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Short Communication

Germline and Somatic Mutation Analysis of *MLH3* in MSI-Positive Colorectal Cancer

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Microsatellite instability (MSI) is characteristic of hereditary nonpolyposis colorectal cancer, and occurs in a subset (10 to 15%) of unselected colorectal cancer cases. In hereditary nonpolyposis colorectal cancer, MSI is caused by defects in five mismatch repair genes, and in sporadic cases the main cause seems to be somatic *MLH1* promoter methylation. Most likely additional hereditary nonpolyposis colorectal cancer genes remain to be discovered. Genes with simple repeats in their coding region are often targets for deletions in MSI-positive tumors. Several genes *(TGFβ RII, IGFIIR, MSH3, MSH6, BAX, MBD4)* with significance in tumorigenesis harbor repeats in their coding regions and are often somatically inactivated because of deletions causing frameshifts. Recently, a novel human mismatch repair gene, *MLH3*, was cloned and shown to be involved in mammalian mismatch repair. To evaluate the possible role of *MLH3* in hereditary cancer, we performed germline single-strand conformation polymorphism-analysis for 52 patients displaying features of inherited colorectal cancer. Forty-six of these had been diagnosed with MSI-positive tumors. No germline mutations were found. Similar to *MSH3* and *MSH6, MLH3* harbors mononucleotide repeats, ie, (A_6)-(A_9), in its coding region, which makes it a putative target for somatic mutations in MSI-positive tumors. To evaluate its somatic inactivation we performed a deletion search focusing on eight exonic *MLH3* mononucleotide repeats in a series of 93 MSI-positive tumors. Somatic deletions were found in 8.6% of the samples, a frequency similar to one detected in neutral noncoding mononucleotide repeats. No evidence of involvement of *MLH3* in MSI tumorigenesis was obtained. *(Am J Pathol 2000, 157:347–352)*

Microsatellite instability (MSI) is a characteristic of hereditary nonpolyposis colorectal cancer and is seen in a subset (10 to 15%) of sporadic colorectal cancers as well.[1–3] It is typically caused by a defect in DNA mismatch repair. To date germline mutations in five mismatch repair genes (*MSH2*,[4] *MLH1*,[5,6] *PMS1* and *PMS2*,[7] and *MSH6*ref 8) have been associated with hereditary nonpolyposis colorectal cancer. Because the causative mutation is found only in a subset of kindreds with MSI-positive colorectal tumors, it is possible that more genes underlying MSI-positive familial colorectal cancer remain to be discovered. In sporadic cases a major cause of MSI seems to be *MLH1* promoter methylation.[9]

Mismatch repair complexes in humans are multiple. Mismatches and insertion/deletion loops are recognized by MSH2/MSH6 heterodimers.[10] In the absence of MSH6 the recognition of insertion/deletion loops can be mediated by MSH2/MSH3 heterodimers, indicating redundant functions of *MSH6* and *MSH3*.[10,11] The actual mismatch repair is performed by the MLH1/PMS2 heterodimer[12] with the help of exonucleases and polymerases.

Recently, a novel mismatch repair gene, *MLH3*, was cloned and shown to be associated with mammalian MSI.[13] The amino terminus of MLH3 contains a predicted ATPase motif and the carboxyl terminus encodes a predicted MLH1 interaction domain.[13] Thus, it is possible that MLH3 replaces PMS2 in mismatch repair complex, and they might have similarly redundant functions as MSH6 and MSH3. The putative MSH2/MSH3-MLH1/MLH3 complex is predicted to address a subset of insertion/deletion loops.[10] Because MLH3 has a greater similarity to yeast Mlh3p than to PMS2, it might have a specific role in human mismatch repair distinct from that of PMS2.[13] *MLH3* is ubiquitously expressed. In the postnatal mouse the highest expression has been detected in epithelial tissues, including colon, skin, small bowel, and

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ventricle.[13] Therefore its function may have importance in maintaining genomic stability in the epithelium of the large bowel.

