# *MSH2* missense mutations alter cisplatin cytotoxicity and promote cisplatin-induced genome instability

## Jill E. Clodfelter, Michael B. Gentry and Karin Drotschmann*

Department of Cancer Biology, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157, USA

## ABSTRACT

**Defects in the mismatch repair protein MSH2 cause tolerance to DNA damage. We report how cancer-derived and polymorphic *MSH2* missense mutations affect cisplatin cytotoxicity. The chemotolerance phenotype was compared with the mutator phenotype in a yeast model system. *MSH2* missense mutations display a strikingly different effect on cell death and genome instability. A mutator phenotype does not predict chemotolerance or *vice versa*. *MSH2* mutations that were identified in tumors (Y109C) or as genetic variations (L402F) promote tolerance to cisplatin, but leave the initial mutation rate of cells unaltered. A secondary increase in the mutation rate is observed after cisplatin exposure in these strains. The mutation spectrum of cisplatin-resistant mutators identifies persistent cisplatin adduction as the cause for this acquired genome instability. Our results demonstrate that *MSH2* missense mutations that were identified in tumors or as polymorphic variations can cause increased cisplatin tolerance independent of an initial mutator phenotype. Cisplatin exposure promotes drug-induced genome instability. From a mechanistical standpoint, these data demonstrate functional separation between MSH2-dependent cisplatin cytotoxicity and repair. From a clinical standpoint, these data provide valuable information on the consequences of point mutations for the success of chemotherapy and the risk for secondary carcinogenesis.**

## INTRODUCTION

Defects in mismatch repair (MMR) proteins promote hereditary [hereditary nonpolyposis colorectal cancer (HNPCC)] and several sporadic forms of cancer (1). The mismatch recognition protein MSH2 and the molecular matchmaker MLH1 are most commonly mutated in MMR-defective tumors. While many of these mutations result in a truncation and loss of the proteins, several single point mutations have been identified that are believed to equally promote carcinogenesis by establishing a mutator phenotype.

In addition to the repair of replication errors, MMR proteins are involved in several other cellular responses. Among these is the response to DNA damage, such as inflicted by most chemotherapeutic agents. A MMR-dependent induction of cell cycle arrest and/or apoptosis is observed. Defects in MMR proteins, therefore, not only result in genome instability, but can also promote tolerance to chemotherapy (2). MMR-deficient cells show increased survival and resistance to treatment with methylating agents and 6-thioguanine (6-TG). In addition, an increased tolerance to the common chemotherapeutic agent cisplatin is observed, which manifests itself in a 2- to 3-fold increase in cell survival after cisplatin exposure (2). Though MMR proteins recognize cisplatinated DNA adducts, they are not involved in the repair of this damage (3). We have recently demonstrated that the repair function of MSH2 is not required for the MMR-dependent induction of cell death after cisplatin exposure, suggesting an uncoupling of both events and a direct role for the MMR protein in damage signaling (4) (F. Salsbury, J. Clodfelter, M. Gentry, T. Hollis and K. Drotschmann, manuscript in preparation).

Though a complete knockout in MMR results in increased cisplatin tolerance, it is unknown how single point mutations in MMR genes affect the response to a chemotherapeutic agent. This is of particular significance, if the missense mutation is identified in tumor cells.

A drug tolerance phenotype can be associated with enhanced susceptibility to DNA damage-induced mutations. As a consequence, MMR-defective cells show a high spontaneous mutation rate after drug exposure (5). This acquired genome instability has been attributed to the growth advantage of MMR-deficient cells after chemotherapeutic treatment, which results in the expansion of cultures of repair-defective cells and the accumulation of mutations in downstream genes. This acquired mutator phenotype may contribute to the development of therapy-related, secondary

---

tumors, as was suggested for the development of acute myeloid leukemia (AML). Fifty percent of therapy-related AML cases show microsatellite instability, a hallmark of MMR defects. Such cases are predominantly observed after chemotherapeutic treatment of primary malignancies with methylating agents, or the treatment of non-malignant cells with immunosuppressiva after organ transplantation (5). Even moderate alkylation tolerance can predispose to AML via MMR defects. It is currently unknown whether exposure to other agents contributes to increased mutagenesis in a similar way. Several single MSH2 mutations were identified in tumor cells. It is unknown how these point mutations affect the development of a supermutator phenotype.

Here, we analyzed single point mutations in MSH2 that are homologous to those identified in tumors, or described as polymorphisms, for their effect on cytotoxicity in response to cisplatin and the development of acquired genome instability. We demonstrate that *MSH2* missense mutations decrease cytotoxicity and confer increased tolerance. This tolerance phenotype is independent of a mutator phenotype. Cisplatin exposure of cells harboring *MSH2* mutations promotes the development of acquired genome instability, independent of an initial cisplatin-independent mutator phenotype. This drug-induced mutator phenotype may be caused by unprocessed cisplatin adducts in DNA. Given the fact that the mutations analyzed here were identified in tumor cells or described as polymorphisms, these data have important implications for the choice and efficacy of cancer treatment.

## MATERIALS AND METHODS

### Strains

All yeast strains used in this study are isogenic and have been described previously (4,6). Expression plasmids have been described previously (4,6). Mutations were introduced by site-directed mutagenesis (Stratagene).

### Immunoblot analysis

Cells were grown in synthetic media lacking uracil overnight at 30°C and harvested by centrifugation. Cell lysis was achieved by the addition of glass beads, and debris was pelleted by centrifugation. The protein concentration of the supernatant was determined and equal amounts separated by gel electrophoresis. Blot analysis was performed using a polyclonal anti-Msh2 antibody (6).

