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**A 10-Mb Paracentric Inversion of Chromosome arm 2p Inactivates *MSH2*
and is Responsible for Hereditary Non Polyposis Colorectal Cancer in a
North-American Kindred**

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A 10-Mb Paracentric Inversion of Chromosome Arm 2p Inactivates *MSH2* and Is Responsible for Hereditary Nonpolyposis Colorectal Cancer in a North-American Kindred

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Genomic deletions of the *MSH2* gene area frequent cause of hereditary nonpolyposis colorectal cancer (HNPCC), a common hereditary predisposition to the development of tumors in several organs including the gastrointestinal and urinary tracts and endometrium. The mutation spectrum at the *MSH2* gene is extremely heterogeneous because it includes nonsense and missense point mutations, small insertions and deletions leading to frameshifts, and larger genomic deletions, the latter representing approximately 25% of the total mutation burden. Here, we report the identification and molecular characterization of the first paracentric inversion of the *MSH2* locus known to cause HNPCC. Southern blot analysis and inverse PCR showed that the centromeric and telomeric breakpoints of the paracentric inversion map within intron7 and toa contig 10 Mb 3' of *MSH2*, respectively. Pathogenicity of the paracentric inversion was demonstrated by conversion analysis. The patient's lymphocytes were employed to generate somatic cell hybrids to analyze the expression of the inverted *MSH2* allele in an *Msh2*-deficient rodent cellular background. The inversion was shown to abolish *MSH2* expression by both northern and western analysis. This study confirms that Southern blot analysis still represents a useful and informative tool to screen for and identify complex genomic rearrangements in HNPCC. Moreover, monoallelic expression analysis represents an attractive approach to demonstrate pathogenicity of unusual mutations in autosomal dominant hereditary conditions.

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INTRODUCTION

Hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch syndrome is a common autosomal dominant cancer syndrome caused by germline mutations in *MSH2* and *MSH6* on chromosome 2, *MLH1* on chromosome 3, and *PMS2* on chromosome7 (Fishel et al., 1993; Bronner et al., 1994; Nicolaides et al., 1994; Akiyama et al., 1997; Miyaki et al., 1997). These genes are responsible for DNA mismatch repair (MMR) and are homologs of the *Escherichia coli* *MutS* and *MutL* MMR genes. Carriers of a germline mutation in one of these genes are at increased risk of developing colorectal, endometrial, and ovarian cancer, transitional cell carcinoma of the ureters and renal pelvis, gastric and small bowel cancer, brain tumors, and sebaceous skin cancer (Lynch, 1999). Clinically, HNPCC is defined by the Amsterdam criteria: at least three cases of pathologically verified colorectal, endometrial, transitional cell, or small bowel cancer should be present within two generations, one of which was diagnosed under the age of 50. Moreover, one pa-

tient must be a first-degree relative of the others and familial adenomatous polyposis (FAP) has to be excluded (Vasen et al., 1999).

In the vast majority of HNPCC families, germline mutations are found in *MSH2* and *MLH1* (Peltonen and Vasen, 1997). These mutations are predominantly single nucleotide substitutions or small insertions or deletions. However, larger genomic deletions have also been recognized as a frequent cause of HNPCC (Wijnen et al., 1998a). Here, we describe the first HNPCC family caused by an inversion within the short arm of chromosome 2 disrupting the *MSH2* gene.

