Characterization of the Mismatch Repair Defect in the Human Lymphoblastoid MT1 Cells

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Abstract

Mutations in repair (MMR) genes predispose to hereditary nonpolyposis colon cancer. Those leading to truncated proteins bring about a MMR defect, but phenotypes of missense mutations are harder to predict especially if they do not affect conserved residues. Several systems capable of predicting the phenotypes of MMR missense mutations were described. We deployed one of these to study the MMR defect in MT1 cells, which carry mutations in both alleles of the hMSH6 gene. In one, an A→T transversion brings about an Asp(1213)Val amino acid change in the highly conserved ATP binding site, whereas the other carries a G→A transition, which brings about a Val(1260)Ile change at a nonconserved site. The hMSH2/hMSH6 (hMutSα) heterodimers carrying these mutations were expressed in the baculovirus system and tested in in vitro MMR assays. As anticipated, the Asp(1213)Val mutation inactivated MMR by disabling the variant hMutSα from translocating along the DNA. In contrast, the recombinant Val(1260)Ile variant displayed wild-type activity. Interestingly, partial proteolytic analysis showed that this heterodimer was absent from MT1 extracts, although both hMSH6 alleles in MT1 cells could be shown to be transcribed with an efficiency similar to each other and to that seen in control cells. The MMR defect in MT1 cells is thus the compound result of one mutation that inactivates the ATPase function of hMutSα and a second mutation that apparently destabilizes the Val(1260)Ile hMSH6 protein in human cells in vivo. (Cancer Res 2005; 65(11): 4525-9)

Introduction

The human lymphoblastoid TK6 cells do not express methylguanine methyltransferase (MGMT) and thus do not remove the cytotoxic lesion O6-methylguanine from their genomic DNA (1). As a consequence, they are highly sensitive to killing by methylating agents of the S9 type, such as the cancer chemotherapeutics procarbazine, dacarbazine, streptozotocine, and temozolomide, agents of the SN1 type, such as the cancer chemotherapeutics that brings about a Val(1260)Ile change at a nonconserved site.

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Materials and Methods

The TK6 and MT1 cell lines (1) were a kind gift of William Thilly (Biological Engineering Division, Massachusetts Institute of Technology, Cambridge, MA). They were cultured as described previously (9). The HeLa cells were obtained from American Type Culture Collection (Molsheim, France) and the HK/MK cells were a kind gift of Minna Nyström-Lahti (University of Helsinki, Helsinki, Finland). The latter cells were grown in modified Eagle's medium supplemented with 10% FCS.

Site-directed mutagenesis and production of the MT1 hMutSα variants. The PCR-based site-directed mutagenesis approach was described previously (9). The primers used in the preparation of the hMSH6/V1260I allele were 5′CTGTGGCGGAAAGATAC3′ and 5′ATTCCTTAGGTTATGGCTAGGCAGCAATATTGTGGAG3′. The hMSH6/V1260I/Blue-script SK vector and the baculovirus vectors expressing hMSH6/D1213V or hMSH6/V1260I were obtained by a strategy identical to that described previously (9). The recombinant proteins were purified as described (9), and analyzed for homogeneity by SDS-PAGE (Fig. 1B).