### Genetic assays

Treatment with cisplatin and cell survival assays were performed as described previously (4). The IC$_{50}$ (concentration resulting in 50% cell death) values and confidence limits were obtained as described previously (4). Briefly, overnight cultures of *Saccharomyces cerevisiae* strains grown in selective media (lacking the respective amino acid for the selection of the ARS-CEN plasmid) were split in half and diluted. At early log phase, one half of the cultures obtained indicated concentrations of cisplatin in selective media. After incubation with (and without) cisplatin for 16–24 h, appropriate dilutions were plated onto selective media without drug and cells allowed to grow at 30°C for 4 days. Wild-type strains were grown in complete media containing all essential amino acids. Colonies

were counted, and the number of colonies after treatment was compared with the total number of colonies without drug and expressed as percent survival. The IC$_{50}$ was determined by fitting parametric response curves using standard least-squares linear models with both linear and quadratic terms for cisplatin dose. For each clone, the following model was fitted:

$Y_i = \beta_0 + \beta_1 x + \beta_2 x^2 + \varepsilon_i$, where $Y_i$ is the % cell death; $\beta_0$, $\beta_1$ and $\beta_2$ are standard least-squares regression coefficients; $x$ is cisplatin dose in μM; and $\varepsilon_i$ is the usual normal error term for the linear regression model. After fitting a model for each cell type, the quadratic formula and estimated regression coefficients were used to solve for $x$ such that $Y = 50\%$. Mutation rate determinations based on fluctuation tests have been described previously (4,6). For the determination of the *CAN1* gene mutations after cisplatin exposure, cells were exposed to the indicated cisplatin concentration overnight and plated onto canavanine containing media. Individual colonies were isolated and the *CAN1* gene PCR-amplified using primers 5′-CAG ACT TCT TAA CTC CTG-3′ and 5′-GGA ATG TGA TTA AAG GTA ATA AAA CG-3′. The PCR product was sequenced using primers Can-SF-1: 5′-ATT CTG TCA CGC AGT CCT; Can-SF-2: GAA CTA GTT GGT ATC ACT; Can-SF-3: CTC AAT CTC GCA CAT CAG; Can-SR-1: TGT CTC CAT GTA AGC CAA; Can-SR-2: ATA TTA TAC CTG GAC CCC; Can-SR-3: ATG AAA AGA CCT GTA CCA. Sequences were compared with the wild-type *CAN1* sequence.

## RESULTS

### Cisplatin cytotoxicity in strains harboring *MSH2* missense mutations

We determined the effect of single *MSH2* point mutations on cisplatin cytotoxicity and addressed its correlation with a mutator phenotype in a yeast model system (6). Numerous *MSH2* missense mutations have been identified in sporadic and hereditary tumors of different origin. In addition, an increasing number of amino acid alterations in MSH2 have been described as genetic variants (6–8) (http://www.insight-group.org; http://www.nfdht.nl). Based on a sequence alignment of MutS proteins (Figure 1A) (9), homologous mutations were introduced into the yeast *MSH2* gene (6). All mutants produce protein levels indistinguishable from wild-type MSH2 (Figure 1B). Figure 1C shows the location of mutations on the MutS crystal structure. *MSH2* missense mutations are randomly distributed over the entire protein. Mutations Y109C (Figure 1D, blue) and R542P are located in the DNA-binding domains, while G770R affects the ATPase domain (red). Other mutations affect regions that are involved in intra- and interdomain contacts (e.g. L402F, Figure 1D).