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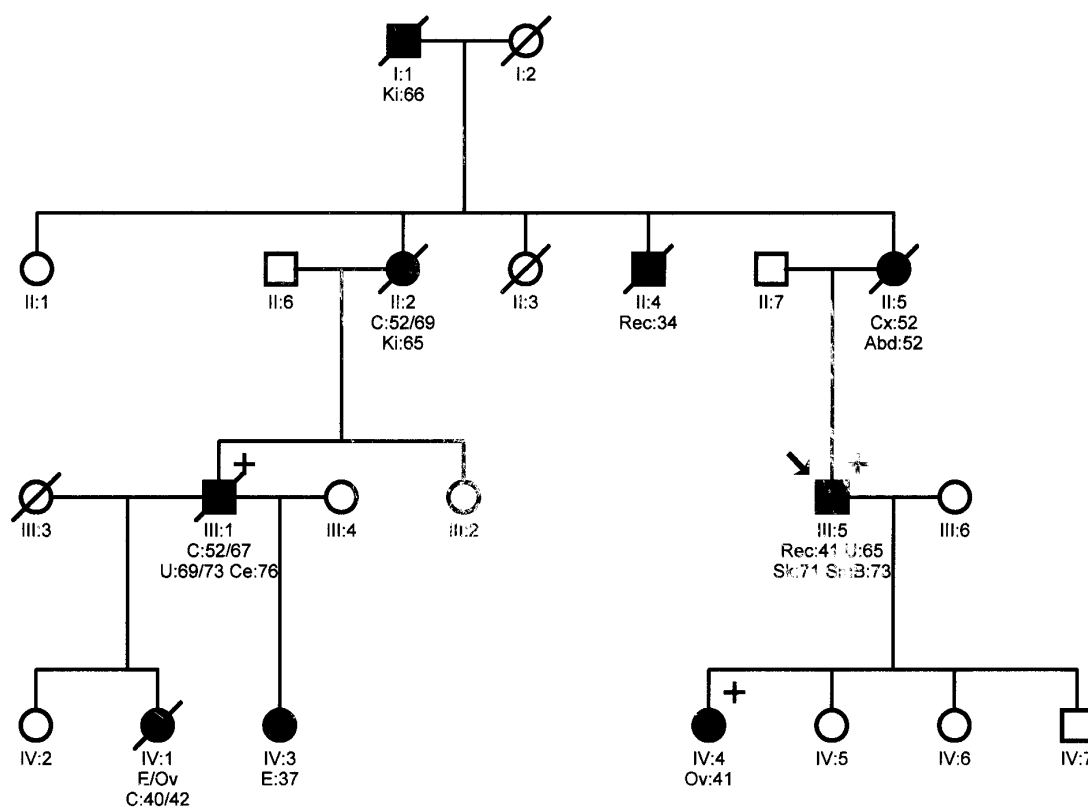


Figure 1. Pedigree of the North-American HNPCC family. Symbols: +, carrier of the paracentric inversion; C, colorectal cancer; Rec, rectal cancer; Ce, cecal cancer; E, endometrial cancer; Ov, ovarian cancer; U, urinary tract cancer; Ki, kidney cancer; SmB, small bowel cancer; Sk, skin cancer; Cx, cervical cancer; Abd, abdominal cancer. The index patient, III:5, is indicated with an arrow.

MATERIALS AND METHODS

The Family

The family described here is Caucasian and is part of the cohort of North-American HNPCC families collected by Dr. Henry Lynch. The kindred fully complies with the clinical Amsterdam criteria for HNPCC (Fig. 1). The index patient is a 77-year-old male (III:5), who had a malignant rectal polyp at age 41, a transitional cell carcinoma of the left kidney at 65, and a small bowel carcinoma at 73. At the age of 71, he was also diagnosed with several sebaceous adenomas and squamous cell carcinomas. One of the index's three daughters developed an endometroid carcinoma of the ovary at age 41 (IV:4). His mother was diagnosed with cervical cancer and died of an abdominal carcinoma at 52 years of age (II:5). Her brother had rectal cancer at 34 and died at 36 (II:4). One of her sisters developed two colon adenocarcinomas at 52 and 69 years, respectively, and a kidney adenocarcinoma

at 65 (II:2). She died at 72 years of age. Her son presented with adenocarcinomas of the colon at age 52 and 67, respectively (III:1). Furthermore, he was diagnosed with transitional cell carcinoma of the right ureter and bladder at 69 and 73 years. At the age of 76, he developed an adenocarcinoma of the cecum and died of cardiac disease at 77. Two of his daughters (from different marriages) also presented with HNPCC-related tumors (IV:1 and IV:3). There was no evidence of miscarriages in this family.

Denaturing Gradient Gel Electrophoresis (DGGE)

Mutation analysis of the *MSH2*, *MLH1*, and *MSH6* genes was performed by DGGE as previously described (Wijnen et al., 1998b, 1999).

Southern Blot Analysis

DNA was extracted from peripheral blood lymphocytes. Conforming to our previously established protocol (Wijnen et al., 1998a), Southern blot analysis of

