HNPCC, molecular and clinical dilemmas

Wagner, A.

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Genes Chromosomes Cancer 2002:35;49-57

GENES, CHROMOSOMES & CANCER 35:49–57 (2002)

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 representsa 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.

INTRODUCTION

Hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch syndrome isa 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 ofa 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 patient must bea 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* (Peltomaki and Vasen, 1997). These mutations are predominantly single nucleotide substitutions or small insertions or deletions. However, larger genomic deletions have also been recognized asa 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.

Supported by: Dutch Cancer Society (KWF).
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Received 13 November 2001; Accepted 12Februar y 2002
DOI 10.1002/gcc.10094

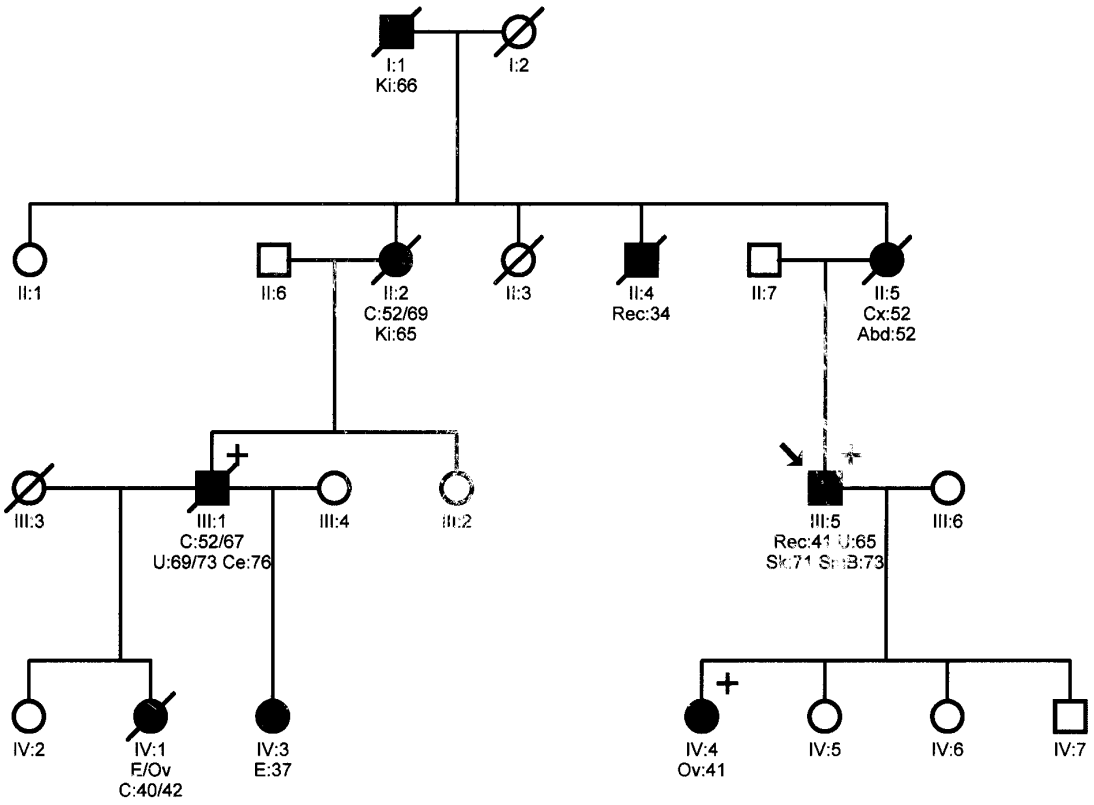

Figure 1. Pedigree of the North-American HNPCC family. Symbols: +, carrier of the paracentric inversion; C, colorectal cancer; Rec, rectal cancer; Ce, cecal cancer; E, endometrial cancer; Ov, ovarian cancer; U, urinary tract cancer; Ki, kidney cancer; SmB, small bowel cancer; Sk, skin cancer; Cx, cervical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

MATERIALS AND METHODS

The Family

The family described here in 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 colonadenocarcinomas 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

