressing tissues, resulting in tissue-specific *MSH2* deficiency (9). Thus, deletions of the last exon of *EPCAM* constitute a distinct class mutation associated with Lynch syndrome. Several investigators have reported families with *EPCAM* deletions (9–13).

In a recent study, Kempers and colleagues (13), established different cancer risks associated with *EPCAM* deletions depending on whether a deletion affects only the *EPCAM* gene or both the *EPCAM* and its neighboring gene *MSH2* (*EPCAM-MSH2*). These risks are then compared with those for Lynch syndrome carriers of a mutation in MMR genes. This is the first study that describes the cumulative cancer risks and cancer profile of *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 MMR gene mutations.

Strikingly, endometrial cancer was observed only in carriers with large *EPCAM* deletions that extended close to the *MSH2* gene. The authors described the cumulative risk of endometrial cancer at 70 years of age in *EPCAM* deletion carriers as being 12%. This risk is much lower than that for *MSH2* mutation carriers (51%) or combined *MSH2-EPCAM* deletion carriers (55%). These data suggest that the risk for endometrial cancer in carriers of *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 *EPCAM* deletions and analyses of the *EPCAM-MSH2* intergenic region for transcription-mediating capacity.

These results highlight, on the one hand, the importance of carrying out 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 break points, 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 *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).

IHC and tumor MSI testing

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

MSI analysis was carried out on matched normal and tumor DNA pairs using the National Cancer Institute/International Collaborative Group on HNPCC reference marker panel (including 2 mononucleotide repeats, Bat-25 and Bat-26, and 3 dinucleotide repeats, D2S123, D5S346, and D17S250). DNA was extracted using the DNeasy Tissue Kit (Qiagen). Fluorescently labeled PCR products were detected using the ABI 3130 Genetic Analyzer and the GeneScan Software. We classified tumors as MSI-positive only if 2 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) with unlabeled 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 Assay (MRC-Holland) was used. The test kits used were SALSA MLPA kits P003 and P008 (MRC-Holland) 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).

Fusion transcript *EPCAM-MSH2*: RNA isolation and reverse transcriptase PCR

The synthesis of cDNA was carried out with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), using DNase-treated RNA in the presence of random primers. The cDNA amplification was carried out with specific primers that encompassed the predicted rearrangement designed for the coding sequences flanking the putative mutation. Short amplicons from reverse transcription PCR (RT-PCR) were sequenced with the same primers: TASCTD1-Ex7-FW, 5′-ggttgtggtgatagcagttg-3′; MSH2-Ex4-Rev, 5′-ggttgaggtcctgataaatg-3′.

Break point characterization and long-range PCR

An array comparative genomic hybridization (aCGH) strategy was used to confirm the extension of deletions identified by MLPA. A specific human aCGH 44K was designed by NIMGenetics for coverage of chromosome 2: 47419322-47580004 (NCBI 36) with a high resolution (500 bp).

On the basis of the information obtained from the aCGH, the interspersed repeats in regions around the break points were examined using the RepeatMasker program (www.repeatmasker.org).

To characterize the rearrangements and determine the exact site of their break points, we carried out long-range PCR of genomic DNA using primers designed to span the putative break points as follows: EPCAM-In6Fw, 5′-TCCCATTTTTAGACCCCAAA-3′; MSH2-In3Rv, 5′ GTGGCTCATGCCTGTAATCC-3′. The Expand Long Template PCR System (Roche Diagnostics GmbH) 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 (Illustra; GE Healthcare UK Limited). Isolated PCR fragments were sequenced by primer walking on both strands using the BigDye V3.1 Terminator Kit (Applied Biosystems) and an automated sequencer.

EPCAM-MSH2 deletion detection

As a diagnostic tool, we designed a multiplex PCR strategy based on 3 primer sequences (1 forward and 2 reverse) to screen these deletions in patients' first-degree relatives. This analysis of genomic DNA produces a unique band sized 705 bp when primers EPCAM-In6Fw and MSH2-In3Rv are used. This situation happens in a wild-type (WT) case and produces an additional band when the third primer EPCAM-In6′Rev (5′-CAATGTGCAAGACACTGATATGAT-3′) is used in deletion carrier samples (Fig. 3). The experimental conditions are supplied as Supplementary Material S1.