MSI-positive tumors acquire somatic deletions and insertions in simple repeated sequences. Several genes involved in tumorigenesis contain mononucleotide repeats in their coding regions. Somatic deletions have been seen in *MSH3*, *MSH6*,[14] *BAX*,[15] *IGFIIR*,[16] *MBD4*,[17,18] and *TGF-β RII*.[19–21]. Frameshift mutations of secondary mutators are presumably induced by primary mutators such as *MLH1* and *MSH2*.[22] Accumulation of somatic mutations in mismatch repair genes such as *MSH3* and *MSH6* is believed to further increase genetic instability. *MSH3* harbors an $(A)_8$ repeat, whereas *MSH6* has a $(C)_8$ repeat. Both genes have confirmed roles in human mismatch repair, and germline *MSH6* mutations are associated with a minor subset of hereditary nonpolyposis colorectal cancer cases. Although these genes do not have prominent roles in hereditary susceptibility to MSI-positive tumors, they seem to be targets for secondary hits. The newly identified mismatch repair gene, *MLH3*, harbors as many as eight mononucleotide repeats, $(A)_6$-$(A)_9$, in its coding region, which makes it an attractive target for somatic deletions in mismatch repair deficient cells.

To test whether germline mutations of *MLH3* predispose to colorectal cancer, we analyzed 52 colorectal cancer patients displaying features of hereditary cancer for all coding exons and exon-intron boundaries by SSCP. To study whether *MLH3* is, similar to *MSH3* and *MSH6*, a target gene for MSI, we performed mononucleotide repeat length analysis on 93 MSI-positive tumor samples for eight polyA repeats, ie, (A_6)-(A_9), residing in the *MLH3* coding region.

Materials and Methods

We analyzed 46 MSI-positive colorectal cancer patients with features of hereditary cancer by genomic SSCP analysis on normal tissue DNA. The MSI status of these samples has been previously determined using BAT26 mononucleotide marker, and *MLH1* and *MSH2* mutations have been excluded by genomic sequencing.[23,24] Of the 46 patients, 43 (93%) had at least one additional first or second degree relative with cancer (in 18 cases colorectal cancer, in six cases endometrial, in eight cases stomach, and in 11 cases some nonhereditary nonpolyposis colorectal cancer) and three (7%) patients were selected based on young age (mean, 40 years).

In addition, we analyzed six MSI-negative patients whose tumors had shown 14q deletions in comparative genomic hybridization and a possible common haplotype in chromosome 14q in loss of heterozygosity-studies (unpublished data). These six MSI-negative patients all had one to four additional first-degree relatives with colorectal cancer.

MLH3 is indicated to have 12 coding exons, one of which is alternatively spliced.[13] The GenBank sequence (AF195657) only includes 11 coding exons. We used the Genscan1.0 exon prediction program available on the web (http://bioweb.pasteur.fr/seqanal/interfaces/genscan.html) and found a 96.9% probability for an exon located between coding exons 5 and 6 (which is the location indicated by Lipkin and colleagues). We performed reverse transcriptase-polymerase chain reaction (RT-PCR) to confirm that our interpretation was correct (data not shown). In this study we call this alternatively spliced exon "6A." This alternative form lacks the MLH1 interaction domain, and its role, if any, in DNA mismatch repair is unclear. All of the exons were amplified separately except exons 8 and 9, because they only have 102 bp of intron sequence in between. Exon 1 was divided into 14 overlapping fragments. Because of the limitations of SSCP analysis, the length of the fragments was always <330 bp (between 158 and 329 bp; mean, 273 bp) (Table 1.).

PCR-reactions were carried out in 20 μl of reaction volume containing 100 ng genomic DNA, 1× PCR buffer (Perkin Elmer Applied Biosystems Division, Foster City, CA), 500 μmol/L of each dNTP (Finnzymes, Espoo, Finland), 1 μmol/L of each primer, and 1 U of AmpliTaqGOLD polymerase (Perkin Elmer). The $MgCl_2$ concentration was 2.75 mmol/L. The following PCR cycles were used for amplification: 95°C for 10 minutes, 40 cycles at 95°C for 45 seconds, corresponding annealing temperature for 45 seconds, and 72°C for 45 seconds (30 seconds for exons 4 and 6A). Final extension was 72°C for 10 minutes. The length of the PCR fragments, primer sequences, and the corresponding annealing temperatures for each fragment are listed in Table 1.