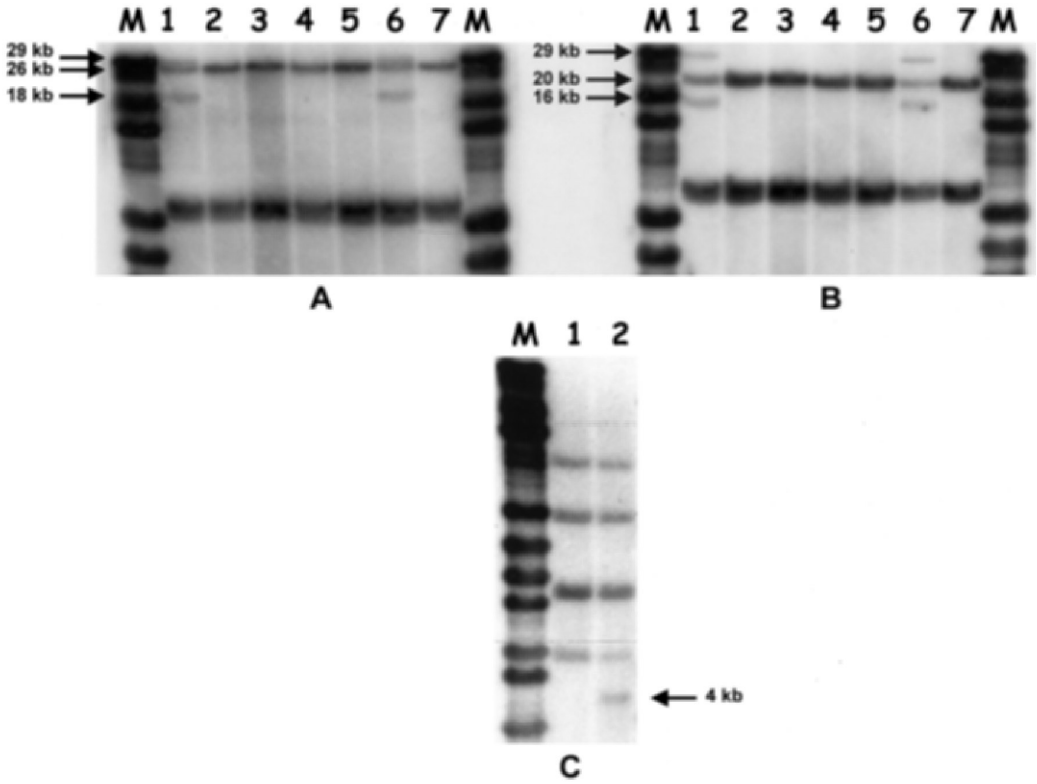

Figure2.Southernblotanalysisoftheindexpatientandotheraffectedandnonaffectedrelativesfrom theHNPCCfamily. *Xba*I(**A**)and *Bsp*I(**B**)digestshybridizedwiththe3aprobe(encompassing *MSH2* exons 7–12).Lane1:indexpatient(III:5);lanes2 –5:unaffected50%riskcarriers;lane6:affectedrelative(III:1);lane 7:normalcontrol.Mindicatesthemolecularweightmarkers.Themolecularweightofthespeci fic *MSH2* bandsisindicated. *Hin*cII(**C**)digestsfromanormalcontrol(lane1)andfromindividualIII:5(lane2)were hybridizedwiththe3aprobe.

MSH2 wasperformedwith *Xba*I, *Eco*RI, *Hin*dIII,and *Nsi*IgenomicDNAdigestsfollowedbyhybridization withthree *MSH2* probes:probe5,encompassingexons1–7;probe3a,forexons7–12;andprobe3b,for exons10–16(seealsoFig.2).Inaddition,digestion withtherestrictionendonucleases *Bsp* 1407I, *Hin*cII, and *Bcl*Iwasperformed.Thelatterfilterswerehybridizedwithseveralexon-specific *MSH2* probes,including exons6to9. *MLH1* and *MSH6* Southern analysiswasperformedwith *Xba*I, *Apa*I, *Hin*dIII,and *Nsi*IgenomicDNAdigestsfollowedbyhybridization withtwo *MLH1* probes(encompassingexons1–12 andexons11–19)andtwo *MSH6* probes(encompassingexons1–4andexons5–10)(Wijnenetal.,1998a).

CytogeneticandFluorescenceInSitu HybridizationAnalyses

Prometaphaseandmetaphasechromosomes wereobtainedfromEBV-transformedlymphoblasticcellsaccordingtostandardprotocols(Dauwerse etal.,1992).Karyotypingwasperformedusing GTG-banding(Seabright,1971)andmulticolorfluorescenceinsituhybridization(MFISH).For MFISH,theCOBRA(forCOmbinedBinaryRAtio)labelingtechniquewithp/qarmdistinctionwas employedaspreviouslydescribed(Tankeetal., 1999;Wiegantetal.,2000).