Mismatch repair assays. Mismatch repair efficiency of the cell extracts was tested in vitro, using an assay described previously (7, 10). The M13mp2 heteroduplexes tested contained either a single G/G mispair or an insertion/deletion loop with two extrahelical bases, 0.2 μg of recombinant wild-type or variant hMutSαs were used in complementation experiments, whereas increasing amounts of the recombinant hMutSα variants were used to test their potential dominant-negative effect (Fig. 2).
Allele-specific oligonucleotide hybridizations. Total RNAs (1.25 μg each) from TK6, MT1, and HeLa cells (11) were used for reverse transcription-PCR (RT-PCR) with the hMSH6-specific primers 5’GTGTGCCAGCGTCAACACCAAATG3’ and 5’GCAATGCCCATATGTCCTAGGCG3’. The PCR products were slot-blotted onto a polyvinylidene difluoride (PVDF) membrane (Hybond-P, Amersham Life Science, Otelfingen, Switzerland) and hybridized with oligonucleotides hyb-A (5’GGTGCTTGATAGTAATGAGAAG3’) and hyb-T (5’GGTGCTTGATTGGTAGATGAGAAG3’), corresponding to the wild-type or mutant sequence at codon 1213, respectively (the mutation site is shown in bold). The filter was rinsed in 3× SSC and baked for 1 hour at 80°C. Prehybridization was done for 1 hour at 62°C in a buffer containing 0.5 mol/L sodium phosphate (pH 6.8), 10% SDS, 1 mmol/L EDTA (pH 8). The hybridizations were done overnight at 62°C, using the above buffer containing a 32P-labeled probe (0.38 pmol/mL). The membrane was washed only at room temperature for 15 minutes and once at 58°C for a further 15 minutes in 3× SSC, 0.1% SDS. It was then dried and autoradiographed.

Restriction fragment length polymorphism. The MT1 cDNA was prepared from 10 μg of total RNA in a reverse transcription mixture containing 5× reverse transcriptase buffer (Promega, Wallisellen, Switzerland), 0.5 mmol/L deoxynucleotide triphosphates, 200 units of Moloney murine leukemia virus reverse transcriptase (Promega), 1 unit/μL RNase inhibitor, and 5 μmol/L oligo(dT) in a total volume of 40 μL at 37°C for 60 minutes. The semiquantitative PCR was done as follows: 1 cycle at 94°C, 4 minutes; 25 cycles at 94°C, 25 seconds, 58°C, 30 seconds, 72°C, 2 minutes; and 1 cycle at 72°C, 7 minutes under nonsaturating conditions. The PCR was done using 0.5 μL of the reverse transcription reaction and the Taq DNA Polymerase kit (Qiagen, Basel, Switzerland) following manufacturer’s instructions. The reaction was done in a total volume of 50 μL. The batch of 20 reactions was washed with the PCR purification kit (Qiagen) and digested with Sphi (1 unit/μL of reaction, Boehringer Mannheim, Rotkreuz, Switzerland). The PCR reactions and the restriction digests were done in parallel for the C1 DV and C1 VI plasmids containing the hMSH6/D1213V and hMSH6/V1260I alleles, respectively. The samples were then loaded on a 1.5% agarose Tris-acetate EDTA (TAE) gel and the bands were quantified using the Kodak Imaging System.

Western blotting and partial proteolyses. Cytoplasmic (50 μg) and nuclear (30 μg) extracts were loaded on a 7.5% SDS-PAGE and electrophoretically transferred onto PVDF membranes. The blots were first blocked in TBST (0.1% Tween 20 in TBS) containing 5% nonfat dry milk for 1 hour at 37°C and then incubated with the respective primary monoclonal antibodies [anti-hMSH6, MAb 2D4 (7); anti-hMSH2, 0.65 μg/mL, NA26, Oncogene Research, Darmstadt, Germany; anti-hMLH1, 0.09 μg/mL, 13271A, Pharmingen, Basel, Switzerland; anti-α-tubulin, 0.03 μg/mL, N357, Amersham] for 1 hour at room temperature. After washing with TBST, the blots were incubated with horseradish peroxidase–conjugated sheep anti-mouse IgG (Jackson, West Grove, PA) for 1 hour at room temperature. After washing with TBST, the blots were incubated with horseradish peroxidase–conjugated sheep anti-mouse IgG (NXA 931, 1:5,000, Amersham) for 1 hour at room temperature. The protein-antibody complexes were detected by ECL (Amersham).

The partial proteolysis experiments were done as described (9). Where nuclear extracts (typically 20 μg) were used, 25 ng of V8 protease were added per micromolar of extract.