SSCP analysis was performed using mutation detection enhancement gel solution (FMC BioProducts, Rockland, Maine). PCR products were run on 0.6× mutation detection enhancement gels, 4 W for 14 to 34 hours. The running buffer was 0.6× Tris borate-ethylenediaminetetraacetic acid. SSCP gels were silver stained according to standard procedure. The SSCP running times for each fragment are indicated in Table 1. The complementary strands were not separated in exon 1 fragment 1 to 14 even after a 40-hour run, and this fragment was analyzed using genomic sequencing. Direct sequencing was performed using the ABI PRISM Dye Terminator or ABI PRISM dRhodamine cycle sequencing ready reaction kit (Perkin Elmer). Cycle sequencing products were electrophoresed on 6% Long Ranger gels (FMC BioProducts) and analyzed on an Applied Biosystems model 373A or 377 automated DNA sequencer (Perkin Elmer).

Tumor DNA of 93 MSI-positive colorectal cancer patients was analyzed for deletions and insertions in eight mononucleotide repeats, ie, (A_6)-(A_9) in the coding region of *MLH3*. New sets of primers for shorter PCR fragments (100 bp or less) were designed to maximize resolution in polyacrylamide gel electrophoresis analysis. For primer sequences see Table 2. The following repeats were analyzed: $(A)_6$ starting at nucleotide 1410 in mRNA, 1576 $(A)_6$, 1861 $(A)_9$, 1961$(A)_6$, 2128 $(A)_8$, 2218 $(A)_6$, 2897 $(A)_6$, and 3796 $(A)_6$. Out of the 93 cases chosen 18 (19%) had an *MLH1* or *MSH2* germline mutation. Thirty-three (35%) out of 93 patients (none with a known germline mutation) were also included in the germline SSCP analysis; 30 because of family history of cancer, and three because they were diagnosed before the age of 50.

Table 1. Primer Sequences, the Length of the PCR Fragments, Annealing Temperatures, and SSCP Running Times for each Fragment