The effects of *MSH2* mutations on cisplatin cytotoxicity were analyzed in a yeast model system (4). Dose-dependent cell survival determines the effect of individual mutations (4). The IC$_{50}$ was used as an indicator for the effect of a particular mutation on cisplatin cytotoxicity. A complete knockout in *MSH2* results in the previously described, typically 2- to 3-fold increase in tolerance of cells to cisplatin treatment (Table 1, 2.4-fold). The effect on cytotoxicity was compared with altered repair activity, as determined in the *CAN1* reporter system (4). This reporter monitors any mutations that inactivate
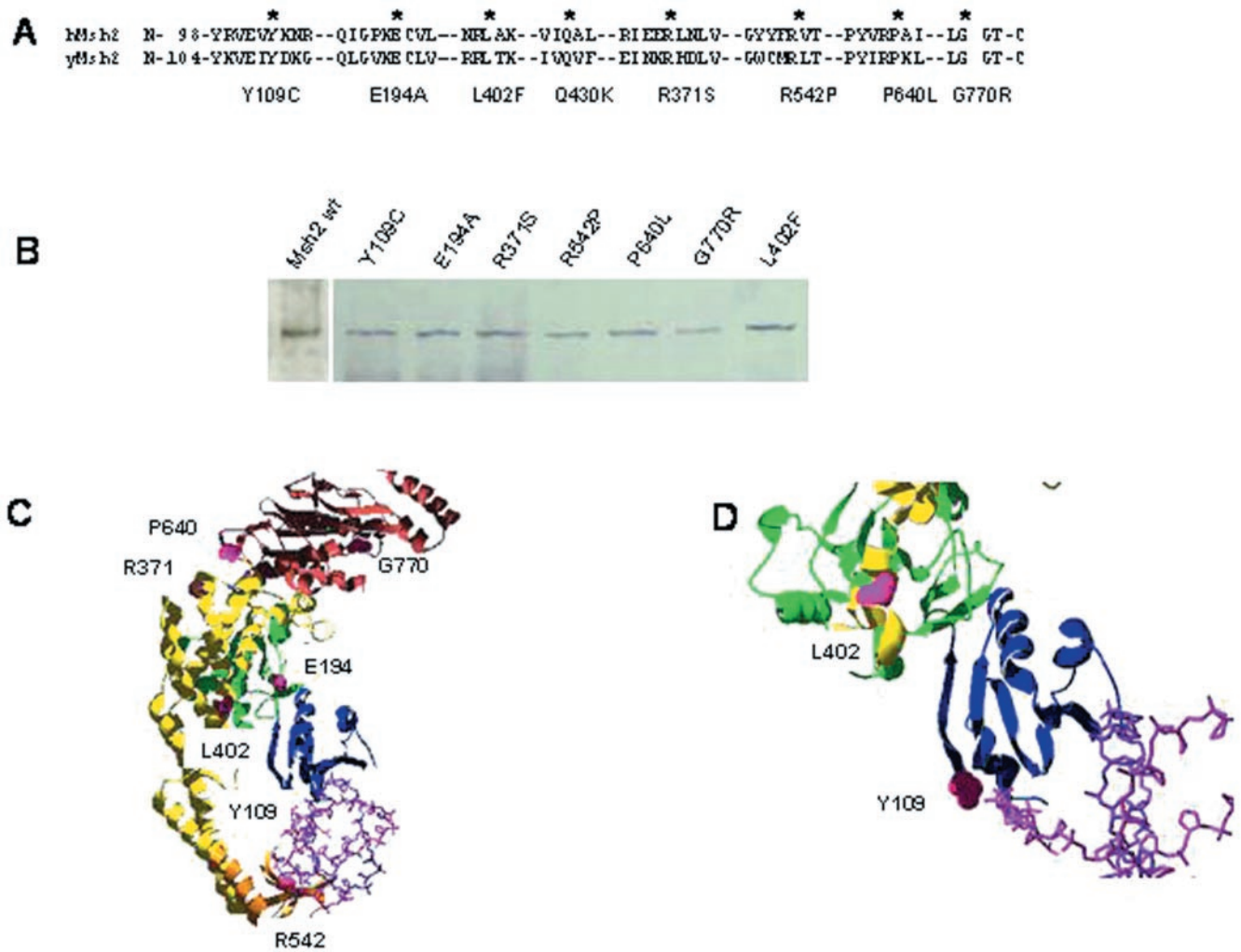
**Figure 1.** Missense mutations in MSH2. (**A**) Alignment of human (hMSH2) and yeast (yMsh2) amino acid sequences indicating the location of point mutations. Stars above the alignment indicate the side of mutation (bold); underneath the diagram single point mutations in the yeast MSH2 protein that were analyzed here are indicated. (**B**) Western blot analysis demonstrating the expression of mutant *MSH2* genes. The corresponding MSH2 mutation is indicated. (**C**) Structure of the MSH2- homologous subunit B of *E.coli* MutS in complex with mismatched DNA (36) with the amino acid alterations indicated at homologous sides. Coloring depicts the domain structure of the protein, with blue: mismatch binding (I), green: connector (II), yellow: core (III), orange: clamp (IV) and red: ATPase/dimerization (V) domains. Amino acid designations are yeast numbers. (**D**) Close-up view of the location of Y109 in the mismatch-binding domain I, and L402 in the core domain III, respectively.

**Table 1.** Effect of *MSH2* missense mutations on DNA damage response and repair

| MSH2 allele | $IC_{50}$ [μM cisplatin] | | | Mutation rate in *CAN1* [$10^{-6}$] | | |
| | $IC_{50}$ | CL | RR | MR | CL | RR |
|---|---|---|---|---|---|---|
| *msh2Δ* | 360 | 330–390 | **2.4** | 15 | 11–19 | **32** |
| MSH2 wt | 150 | 130–170 | **<u>1</u>** | 0.47 | 0.37–0.66 | **<u>1</u>** |
| Separation-of-function: increased cisplatin tolerance without a mutator phenotype | | | | | | |
| Y109C | 290 | 240–340 | **1.9** | 1.2 | 0.25–4.1 | **2.6** |
| L402F | 250 | 190–310 | **1.7** | 3.6 | 1.4–5.5 | **7.7** |
| Separation-of-function: increased mutator phenotype with unaltered cisplatin cytotoxicity | | | | | | |
| G770R | 130 | 120–140 | **0.87** | 19 | 14–25 | **40** |
| R371S | 180 | 170–190 | **1.2** | 39 | 20–54 | **83** |
| P640L | 200 | 130–260 | **1.3** | 24 | 20–34 | **51** |
| Increased cisplatin tolerance and a mutator phenotype | | | | | | |
| R542P | 300 | 250–340 | **2** | 19 | 13–26 | **40** |
| No/weak effect on cisplatin cytotoxicity and mutation rate | | | | | | |
| E194A | 130 | 101–150 | **0.87** | 4.4 | 2.4–7.2 | **9.4** |

CL, 95% confidence limits; RR, relative rate (bold); compared with wild type (underlined); MR, mutation rate.

the arginine permease gene; inactive MMR results in a 32-fold increase in the mutation rate (Table 1). Non-overlapping, 95% confidence limits between different strains indicate statistical significance.