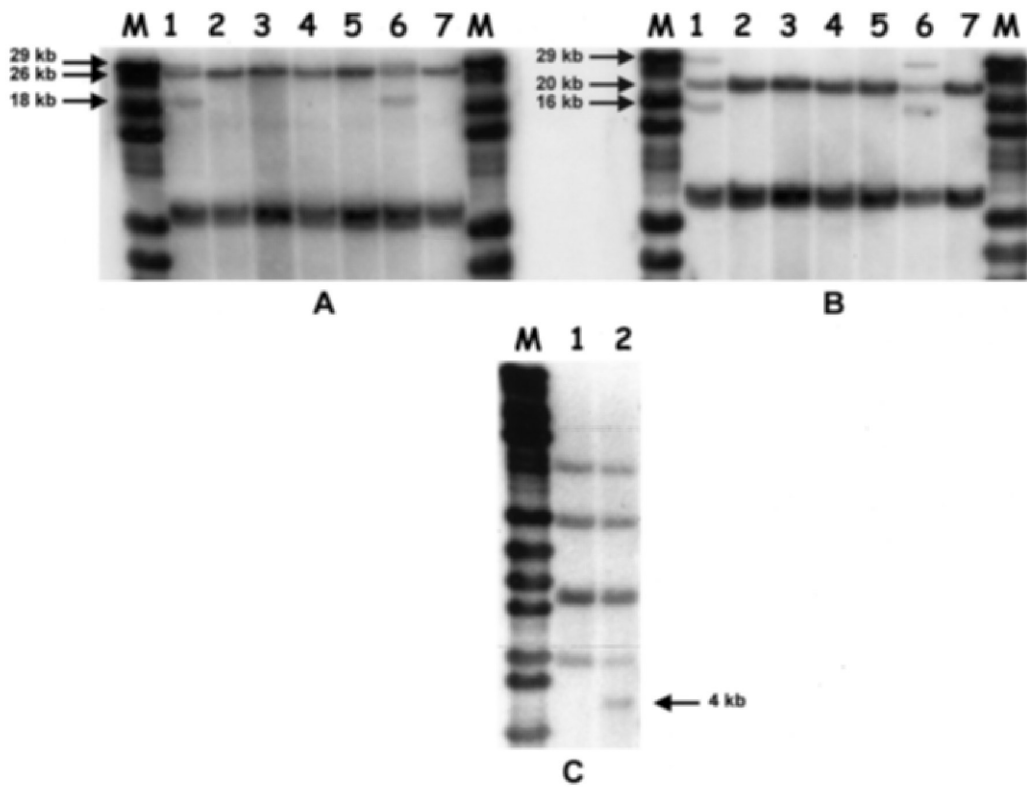


Figure 2. Southern blot analysis of the index patient and other affected and nonaffected relatives from the HNPCC family. *Xba*I (A) and *Bsp*I (B) digests hybridized with the 3a probe (encompassing *MSH2* exons 7–12). Lane 1: index patient (III:5); lanes 2–5: unaffected 50% risk carriers; lane 6: affected relative (III:1); lane 7: normal control. M indicates the molecular weight markers. The molecular weight of the specific *MSH2* bands is indicated. *Hinc*II (C) digests from a normal control (lane 1) and from individual III:5 (lane 2) were hybridized with the 3a probe.

MSH2 was performed with *Xba*I, *Eco*RI, *Hind*III, and *Nsi*I genomic DNA digests followed by hybridization with three *MSH2* probes: probe 5, encompassing exons 1–7; probe 3a, for exons 7–12; and probe 3b, for exons 10–16 (see also Fig. 2). In addition, digestion with the restriction endonucleases *Bsp* 1407I, *Hinc*II, and *Bcl*I was performed. The latter filters were hybridized with several exon-specific *MSH2* probes, including exons 6 to 9. *MLH1* and *MSH6* Southern analysis was performed with *Xba*I, *Apa*I, *Hind*III, and *Nsi*I genomic DNA digests followed by hybridization with two *MLH1* probes (encompassing exons 1–12 and exons 11–19) and two *MSH6* probes (encompassing exons 1–4 and exons 5–10) (Wijnen et al., 1998a).

Cytogenetic and Fluorescence In Situ Hybridization Analyses

Prometaphase and metaphase chromosomes were obtained from EBV-transformed lymphoblas-

tic cells according to standard protocols (Dauwerse et al., 1992). Karyotyping was performed using GTG-banding (Seabright, 1971) and multicolor fluorescence in situ hybridization (MFISH). For MFISH, the COBRA (for COmbined Binary Ratio) labeling technique with p/q arm distinction was employed as previously described (Tanke et al., 1999; Wiegant et al., 2000).

Inverse Polymerase Chain Reaction (IPCR)

IPCR was performed as previously described (Fodde et al., 1990) (Fig. 3). A 5- μ g sample of genomic DNA extracted from lymphoblastoid cells was digested with *Hinc*II. The digested DNA was extracted by phenol-chloroform, ethanol-precipitated, diluted to a concentration of 1 μ g/ml in ligation buffer (Fermentas, St. Leon-Rot, Germany), and ligated overnight at 16°C with 1 U/ μ l ligase (Fermentas).