Results and Discussion

As shown in Fig. 1A, one of the hMSH6 mutations in MT1 cells affects a highly conserved aspartate residue (D1213) that was thought to be required for the coordination of a magnesium ion within the ATP binding site of hMSH6. This mutation indeed caused a MMR defect through inactivation of the translocating function of hMutSα (9). The phenotype of the V1260I mutation could not be predicted. This substitution is conservative and affects a nonconserved residue of the polypeptide. To understand the reason underlying the MMR defect of MT1 cells, we decided to coexpress both these hMSH6 mutants with the wild-type hMSH2 and to test the activity of the recombinant hMutSα variants in the in vitro MMR assay.

Following infection of SF9 cells with the recombinant baculovirus vectors, both heterodimers, hMSH2/hMSH6-D1213V (hMutSαDV) and hMSH2/hMSH6-V1260I (hMutSαVI), could be obtained in nearly homogeneous form in yields comparable with the wild-type hMutSα (Fig. 1B). As shown previously (9), addition of the wild-type factor to the MMR-deficient MT1 extracts restored their ability to correct a substrate carrying a G/G mispair, whereas addition of hMutSαDV failed to do so. In contrast, the hMutSαVI factor was fully MMR-proficient at the concentration tested (Fig. 2A). Similar results were obtained when the recombinant hMutSα variants were used to complement extracts of the human colon cancer cell line HCT15, which carries truncating mutations in both alleles of hMSH6 (data not shown). These results confirmed our expectation that the V→I amino acid change might not have an adverse effect on the biological activity of the hMSH6 variant.
To eliminate the possibility that the hMSH6 gene in MT1 cells harbored another, as yet unidentified mutation, we resequenced the entire genomic locus. As no new mutations were found, we considered the possibility that the hMutSoDV heterodimer might have an inhibitory effect on MMR. This is not the case, as addition of increasing amounts of the latter factor to the MMR-proficient TK6 extracts did not show a significant dominant-negative effect in vitro, except at concentrations significantly higher than those of the wild-type hMutSα present in these extracts. (With the help of Western blots, we estimated that 50 μg of TK6 cell extract used in the in vitro repair assay contain ~0.2 μg of hMutSα.) Furthermore, at these high concentrations, hMutSoVI displayed a similar inhibitory effect (Fig. 2B).

We next decided to test whether the MMR-deficient phenotype of MT1 cells could be explained by lower expression of the hMSH6 V1260I allele in vivo. In the first experiment, we designed oligonucleotide probes specific for the wild-type sequence at this

Figure 2. In vitro mismatch and insertion/deletion loop repair efficiency of TK6 and MT1 extracts. A, TK6 extracts (50 μg) repaired both a G/G mismatch and an insertion/deletion loop with two extrahelical bases (Δ2). Identical quantities of MT1 extracts were unable to repair a G/G mismatch. MMR could be restored in MT1 extracts by the addition of 0.2 μg of either recombinant wild-type hMutSα (+Sα) or the hMSH2/hMSH6 V1230I complex (+VI), but not by the hMSH2/hMSH6 D1213V complex (+DV). Efficiency of insertion/deletion loop repair, known to involve the hMSH2/hMSH3 heterodimer, was similar to that observed in TK6 extracts. (The amount of wild-type hMutSα present in 50 μg of TK6 extract was estimated by Western blots to be ~0.2 μg.) B, G/G MMR was done in the presence of increasing amounts of the recombinant D1213V or V1260I complexes (0.3, 0.6, 1, 1.5, 2, and 4 μg) to 50 μg of extract from the MMR-proficient cell line TK6. This experiment shows that the V1260I heterodimer does not have a dominant-negative effect as it inhibits the MMR assay only at a concentration 20× higher than that present in MMR-proficient cell extracts. At this high concentration, the V1260I variant was also inhibitory.