Most prominent changes in cisplatin cytotoxicity are observed with mutations Y109C and L402F (Table 1). Mutation Y109C shows a significant increase in the tolerance to cisplatin exposure. The $IC_{50}$ of 290 μM has overlapping confidence limits with the knockout strain, suggesting that the response in both strains is indistinguishable (Table 1). In contrast, this mutation does not result in a mutator phenotype, and the mutation rate is not significantly different from the wild-type strain (2.6-fold increase, with overlapping confidence limits to wild type). The Y109C mutation is homologous to the Y103C mutation in human MSH2, which was identified in association with HNPCC (http://www.insight-group.org; http://www.nfdht.nl).

The L402F mutation results in an increase in cisplatin tolerance, which is significantly different from the wild-type response (Table 1, $IC_{50}$ of 250 μM). In contrast, the mutant strain shows a weak mutator phenotype (7.7-fold elevated over wild type; compared with 32-fold for the knockout strain). The corresponding mutation L390F in human MSH2 was described as a genetic variation in the general population with an allele frequency of 0.005 (8).

These effects are in contrast to consequences observed for MSH2-G770R. The cisplatin cytotoxicity in this strain is unaltered when compared with the wild type (130 μM compared with 150 μM for the wild-type strain). The G770R mutation confers a strong mutator phenotype (40-fold) that is indistinguishable from a complete knockout strain (Table 1, 32-fold). G770R affects a residue in the ATPase domain of MSH2 (Figure 1C). The corresponding mutation in human MSH2 is associated with HNPCC (http://www.insight-group.org; http://www.nfdht.nl). Similarly, mutations R371S and P640L do not significantly alter cisplatin cytotoxicity (1.2- and 1.3-fold, respectively, with overlapping confidence limits with wild type). The mutation rate is considerably elevated in both strains (83- and 51-fold, respectively, Table 1). The homologous human mutations are found in association with HNPCC [(10,11), http://www.insight-group.org; http://www.nfdht.nl].

Mutation R542P significantly increases both cisplatin resistance ($IC_{50}$ of 300 μM with confidence limits overlapping with the *msh2Δ* strain) and the mutation rate of the cell (40-fold; Table 1). The homologous mutation was identified in a patient with ovarian cancer suggestive of HNPCC (12). Mutation E194A does not significantly alter cisplatin cytotoxicity and displays a weak mutator phenotype.

These data demonstrate that single point mutations in MSH2 can alter cisplatin cytotoxicity. Individual mutations affect the cytotoxicity differently. No obvious association with the location of the mutation in the protein is observed. The chemotolerant phenotype is not correlated with a mutator phenotype.

### A cisplatin-induced mutator phenotype in strains harboring Y109C and L402F

We next determined whether the presence of a single point mutation in *MSH2* promotes the development of acquired genome instability after cisplatin exposure. The mutation rate of cisplatin-tolerant clones was analyzed in the *CAN1* reporter system. The mutator phenotype of cisplatin-tolerant clones (Table 2) was compared with the mutation rate without exposure to identify a cisplatin-induced change (Table 1). Exposure of the wild type strain to cisplatin does not alter the mutation rate (mutation rates 0.47 and $0.37 \times 10^{-6}$, respectively, Table 2). Mutations MSH2-Y109C and L402F, respectively, which confer cisplatin tolerance and no or a weak mutator phenotype in the absence of cisplatin exposure (Table 1), show a significant cisplatin-induced mutator phenotype. After drug exposure, cisplatin-tolerant clones harboring MSH2-Y109C show a significant mutator phenotype that is 5.2-fold elevated when compared with the initial mutation rate (compare 6.2 with $1.2 \times 10^{-6}$; Table 2). This acquired genome instability results in an overall 13-fold elevation in the mutation rate when compared with the wild-type rate (without exposure). MSH2-L402F weakly elevates the mutation rate in the absence of cisplatin ($3.6 \times 10^{-6}$; 7.7-fold above wild-type levels; Tables 1 and 2). Cisplatin-resistant clones of this strain display a significant 2.5-fold increase in the mutation rate when compared with this initial rate. This causes an overall 19-fold elevation, resulting in a mutation rate that is indistinguishable from a complete knockout strain (compare $9 \times 10^{-6}$ for L402F with $15 \times 10^{-6}$ for *msh2Δ*, with overlapping confidence limits, Table 2). Both strains harboring Y109C and L402F, respectively, promote acquired genome instability.

The already strong mutator phenotype of cells harboring G770R (Table 1) is further elevated by exposure to cisplatin and reaches a mutation rate that goes beyond the knockout

**Table 2.** Drug-induced supermutator phenotype

| | Without exposure | | | After cisplatin (340 μM) | | |
|---|---|---|---|---|---|---|
| | MR $10^{-6}$ | CL $10^{-6}$ | RR | MR $10^{-6}$ | CL $10^{-6}$ | RR |
| *msh2Δ* | 15 | 11–19 | **32** | ND | | |
| MSH2 wt | 0.47 | 0.37–0.66 | **1** | 0.37 | 0.31–0.45 | **0.8** |
| Y109C | 1.2 | 0.25–4.1 | **2.6** | 6.2 | 5.9–9.6 | **13 (5.2x)** |
| L402F | 3.6 | 1.4–5.5 | **7.7** | 9.0 | 7.5–12 | **19 (2.5x)** |
| G770R | 19 | 14–25 | **40** | 41 | 26–47 | **87 (2.1x)** |
| R371S | 39 | 20–54 | **83** | 29 | 26–43 | **62** |
| P640L | 24 | 20–34 | **51** | 24 | 20–30 | **51** |
| R542P | 19 | 13–26 | **40** | 24 | 19–29 | **51** |
| E194A | 4.4 | 2.4–7.2 | **9.4** | 6.5 | 4.6–7.4 | **14** |

MR, mutation rate; CL, 95% confidence limits; RR, relative rate (bold); compared with wild type (underlined).

strain (compare $41 \times 10^{-6}$ with $15 \times 10^{-6}$ for *msh2*Δ, Table 2). None of the strains harboring any of the other mutations, independent of their initial mutation rate or cisplatin tolerance, promotes a cisplatin-induced mutator phenotype.