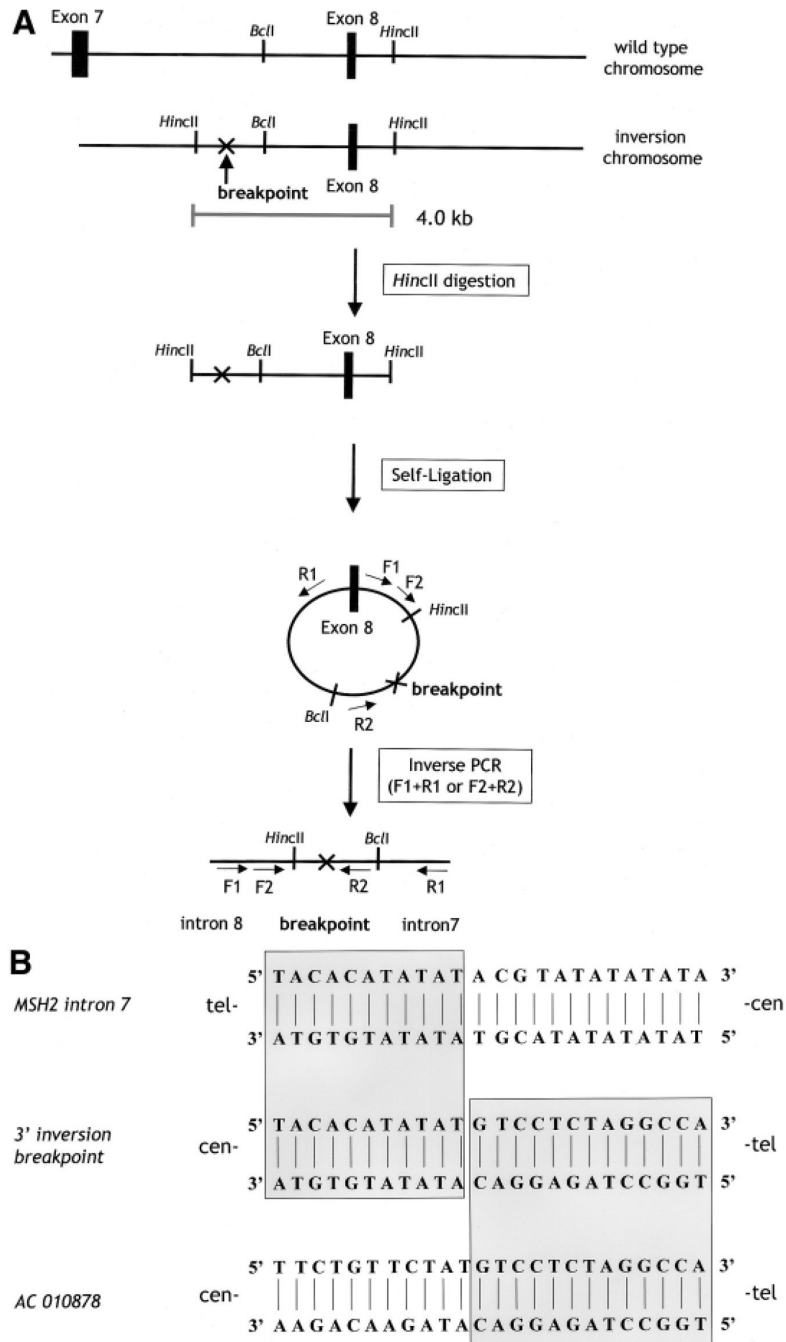


Figure 3. Cloning and characterization of the 3' (telomeric) inversion breakpoint by inverse-PCR. **A:** The inverse-PCR procedure. Genomic DNA from the index patient (III:5) was digested by *HincII*. The latter results in an aberrant 4.0-kb fragment encompassing *MSH2* exon 8 and the 3' breakpoint as indicated. The digested DNA was ligated as described under Materials and Methods. The ligated DNA was employed in a PCR reaction with primer sets F1/R1 and F2/R2, indicated by arrows. In both cases, only DNA from carriers of the paracentric inversion resulted in the expected inverse-PCR products. The latter encompass the 5' end of *MSH2* intron 8, the original *HincII* site, the inversion breakpoint, and the 3' end of *MSH2* intron 7. **B:** Both the sense and antisense strands of these sequences around the 3' (telomeric) inversion breakpoint are depicted. The alignment shows (from top to bottom) the wild-type *MSH2* intron 7 sequence, the 3' (telomeric) breakpoint obtained by inverse PCR, and the sequence of the contig AC010878, located 10 Mb 3' (telomeric) to *MSH2*. The shaded boxes indicate the homologies among the three aligned sequences. The orientation of both the centromere and telomere relative to the reported sequences are indicated. This sequence of *MSH2* intron 7 is depicted in the reversed direction relative to the other two sequences.