InversePolymeraseChainReaction(IPCR)

IPCRwasperformedaspreviouslydescribed (Foddeetal.,1990)(Fig.3).A5- µgsampleof genomicDNAextractedfromlympoblastoidcells wasdigestedwith *Hin*cII.ThedigestedDNAwas extractedbyphenol-chloroform,ethanol-precipitated,dilutedtoaconcentrationof1 µg/mlin ligationbuffer(Fermentas,St.Leon-Rot,Germany),andligatedovernightat16°Cwith1U/ µl ligase(Fermentas).

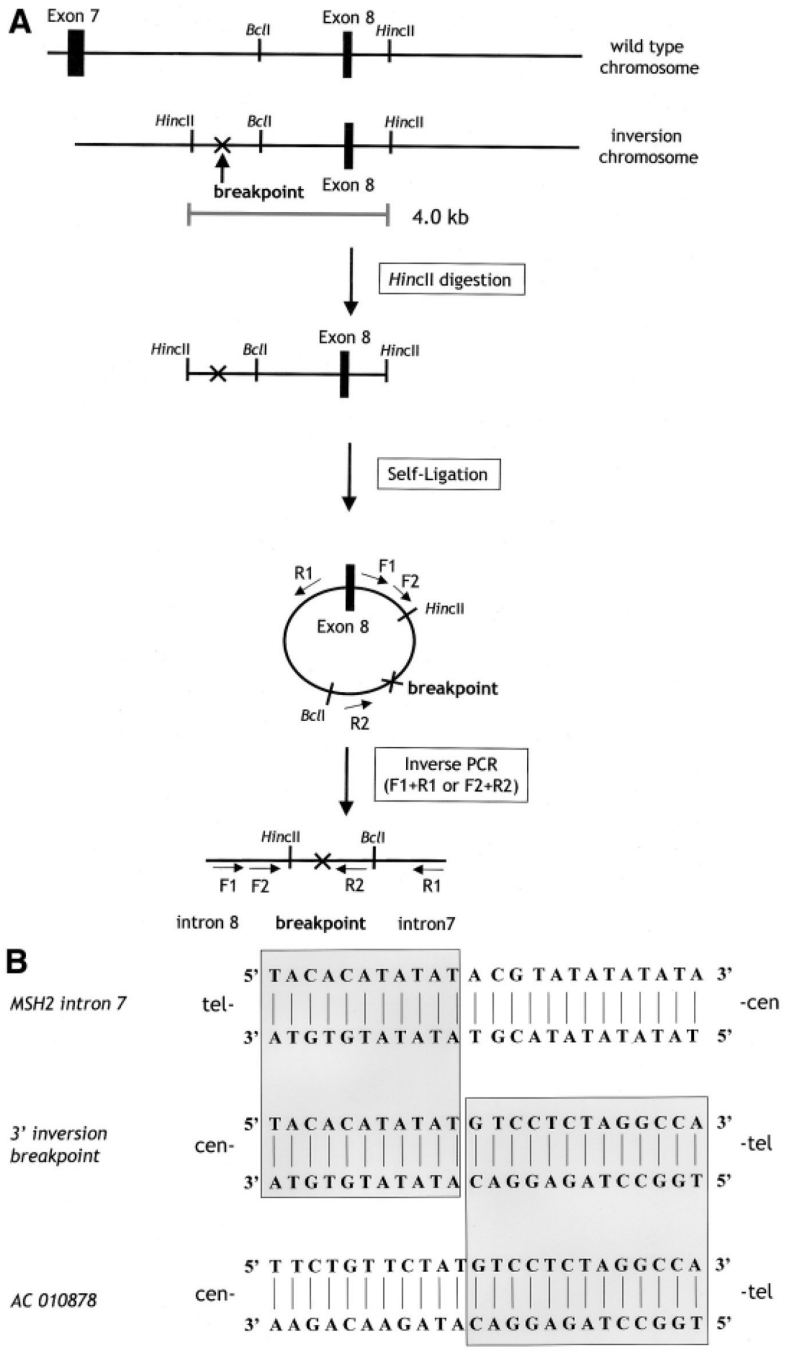

Figure3.Cloningandcharacterizationofthe3′(telomeric)inversion breakpointbyinverse-PCR. **A:** Theinverse-PCRprocedure.GenomicDNA fromtheindexpatient(III:5)wasdigestedby *Hinc*II.Thelatterresultinan aberrant4.0-kbfragmentencompassing *MSH2* exon8andthe3′breakpointas indicated.ThedigestedDNAwasligatedasdescribedunderMaterialsand Methods.TheligatedDNAwasemployedinaPCRreactionwithprimer setsF1/R1andF2/R2,indicatedbyarrows.Inbothcases,onlyDNAfrom carriersoftheparacentricinversionresultedintheexpectedinverse-PCR products.Thelatterencompassthe5′ endof *MSH2* intron8,theoriginal *Hinc*IIsite,theinversionbreakpoint, andthe3′endof *MSH2* intron7. **B:** Boththesenseandantisensestrandsof thesequencesaroundthe3′(telomeric)inversionbreakpointaredepicted.Thealignmentshows(fromtop tobottom)thewild-type *MSH2* intron 7sequence,the3′(telomeric)breakpointobtainedbyinversePCR,andthe sequenceofthecontigAC010878,located10Mb3′(telomeric)to *MSH2*. Theshadedboxesindicatethehomologiesamongthethreealignedsequences.Theorientationsofboththe centromereandtelomererelativeto thereportedsequencesareindicated. Thesequenceof *MSH2* intron7isdepictedinthereversedirectionrelative totheothertwosequences.

Aforwardprimer(F1)wasdesignedatthe3′end ofexon8(5′-AGAAACAACTTTAGATATGG-3′),whereasthereverseprimer(R1)wasdeveloped 3′ofthe *Bcl*Isiteatposition13,392withinintron7 (5′-AGACTTTGTAAAGCTACATTC-3′)(Fig. 3).High-fidelityPCR(RocheMolecularDiagnosticSystems,Alameda,CA)wasperformedwith theseprimers.TheIPCRproductwasanalyzedby agarosegelelectrophoresis.Anapproximately 2.0-kbbandwasobservedintheindexDNAbut