### The mutational spectrum of acquired genome instability

To analyze whether the cisplatin-induced genome instability observed for mutations Y109C and L402F is due to an accumulation of unprocessed, cisplatin-induced mutations, the mutation spectrum was determined. Individual, cisplatin-tolerant mutator clones from strains harboring either mutation (Table 2) were isolated. The reporter gene *CAN1* was sequenced to determine the nature of inactivating mutations. The sequence was compared with the wild-type sequence for the *CAN1* gene. In addition, the mutation spectrum was compared with the published mutation spectrum of an *msh2*Δ strain without drug exposure (13). The overall distribution of insertion/deletion and base substitution mutations is unaltered when compared with the described spectrum for the *msh2*Δ strain (13). A total of 83 and 75% of cisplatin-resistant clones of cells harboring MSH2-Y109C and L402F, respectively, are insertion/deletions [as compared with 85% for the *msh2*Δ strain (13)]. The spectrum of mutations prevalent in Y109C or L402F expressing clones without cisplatin exposure is indistinguishable from the *MSH2*-defective strain. One nucleotide deletions in a stretch of six adenines, and a 1 nt addition in a run of six thymidines are observed (data not shown). These mutations are commonly found in *MSH2*-deficient strains (13). The mutation spectrum of cisplatin-tolerant cells harboring either one of the point mutations shows considerable differences (Table 3). Mutations in previously undescribed sequence contexts are observed. The spectrum of cisplatin-resistant clones of Y109C primarily shows a 1 nt deletion in two adjacent guanines. In addition, the deletion of 4 nt (ΔAAGT) is observed. Both sequence contexts are targets for the adduction with cisplatin, which crosslinks two adjacent purines, primarily GpG, but also ApG. Similarly, mutation L402F generates a 1 nt deletion within two adjacent guanines, though at a different site. An additional thymine deletion is observed directly downstream of a guanine dinucleotide repeat (Table 3). The only base substitution observed in the L402F mutant strain is a G to T mutation that alters the cisplatin target GA to a TA, consistent with errors occurring at the site of the cisplatin-intrastrand crosslink. These data demonstrate that cisplatin-resistant clones of MSH2-Y109C and L402F accumulate mutations at sites of potential cisplatin adduction.

**Table 3.** Mutation spectrum in *CAN1* of cisplatin-resistant clones

| Mutant | Insertion/deletion | Base substitution |
|---|---|---|
| Y109C | | |
| ΔG | GG → G | C → T |
| ΔA | A6 → A5 | |
| +TT | T3 → T5 | |
| ΔAAGT | | |
| L402F | | |
| ΔG | GG → G | TGA → TTA |
| ΔT | GGTTA → GGTA | |
| +T | T6 → T7 | |

## DISCUSSION

We demonstrate that single point mutations in the MMR protein MSH2 have the potential to significantly increase tolerance to the chemotherapeutic agent cisplatin and promote a cisplatin-induced mutator phenotype.

Amino acid alterations MSH2-Y109C and L402F represent separation-of-function mutants that demonstrate that (i) single point mutations can increase cisplatin tolerance and cell survival after exposure to cisplatin; (ii) this cisplatin tolerance is independent of a mutator phenotype; and (iii) exposure of these mutants to cisplatin induces a mutator phenotype.

The missing correlation between genome instability and increased cisplatin tolerance demonstrates that the MSH2-dependent cell death pathway that is initiated after exposure to cisplatin is functionally independent of repair events initiated by the protein. This is supported by earlier findings that showed that a mutation in the ATPase domain of MSH2 leaves the cell MMR defective, but does not alter cisplatin cytotoxicity in both a yeast and mouse model (4,14). Similarly, a point mutation in yeast and mouse *MSH6* showed separation-of-function between repair and damage response (15).

A complete MMR deficiency results in a weak, 2- to 3-fold increase in cisplatin tolerance, which is observed in all tested systems [Table 2; (4,16–18); F. Salsbury, J. Clodfelter, M. Gentry, T. Hollis and K. Drotschmann, manuscript in preparation]. Though an apparently small effect, it results in clonal selection of MMR-defective cells and contributes to secondary tumor growth (19). Furthermore, this chemotolerant phenotype has been observed consistently in all MMR-deficient cell lines tested to date. Both observations point to the biological significance of this chemotolerant phenotype (2).