A forward primer (F1) was designed at the 3' end of exon 8 (5'-AGAAACAACCTTTAGATATGG-3'), whereas the reverse primer (R1) was developed 3' of the *BclI* site at position 13,392 within intron 7 (5'-AGACTTTGTAAAGCTACATTC-3') (Fig.

3). High-fidelity PCR (Roche Molecular Diagnostic Systems, Alameda, CA) was performed with these primers. The PCR product was analyzed by agarose gel electrophoresis. An approximately 2.0-kb band was observed in the index DNA but

not in the normal control. This band was excised and purified using a nucleotrap kit (Machery-Nagel, Düren, Germany). The purified product was cloned by TOPO-cloning (Invitrogen, La Jolla, CA) and sequenced using the same F1 and R1 primers. Based on the newly obtained sequences, new primers were designed, that is, a forward primer (F2) in intron 8 (5' -TCAAGCTATCCTC-CGGCCTC-3') and a reverse primer (R2) in intron 7 (5' -GGATGCAGTGAACAGGGAAC-3') (Fig. 3). The IPCR procedure was then repeated with F2 and R2, and the patient-specific PCR product was sequenced.

This sequence also enabled us to develop additional primers around the 5' (centromeric) breakpoint (Fig. 4): a forward primer (F3, 5' -TGCTGACATGGCCCATTCCTAC-3') within the contig AC010878, and a reverse primer (R3, 5' -TCCC-AGTTTA ACTCTAGCAG-3') in intron 7. PCR amplification with these primers resulted in a fragment of approximately 900 bp in the index patient (III:5) and in two other affected individuals (III:1 and IV:4) but not in a normal control (see also Fig. 4C). This fragment was again excised and purified by the nucleotrap procedure and sequenced using the PCR forward and reverse primers.

Monoallelic Expression Analysis

To allow RNA and protein expression analysis of the *MSH2* allele carrying the inversion, somatic cell hybrids containing the wild type and rearranged chromosome 2p from the index patient were generated in a *Msh2*-deficient murine background (GMP Conversion Technologies, Waltham, MA) (Papadopoulos et al., 1995; Yan et al., 2000). Somatic cell hybrids were genotyped by CA repeat markers around the *MSH2* gene on chromosome arm 2p and selected for monoallelic expression analysis. Total RNA and protein samples were isolated from the different hybrid cell lines, and northern and western blot analyses were carried out according to conventional procedures. Probe 3b (*MSH2* exons 10–16) was employed for the northern analysis, whereas a rabbit polyclonal antibody raised against amino acids 402–737 of human *MSH2* was used for the western analysis (Smit et al., 2000).

RESULTS AND DISCUSSION

Southern Analysis

DGGE analysis of *MSH2*, *MLH1*, and *MSH6* did not reveal any mutation in the index patient. Therefore, Southern blot analysis was performed.

MLH1 and *MSH6* hybridizations did not show any aberrant fragments. After hybridization with the *MSH2* 5' probe (encompassing exons 1–7), an aberrant band of approximately 30 kb was seen in the *Xba*I digest (not shown). Hybridization with the *MSH2* 3a probe (exons 7–12) revealed two aberrant fragments of 29 and 18 kb, respectively (Fig. 2A). Based on the decreased intensity of the wild-type 26-kb fragment encompassing *MSH2* exons 7 and 8, we assumed that the genomic rearrangement involved this segment of the gene. Hybridization with exon 7- and exon 8-specific probes indicated that the 29-kb band contained exon 7, whereas the 18-kb fragment encompassed exon 8 of *MSH2*. This was confirmed by digestion with *Bsp*1407I: in addition to the wild-type 20-kb fragment containing exons 7 and 8, hybridization with both the 3'a-specific and the exon 7- and exon 8-specific probes revealed two fragments of 29 and 16 kb, encompassing exon 7 and exon 8, respectively (Fig. 2B). These results are indicative of a complex rearrangement encompassing both exon 7 and exon 8 in the mutated allele. The normal intensity of all other digestion fragments suggested that no other *MSH2* exon was involved in the rearrangement (data not shown). This also indicated that the genomic rearrangement was likely to be a translocation or inversion with a breakpoint in intron 7. The aberrant restriction site closest to the breakpoint appeared to be a *Hinc*II site at position 11,738 in intron 7 (Fig. 2C). *Bcl*I digestion showed no aberrant fragments after hybridization with an exon 8-specific probe, indicating that the breakpoint had to map 5' to the *Bcl*I site located at position 13,392 within intron 7. Given that no other aberrant fragments were found after hybridization with any of the other *MSH2* probes, the other breakpoint had to map outside the *MSH2* gene.