![Figure 2](image-url)

Figure 3. Analysis of mRNA expression from both hMSH6 alleles in MT1 cells. A, allele-specific oligonucleotide hybridizations with total cDNA from MT1 cells. The oligonucleotide probes hyb-A and hyb-T were designed to hybridize specifically to the wild-type hMSH6 or to the mutant hMSH6-D1213V cDNA, respectively. The experiment showed that the 32P-labeled 24-mer oligonucleotide hyb-A annealed specifically to the wild-type hMSH6 cDNA from HeLa or TK6 cells. It also hybridized with two independent cDNA preparations from MT1 cells, albeit with only ~50% efficiency of the control HeLa and TK6 cDNAs. In contrast, the 32P-labeled 24-mer oligonucleotide hyb-T failed to hybridize with HeLa or TK6 cDNA, but annealed to the two MT1 cDNA preparations with an efficiency similar to that seen with the hyb-A probe. The amounts of radioactivity in the hyb-A and hyb-T spots annealed to MT1 cDNA were similar, which indicates that both hMSH6 transcripts were present in the total mRNA isolated from MT1 cells in roughly equal amounts. B, RFLP in MT1 hMSH6 cDNA. The A→T transversion mutation in the hMSH6-V1260I allele generates a new SspI restriction site. Thus, a 2,615 bp PCR amplicon spanning the hMSH6 codon 1260 should produce two fragments, of 2,126 and 489 bp, in DNA amplified from the D1213V allele, whereas the V1260I amplicon should be cut twice, to produce fragments of 1,853, 489 and 273 bp. (Due to space limitations, only the upper part of the gel is shown). Lane 1, undigested PCR amplicon from the D1213V allele (the PCR template in this reaction was plasmid C1DV carrying the hMSH6 D1213V insert); lane 2, D1213V amplicon digested with SspI; lane 3, undigested amplicon from the V1260I allele (the PCR template in this reaction was a plasmid C1V1260I carrying the hMSH6 V1260I insert); lane 4, V1260I amplicon digested with SspI; lane 5 to 7, digestion with SspI of 2,615 bp PCR amplicons obtained with MT1 total cDNA as template after 5 (lane 7), 20 (lane 6), and 30 (lane 5) PCR cycles. M, molecular size marker. This panel shows that the two larger fragments were amplified in similar amounts, which indicated that the cDNAs from both hMSH6 alleles were present in the total MT1 cDNA in similar quantities. The figure shows a photograph of a 1% TAE agarose gel.

![Figure 3](image-url)
hMSH6-DV allele with SspI gave rise to two fragments, of 2,126 and 489 bp (Fig. 3B, lane 2). Digestion of the corresponding fragment (Fig. 3B, lane 3) amplified from the C6VI plasmid that carries the hMSH6-VI cDNA insert yielded three fragments, of 1,853, 489, and 273 bp (Fig. 3B, lane 4). RT-PCR amplification of total mRNA isolated from MT1 cells, using the same primers as in the above control reactions followed by digestion with SspI, gave rise to four fragments, of 2,126, 1,853, 489, and 273 bp. As the PCR reactions were done under nonsaturating conditions, and as the relative intensity of the two longer bands was similar after 5, 20, and 30 PCR cycles (Fig. 3B, lanes 7, 6, and 5, respectively), we concluded that both alleles were transcribed with similar efficiency. Although both the methods used here are only semiquantitative, they indicate that the transcript levels of the two hMSH6 alleles present in MT1 cells are similar.