It was previously shown that exposure of MMR-deficient cancer cell lines to chemotherapeutic agents, such as methyl methanesulfonate (MMS), MNNG or 6-TG (20,21), results in induced genome instability. We demonstrate that different *MSH2* point mutations can promote the development of a cisplatin-induced mutator phenotype (Table 2). An increased cisplatin tolerance phenotype is not sufficient for or predictive of the induced genome instability phenotype. This is evident from the cisplatin-induced mutator phenotype observed for G770R. This mutation does not promote cisplatin tolerance, but shows a significant increase in the mutation rate after cisplatin treatment, despite the already high initial mutation rate (Table 2). On the other hand, mutation R542P, which confers a cisplatin tolerance phenotype indistinguishable from the knockout strain (Table 1) and a strong mutator phenotype, does not promote cisplatin-induced genome instability (Table 2). Neither an initial mutator nor a cisplatin-tolerant phenotype appears to be predictive of cisplatin-induced genome instability. Products of MNNG and 6-TG are suggested to create mismatches in DNA that are processed by MMR proteins. Lack of repair or the generation of single-strand breaks is one hypothesis to explain induced genome instability in the MMR-deficient cell lines for these types of damage. Our data demonstrate that the mutagenic effect of chemical exposure can be independent of an initial mutator phenotype, and thereby appears to be independent of the repair function. In the case of MMS, the mutagenic effect was attributed to the generation of and failure to processing of abasic sites in the MMR-defective background. The overall level of increase in the

mutator phenotype is comparable with the one we describe here (22). The mutation spectrum for MMS-induced genome instability identified primarily transversions. In contrast to MMS, cisplatin adducts are not subject to BER and are not expected to generate abasic sites. In addition, our data here identify primarily frameshift mutations at target sequences for cisplatin adduction (Table 3). Both, our study and the one by Glaab *et al.* (20) demonstrate that MMR-dependent cytotoxicity and mutagenicity can function independent from each other.

The *MSH2* mutations that promote induced genome instability are randomly distributed over the protein with no obvious 'clustering'. Hence, no predictions of their phenotypes can be made based on the nature of the mutation. We cannot exclude that the point mutations confer a weak or 'silent' mutator phenotype that will become more important under conditions when the mutational burden of the cell is increased. Recent studies suggested that the amount of DNA damage may be crucial in the response of cells harboring mutated MMR proteins (23). Mutant proteins may retain different capacities for the processing of different substrates, and a functional 'dose response' may be observed. However, MMR proteins are not suggested to be involved in the direct repair of cisplatin adducts (3). If the induced mutator phenotype of MSH2-Y109C and MSH2-L402F, respectively, was due to a saturation of the (reduced) repair capacity of these mutants after cisplatin exposure, the mutation spectrum should represent more random changes in the DNA. Though we observe some mutations in sequences outside of cisplatin targets, the overall mutation spectrum is shifted toward cisplatin target sequences (Table 3). This would suggest that the induced mutation rate is due to alterations specific for these sequences. Together with the cell survival studies (Table 1), our data suggest that the mutant proteins are unable to eliminate damaged cells via a cell death pathway, cisplatin adducts are retained in the DNA and can be subject for aberrant or translesion bypass. In addition, mutant proteins may have a 'hidden' defect in the antirecombination function, which under massive DNA damage would result in uncontrolled recombinational events (see below).

Owing to their ability to recognize DNA damage, MMR proteins may contribute to replication blockage and thereby initiate a cell death pathway (2). If the damage-sensing and signaling function is disrupted, increased translesion bypass by error-prone polymerases might occur. It was previously shown that the net bypass of cisplatin adducts is increased in MMR-deficient cell lines, and that this increased bypass is correlated with increased drug resistance in these cultures (24). This observation supports the hypothesis that mutations that alter the DNA damage-sensing ability of the protein will prevent blockage and promote increased lesion bypass. This switch would then result in an increase in the mutational burden in the cell rather than cell death. Importantly, we demonstrate here that single point mutations in MSH2, which are either derived from tumor tissue or were identified as polymorphisms, can influence and promote this effect. The mutation spectra (Table 3) are consistent with the insertion specificity of several of the translesion polymerases shown to process cisplatin-containing DNA damage (25,26). Polymerases β and η were shown to generate mutations that are identical to the ones determined here. The lack of an efficient MMR-dependent replication block might result in increased translesion bypass by error-prone polymerases and result in the increased mutational burden. The relatively small increase in the mutation rate may be due to the observation that polymerase η can bypass some cisplatin adducts in an error-free translesion synthesis step (27). In addition, lack of MMR proteins might provide additional access to the lesion for the correction of the damage by nucleotide excision repair (3). The functional implications of the missense mutations for which a cisplatin-induced mutator phenotype is observed support this hypothesis. These mutations affect regions involved in DNA binding or ATPase, or affect structurally important areas which may have consequences for DNA–ATP interactions.

In addition to translesion synthesis, recombination bypass of DNA damage can be initiated. It was previously shown that low doses of MNNG can induce intrachromosomal recombination. Zhang *et al.* (28) demonstrated that MMR proteins are required for the recombination event. The recombination event was found to be stimulated by futile repair cycles and hence requires functional repair activity (28). Our data demonstrate that the repair function of the proteins is not required for MMR-mediated cisplatin cytotoxicity. The induction of recombination by futile repair cycles of cisplatin-containing DNA is hence unlikely.