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 (Fig. 1). Clinicopathologic features, molecular findings of the index patients, and sample numbers are listed in Supplementary Material S2.

MSH2/*EPCAM* genomic rearrangement analyses

The 10 families tested negative for point mutations in *MSH2* were screened for the presence of large genomic rearrangements in *EPCAM*/*MSH2* using MLPA (Fig. 1). MLPA detected 2 different rearrangements in *MSH2*, involving the deletion of exon 7 and exons 4 to 8, in 3 and 4 unrelated families, respectively, using the SALSA MLPA Kit P003 MSH2/MLH1 (Table 1). Also, a new deletion encompassing *EPCAM-MSH2* in 2 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 to 8, were previously confirmed through an RT-PCR analysis and sequencing (16). The exact break points were determined in a companion article by Pérez-Cabornero and colleagues (in this issue). Both of them are positioned within Alu elements (Table 1).

To confirm the extension of multiexonic *MSH2* deletion involving exons 1 to 3 in the 2 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 1 or 2 *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

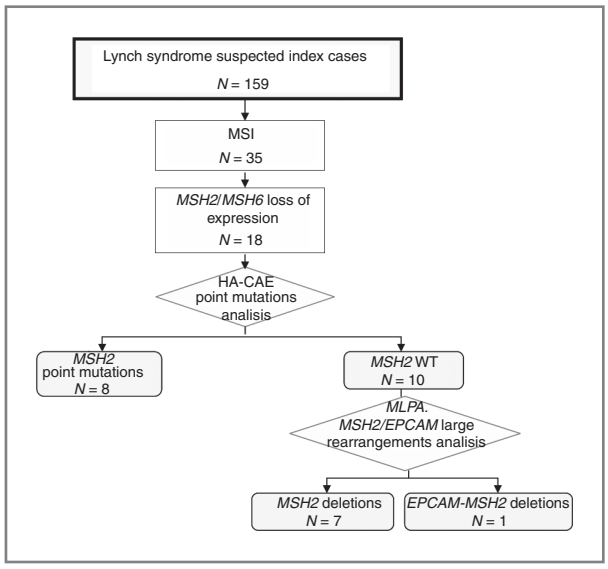

Figure 1. Schematic overview of the *MSH2* and *EPCAM* genetic analysis.

Table 1. Characteristics of the *EPCAM/MSH2* deletions

Gene	Exon involved	Family code	Deletions	Break point homology (bp)	Type of repeat element	Detection method	Confirmation method Characterization method
MSH2	4–8	VA-17	c.646-1019_1386+2420del	8	Alu-Sq/Alu-Sp	MLPA MSH2/MLH1 kit P003	Junction fragment, RNA
		VA-20	c.646-1019_1386+2420del				
		VA-32	c.646-1019_1386+2420del				aCGH, sequencing, junction fragment
		VA-134	c.646-1019_1386+2420del				
	7	VA-4	c.1077-3513_1276+5655del	6	Alu-Y/Alu-Sg	MLPA MSH2/MLH1 kit P003	Junction fragment, RNA
		VA-169	c.1077-3513_1276+5655del				
		VA-247	c.1077-3513_1276+5655del				CGH, sequencing, junction fragment
EPCAM-MSH2	Exons 8–9 of *EPCAM* and exons 1–3 of *MSH2*	VA-25	c.859-1860_MSH2:646-254del	3	Alu-Y/Alu-Sx	MLPA MSH2/MLH1 kit P003 MLPA MSH6/PMS2 kit P008	Junction fragment, RNA, fusion transcript CGH, sequencing, junction fragment

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). An aCGH 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 break points are located within 2 interspersed elements, one Alu-Y and an Alu-Sx 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 1to 3 of *MSH2* have been described in the Leiden Open Variation Database (http://chromium.liacs.nl/LOVD2/colon_cancer/home.php) and their break points have still not been investigated.

We designed a PCR test based on the discrimination of deletion or WT alleles to screen this complex deletion in first-degree relatives of the VA-25 family (Fig. 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 Lynch syndrome. There were 6 cancers associated to the Lynch syndrome spectrum in 2 consecutive generations, 2 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 Lynch syndrome.

