

# A Human Cell-Based Assay to Evaluate the Effects of Alterations in the *MLH1* Mismatch Repair Gene

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## Abstract

**We describe a new approach to investigate alterations in the human *MLH1* mismatch repair (MMR) gene. This is based on complementation of the phenotype of a *MLH1*-defective subclone of the ovarian carcinoma A2780 cells by transfection of vectors encoding altered *MLH1* proteins. Measurements of resistance (tolerance) to methylating agents, mutation rate at *HPRT*, microsatellite instability (MSI), and steady-state levels of DNA 8-oxoguanine were used to define the MMR status of transfected clones. The approach was validated by transfecting cDNA of wild-type (WT) *MLH1*, cDNAs bearing two previously identified polymorphisms (I219V and I219L) and two with confirmed hereditary nonpolyposis colorectal cancer (HNPCC) syndrome mutations (G224D and G67R). A low-level expression of two *MLH1* polymorphisms partially reversed methylation tolerance and the mutator phenotype, including MSI. Higher levels of I219V resulted in full restoration of these properties to WT. Increased expression of I219L did not fully complement the *MLH1* defect, because there was a simultaneous escalation in the level of oxidative DNA damage. The findings confirmed the important relationship between deficient MMR and increased levels of oxidative DNA damage. Mutations from Italian HNPCC families (G224D, G67R, N635S, and K618A) were all ineffective at reversing the phenotype of the *MLH1*-defective A2780 cells. One (K618A) was identified as a low penetrance mutation based on clinical and genetic observations. (Cancer Res 2006; 66(18): 9036-44)**

## Introduction

Colorectal cancer remains one of the most common fatal cancers in developed countries in which it represents a significant public health problem. One approach to reducing colorectal cancer mortality is to identify individuals predisposed to the disease and to target preventive measures to this group. Familial aggregation of colorectal, uterine, and other cancers constitutes the autosomally dominant hereditary nonpolyposis colorectal cancer (HNPCC) syndrome. HNPCC is associated with defective DNA mismatch repair (MMR) that is a consequence of germ-line mutations in the *MLH1*, *MSH2*, or, occasionally, *MSH6* or *PMS2* genes. The majority of HNPCC patients are heterozygous for a mutated MMR gene, and

MMR is functional in normal somatic cells. Cancer development is thought to be the consequence of inactivation or loss of the wild-type (WT) allele.

MMR reverses replication errors that escape proofreading by replicative DNA polymerases. In MMR-defective cells, both base-to-base mismatches and insertion/deletion loops, which are precursors of missense and frameshift mutations, are left uncorrected. This results in increased spontaneous somatic mutation rates in both expressed and nonexpressed sequences. This mutator effect is particularly marked in nonexpressed sequences comprising multiple simple repeats (microsatellites), and the characteristic microsatellite instability (MSI) is diagnostic for MMR-defective tumors (1, 2).

MMR is also involved in the processing of *O*<sup>6</sup>-methylguanine (*O*<sup>6</sup>-meGua) and 6-methylthioguanine (me6-TG), DNA adducts induced by methylating agents and thiopurines, respectively (3). *O*<sup>6</sup>-meGua- or me6-TG-containing base pairs are recognized by MutS $\alpha$ , the MSH2/MSH6 heterodimer that comprises the major MMR recognition complex, and subsequently processed by MMR. This processing does not result in lesion repair, however. Instead, it causes cell death and MMR-defective cells are characteristically extremely resistant to killing by methylating agents, such as *N*-methyl-*N*-nitrosourea (MNU) and thiopurines. Although the precise connection between MMR processing of damaged DNA bases and cell death is not completely understood, this methylation tolerance phenotype has been consistently identified in cells with *MSH2*, *MLH1*, *MSH6*, or *PMS2* defects. *MSH3* is the single exception.

Identifying HNPCC families and defining the role of MMR gene mutations in colorectal cancer predisposition is not always easy. There are several reasons for this, including deficiencies in family information, lack of pathologic features, and incomplete gene penetrance. There is, therefore, a particular need for assays that can define the functional significance of MMR gene variants that have been identified in genetic screens. A few such assays have been established and used to examine HNPCC-related *MLH1* mutations and polymorphisms. The first of these is the "dominant-negative" mutator test in *Saccharomyces cerevisiae* in which expression of a WT, but not of a mutant, human *MLH1* cDNA induces a mutator phenotype (4). In a similar approach, analysis of the mutator phenotype conferred either by *MLH1* overexpression or by homozygous or heterozygous *mlh1* mutations was investigated in yeast (5). The second is a biochemical assay that measures the degree of interaction between *MLH1* and *PMS2* proteins in MMR-defective strains complemented with plasmids expressing *MLH1* cDNAs (6–10). The activities of *MLH1* variants have also been examined by looking at MMR correction using recombinant proteins or following transient transfection of *MLH1* cDNA into 293T human embryonic kidney cells (11–13).

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doi:10.1158/0008-5472.CAN-06-1896

Here, we describe a new approach to determine the significance of alterations in *MLH1* that are identified by genetic screens in HNPCC families. It has been validated with a WT *MLH1* sequence, with *MLH1* mutations described previously in Italian families, and with two known polymorphic variants. The assays have also been used to identify a previously unassigned variant as an inactivating *MLH1* mutation. The approach involves stable expression of the altered cDNA in a *MLH1*-defective clone of A2780 human ovarian carcinoma cells that contains an epigenetically silenced *MLH1* gene (14). These A2780MNU-clone1 cells manifest the pronounced mutator phenotype and extensive tolerance to methylation damage characteristic of MMR-defective cells (15). Stable transfection of WT *MLH1* cDNA or cDNAs containing polymorphic variants reverts both these phenotypes, whereas mutant *MLH1* cDNAs do not. In addition to classifying *MLH1* changes, this new assay permits a quantitative evaluation of the consequences of the expression of variant *MLH1* proteins.

## Materials and Methods

**Molecular characterization of HNPCC families and controls.** The *MLH1* mutations of this study were identified in Italian families enrolled in the Registry of Hereditary Colorectal Cancer at the Istituto Nazionale Tumori. Screening of *MLH1* was done on genomic DNA purified from peripheral blood leukocytes by PCR amplification of all exons followed by either single-strand conformation polymorphism (16) or denaturing high-performance liquid chromatography (DHPLC; ref. 17). To characterize nucleotide alterations, anomalous PCR fragments were sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and examined on ABI PRISM 3100 DNA Sequencer (Applied Biosystems) using the Sequencing Analysis software. The occurrence of the identified mutations in the DNA of probands' relatives was assessed by direct sequencing.

MSI in colorectal cancer DNA was investigated using the five-marker panel recommended by international guidelines (18). Tumor and matched normal DNAs were amplified by PCR using fluorescent primers followed by gel electrophoresis on the 377 DNA Sequencer (Applied Biosystems) and fragments were analyzed using GeneScan and Genotyper softwares (19). A sample was considered MSI positive when at least two microsatellites showed altered electrophoretic pattern in tumor compared with normal DNA. DHPLC was also applied to screen for selected *MLH1* variants in healthy controls. These were blood donors of both sexes, Italian ancestry, >50 years old, and with no personal history of cancer.