Primer sequence	Length, bp	Annealing temperature, °C	SSCP, hours
MLH3 F1-1: TCC AGT CAG AGA AGG AAA CCA MLH3 R1-1: CGA GTG GCA TTT ACT GGT GA	294	58	22
MLH3 F1-2: TGT AGA GAA AGT GGG AAA TCG TT MLH3 R1-2: ACA GGA AGC TGG TAA AAT AGG TT	276	60	22
MLH3 F1-3: CTG ATG TGA CTA GAG CAA GCG MLH3 R1-3: AGC CAC TAA GCT CAA ACT CTT TAT	309	60	22
MLH3 F1-4: GGG AAA GTC CCA AAA GCT AA MLH3 R1-4: CAT CAT ACT CAC AGA ATT GGC AC	305	60	22
MLH3 F1-5: ATG TGC AGT GCC AAT TCT GT MLH3 R1-5: GAA ATT GCT CCT CTC ATC GG	245	60	22
MLH3 F1-6: TCA GGT GAG GAT ATT AAG GAA TTT MLH3 R1-6: TTT GTT TTG TAA AGA TGG CTC TG	312	61	34
MLH3 F1-7: CCA GGC CAT AGC AAA ATG AC MLH3 R1-7: GCA GCC ATG CCA TTA ACA G	275	60	30
MLH3 F1-8: TGG GCA GGA TCT AGA AAT ATG G MLH3 R1-8: TGA ATG TTC TGT TTC AGT TGA TTT	316	60	22
MLH3 F1-9: GGG CGA GTT AAA TTA TGT TCC A MLH3 R1-9: GCT ACC TTC CTG AAA AGC AGA A	309	60	22
MLH3 F1-10: GGC TAG AGA ATG AAC CTA CAG CA MLH3 R1-10: CTT GAA GAC TGA GAT TGG TAG TGA	312	60	22
MLH3 F1-11: TGG GAA GGT TGA AAA TCC TC MLH3 R1-11: TCA AGG TCC AAA GGT TTT CTA TT	318	60	22
MLH3 F1-12: TCC TAT GAC CCT GAA GGA GTT MLH3 R1-12: AGG AAT TAT CCT GTG TGG CAG	293	60	22
MLH3 F1-13: CAG AGA ATG GTG TCA TCC CAA MLH3 R1-13: CCA TTT TGG TCA CCT GTG G	265	60	30
MLH3 F1-14: ATC CGG TAG AAG ATG CCA CA MLH3 R1-14: CCT TGT CCA GCA TTC CCA T	320	60	Does not separate
MLH3 F2: TGT CTT GAC TCA GTT TGT GCA G MLH3 R2: ACG ATG TGT ACT GTG TGC CC	247	62	22
MLH3 F3: TGG TTC TGG ATG CCA ACT TT MLH3 R3: ATT TCA GTC TGG GCA ACA GG	229	59	26
MLH3 F4: CAA TTA TAT TTT GCT GAG TC MLH3 R4: ATG AGA TTT TGA AGT TAA TC	158	52	14
MLH3 F5: CCC AGT CTC AAA GAA AGG AGT G MLH3 R5: AGC TGG TTA GTC ATT CAG GC	239	57	20
MLH3 F6: TTC CCT TCC TAC TCT TAA CCC A MLH3 R6: TGT AAC CTC TCT TGG TCT CAT CTG	265	59	20
MLH3 F7: TTT GGA ACC AGT AGT GAA GTG C MLH3 R7: CAG CAA TTT CCT TAA CAT CTG C	267	62	20
MLH3 F8/9: CGT AGA TTA AAG CCG ATT TTC MLH3 R8/9: TGT ACC CTC TGC CTC TTT CG	329	59	22
MLH3 F10: GTC AGC ATT GGT TTC CCA CT MLH3 R10: AAA CTT TGC TCC CTC CTG CT	251	59	20
MLH3 F11: GCC CAG CCT GTA TGC TAC CT MLH3 R11: CAG TGA CAC TCC CTT TGT TCC	226	59	20
MLH3 6AF: CAT GAT GGT TGT CGT CTT GC MLH3 6AR: GGT GTA CTG ATT CTG CTG GGA	185	60	14

PCR reactions were carried out in 10 μl of reaction volume containing 50 ng genomic DNA, 1× PCR buffer (Perkin Elmer), 1 μmol/L of each primer (2 μmol/L of primers 1–6s, 1–7s, and 1–9as), dCTP at 20 μmol/L, 200 μmol/L each of dATP, dGTP, dTTP (Amersham Pharmacia Biotech, Piscataway, NJ), 0.7 μCi [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK), and 0.5 units of AmpliTaqGOLD polymerase (Perkin Elmer). The $MgCl_2$ concentration was 3.75 mmol/L (1.25 mmol/L for exon 1–8s). The following PCR cycles were used for amplification: 95°C for 10 minutes, 37 cycles of 95°C for 45 seconds, for annealing conditions see Table 2, and 72°C for 1 minute (45 seconds for exon 1–8). Final extension was 72°C for 10 minutes. Ten μl of PCR-product was mixed with 7 μl of loading buffer and run on 6.7% polyacrylamide gels, with 0.6× TBE as a running buffer, at a constant power of 80 W. All aberrant bands were sequenced in both directions as previously described. In all aberrant cases the corresponding normal DNA was analyzed to confirm the somatic nature of the changes.

To determine the polyA background mutation rate in MSI-positive colorectal tumors, we analyzed an $(A)_8$ and an $(A)_9$ repeat in the intron of *OBR*-gene, coding for leptin receptor (GenBank accession codes U62509 and U62513, respectively) by radioactive polyacrylamide gel electrophoresis in the 93 tumors. All aberrant bands as well as the corresponding normal DNA were sequenced

Table 2. Primer Sequences, the Length of the PCR Fragments, and Annealing Temperature for PAGE Analysis