As the MMR defect of MT1 cells is not linked to the lower expression of the hMSH6-V1260I allele, we decided to analyze the protein extracts of these cells. Unexpectedly, Western blot analysis of both cytoplasmic and nuclear MT1 extracts showed that the steady-state levels of hMSH6 in these cells were lower than those found in the MMR-proficient parental cell line TK6 (Fig. 4). The hMSH2 levels in MT1 were also slightly reduced compared with TK6, possibly due to the reduced stability of this protein in the absence of hMSH6. As both extracts contained similar amounts of hMLH1 or β-tubulin, this result suggested that the hMSH6 mutations present in MT1 destabilize the polypeptide. We decided to test this hypothesis by subjecting the recombinant hMutSoDV and hMutSoVI variants to partial proteolysis with V8 protease. Interestingly, the hMutSoDV and the hMutSoVI variants produced different sets of fragments following partial digestion with V8, especially in the presence of ATP and magnesium (Fig. 4B; ref. 9). In particular, the wild-type and the hMutSoVI factors yielded a ~50 kDa fragment, which was absent from the hMutSoDV partial digests. We decided to make use of this finding in the analysis of the extracts of TK6 and MT1 cells. As shown in Fig. 4B, the partial proteolysis pattern of hMSH6 present in extracts of the MMR-proficient TK6 line was similar to that seen in the purified recombinant wild-type factor or in hMutSoVI. In contrast, hMSH6 present in MT1 extracts yielded no ~50 kDa fragment, which indicated that only the hMutSoDV factor was present in these extracts. These results lead us to conclude that the MMR defect of MT1 cells is brought about by decreased stability of the MMR-proficient hMutSoVI factor, such that the only mismatch binding heterodimer present in these cells is hMutSoDV, which has a defective ATPase. We were unable to establish whether the instability of the hMutSoVI factor was due to its degradation in the proteasome. Treatment of MT1 cells with the proteasome inhibitor MG132 resulted in cell death within 24 hours, even at 100 nmol/L concentration of the drug. No increase in the amount of hMSH6 relative to hMLH1 was noted in Western blots of the extracts made 16 hours posttreatment (data not shown); however, it must be pointed out that the steady-state levels of all MMR proteins could be seen to decline in these cells, most likely because of their growth arrest as noted previously (12).

The combination of a mutation in one hMSH6 allele that cripples the ATPase activity of its polypeptide product and a mutation in the second allele that sensitizes the variant protein to degradation fully explains the MMR defect in the MT1 cell line. However, the sensitivity of the V1260I hMSH6 recombinant protein to proteolysis was not apparent in the *baculovirus* system nor was it degraded upon addition to the TK6 or MT1 cell extracts during the *in vitro* MMR assays (Fig. 2). This suggests that the instability of the V1260I hMSH6 mutant might be linked to aberrant protein folding in human *cells in vivo*.

With the possible exception of the system described by Trojan et al. (13), the underlying cause of the MMR defect in MT1 cells would have most likely escaped detection in assays developed specifically to study the phenotypic effects of missense mutations in *MMR* genes (14–20). This present work thus serves as a cautionary tale for the phenotypic analysis of hereditary nonpolyposis colorectal carcinoma missense mutations and emphasizes the importance of studying MMR protein variants that fail to give unambiguous results in currently available functional assays also by alternative experimental techniques such as those described above.

**Addendum**

As mentioned in "Results and Discussion," sequence analysis of the hMSH6 gene in MT1 cells confirmed the presence of the two previously-described mutations (3), which bring about changes in...
amino acid residues 1213 and 1260 that were the subject of this study. However, when we deployed newly-available primer sets to re-sequence the G+C rich exon 1 in both forward and reverse directions, we identified an insertion mutation at nucleotide position 114 (c.114dupC). The frameshifted hMSH6 open reading frame comes to a stop codon several amino acid residues downstream. The insertion must be in the allele carrying also the Val1260Ile missense mutation, as no protein encoded by this allele was detected in MT1 cell extracts (Fig. 4). The MMR-deficient phenotype of MT1 cells is thus caused by an A→T transversion mutation in one allele of hMSH6 that brings about the ATPase-inactivating D1213V amino acid change, and by an insertion of a cytidine in the second allele, which results in a premature termination of translation of the polypeptide.

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