Additional Southern blot analysis of individual III:1, IV:4, and several unaffected relatives showed co-segregation of the rearrangement with the disease in this family (not shown).

Cytogenetic and FISH Analyses

Karyotype analysis of the index patient by GTG-banding and PQ-COBRA-FISH did not show either any apparent translocation or an inversion concerning chromosome 2. This indicated that the alleged genomic rearrangement had to be either a small inter- or intrachromosomal translocation, or a paracentric inversion (data not shown).

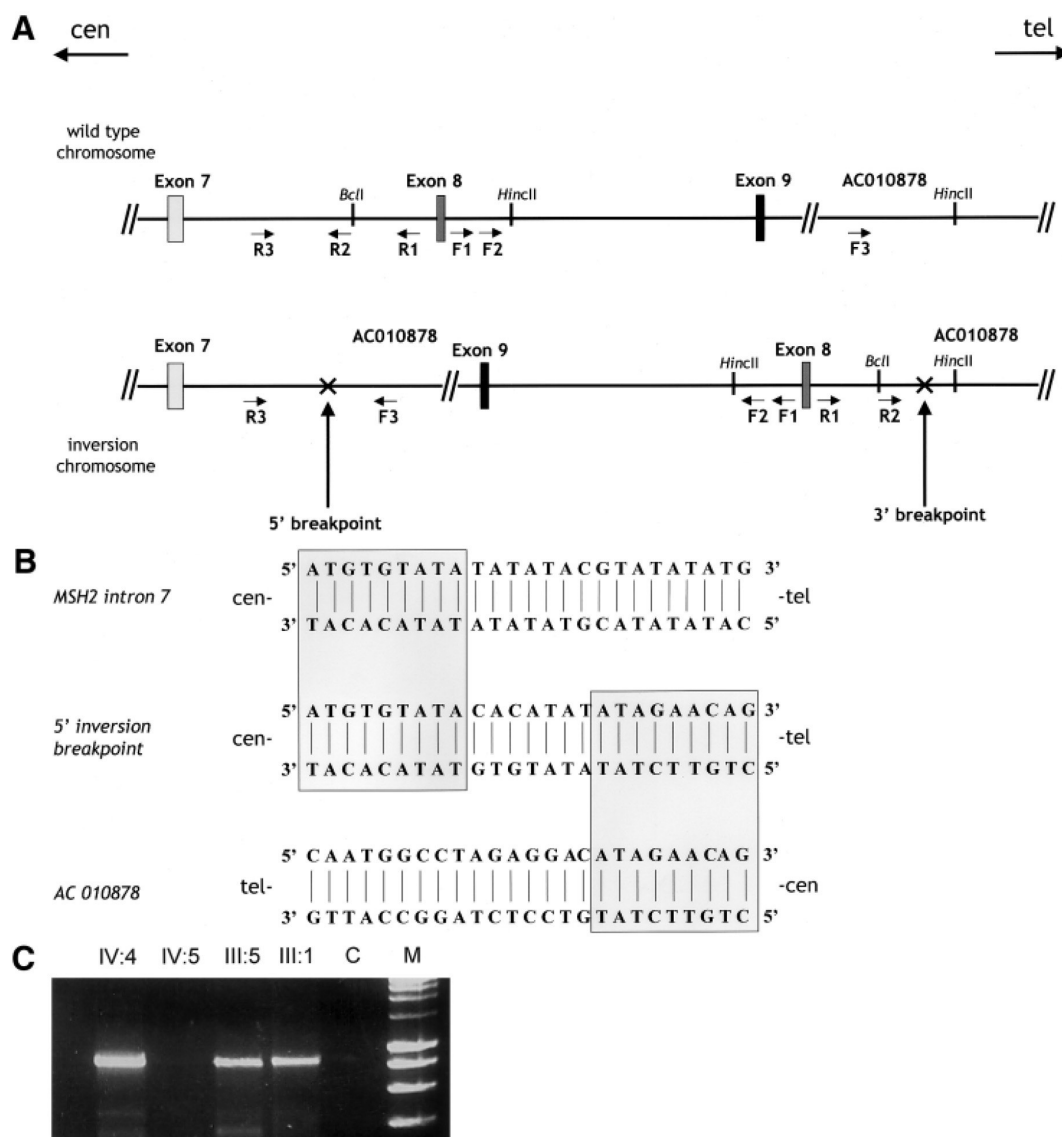


Figure 4. Cloning and characterization of the 5' (centromeric) inversion breakpoint. **A:** Scheme of the wild-type and mutated *MSH2* alleles. Only the part of the chromosome 2 region involved in the paracentric inversion is shown. Shaded and black boxes indicate exons 7, 8, and 9 of the *MSH2* gene, respectively. Only the *HincII* and *BclI* restriction sites and the 5' (centromeric) and 3' (telomeric) breakpoints are depicted for the sake of clarity. AC010878 is the contig located 10 Mb 3' of *MSH2*. The primer set employed to amplify and sequence both breakpoints (F1/R1, F2/R2, F3/R3) are indicated by arrows. **B:** Both the sense and antisense strands of these sequences around the 5' (centromeric) inversion breakpoint are depicted. The

alignment shows (from top to bottom) the wild-type *MSH2* intron 7 sequence, the 5' breakpoint obtained by PCR with primers F3 and R3, and the sequence of the contig AC010878, located 10 Mb 3' (telomeric) to *MSH2*. The shaded boxes indicate the homologies among the three aligned sequences. The orientation of both the centromere and telomere relative to the reported sequences are indicated. This sequence of the AC010878 contig is depicted in the reverse orientation relative to the other two sequences. **C:** 5' Breakpoint-specific PCR results obtained with primers F3 and R3 (see A) in affected (IV:4, III:5, III:1) and nonaffected (IV:5) individuals.

IPCR

To discriminate among the above possibilities, we set out to characterize the intron 7 breakpoint by IPCR (Fig. 3A). Based on the Southern blot

analysis, *HincII* digestion results in a 4-kb fragment encompassing exon 8 and the breakpoint of the genomic rearrangement within intron 7 (Fig. 2C). The IPCR procedure enabled us to obtain a