**Plasmid and cloning procedure.** Missense mutations were introduced by site-directed mutagenesis using the Stratagene QuickChange kit (La Jolla, CA), into the vector pCMV-Bam-Neo, which contains a full-length WT *MLH1* cDNA. PCRs were carried out using primers (0.1  $\mu\text{mol/L}$ ) containing the desired mutation, 2.5 units Taq DNA polymerase, and 3 to 15 ng plasmid DNA. The 1.9-kb *Bst*XI fragments containing the desired mutation were subcloned in a new copy of the vector to avoid the risk of random mutations introduced by PCR. Digestions with *Eco*RI were used to verify the correctness of insertions and all the constructs were verified by direct DNA sequencing.

**Cell cultures, DNA transfection, and Western blotting.** Cells were grown in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS, 100 units/mL penicillin, and 100  $\mu\text{g/mL}$  streptomycin (Life Technologies) at 37°C in a 5% CO<sub>2</sub> atmosphere. A2780MNU-clone1 cells were transfected (LipofectAMINE; Life Technologies) with pCMV-Bam-Neo vectors containing WT and/or mutated *MLH1* cDNA and selected for G418 (100  $\mu\text{g/mL}$ ) resistance (Life Technologies). Individual clones were isolated 15 to 20 days later. *MLH1* expression was monitored by Western blotting. Extracts of mammalian cells were separated on 8.5% SDS polyacrylamide gels, transferred to nitrocellulose membrane using a Trans-Blot cell apparatus (Bio-Rad, Hercules, CA), and probed overnight with anti-*MLH1* (BD PharMingen, San Diego, CA) antibody followed by the appropriate secondary antibody. Blots were developed using the enhanced chemilumi-

nescence detection reagents (GE Healthcare, Chalfont St. Giles, United Kingdom). The antibodies against PMS2 and proliferating cell nuclear antigen (PCNA) were obtained from BD PharMingen. Phenotypic characterization of *hMLH1*-expressing clones was done shortly following transfection (5-10 passages).

**MNU survival.** To measure cell survival, 100 cells were pretreated for 2 hours with *O*<sup>6</sup>-benzylguanine (Sigma, St. Louis, MO) in complete medium and then exposed to MNU (Sigma) in PBS and 20 mmol/L HEPES (pH 7.4) for 30 minutes at 37°C. After 1 week, surviving colonies were fixed with methanol, stained with Giemsa, and counted.

**Mutation rate analysis at the *HPRT* gene.** Cells were plated at low density (100 per dish) and grown in complete medium to a density of  $0.4 \times 10^6$  to  $1 \times 10^6$  per dish before plating the entire culture (50-60 independent cultures) into medium supplemented with 6-TG (5  $\mu\text{g/mL}$ ; Sigma). The mutation rate was calculated as  $\mu = MC^{-1} \ln 2$ , where  $C$  is the number of cells at selection time and  $M$  is  $-\ln(P_0)$ , where  $P_0$  is the proportion of cultures with no mutants.

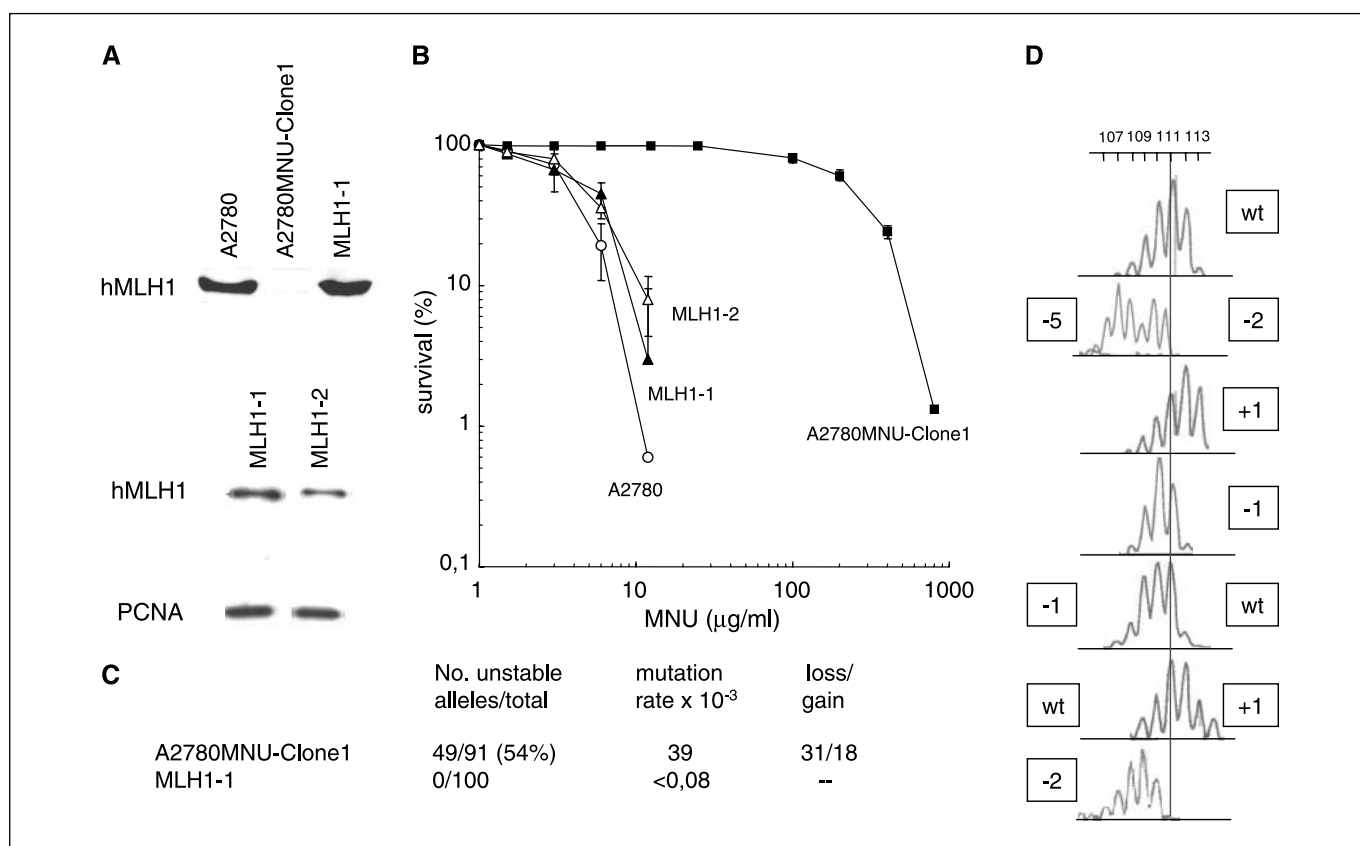
**Microsatellite instability.** Genomic DNA was isolated from subclones of A2780-derived cell lines. Ninety-six well plates were seeded at a density of <1 cell per well and DNA was prepared from  $\sim 2 \times 10^4$  cells per well. DNA samples (10 ng) were used in PCRs using primers for *BAT26* (2 pmol/ $\mu\text{L}$ ) and deoxynucleotide triphosphates (200 mmol/L) in a reaction buffer containing 0.5 units Taq polymerase (Applied Biosystems). Amplification products (10  $\mu\text{L}$ ) were digested with 0.4 units T4 DNA polymerase (Roche, Indianapolis, IN) for 30 minutes at 37°C, denatured in deionized formamide for 2 minutes at 95°C, and analyzed with the ABI PRISM 310 automatic sequencer by GeneScan.