notinthenormalcontrol.Thisbandwasexcised andpurifiedusinganucleotrapkit(Machery-Nagel,Du¨ren,Germany).Thepurifiedproductwas clonedbyTOPO-cloning(Invitrogen,LaJolla, CA)andsequencedusingthesameF1andR1 primers.Basedonthenewlyobtainedsequences, newprimersweredesigned,thatis,aforward primer(F2)inintron8(5′-TCAAGCTATCCTC-CGGCCTC-3′)andareverseprimer(R2)inintron 7(5′-GGATGCAGTGAACAGGGAAC-3′)(Fig. 3).TheIPCRprocedurewasthenrepeatedwith F2andR2,andthepatient-specificPCRproduct wassequenced.

Thissequencealsoenabledustodevelopaddi-tionalprimersaroundthe5′ (centromeric)break-point(Fig.4):aforwardprimer(F3,5′-TGCTGA-CATGGCCCATTCCTAC-3′)withinthecontig AC010878,andareverseprimer(R3,5′-TCCC-AGTTTAACTCTAGCAG-3′)inintron7.PCR amplificationwiththeseprimersresultedinafrag-mentofapproximately900bpintheindexpatient (III:5)andintwootheraffectedindividuals(III:1 andIV:4)butnotinanormalcontrol(seealsoFig. 4C).Thisfragmentwasagainexcisedandpurified bythenucleotrapprocedureandsequencedusing thePCRforwardandreverseprimers.

MonoallelicExpressionAnalysis

ToallowRNAandproteinexpressionanalysisof the *MSH2* allelecarryingtheinversion,somaticcell hybridscontainingthewildtypeandrearranged chromosome2pfromtheindexpatientweregen-eratedina *Msh2*-deficientmurinebackground (GMPConversionTechnologies,Waltham,MA) (Papadopoulosetal.,1995;Yanetal.,2000).So-maticcellhybridsweregenotypedbyCArepeat markersaroundthe *MSH2* geneonchromosome arm2pandselectedformonoallelicexpression analysis.TotalRNAandproteinsampleswereiso-latedfromthedifferenthybridcelllines,and northernandwesternblotanalyseswerecarriedout accordingtoconventionalprocedures.Probe3b (*MSH2* exons10–16)wasemployedforthenorth-ernanalysis,whereasarabbitpolyclonalantibody raisedagainstaminoacids402–737ofhuman MSH2wasusedforthewesternanalysis(Smitset al.,2000).