In response to cisplatin, MMR proteins exhibit an antirecombination effect; a defect in this mechanism contributes to increased damage tolerance in MMR-defective tumors (29). Increased recombinational bypass of cisplatin lesions results in elevated sister chromatid exchange. This was observed in MMR-deficient ovarian cancer cell lines (29). However, another study demonstrated that MMR proteins are not involved in the generation of chromosomal aberrations induced by cisplatin (30). No gross rearrangements are observed after the treatment of cells with cisplatin in our study (Table 3). In addition, cisplatin-induced recombination in a *mutS* strain is indistinguishable from the wild-type response (31). Until recently, no mutants had been identified that would separate the functional requirements for the MMR-dependent antirecombination effect from those in repair (32). However, recent studies investigating the MutS antirecombination effect on cisplatinated DNA in *Escherichia coli* (23) identified a MutS mutant that lost this antirecombination effect. The loss of this effect was associated with increased cisplatin resistance, but did not affect MNNG sensitivity or the repair function. Similarly, Durant *et al.* (29) demonstrated that MMR proteins in yeast inhibit the recombinational bypass of cisplatin adducts. Mutants in either *rad52* or *rad1* reverse the increased resistance found in the MMR-deficient strains. Loss of MMR proteins hence abolishes the antirecombination effect in yeast and results in increased RAD52/RAD1-dependent recombinational bypass of damage.

In the light of our results, mutations L402F and Y109C may exhibit a (partial) defect in the antirecombination function of the protein. With increasing concentrations of cisplatin damage, increased, aberrant recombinational bypass may be observed. If this was the case, an additional mutation in recombination would be predicted to reverse the effects observed for these point mutations. To address this question and elucidate the precise mechanism(s) behind the results described here, further biochemical and genetic analyses of the mutants have been initiated in the laboratory.

Different hypotheses have been put forward to address the mechanism of MMR-dependent damage response. Futile cycles of repair have primarily been suggested for the response to alkylation damage. This hypothesis is based on the observation that MMR-dependent cell cycle arrest to alkylation damage occurs after the second S phase. However, this observation is largely dependent on the dose of the chemotherapeutic agent (2,5). This mechanism requires the repair function of these proteins.

The concentration-dependent response to MNNG suggested the different participation of MMR proteins in response pathways to different levels of damage. At low dosage, $O^6$meG/T mismatches are generated, resulting in delayed response. Processing of these mismatches is absolutely dependent on MMR, which presumably initiates futile cycles of repair. At high doses of MNNG, DNA damage signaling becomes too rapid for replication-association, and cell cycle arrest is largely independent of MMR; however, cell killing remains to be dependent on MMR (33). This observation can be explained by either (i) MMR function as DNA damage sensors of high density $O^6$meG/C in DNA and direct signaling or (ii) processing of $O^6$meG/C initiated, unprocessed AP sites.

The hypothesis of direct signaling suggests a more direct involvement of MMR proteins in the induction and recruitment of proapoptotic proteins in response to DNA damage and the initiation of apoptosis. MMR proteins can contribute to replication fork blockage, or the block of transcription or repair. A prediction of this model is that the repair function of the proteins would not be required for the damage-induced cell death. Several pieces of evidence support this hypothesis for the processing of cisplatin. A repair-deficient mutation in *MSH6* was shown to retain wild-type response to several chemotherapeutic agents (15,34). R. Fishel suggested that the 'sliding clamp model' supports an ATP-dependent, direct signaling mechanism (35).

We [(4), F. Salsbury, J. Clodfelter, M. Gentry, T. Hollis and K. Drotschmann, manuscript in preparation)] and others (14) were able to identify separation-of-function mutations in *MSH2* that distinguish damage response from repair. The data presented here support this earlier observation. Here, we demonstrate that single point mutations in *MSH2* can affect repair or cisplatin sensitivity, but not necessarily both. This separation-of-function suggests a repair-independent mechanism of cell death signaling by MMR proteins. Whether this is true for different types of damage remains to be determined.

The *MSH2* missense mutations are homologous to mutations identified in tumors with suggestions for a causative effect on carcinogenesis. Other mutations were described as genetic variations in the general population. Our data demonstrate that such single point mutations can contribute to genome instability, a prerequisite for carcinogenesis, or increased cell survival after cisplatin exposure, a prerequisite for failure of chemotherapy and clonal expansion of mutant cells. The lack of a correlation between both phenotypes requires knowledge of the individual effects for applicability in the clinical setting, with the goal to improve the efficacy of chemotherapy. Clonal selection of clones that are not initial mutators will be observed after cisplatin treatment. If these clones promote a drug-induced mutator phenotype, even primary cancers that are not suggestive of MMR defects will be at risk for secondary carcinogenesis. The association of chemotolerance with cisplatin-induced genome instability that is promoted by these point mutations cautions about the treatment of tumors of certain genetic backgrounds with chemotherapeutic agents. Whether the effects are transferable to the treatment with chemotherapeutics other than cisplatin remains to be determined. Current literature suggests differential MMR-dependent cell death in dependence of the nature and dose of damage. A comparison to the response to other drugs is underway.

The observation that mutations that were described as genetic variations in the general population can promote increased cell survival after cisplatin exposure, suggests that the presence of such polymorphisms will modulate the individual's capacity to process exposure to DNA damage. This may be potentially important for the exposure to therapeutic, as well as environmental agents.

Taken together, we demonstrate here that tumor-derived, as well as polymorphic missense mutations in *MSH2* can alter cisplatin cytotoxicity and result in a tolerance phenotype that is independent of an effect on genome stability. This chemotolerance can be associated with a drug-induced mutator phenotype that significantly increases the initial mutation rate. Data presented here have important implications for the treatment of cancers with single point mutations in a MMR protein and the prevention of tolerance and secondary carcinogenesis. In addition, our results caution that the presence of polymorphic mutations with unknown functional consequences may significantly alter the response to clinical and environmental mutagens.