**8-Oxoguanine determinations.** 8-Oxoguanine (8-oxoG) was measured by HPLC with electrochemical detection (HPLC/EC) as described previously following DNA extraction, RNase treatment, and enzymatic hydrolysis (20). Briefly, DNA was resuspended in Tris-EDTA, incubated with RNases A and T1 at 37°C for 1 hour, and precipitated with ethanol. Samples were digested at 37°C with nuclease P1 (Roche; 2 hours) and alkaline phosphatase (Roche; 1 hour). Enzymes were precipitated by CHCl<sub>3</sub> addition and the upper layer was stored for analysis of 8-oxoG at -80°C under N<sub>2</sub>. The DNA was analyzed by HPLC/EC (Coulochem I, ESA, Inc., Chelmsford, MA) using a C18 250  $\times$  46 mm, 5  $\mu\text{m}$  Uptisphere column (Interchim, Montlucion, France) with a C18 guard column. The eluent was 50 mmol/L ammonium acetate (pH 5.5) containing 9% methanol at a flow rate of 0.7 mL/min. The potentials applied were 150 and 400 mV for E1 and E2, respectively. The retention time of 8-oxoG was  $\sim 23$  minutes. Deoxyguanosine was measured in the same run of corresponding 8-oxoG with a UV detector (model SPD-2A, Shimadzu, Milano, Italy) at 256 nm; the retention time was  $\sim 17$  minutes.

## Results

**Transfection of WT *MLH1* cDNA into *MLH1*-deficient A2780 cells.** A2780MNU-clone1 was isolated following treatment of A2780 ovarian carcinoma cells with MNU and displays the extreme MNU resistance and tolerance to 6-TG associated with defective MMR (14). A2780MNU-clone1 cells lack detectable expression of *MLH1* (Fig. 1A) and cell extracts are defective in mismatch correction (14). *MLH1* expression in A2780MNU-clone1 is abrogated by cytosine methylation and can be partially reactivated by azadeoxycytidine treatment, and expression of a transfected WT *MLH1* cDNA restores full MMR capacity to A2780MNU-clone1 (14). A2780MNU-clone1 was transfected with a WT *MLH1* cDNA and six independent transfectants were isolated. Western blotting indicated that five of six contained *MLH1* levels comparable with those of WT A2780 cells (an example is shown in Fig. 1A). In one case (*MLH1*-2), expression was closer to 50% of the normal level (Fig. 1A). *MLH1* cDNA expression did not alter the growth rate or the cloning efficiency and was stable even in the absence of G418 selection.

A2780MNU-clone1 is 100-fold more resistant to MNU than A2780 (Fig. 1B). WT levels of *MLH1* in *MLH1*-1 cells almost completely



**Figure 1.** Survival of A2780, the MLH1-defective A2780MNU-clone1, MLH1-1, and MLH1-2 cells following exposure to increasing concentrations of MNU. *A*, top, levels of MLH1 in extracts (20  $\mu\text{g}$ ) of A2780, A2780MNU-clone1, and MLH1-1; bottom, levels of MLH1 in extracts (20  $\mu\text{g}$ ) of MLH1-1 and MLH1-2. *B*, survival of A2780MNU-clone1 ( $\blacksquare$ ), A2780 ( $\circ$ ), MLH1-1 ( $\blacktriangle$ ), and MLH1-2 ( $\triangle$ ) cells after treatment with increasing MNU concentrations in the presence of *O*<sup>6</sup>-benzylguanine. *C*, MSI at the *BAT26* locus in A2780MNU-clone1 and MMR-proficient MLH1-1 cells were calculated as indicated in Materials and Methods. Number of unstable alleles, mutation rates, and loss/gain frequency. *D*, examples of MSI at *BAT26* in A2780MNU-clone1. Numbers, size of the alleles. Alterations are shown next to the appropriate panel.

reversed this phenotype and their MNU sensitivity was comparable with that of A2780 (Fig. 1*B*). Similar results were obtained with the other clones expressing normal levels of MLH1 (data not shown). The survival of MLH1-2 after MNU treatment is similar to that of MLH1-1 (Fig. 1*B*), indicating that the 2-fold difference in MLH1 expression between these clones was not reflected in a differential MNU sensitivity. Thus, restoration of WT MLH1 reverses the methylation tolerance of A2780MNU-clone1 and half of the normal level of MLH1 is sufficient to restore full MNU sensitivity.

Because MLH1-1 was typical of the clones expressing a transfected WT MLH1, it was used to investigate the effect of MLH1 restoration on the mutator phenotype. The mutation rate at the *BAT26* mononucleotide repeat in MLH1-1 was  $<0.08 \times 10^{-3}$  per cell per generation and we observed no changes in *BAT26* allele length among 50 subclones. In contrast, the mutation rate at this locus in MLH1-defective A2780MNU-clone1 cells was  $39 \times 10^{-3}$  per cell per generation (Fig. 1*C*). Among the mutations, there was a predominance of single nucleotide frameshifts, with an excess of losses over gains (Fig. 1*D*). Thus, the WT level of MLH1 in MLH1-1 reversed the mutator effect at *BAT26* by a factor of  $\sim 500$ -fold.

The reduced *BAT26* instability in MLH1-1 was paralleled by significant changes in mutation rates at other loci. Reexpression of MLH1 produced a 40-fold reduction in the spontaneous mutation rate at *HPRT*, which declined from a mean of  $1.8 \times 10^{-6}$  in A2780MNU-clone1 to  $4.5 \pm 2.0 \times 10^{-8}$  in MLH1-1 (Fig. 2*A* and *B*).

The latter value is similar to the spontaneous *HPRT* mutation rate in A2780 cells ( $1.7 \pm 1.2 \times 10^{-8}$ ). Consistent with its ability to reverse methylation tolerance, 50% of normal MLH1 in MLH1-2 was also sufficient to reverse the mutator phenotype, and the spontaneous mutation rate in MLH1-2 cells was reduced to  $4.8 \pm 2.1 \times 10^{-8}$ , a value closely similar to that of MLH1-1 (Fig. 2*B*).