RESULTSANDDISCUSSION

SouthernAnalysis

DGGEanalysisof *MSH2*,*MLH1*, and *MSH6* did notrevealanymutationintheindexpatient. Therefore,Southernblotanalysiswasperformed. *MLH1* and *MSH6* hybridizationsdidnotshowany aberrantfragments.Afterhybridizationwiththe *MSH2* 5′ probe(encompassingexons1–7),anab-errantbandofapproximately30kbwasseeninthe *Xba*Idigest(notshown).Hybridizationwiththe *MSH2* 3aprobe(exons7–12)revealedtwoaberrant fragmentsof29and18kb,respectively(Fig.2A). Basedonthedecreasedintensityofthewild-type 26-kbfragmentencompassing *MSH2* exons7and 8,weassumedthatthegenomicrearrangement involvedthissegmentofthegene.Hybridization withexon7-andexon8-specificprobesindicated thatthe29-kbbandcontainedexon7,whereasthe 18-kbfragmentencompassedexon8of *MSH2*. Thiswasconfirmedbydigestionwith *Bsp*1407I:in additiontothewildtype20-kbfragmentcontain-ingexons7and8,hybridizationwithboththe 3′a-specificandtheexon7-andexon8-specific probesrevealedtwofragmentsof29and16kb, encompassingexon7andexon8,respectively(Fig. 2B).Theseresultsareindicativeofacomplexre-arrangementencompassingbothexon7andexon8 inthemutatedallele.Thenormalintensityofall otherdigestionfragmentssuggestedthatnoother *MSH2* exonwasinvolvedintherearrangement (datanotshown).Thisalsoindicatedthatthe genomicrearrangementwaslikelytobeatranslo-cationorinversionwithabreakpointinintron7. Theaberrantrestrictionsiteclosesttothebreak-pointappearedtobea *Hin*cIIsiteatposition 11,738inintron7(Fig.2C). *Bcl*Idigestionshowed noaberrantfragmentafterhybridizationwithan exon8-specificprobe,indicatingthatthebreak-pointhadtomap5′ tothe *Bcl*Isitelocatedat position13,392withinintron7.Giventhatnoother aberrantfragmentswerefoundafterhybridization withanyoftheother *MSH2* probes,theother breakpointhadtomapoutsidethe *MSH2* gene.

AdditionalSouthernblotanalysisofindividual III1,IV4,andseveralunaffectedrelativesshowed co-segregationoftherearrangementwiththedis-easeinthisfamily(notshown).

CytogeneticandFISHAnalyses

KaryotypeanalysisoftheindexpatientbyGTG-bandingandPQ-COBRA-FISHdidnotshowei-theranyapparenttranslocationoraninversioncon-cerningchromosome2.Thisindicatedthatthe allegedgenomicrearrangementhadtobeeithera smallinter-orintrachromosomaltranslocation,ora paracentricinversion(datanotshown).