proximately 2-kb PCR product containing the 5' end of intron 8, the *HincII* site used for digestion and ligation, 60 base pairs of an unknown sequence, the breakpoint, and the 3' end of intron 7 (Fig. 3B). The IPCR product was not observed when genomic DNA from a healthy individual was subjected to the procedure. By use of the Basic Local Alignment Search Tool (BLAST) database of the National Center of Biotechnology Information, a perfect match was found between the unknown sequence and a contig (AC010878) located 10 Mb 3' of *MSH2*. The latter finding indicated that the genomic rearrangement in this kindred is likely to be a 10-Mb inversion of exons 8 to 16 of the *MSH2* gene (Fig. 4A). To test this hypothesis, PCR primers (F3 and R3) were designed within AC010878 and in *MSH2* intron 7, to allow amplification and sequencing of the 5' (centromeric) breakpoint of the 10-Mb inversion (Fig. 4A). The approximately 900-bp PCR product obtained using these primers encompasses sequences derived from AC010878 (in the reverse direction relative to the sequence found in the 3' telomeric breakpoint sequence), the 5' centromeric breakpoint, and 5' intron 7 sequences (again, in the reverse orientation relative to the 3' telomeric breakpoint sequence) (Fig. 4B). Moreover, the combination of these primers resulted in the 900-bp product only when DNA from the index patient or the other two available affected individuals (III:1 and IV:4 in Fig. 1) from the affected kindred were employed (Fig. 4C). This proved that the rearrangement in this family was indeed a paracentric inversion of about 10 Mb, including exons 8 to 16 of the *MSH2* gene.

Genomic rearrangements were previously described in *MSH2*, as well as in other cancer-susceptibility genes like *APC*, *MLH1*, *BRCA1*, *TP53*, *NF2*, and *STK11* (vander Luijt et al., 1995; Petrij-Bosch et al., 1997; Jenne et al., 1998; Slebos et al., 1998; Wijnen et al., 1998a; Puget et al., 1999; Legoux et al., 2000; Chan et al., 2001). These rearrangements may result from nonhomologous breakage and reunion events but also from homologous recombination between repetitive sequences dispersed throughout the genome, like Alu repeats (Nystrom-Lahti et al., 1995; Mauillon et al., 1996; Slebos et al., 1998). Also, genomic inversions have been described in hemophiliacs resulting from unequal alignments between small-sequence homologies (Lakich et al., 1993). Computer analysis (BLAST) of intron 7 of the *MSH2* gene revealed many repetitive sequences, among which were Alu and simple-sequence repeats (not shown). During our search for mutations in HNPCC families, we