Thus, a full or half complement of transfected WT MLH1 efficiently reverses the methylation tolerance and genome instability of A2780MNU-clone1.

**MLH1 cDNA containing missense mutations.** We analyzed four missense mutations (199G>A, 731G>A, 1852AA>GC, and 1904A>G) that have been identified in Italian HNPCC families (Table 1). The pathologic significance of these changes is not immediately evident, although none has been observed in >200 healthy Italian controls. A causative role in familial colorectal cancer was indirectly suggested by their association with tumor MSI, which was detected in all mutation carriers analyzed, with the exception of 1904A>G that could not be tested for lack of tumor samples.

Each of the mutations was introduced by site-directed mutagenesis of the WT *MLH1* cDNA and transfected into A2780MNU-clone1. The transfection efficiencies of the mutant constructs were comparable with that of the WT, although Western blotting of several clonal isolates of transfectants indicated large variations in the amount of MLH1 expressed (data not shown).

For each mutant cDNA, clones containing the highest level of MLH1 were analyzed further. In two cases (199G>A and 1904A>G), the steady-state level of mutated MLH1 was significantly lower than WT (Fig. 3A) and this was accompanied by reduced levels of PMS2, consistent with the known instability of the PMS2 protein in the absence of its partner (21–23). The clone expressing the 1852AA>GC mutation had normal levels of both MLH1 and PMS2, indicating that the mutated MLH1 was expressed at normal levels, was stable, and could interact normally with PMS2.

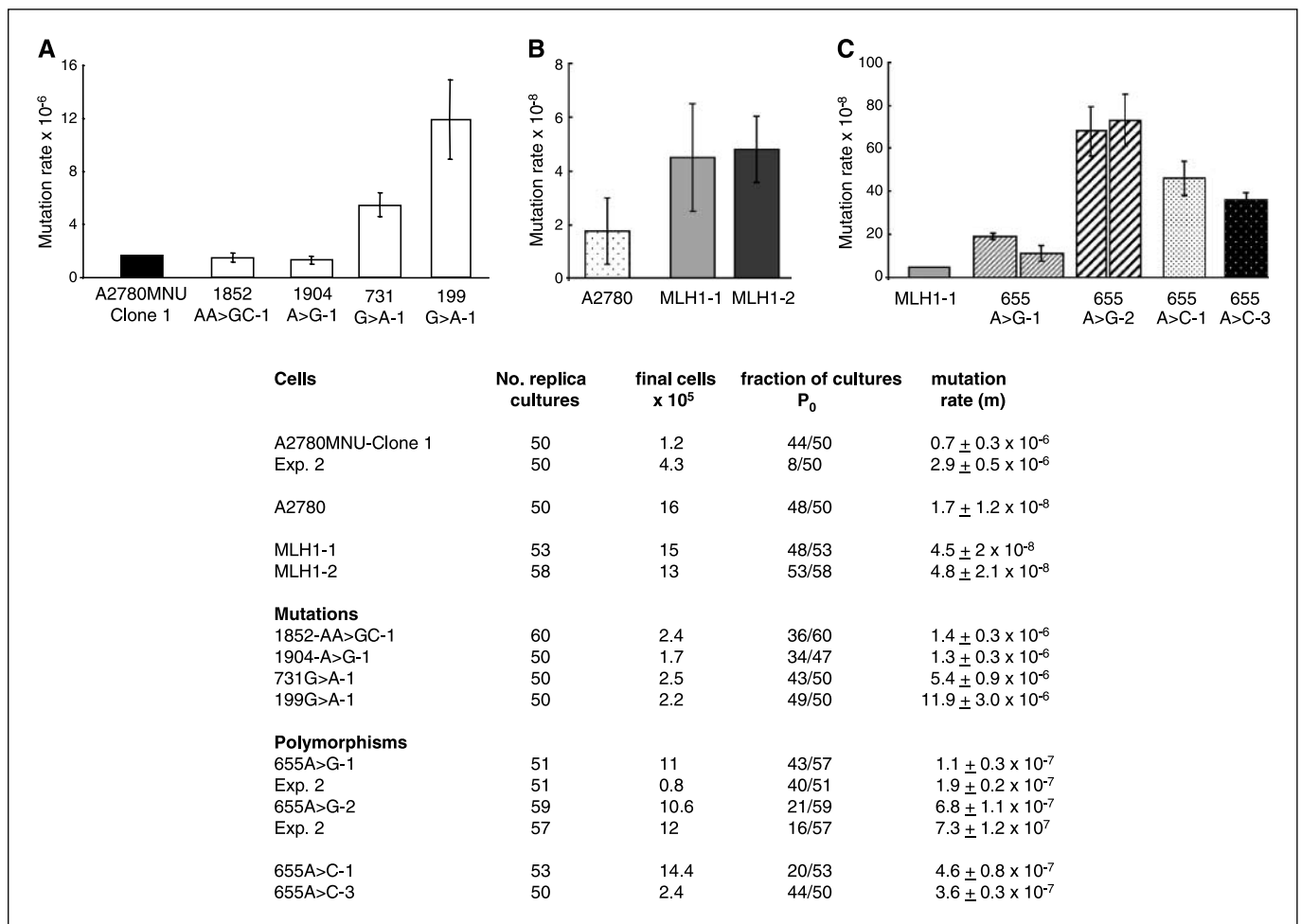
None of the mutant MLH1 proteins had a detectable effect on the phenotype of A2780MNU-clone1. All four mutated proteins were associated with the same degree of MNU tolerance (Fig. 3B) irrespective of their level of expression. Similarly, the mutator phenotype of the recipient cells was unaffected by any of the four mutant MLH1 proteins. *HPRT* mutation rates in clones expressing each of the mutated MLH1 proteins were comparable with (or even higher in 199G>A) that of A2780MNU-clone1 (Fig. 2A).

Thus, A2780MNU-clone1 cells used as recipients for transfected variants of MLH1 provide a new assay capable to identify mutated forms of this protein because of their inability to reverse the methylation tolerance of MMR-defective cells.

**Expression of MLH1 polymorphisms.** The effect of two reported MLH1 polymorphisms (24, 25) on the phenotype of A2780MNU-clone1 was also examined. Expression of 655A>G was detectable in 2 (of 10) transfected clones, which were designated 655A>G-1 and 655A>G-2. Their steady-state levels of MLH1 were 80% and 20% of WT, respectively (Fig. 4B). The levels of the PMS2 protein, like those of the respective MLH1 proteins, were high in 655A>G-1 cells and low in 655A>G-2.

The high-level expression of MLH1 in 655A>G-1 cells was associated with full reversion of methylation tolerance (Fig. 4A). In addition, the spontaneous *HPRT* mutation rate in 655A>G-1 was reduced 12-fold to  $1.5 \times 10^{-7}$  (Fig. 2C). Both of these findings indicate that an almost normal level of a transfected 655A>G-1 MLH1 allele confers restoration of functional MMR.