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 with 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 approximately 47 years compared with approximately 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 with 33 and approximately 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 with approximately 51 years for those in the study with an *MSH2* deletion.

When compared with patients with point mutations, *MSH2* deletion patients had a 2.5 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 (10) of 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 MSI (8 of 35) who were previously screened negative for point mutations in *MLH1*, *MSH2*, and *MSH6* genes. MLPA detected 2 different rearrangements in *MSH2* in 7 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, 2 new rearrangements encompassing exon 7 and exon 4 to 8 deletion in *MSH2* were detected in 3 and 4 nonrelated families, respectively, and an additional deletion affecting both *EPCAM* and its neighboring gene *MSH2* (*EPCAM-MSH2*). However, the 2 rearrangements involving the *MSH2* gene occurred in multiple kindred, and, in an article to be published in parallel, we will show evidence of a common origin in the 2 deletion of *MSH2* (data published in Pérez-Cabornero and colleagues (in this issue).

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 with *MLH1*, depends on the studied population. Several studies have shown that these rearrangements correspond 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), although a higher frequency of rearrangements was found in a Basque Country population (~25%; ref. 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; ref. 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 break point 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

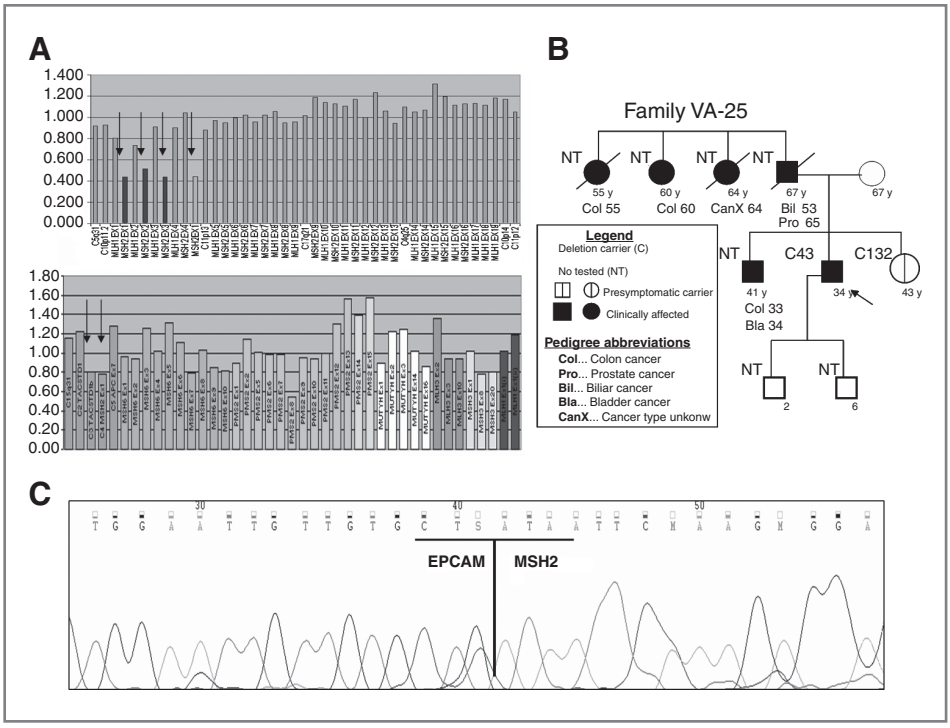

Figure 2. A, representation of MLPA results of index subject from families VA-25. Exon 1 from the *MSH2* gene is analyzed in duplicate with 2 synthetic probe mixes. This probe was also present in the SALSA P008 Kit used for routine *EPCAM* diagnostics. The bar diagram is also shown. A peak reduction 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 (C43), 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 C43, in whom exon 7 of *EPCAM* is fused to exon 4 of *MSH2*, was previously carried out.