Primer sequence	Length, bp	Annealing temperature
MLH3 F1-6s: CTG CAG AAA ACG TAA ACA CAC A MLH3 R1-6s: CCT GGA CCA CCT GAT TCA TAA	100	54°C, 1 minute
MLH3 F1-7s: AAT TGT AGC ATC AGA AGC TGG AG MLH3 R1-7s: AAA CTG GTT CCA CAC GGA TT	90	54°C, 1 minute
MLH3 F1-8s: TGC AAC AAC ATT ATG GGG AGT MLH3 R1-8s: TCG CCC ATA ACT AAA AAC ATT TC	100	60°C, 45 seconds
MLH3 F1-9as: TGT TCC ACT GGC TTT ATA ACT CA MLH3 R1-9as: TGG GAC CAG GTC TAA CAT AAT TT	97	54°C, 1 minute
MLH3 F1-9bs: CAT CAA AGA TTT AGC CAG CAC T MLH3 R1-9bs: GGT TCA TTC TCT AGC CCA TAA CTT	99	54°C, 45 seconds
MLH3 F1-10s: CAG CAA CTT ATA CAA TGT TTT CTG C MLH3 R1-10s: GGA AAG AGG GGG ATG TAT CA	90	54°C, 45 seconds
MLH3 F1-12s: CAG CAA GTT GTG CAG TGT GTT MLH3 R1-12s: GCA GAA TCT GAT GTT GGG ATG	99	55°C, 45 seconds
MLH3 F6s: TTC CTA CGA GAA GCA ACA GG MLH3 R6s: TTC CTC TGT CAC TGT TAT CTC TAG C	94	54°C, 50 seconds

in both directions. Primer sequences and PCR conditions are available on request.

Results

No germline mutations were detected in any of the 52 normal tissue samples analyzed. Multiple aberrant SSCP bands were discovered, and sequencing revealed several polymorphisms. Some of the aberrant SSCP bands are shown in Figure 1, and the corresponding sequences are shown in Figure 2. In exon 1 fragment 1 to 6, three samples showed aberrant bands. Genomic sequencing revealed a missense type of change 1258G→A (Val→Ile) (allele frequency, 2.9%). The same change was seen in three out of 56 cancer-free controls (allele frequency, 2.7%) confirming its benign nature. In exon 11 a silent change 4377G→A (Gln→Gln) was detected in 28 patients in heterozygous form and in 10 patients in homozygous form, giving an allele frequency of 46%. The same change was seen in 30 out of 57 and in 13 out of 57 cancer-free controls in heterozygous and homozygous forms, respectively (allele frequency, 49%). In the intron between exons 9 and 10 (exon 9 + 55) a heterozygous G→C change was found in three samples (allele frequency, 2.9%). When sequencing the PCR product of these three samples showing aberrant bands in SSCP, also a A→G change was discovered in the intron between exons 8 and 9 (exon 8 + 66). This change was not visible on SSCP, because other samples not showing aberrant bands were found to have it. Out of seven sequenced samples, five were heterozygous for this variant, and one was homozygous. In analysis of exons 3 and 7 some aberrant SSCP bands were seen (five in exon 3 and one in exon 7). Sequencing did not reveal any changes in the coding region or in the splice sites.

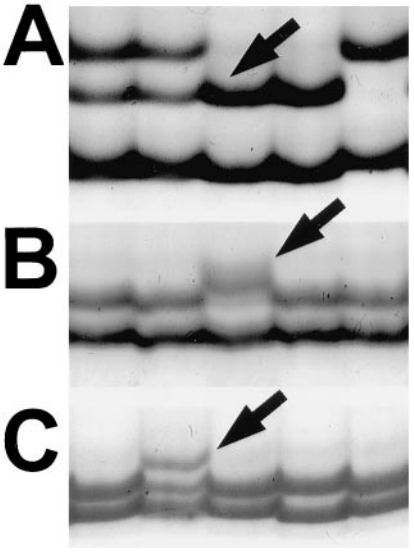

Figure 1. Some of the aberrant SSCP bands are shown here. **A:** *MLH3* exon 11, 4377G→A (Gln→Gln), **arrow** points at the mutant band. **B:** *MLH3* exon 1, fragment 1 to 6, 1258G→A (Val→Ile). **C:** MLH3 exon 8/9, a G→C in the intron between exons 8 and 9.