In contrast to the effects of high-level expression of the 655A>G polymorphism, a low level of the same polymorphic MLH1 had a modest effect. There was a significant decrease in the extent of methylation tolerance in 655A>G-2 and the MNU resistance relative to MLH1-1 (in which MMR is fully corrected by expression of WT MLH1) was reduced 15-fold (Fig. 4A). The mutation rate at *BAT26* was 10-fold lower in 655A>G-2 than in MLH1-clone1 ( $4.1 \times 10^{-3}$



**Figure 2.** Spontaneous mutation rates at the *HPRT* gene in untransfected and *MLH1*-transfected A2780 clones. **A**, *HPRT* mutation rates in A2780MNU-clone1 (black column). Mutation rates of 1852AA>GC-1, 1904A>G-1, 731G>A-1, and 199G>A-1 (white columns). Columns, mean of two experiments; bars, SD. **B**, spontaneous mutation rates at the *HPRT* locus in A2780 (dotted white column), MLH1-1 (dotted light gray column), and MLH1-2 (dotted dark gray column). **C**, *HPRT* mutation rate in 655A>G-1 (fine-patterned column), 655A>G-2 (coarse-patterned columns), 655A>C-1 (dotted white column), and 655A>C-3 (dotted black column). Number of cultures, final number of cells at time of selection, fraction of cultures with no mutants, and mutation rates at the *HPRT* gene in untransfected and MLH1-expressing A2780MNU-clone1 clones. The mutation rate was calculated as  $\mu = MC^{-1} \ln 2$  as indicated in Materials and Methods.

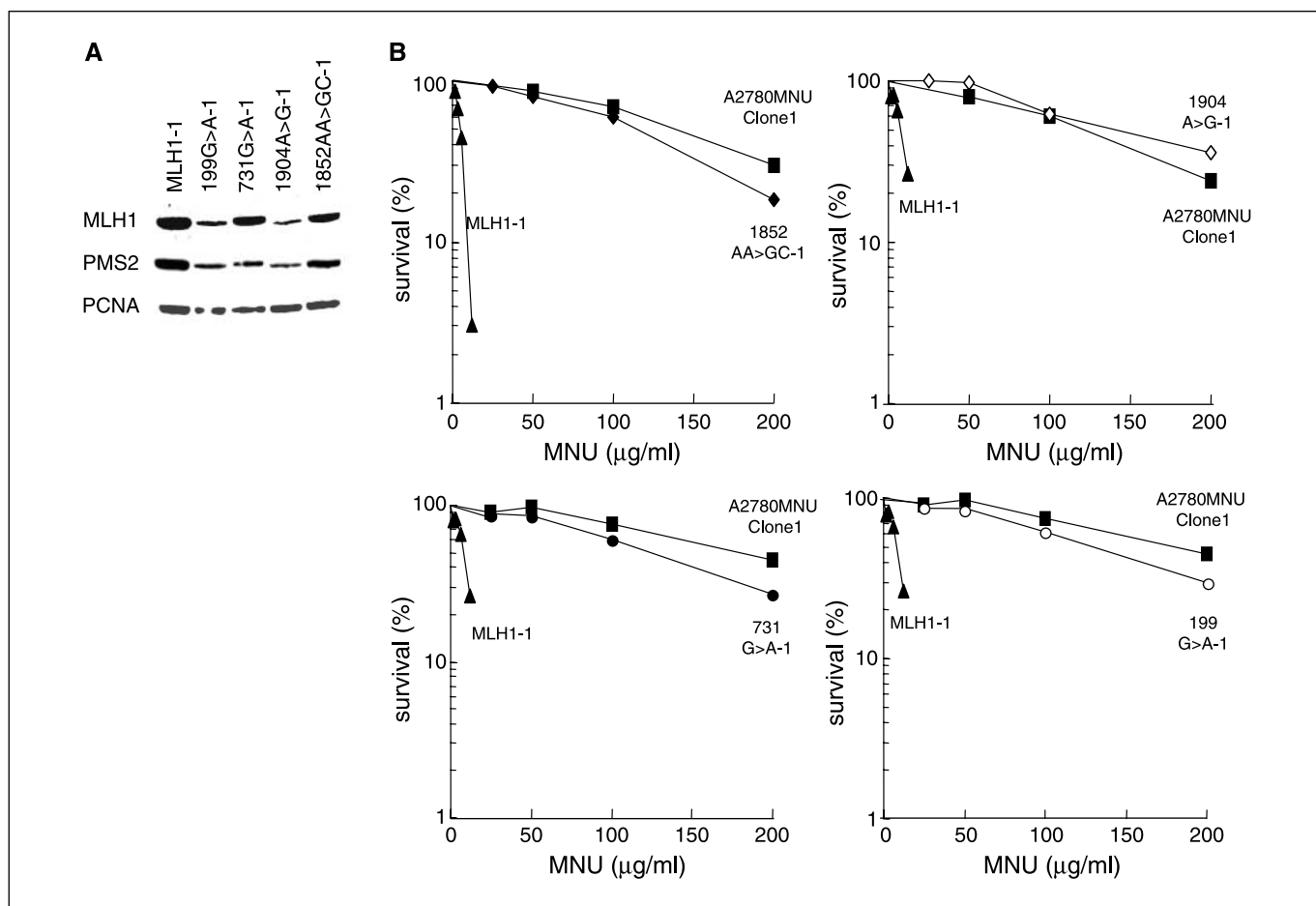
**Table 1.** Characterization of *hMLH1* mutations in colon cancer patients

Mutation	Exon	Codon	DNA change	Protein change	No. families	Frequency in healthy controls	Tumor phenotype	
							Colorectal cancer tested	MSI
199G>A G67R	2	67	GGG>AGG	Gly-to-Arg	1	0/220	1	MSI-H
731G>A G244D	9	244	GGT>GAT	Gly-to-Asp	3	0/220	2	MSI-H
1852-3AA>GC K618A	16	618	AAG>GCG	Lys-to-Ala	2	0/220	1	MSI-H
1904A>G N635S	17	635	AAC>AGC	Asn-to-Ser	1	0/220	0	NI
Polymorphism								
655A>G I219V	8	219	ATC>GTC	Ile-to-Val				
655A>C I219L	8	219	ATC>CTC	Ile-to-Leu				