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

	MSH2		*EPCAM-MSH2*
	Point mutations	**Deletions**	**Deletions**
Families	8	7	1
Mutation carriers	19	15	1
CRC[a]	23	25	4
Carriers affected	17 (4 females)	13 (2 females)	1
Mean (SD) age at diagnostic, y,			
Men	46.71 (6.24)	38.29 (9.01)	33.5 (0.71)
Female	50.00 (23.72)	33.00 (6.89)	57.5 (3.54)
Endometrial cancer	4	6	0
Female carriers affected	4	5	–
Mean (SD) age at diagnostic, y	43.00 (1.41)	50.75 (7.37)	–
Ratio of colonic to endometrial cancer in women patients	1:1	1:2.5	4:0

[a]Included the carrier mutation probands and their relatives.

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 to carrying out techniques for the characterization of these mutations (such as MLPA, RT-PCR, aCGH, and sequencing), we proposed

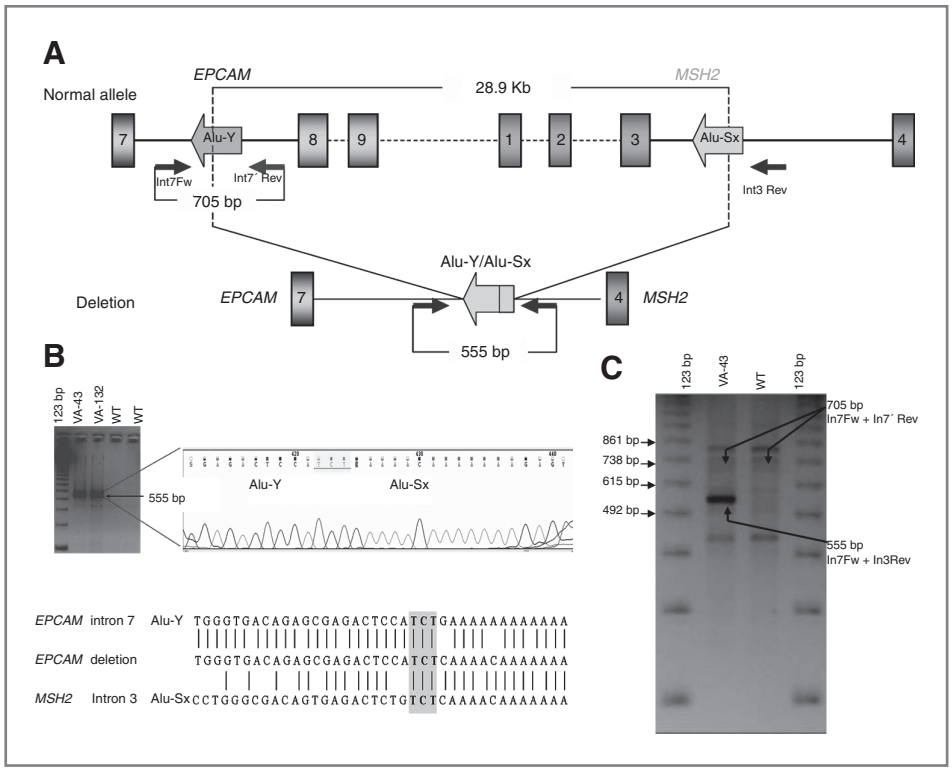

Figure 3. A, structural organization of the *EPCAM-MSH2* locus. Exons from both *EPCAM* and *MSH2* genes are depicted as rectangular vertical boxes. The positions of the long-range PCR primers are indicated by horizontal arrows. Alu sequences involved in the deletion are marked in a big arrow (Alu-Y/Aly-Sx). B, long-range PCR, using primers flanking the deletion, detects a WT fragment (555 bp) in 2 carrier VA-25 family members (C43 and C132). The underlined bases show the break point. The sequence alignment of the region encompassing the break point is shaded in gray. C, diagnostic PCR tool in which the WT band 705 bp appears in both control and deletion carrier DNA, whereas deleted fragment 555 bp is amplified only in the deletion carrier DNA. The additional bands in the PCR are nonspecific products.

that the mutation screening algorithm should begin with MLPA and not with conventional screening/scanning methods, especially in cases in which 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 vary, depending on the type of MMR 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 such as Kempers and colleagues (13), who 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.

Disclosure of Potential Conflicts of interest

No potential conflicts of interest were disclosed.

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