Somatic deletions were detected in eight out of 93 (8.6%) MSI-positive tumor samples. Seven patients showed a 1-bp deletion in the $(A)_9$ tract in exon 1 (fragment 1–8s, starting at nucleotide 1861). One patient showed a deletion of 1-bp in the $(A)_8$ tract in exon 1 (fragment 1–9bs, starting at nucleotide 2128). Deletions of 1 bp in these polyA tracts cause frameshifts and predicted premature stop at codons 609 and 679, respectively. None of these mutations were present in the corresponding normal DNA. One of these eight patients also has a germline *MLH1* mutation. An example sequence of a deletion in the $(A)_9$ tract is shown in Figure 3. During the sequencing of aberrant bands a 1234A→G (Lys→Glu) change was seen in one out of 13 samples in exon 1 (within fragment 1–6) (allele frequency 3.8%). The change was present both in the tumor as well as the normal DNA of the patient. The same change was seen in two out of 92 (allele frequency, 1.1%) cancer-free controls. Also the fact that the patient showing this change has a germline *MLH1* mutation favors the benign nature of this change.

Somatic deletions in the intronic *OBR* $(A)_8$ and $(A)_9$ repeats were seen in three out of 93 (3.2%), and in three out of 93 (3.2%) tumors, respectively. None of these mutations were present in the corresponding normal DNA.

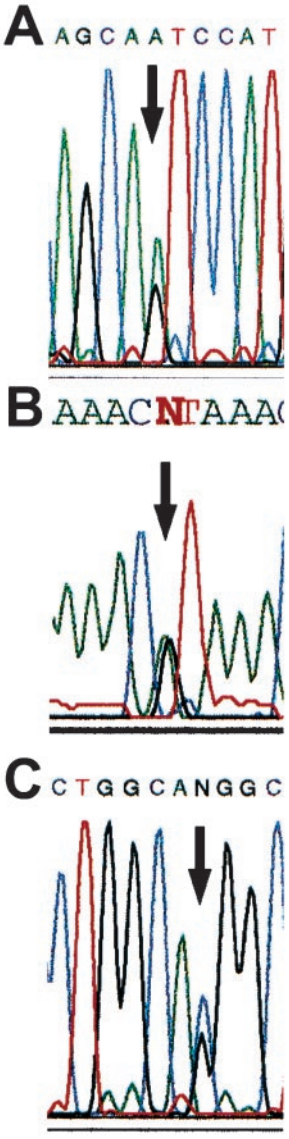

Figure 2. Direct sequencing results of the polymorphisms presented in Figure 1. **A:** *MLH3* exon 11, 4377G→A (Gln→Gln), **arrow** points at a heterozygous change. **B:** *MLH3* exon 1 fragment 1 to 6, 1258G→A (Val→Ile). **C:** MLH3 exon 8/9, a G→C in the intron between exons 8 and 9.

Discussion

Recently, a novel *mutL* homologue, *MLH3*, was cloned.[13] An association between *MLH3* and human DNA mismatch repair was proposed based on the following evidence: first, MLH3 is predicted to have an MLH1 interaction domain. Second, overexpressed dominant-negative MLH3 induces MSI in mammalian cell culture.[13] Third, MLH3 is predicted to be involved in mismatch repair in yeast.[13]

An interesting question is whether MLH3 has a role in MSI tumorigenesis *in vivo*. The predicted MLH1 interaction domain indicates that MLH3 might replace PMS2 in the mismatch repair complex. Very few germline mutations have been found in *PMS2*, and *Pms2*$^{-/-}$ mice do not display colon cancer susceptibility.[25] However, MLH3 does not show great similarity to PMS2, so it may have a different role in mismatch repair.