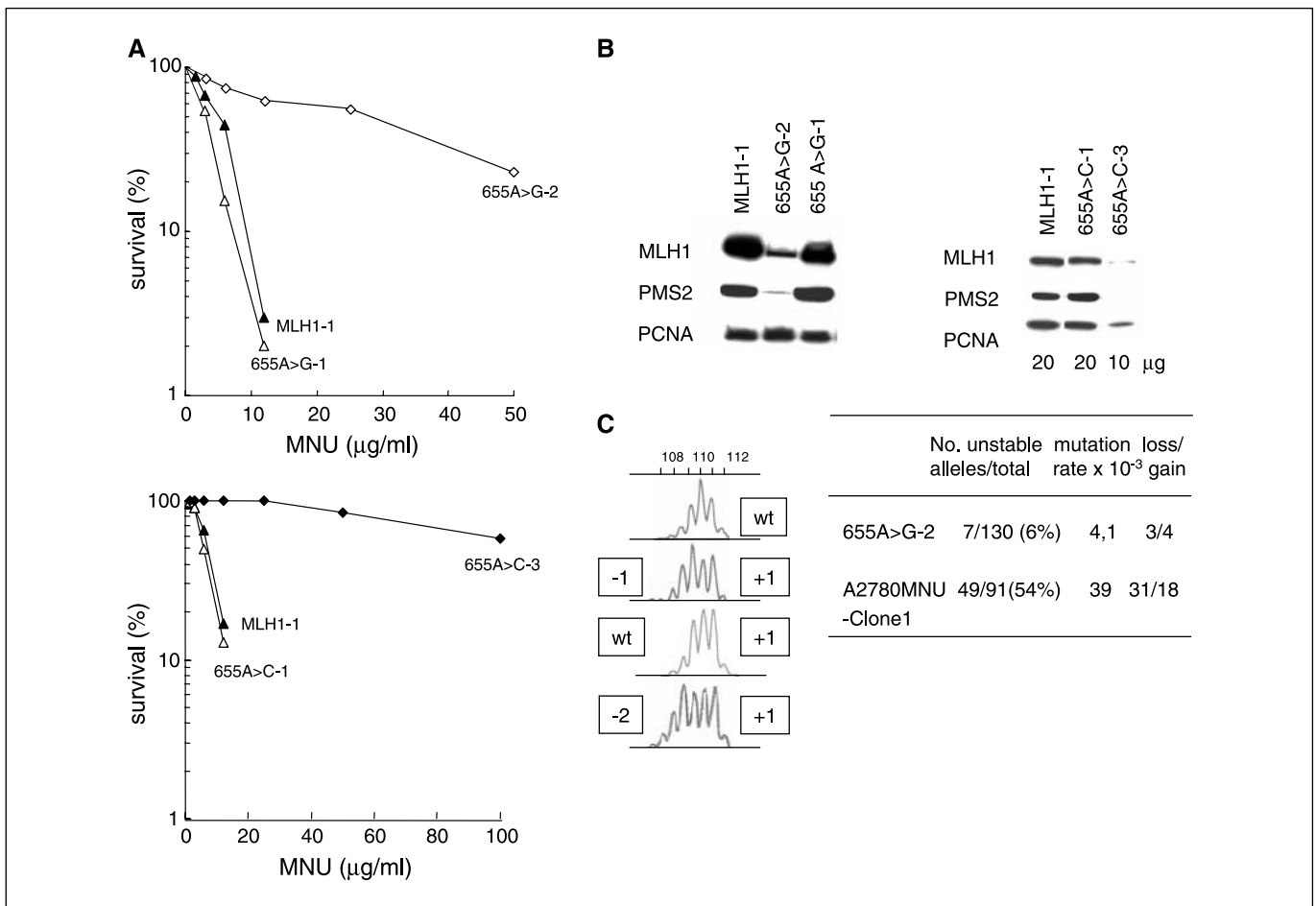
NOTE: NI, no information available.

versus  $39 \times 10^{-3}$  per cell per generation). Nevertheless, it remained 50-fold higher than that of MMR-proficient MLH1-1 (Figs. 2A and 4C). In accordance with this moderate effect on the mutator phenotype, the spontaneous *HPRT* mutation rate was also 15-fold higher in 655A>G-2 than in MLH1-1 cells (a mean of  $7 \times 10^{-7}$  versus  $4.5 \times 10^{-8}$  per cell per generation, respectively; Fig. 2C).

We conclude that, when expressed at high level, the 655A>G MLH1 polymorphism behaves like WT and reverts the methylation tolerance and mutator phenotype associated with defective MMR. At 20% of expression, the same protein only partially reverses the phenotype and is associated with significant residual genetic instability.



**Figure 3.** MNU survival in A2780MNU-clone1 expressing different MLH1 missense mutations. A, levels of the MLH1, PMS2, and PCNA proteins in extracts (20 µg) of MLH1-1 and the transfectants containing the mutated *MLH1* cDNA (1852AA>GC-1, 1904A>G-1, 199G>A-1, and 731G>A-1). B, MNU survival in A2780MNU-clone1 (■), MLH1-1 (▲), 1852AA>GC-1 (◆), 1904A>G-1 (◇), 731G>A-1 (●), and 199G>A-1 (○).



**Figure 4.** Characterization of polymorphic MLH1. *A, top*, survival of MLH1-1 ( $\blacktriangle$ ), 655A>G-1 ( $\triangle$ ), and 655A>G-2 ( $\diamond$ ) clones treated with MNU; *bottom*, survival of MLH1-1 ( $\blacktriangle$ ), 655A>C-1 ( $\triangle$ ), and 655A>C-3 ( $\blacklozenge$ ) following MNU exposure. *B*, levels of the MLH1, PMS2, and PCNA proteins in extracts of MLH1-1, 655A>G-1 and 655A>G-2 (20  $\mu$ g), and 655A>C-1 and 655A>C-3 (10-20  $\mu$ g). *C*, examples of MSI at *BAT26* in 655A>G-2 subclones. Number of clones with -1/-2 changes (loss) or +1 changes (gain). Alterations are shown next to the appropriate panel. *Numbers*, size of the alleles; *numbers inside parenthesis*, number of unstable clones.

Two clones expressing a second putative polymorphism (25), 655A>C, were also compared. MLH1 was present in 655A>C-1 at  $\sim 100\%$  of the WT level, whereas it was expressed at 10% of WT in 655A>C-3 (Fig. 4B). The levels of PMS2 paralleled MLH1 expression and were high in 655A>C-1 and low in 655A>C-3 (Fig. 4B), indicating that this altered MLH1 can stably interact with PMS2. Consistent with its designation as a polymorphism, a high MLH1 level in 655A>C-1 was accompanied by full reversion of methylation tolerance (Fig. 4A). As expected, the low level of MLH1 in 655A>C-3 was associated with an unaltered MNU resistance (Fig. 4A) and these cells retained a strong mutator phenotype (Fig. 2C).

Unexpectedly, the *HPRT* mutation rate of 655A>C-1 remained high. At  $\sim 5 \times 10^{-7}$  per cell per generation, this rate was only slightly lower than that of the parental MMR-defective cells (Fig. 2C). Thus, although the 655A>C MLH1 protein behaves as expected for a harmless polymorphism and fully reverses the methylation-tolerant phenotype, it does not have a significant effect on the mutator phenotype even when highly expressed. Because this behavior is unexpected, we confirmed that MMR was fully active in 655A>C-1 by analyzing their sensitivity to 6-TG. High-level 655A>C MLH1 expression restored full 6-TG sensitivity and 655A>C-1 cells were as sensitive as MLH1-1 cells to the thiopurine (data not shown). The anomalous behavior of 655A>C-1

with regard to spontaneous mutation rates was investigated in more detail.