identified four genomic deletions of *MSH2* with a breakpoint within intron 7 (unpublished observations). However, because the deletion breakpoints in these families, including the one studied here, appear to be located at different sites along intron 7, no actual recombination hot spot can be pinpointed. We did not find Alu repeats at or in the flanking regions of the breakpoints of the inversion reported here. Hence, a nonhomologous breakage and reunion recombination event is the most likely cause of the genomic rearrangement observed in this family. Notably, during the recombination process, nine base pairs were lost from intron 7, whereas an additional seven base pairs were found at the 5' (centromeric) breakpoint (Figs. 3B and 4B). The latter does not result from a PCR artifact, given that sequence analysis of the 5' breakpoint of the affected individuals III:1 and IV:4 confirmed the presence of the 9-bp deletion and the 7-bp insertion (data not shown). To elucidate the recombination mechanism underlying the paracentric inversion, we searched the entire intron 7 and AC010878 sequences for the presence of the 7-bp insertion. Because of the presence of two dinucleotide repeats within these short sequences, several hits were obtained by BLAST in both genomic sequences. The latter does not allow us to infer the exact recombination mechanism leading to the present rearrangement.

Monoallelic Expression Analysis

To confirm the pathogenicity of the newly characterized genomic rearrangement, *MSH2* expression was analyzed by northern and western blot of somatic cell hybrids containing the wild-type and inversion chromosomes 2 from the index patient (GMP Conversion Technologies) (Papadopoulos et al., 1995; Yan et al., 2000). The murine cell line employed for this aim was *Msh2*-deficient and was therefore particularly suited to analyze expression of the human *MSH2* gene without the interference of the highly homologous and ubiquitously expressed mouse homolog. Neither *MSH2* mRNA nor *MSH2* protein was detectable in the hybrid containing the rearranged chromosome arm 2p, whereas the full-length allele was observed in the hybrid containing the wild-type allele (Fig. 5). Hence, the paracentric chromosome arm 2p inversion represents a true null allele because it disrupts the *MSH2* gene and its expression.

In conclusion, we report a novel 10-Mb paracentric inversion of chromosome 2 responsible for HNPCC in a North American family. In agreement with previous reports (Wijnen et al., 1998a), this

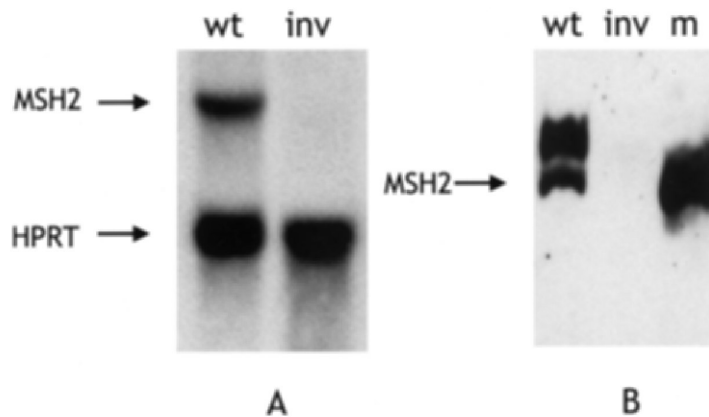


Figure 5. Northern and Western analysis of somatic cell hybrids containing the wild-type and inversion chromosome 2p. Somatic cell hybrids were generated by GMP conversion technology from an EBV-lymphoblastoid cell line derived from the index patient. Individual hybrids were screened by CA repeat markers flanking the *MSH2* gene on chromosome arm 2p, to discriminate between those carrying the wild-type (wt) and inversion (inv) allele. One of each was employed for expression analysis by Northern (A) and Western (B) blot. Probe 3b (*MSH2* exons 10–16) was employed for the Northern analysis together

with an *HPRT* control probe, whereas a rabbit polyclonal antibody raised against amino acids 402–737 of human *MSH2* was used for the Western analysis (Smit et al., 2000). "m" indicates a mouse cellular lysate employed as a control for the detection of the mouse *Msh2* protein. The recipient mouse cell line employed to generate the somatic cell hybrids is *Msh2*^{-/-}. Therefore, the 100-kDa band corresponding to the human *MSH2* protein is detectable only in the hybrid containing the wild-type *MSH2* allele.

study confirm that the *MSH2* gene is susceptible to genomic rearrangements such as deletions and inversions. In view of the latter, Southern blot analysis still represents a useful and informative tool to identify and characterize disease-causing complex genomic rearrangements in HNPCC families, and to facilitate presymptomatic diagnosis and patient care. Moreover, the generation of somatic cell hybrids containing the alleged mutant chromosome arm 2p allele in a *Msh2*-deficient murine cellular background greatly facilitates the analysis of its pathogenicity.

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