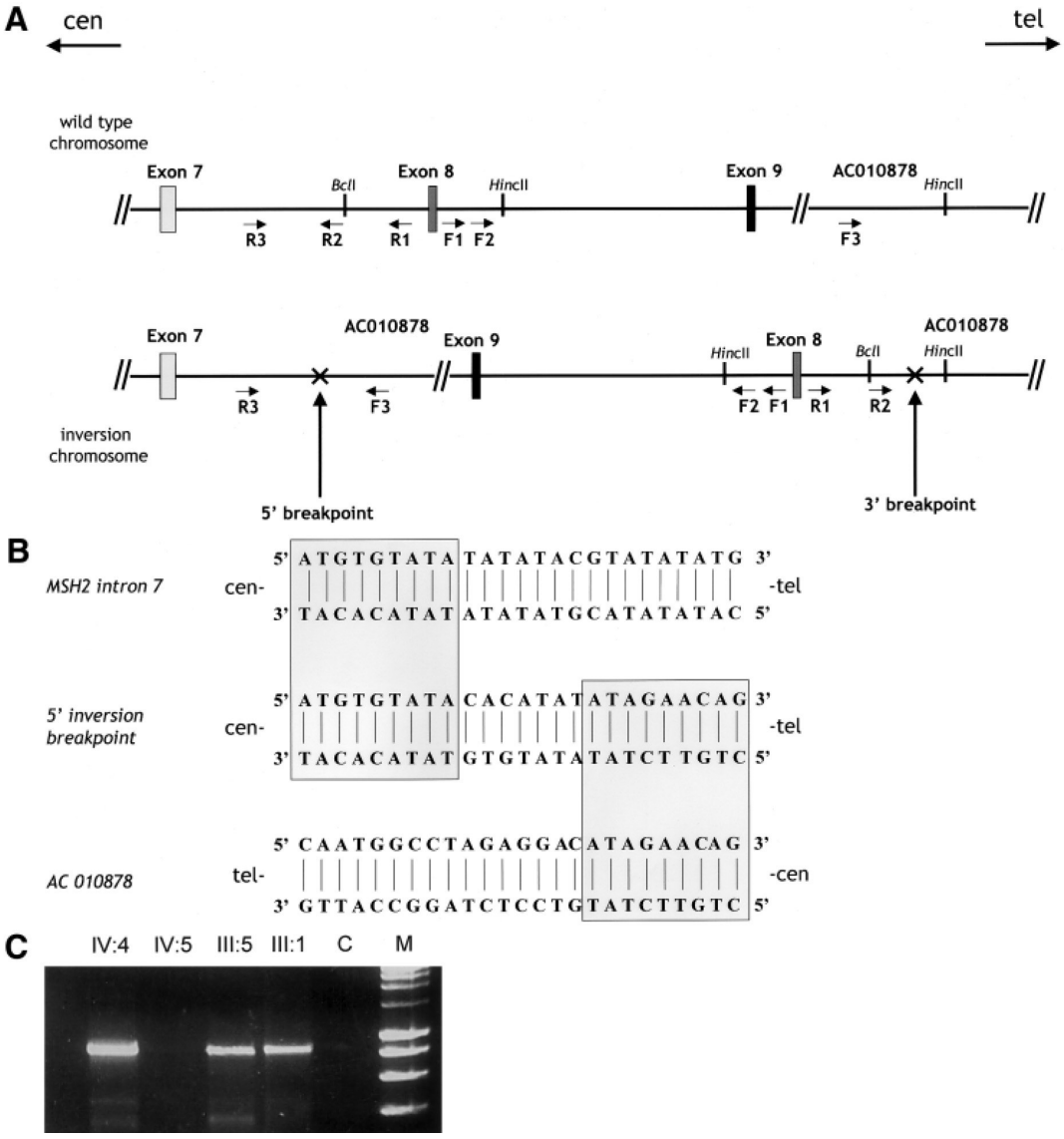

Figure4.Cloningandcharacterizationofthe5′(centromeric) inversionbreakpoint. **A:** Schemeofthewild-typeandmutated *MSH2* alleles.Onlythepartofthechromosome2regioninvolvedinthe paracentricinversionisshown.Shadedandblackboxesindicateexons 7,8,and9ofthe *MSH2* gene,respectively.Onlythe *Hin*cIIand *Bcl*I restrictionsitesandthe5′(centromeric)and3′(telomeric)break-pointsaredepictedforthesakeofclarity.AC010878isthecontig located10Mb3′of *MSH2*. Theprimersetsemployedtoamplifyand sequencebothbreakpoints(F1/R1,F2/R2,F3/R3)areindicatedby arrows. **B:** Boththesenseandantisensestrandsofthesequences aroundthe5′(centromeric)inversionbreakpointaredepicted.The alignmentshows(fromtoptobottom)thewild-type *MSH2* intron7 sequence,the5′breakpointobtainedbyPCRwithprimersF3andR3, andthesequenceofthecontigAC010878,located10Mb3′(telomeric) to *MSH2*. Theshadedboxesindicatethehomologiesamongthethree alignedsequences.Theorientationsofboththecentromereandtelo-mererelativetothereportedsequencesareindicated.Thesequenceof theAC010878contigisdepictedinthereverseorientationrelativeto theothertwosequences. **C:** 5′ Breakpoint-specificPCRresultsob-tainedwithprimersF3andR3(seeA)inaffected(IV:4,III:5,III:1)and nonaffected(IV:5)individuals.