**Steady-state levels of DNA 8-oxoG.** The mutator phenotype of MMR-defective cells is influenced by oxidative stress (20). MMR prevents the accumulation of the promutagenic base 8-oxoG in DNA, and MMR-defective A2780MNU-clone1 cells accumulate 8-oxoG in their DNA (26). Consistent with our previous observations, restoration of MMR by a transfected WT MLH1 (MLH1-1) reduced the level of DNA 8-oxoG to approximately half (Fig. 5A). A high level of the 655A>G MLH1 in 655A>G-1 had a similar effect on DNA 8-oxoG, indicating that these cells are functionally WT for MMR. The low level of the same protein in 655A>G-2 cells was not associated with a significant reduction in DNA 8-oxoG (Fig. 5A).

The findings for the 655A>C polymorphism were in stark contrast. The DNA 8-oxoG content of 655A>C-3 cells was comparable with that for uncorrected A2780MNU-clone1 cells (Fig. 5A). This is consistent with their expression of a very low level of a functional polymorphic MLH1. In contrast, 655A>C-1 cells, in which a high level of the same MLH1 should render MMR fully active, contained around twice more DNA 8-oxoG than the uncorrected A2780MNU-clone1 cells. This suggested that the 655A>C-1 clone may have a deranged oxidative metabolism and suffer atypically high levels of endogenous oxidative DNA damage

with which their restored MMR is unable to cope. To examine this possibility, we compared the levels of endogenous reactive oxygen species (ROS) in 655A>C-1 and A2780MNU-clone1 cells. Cytofluorimetric analysis by dichlorofluorescein staining indicated that 655A>C-1 cells contained 30% more ROS in comparison with their parental cells (data not shown). These data indicate that the anomalous properties of the 655A>C-1 transfectant reflect the unexpectedly high levels of oxidative damage in the recipient cells rather than the properties of the encoded MLH1 itself.

Consistent with expectation and with their failure to restore active MMR, there was no reduction in DNA 8-oxoG in any of the clones expressing a mutated MLH1 (Fig. 5B).

These data confirm the relationship between defective MMR and buildup of DNA 8-oxoG. They also indicate that variations in the endogenous levels of oxidative damage among clonal isolates of a transfected population might subtly influence their mutator phenotype.

## Discussion

Restoration of MMR in human tumor cell lines is generally accomplished by introducing an extra copy of the relevant human

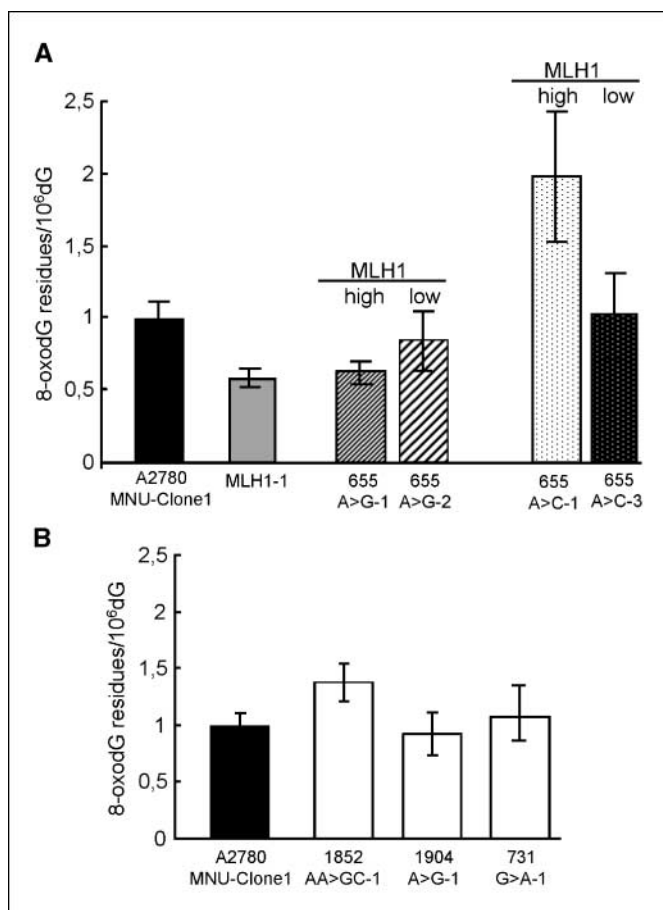
chromosome (chromosomes 2 and 3 for MSH2/MSH6 and MLH1, respectively; refs. 27, 28). The MLH1-defective clone 1 variant of the A2780 ovarian carcinoma cell line provides one of a very few examples of cell lines in which MMR deficiency is fully corrected by a transfected cDNA (14, 29). We have suggested that stable expression of MLH1 cDNA in clone 1 might be related to its dominant-negative p53 mutation that distinguishes it from most MMR-defective human tumor cell lines (15). Another example is the MMR-defective mouse cells complemented with human cDNAs (30). We show here that stable expression of WT *MLH1* cDNA in A2780MNU-clone1 reverts several aspects of the phenotype associated with inactive MMR. In particular, MLH1 expression restores WT levels of methylating agent sensitivity and reverses the mutator phenotype.

A relatively low level (~50% of normal) of MLH1 was sufficient to restore a WT phenotype. This reversed the mutator phenotype and MSI, indicating efficient correction of replication errors. It also promoted efficient processing of MNU-induced *O*<sup>6</sup>-meGua-containing base pairs and restored MutL $\alpha$ -mediated sensitivity to a methylating agent. These data are consistent with the properties of 293T cells expressing variable levels of WT MLH1 (29).

Measurements of MNU sensitivity, mutator phenotype, and DNA oxidation levels in transfected A2780MNU-clone1 cells correctly differentiated a series of known MLH1 mutations and polymorphisms. For four of four presumptive mutations, there was no reversion of methylation tolerance and the transfected cells retained their strong mutator phenotype. Expression of these mutant MLH1 proteins in clone 1 cells was also associated (with a single exception) with low levels of PMS2 and with high steady-state levels of DNA 8-oxoG. These findings are consistent with the involvement of these mutations in the pathogenesis of colorectal cancer. For the G67R (199G>A) variant, our data agree with findings from other approaches to mutation identification. The G67R amino acid change introduces a positive charge in the conserved ATP-binding motif of the gene. This variant is not found in the general population and is detected in HNPCC families (31). G67R was previously designated mutant in a yeast-based assay in which overexpression of the WT MLH1 induces a dominant-negative phenotype (4, 5). The same variant MLH1 was unable to interact with PMS2 and EXOI in a yeast two-hybrid assay (7, 8). *In vitro* MMR assays also showed that this mutation inactivates hMutL $\alpha$  (13). Although we have no information about the segregation of the G67R allele in the Italian family in which it was identified, the only tumor from this family that could be examined have high MSI levels. This is consistent with our identification of this variant MLH1 as mutant.