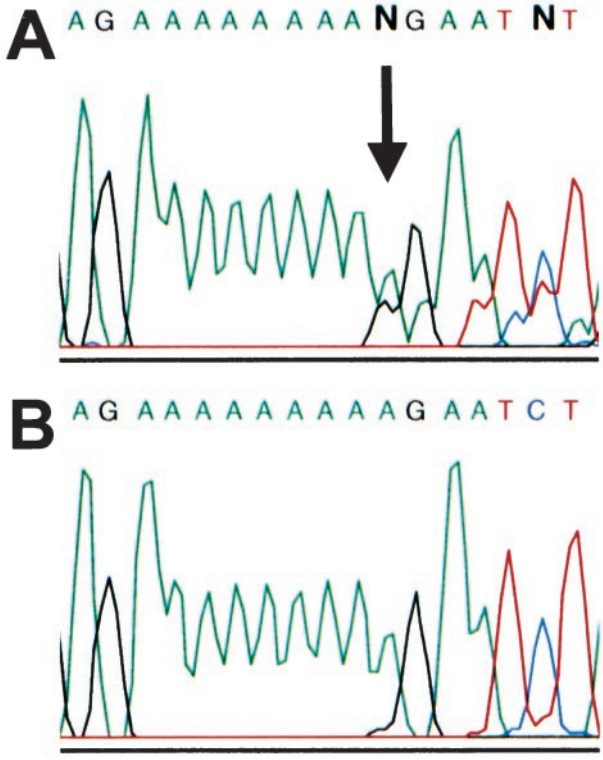

Figure 3. Direct sequencing results of a somatic deletion in the $(A)_9$ repeat in *MLH3* exon 1 (starting at nucleotide 1861). **A:** Tumor DNA displays a 1-bp deletion causing frameshift. The **arrow** depicts the deletion point. **B:** The corresponding normal DNA shows wild-type sequence.

To study whether *MLH3* is involved in MSI-positive colorectal tumorigenesis, we performed germline mutation analysis by SSCP on 46 samples from MSI-positive colorectal cancer patients with features of hereditary cancer. No deleterious mutations were found in any of the samples. Six MSI-negative familial colon cancer patients whose tumors had shown 14q deletions (data not shown) did not show germline mutations either.

MLH3 harbors eight polyadenine repeats in its coding sequence, ie, six discrete $(A)_6$ repeats, one $(A)_8$-repeat, and one $(A)_9$-repeat. These repeats provide a tool for evaluating the possible role of *MLH3* in somatic tumorigenesis. Analysis of 93 MSI-positive colorectal carcinomas revealed somatic deletions in eight (8.6%) patients in the mononucleotide repeats in the coding region of *MLH3*. For comparison, 90 out of these 93 MSI-positive samples had previously been analyzed for somatic *TGFβ RII* deletions and insertions, and 87% had shown deletions in the $(A)_{10}$ tract (data not shown).

Analysis of the intronic $(A)_8$ and $(A)_9$ repeats in the *OBR*-gene revealed somatic deletions in three out of 93 (3.2%), and in three out of 93 (3.2%) tumors, respectively. These intronic mutations most likely have no impact on tumor development, and are thus not selected for. Therefore they serve as an ideal tool for evaluating the background mutation rate in MSI tumors. The frequency of *MLH3* deletions (8.6%) is similar to the background mutation frequency in equal-sized repeats in the intron of the *OBR* gene (six out of 93, 6.5%). Our data thus suggest that the deletions observed in *MLH3* are merely a conse-

quence of the MSI phenotype rather than selected events driving tumor progression.

It seems that *MLH3* is not frequently involved in MSI-positive colorectal cancer as a hereditary or somatic component. Redundant function of MLH3 and PMS2 may explain the rarity of mutations, like in the case of MSH6 and MSH3.[10] The absence of frequent somatic *MLH3* changes even though the sequence provides abundant targets for MSI emphasizes the significance of the previous finding of somatic *MSH3* deletions in 39 to 57%[14,26] and *MSH6* deletions in 30%[14] of MSI-positive colorectal cancers. Also another mismatch repair gene, *MBD4*, has been shown to acquire somatic deletions in its $(A)_{10}$ tract in 25 to 40% of MSI-positive tumors.[17,18] In light of the *MLH3* results *MSH3*, *MSH6*, and *MBD4* indeed appear as important target genes of MSI. Presumably somatic deletions occur also in *MLH3*, but unlike *MSH3*, *MSH6*, and *MBD4* deletions, these are not selected for. This data supports the hypothesis that the effect of the putative MLH1/MLH3 complex to insertion/deletion loop repair is not significant.[10]

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