IPCR

Todiscriminateamongtheabovepossibilities, wesetouttocharacterizetheintron7breakpoint byIPCR(Fig.3A).BasedontheSouthernblot analysis, *Hin*cIIdigestionresultsina4-kbfragment encompassingexon8andthebreakpointofthe genomicrearrangementwithinintron7(Fig.2C). TheIPCRprocedureenabledustoobtainanap-

proximately2-kbPCRproductcontainingthe5′ endofintron8,the *Hin*cIIsiteusedfordigestion andligation,60basepairsofanunknownsequence,thebreakpoint,andthe3′ endofintron7 (Fig.3B).TheIPCRproductwasnotobserved whengenomicDNAfromahealthyindividualwas subjectedtotheprocedure.ByuseoftheBasic LocalAlignmentSearchTool(BLAST)database oftheNationalCenterofBiotechnologyInformation,aperfectmatchwasfoundbetweentheunknownsequenceandacontig(AC010878)located 10Mb3′ of *MSH2*. Thelatterfindingindicated thatthegenomicrearrangementinthiskindredis likelytobea10-Mbinversionofexons8to16of the *MSH2* gene(Fig.4A).Totestthishypothesis, PCRprimers(F3andR3)weredesignedwithin AC010878andin *MSH2* intron7,toallowamplificationandsequencingofthe5′ (centromeric) breakpointofthe10-Mbinversion(Fig.4A).The approximately900-bpPCRproductobtainedusing theseprimersencompassessequencesderived fromAC010878(inthereversedirectionrelativeto thesequencefoundinthe3′ telomericbreakpoint sequence),the5′ centromericbreakpoint,and5′ intron7sequences(again,inthereverseorientationrelativetothe3′ telomericbreakpointsequence)(Fig.4B).Moreover,thecombinationof theseprimersresultedinthe900-bpproductonly whenDNAfromtheindexpatientortheothertwo availableaffectedindividuals(III:1andIV:4inFig. 1)fromtheaffectedkindredwereemployed(Fig. 4C).Thisprovedthattherearrangementinthis familywasindeedaparacentricinversionofabout 10Mb,includingexons8to16ofthe *MSH2* gene.

Genomicrearrangementswerepreviouslydescribedin *MSH2,* aswellasinothercancer-susceptibilitygeneslike *APC,MLH1,BRCA1,TP53,NF2,* and *STK11* (vanderLuijtetal.,1995;Petrij-Bosch etal.,1997;Jenneetal.,1998;Slebosetal.,1998; Wijnenetal.,1998a;Pugetetal.,1999;Legoixet al.,2000;Chanetal.,2001).Theserearrangements mayresultfromnonhomologousbreakageandreunioneventsbutalsofromhomologousrecombinationbetweenrepetitivesequencesdispersed throughoutthegenome,likeAlurepeats(Nystrom-Lahtietal.,1995;Mauillonetal.,1996;Slebosetal.,1998).Also,genomicinversionshave beendescribedinhemophiliaresultingfromunequalalignmentsbetweensmall-sequencehomologies(Lakichetal.,1993).Computeranalysis (BLAST)ofintron7ofthe *MSH2* generevealed manyrepetitivesequences,amongwhichwereAlu andsimple-sequencerepeats(notshown).During oursearchformutationsinHNPCCfamilies,we identifiedfourgenomicdeletionsof *MSH2* witha breakpointwithinintron7(unpublishedobservations).However,becausethedeletionbreakpoints inthesefamilies,includingtheonestudiedhere, appeartobelocatedatdifferentsitesalongintron 7,noactualrecombinationhotspotcanbepinpointed.WedidnotfindAlurepeatsatorinthe flankingregionsofthebreakpointsoftheinversion reportedhere.Hence,anonhomologousbreakage andreunionrecombinationeventisthemostlikely causeofthegenomicrearrangementobservedin thisfamily.Notably,duringtherecombinationprocess,ninebasepairswerelostfromintron7, whereasanadditionalsevenbasepairswerefound atthe5′ (centromeric)breakpoint(Figs.3Band 4B).Thelatterdoesnotresultfromapcrartifact, giventhatsequenceanalysisofthe5′ breakpointof theaffectedindividualsIII:1andIV:4confirmed thepresenceofthe9-bpdeletionandthe7-bp insertion(datanotshown).Toelucidatetherecombinationmechanismunderlyingtheparacentricinversion,wesearchedtheentireintron7and AC010878sequencesforthepresenceofthe7-bp insertion.Becauseofthepresenceoftwodinucleotiderepeatswithintheseshortsequences,several hitswereobtainedbyBLASTinbothgenomic sequences.Thelatterdoesnotallowustoinferthe exactrecombinationmechanismleadingtothe presentrearrangement.