The second mutation G244D (731G>A) has been found in three Italian families. In one of these families, three affected individuals could be tested and segregation of the mutation with the disease was observed. The mutation was not found in healthy controls, and colorectal cancers from two affected carriers were MSI-H. The altered MLH1 was associated with a mutator phenotype in yeast (5), the interaction with its partner was defective in the two-hybrid assay (7), and human 293T cell extracts expressing the mutated protein were defective in MMR (12). Together with these observations, our data confirm the probable pathogenicity of this mutation.

The third mutation, K618A (1852-3AA>GC), conferred only a partial defect in its interaction with MLH1 partners in the yeast two-hybrid assay (7). Unusually, MLH1 and PMS2 levels in A2780MNU-clone1 cells expressing the K618A mutation were



**Figure 5.** Steady-state levels of DNA 8-oxoG in untransfected and MLH1-transfected A2780MNU-clone1. A, A2780MNU-clone1 (black columns), MLH1-1 (gray column), 655A>G-1 (fine-patterned column), 655A>G-2 (coarse-patterned column), 655A>C-1 (dotted white column), and 655A>C-3 (dotted black column). B, A2780MNU-clone1 (black column) and 1852AA>GC-1, 1904A>G-1, and 731G>A-1 (white columns). DNA was extracted from exponentially growing cells, and 8-oxoG was determined by HPLC/EC as described in Materials and Methods. Columns, mean of six independent determinations; bars, SD.



comparable with WT, and *in vitro* MMR assays indicate that the K618A variant MLH1 retains some function (13). Expression of K618A was unable to reverse either methylation tolerance or the mutator phenotype of A2780MNU-clone1, indicating that, despite a high level of hMutL $\alpha$ , this variant MLH1 is unable to participate in MMR *in vivo*. The K618A variant has been suggested to be associated with an increased risk of multiple adenomas (32) and has been identified in early-onset Scottish colorectal cancer patients (33). In our genetic survey, the variant was never detected in healthy controls and was observed in two unrelated HNPCC patients both affected with colorectal cancer. In one case, the tumor DNA was available and tested positive for MSI. However, the K618A variant was not present in two other affected members of the same family. The other patient inherited the variant from the unaffected father and, in addition, carried a second *MLH1* mutation *in trans*, an in-frame deletion (1852-1854delAAG) leading to the loss of a lysine residue (K618del). This mutation (also called *MLH1* del616) has been shown previously to be clearly deleterious (34). Biallelic HNPCC-related *MLH1* mutations are associated with a very severe phenotype, characterized by early-onset gastrointestinal cancers, childhood hematologic malignancies, and signs of neurofibromatosis type 1 (35–37). Although our patient had neither leukemia nor neurofibromatosis type 1 symptoms, it is noteworthy that he was diagnosed with a colorectal cancer at a very young age (28 years). These observations suggest that the K618A variant has a significant pathogenetic involvement and are consistent with the results of the *in vitro* assays that unequivocally identify K618A as nonfunctional. In addition, its absence in unaffected controls is consistent with its being a cancer-predisposing allele. The carrier father is, however, unaffected, suggesting that K618A might be a low penetrance mutation. The lack of segregation with the disease in the second family is also consistent with a low penetrance HNPCC mutation (38). Levels of expression of the WT *MLH1* allele should therefore be monitored with care in the carriers of the K618A mutation because their variation might have major consequences for the stability of the genome.

The N635S (1904A>G) variant has never been reported previously in familial colorectal cancer (<http://www.insight-group.org>). The effect of the *MLH1* alteration on the genetic stability of colorectal cancer in this family is currently unknown. In our assays, 1904A>G expression was associated with retention of methylation tolerance and a strong mutator phenotype, indicating that this variant allele encodes an inactive protein. We conclude that the N635S *MLH1* is most likely a pathogenic variant that is associated with the development of colorectal cancer.

The incidence of the I219V (655A>G) *MLH1* variant ranges from 3% to 34% in different geographic regions (24, 31, 39, 40). When expressed in A2780MNU-clone1, I219V restored MNU sensitivity and genetic stability. It also reduced the steady-state level of DNA 8-oxoG. These observations, together with similar findings in yeast (4, 7, 8) and *in vitro* assay of MMR by human cell extracts (12, 13), are all consistent with full retention of MMR activity and a

designation of I219V as a silent polymorphism. Low levels of I219V *MLH1* are apparently associated with a partial defect in MMR as indicated by incomplete restoration of MNU sensitivity, accumulation of DNA 8-oxoG, and intermediate genetic instability. The partial phenotype of these cells suggests that these biological end points constitute very sensitive indicators of inefficient MMR.

Although the I219V polymorphism is not associated with an increased risk of colorectal cancer (39) and behaves as WT in our assays, it may not be completely without effect. When combined with genotypes known to influence childhood acute lymphoblastic leukemia susceptibility (GSTM1-null and CYP2A1\*2A or CYP2E1\*5), homozygosity causes a significant additional risk (41). Homozygosity for the I219V polymorphism is also associated with ulcerative colitis refractory to treatment with 6-mercaptopurine or azathioprine (42), suggesting that MMR capacity is compromised (3).

The I219L (655A>C) variant (25) was also confirmed as a polymorphism. This was also inferred from the yeast two-hybrid system (7). In the transfectant (655A>C1) that expressed a normal level of the I219L protein, we observed the high level of spontaneous mutagenesis typical of inefficient MMR despite a full reversion of methylation tolerance. A large part of the mutator phenotype in MMR-defective human and mouse cells depends on oxidized DNA purines (20). In that particular transfectant, we noted a significant accumulation of DNA 8-oxoG to a level even greater than that of parental A2780MNU-clone1 cells. This was accompanied by an aberrantly high level of cellular ROS. It seems that this particular clone of transfectant A2780MNU-clone1 cells had an altered oxidative metabolism that exacerbated its mutator effect despite the presence of sufficient MMR activity to confer methylation sensitivity.

In conclusion, this approach provides a new tool with which to investigate the effect of alterations in the *MLH1* gene. These assays also provided further evidence for the importance of oxidative DNA damage in MMR-defective cells and correctly classified four missense *MLH1* substitutions, including two previously reported pathogenic mutations (G67R and G244D) and two neutral polymorphisms (I219V and I216L). The *in vitro* assays unequivocally identified the K618A change as an inactivating mutation. The segregation of this mutation in the two Italian families is, however, inconsistent with a simple causative role in HNPCC. Our findings illustrate the importance of combining genetic and clinical observations with data from *in vitro* assays.

## Acknowledgments

Received 5/24/2006; revised 7/14/2006; accepted 7/20/2006.

**Grant support:** Associazione Italiana per la Ricerca sul Cancro/Federazione Italiana Ricerca sul Cancro and Ministero della Salute (M. Bignami and P. Radice).

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We thank Peter Karran for helpful suggestions, Claudia Foglia and Donata Penso for technical support, and Augusto Ravagnani and Paola Sala for providing biological samples of blood donors and clinical data.

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