MonoallelicExpressionAnalysis

Toconfirmthepathogenicityofthenewlycharacterizedgenomicrearrangement, *MSH2* expressionwasanalyzedbynorthernandwesternblotsof somaticcellhybridscontainingthewild-typeand inversionchromosomes2fromtheindexpatient (GMPConversionTechnologies)(Papadopoulosetal.,1995;Yanetal.,2000).Themurinecellline employedforthisaimwas *Msh2*-deficientandwas thereforeparticularlysuitedtoanalyzeexpression ofthehuman *MSH2* genewithouttheinterference ofthehighlyhomologousandubiquitouslyexpressedmousehomolog.Neither *MSH2* mRNA norMSH2proteinwasdetectableinthehybrid containingtherearrangedchromosomearm2p, whereasthefull-lengthallelewasobservedinthe hybridcontainingthewild-typeallele(Fig.5). Hence,theparacentricchromosomearm2pinversionrepresentsatruenullallelebecauseitdisrupts the *MSH2* geneanditsexpression.

Inconclusion,wereportanovel10-Mbparacentricinversionofchromosome2responsiblefor HNPCCinaNorthAmericanfamily.Inagreement withpreviousreports(Wijnenetal.,1998a),this

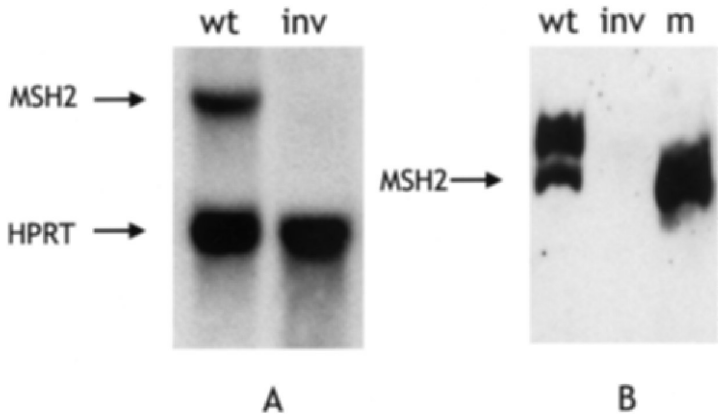

Figure5.Northernandwesternanalysisofsomaticcellhybrids containingthewild-typeandinversionchromosome2p.Somaticcell hybridsweregeneratedbyGMPconversiontechnologyfromanEBV-lymphoblastoidcelllinederivedfromtheindexpatient.Individualhybridswerescreenedby CArepeatmarkers flankingthe *MSH2* geneon chromosomearm2p,todiscriminatebetweenthosecarryingthe wild-type(wt)andinversion(inv)allele.Oneofeachwasemployedfor expressionanalysisbynorthern(**A**)andwestern(**B**)blot.Probe3b (*MSH2* exons10 –16)wasemployedforthenorthernanalysistogether withan *HPRT* controlprobe,whereasarabbitpolyclonalantibody raisedagainstaminoacids402 –737ofhumanMSH2wasusedforthe westernanalysis(Smitsetal.,2000). "m" indicatesamousecellular lysateemployedascontrolforthedetectionofthemouseMsh2 protein.Therecipientmousecelllineemployedtogeneratethesomatic cellhybridsis $Msh2^{-/-}$.Therefore,the100-kDabandcorrespondingto thehumanMSH2proteinisdetectableonlyinthehybridcontainingthe wild-type *MSH2* allele.

studyconfirmsthatthe *MSH2* geneissusceptible togenomicrearrangementssuchasdeletionsand inversions.Inviewofthelatter,Southernblot analysisstillrepresentsausefulandinformative tooltoidentifyandcharacterizedisease-causing complexgenomicrearrangementinHNPCCfamilies,andtofacilitatepresymptomaticdiagnosis andpatientcare.Moreover,thegenerationofsomaticcellhybridscontainingtheallegedmutant chromosomearm2palleleina *Msh2*-deficientmurinecellularbackgroundgreatlyfacilitatestheanalysisofitspathogenicity.

ACKNOWLEDGMENTS

WethankProf.B.VogelsteinandProf.R.D. Kolodnerforprovidinguswiththe PACclones encompassing *MSH2*.

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