## REFERENCES

1. Jacob,S. and Praz,F. (2002) DNA MMR defects: role in colorectal carcinogenesis. *Biochimie*, **84**, 27–47.
2. Stojic,L., Brun,R. and Jiricny,J. (2004) Mismatch repair and DNA damage signalling. *DNA Repair*, **3**, 1091–1101.
3. Moggs,J.G., Szymkowski,D.E., Yamada,M., Karran,P. and Wood,R.D. (1997) Differential human nucleotide excision repair of paired and mispaired cisplatin-DNA adducts. *Nucleic Acids Res.*, **25**, 480–491.
4. Drotschmann,K., Topping,R.P., Clodfelter,J.E. and Salsbury,F.R. (2004) Mutations in the nucleotide-binding motif of MutS homologs uncouple cell death from cell survival. *DNA Repair*, **1**, 743–753.
5. Karran,P., Offman,J. and Bignami,M. (2003) Human mismatch repair, drug-induced DNA damage, and secondary cancer. *Biochimie*, **85**, 1149–1160.
6. Drotschmann,K., Clark,A.B. and Kunkel,T.A. (1999) Mutator phenotypes of common polymorphisms and missense mutations in *MSH2*. *Curr. Biol.*, **9**, 907–910.
7. Konishi,M., Kikuchi-Yanoshita,R., Tanaka,K., Muraoka,M., Onda,A., Okumura,Y., Kishi,N., Iwama,T., Mori,T., Koike,M. *et al.*, (1996) Molecular nature of colon tumors in hereditary nonpolyposis colon cancer, familial polyposis, and sporadic colon cancer. *Gastroenterology*, **111**, 307–317.
8. Mohrenweiser,H.W., Xi,T., Vazquez-Matias,J. and Jones,I.M. (2002) Identification of 127 amino acid substitution variants in screening

37 DNA repair genes in humans. *Cancer Epidemiol. Biomarkers Prev.*, **11**, 1054–1064.

9. Obmolova,G., Bam,C., Hsieh,P. and Yang,W. (2000) Crystal structures of MMR protein MutS and its complex with a substrate DNA. *Nature*, **407**, 703–710.

10. Leach,F.S., Nicolaides,N.C., Papadopoulos,N., Liu,B., Jen,J., Parsons,R., Peltomaki,P., Sistonen,P., Aaltonen,L.A. and Nystrom-Lahti,M. (1993) Mutations of a mutS homolog in hereditary nonpolyposis colorectal cancer. *Cell*, **75**, 1215–1225.

11. Liu,B., Parsons,R.E., Hamilton,S.R., Petersen,G.M., Lynch,H.T., Watson,P., Markowitz,S., Willson,J.K., Green,J. and de la Chapelle,A. (1994) hMSH2 mutations in hereditary nonpolyposis colorectal cancer kindreds. *Cancer Res.*, **54**, 4590–4594.

12. Orth,K., Hung,J., Gazdar,A., Bowcock,A., Mathis,J.M. and Sambrook,J. (1994) Genetic instability in human ovarian cancer cell lines. *Proc. Natl Acad. Sci. USA*, **91**, 9495–9499.

13. Marsischky,G.T., Filosi,N., Kane,M.F. and Kolodner,R. (1996) Redundancy of *Saccharomyces cerevisiae MSH3* and *MSH6* in *MSH2*-dependent mismatch repair. *Genes Dev.*, **10**, 407–420.

14. Lin,D.P., Wang,Y., Scherer,S.J., Clark,A.B., Yang,K., Avdievich,E., Jin,B., Werling,U., Parris,T., Kurihara,N. *et al.* (2004) An Msh2 point mutation uncouples DNA mismatch repair and apoptosis. *Cancer Res.*, **64**, 517–522.

15. Yang,G., Scherer,S.J., Shell,S.S., Yang,K., Kim,M., Lipkin,M., Kucherlapati,R., Kolodner,R.D. and Edelmann,W. (2004) Dominant effects of an Msh6 missense mutation on DNA repair and cancer susceptibility. *Cancer Cell*, **6**, 139–150.

16. Fink,D., Nebel,S., Aebi,S., Zheng,H., Cenni,B., Nehme,A., Christen,R.D. and Howell,S.B. (1996) The role of DNA MMR in platinum drug resistance. *Cancer Res.*, **56**, 4881–4886.

17. Aebi,S., Kurdi-Haidar,B., Gordon,R., Cenni,B., Zheng,H., Fink,D., Christen,R.D., Boland,C.R., Koi,M., Fishel,R. and Howell,S.B. (1996) Loss of DNA MMR in acquired resistance to cisplatin. *Cancer Res.*, **56**, 3087–3090.

18. Drummond,J.T., Anthoney,A., Brown,R. and Modrich,P. (1996) Cisplatin and adriamycin resistance are associated with MutLa and MMR deficiency in an ovarian tumor cell line. *J. Biol. Chem.*, **21**, 19645–19648.

19. Fink,D., Nebel,S., Norris,P.S., Baergen,R.N., Wilczynski,S.P., Costa,M.J., Haas,M., Cannistra,S.A. and Howell,S.B. (1998) Enrichment for DNA mismatch-repair deficient cells during treatment with cisplatin. *Intl. J. Cancer*, **77**, 741–746.

20. Glaab,W.E., Risinger,J.I., Umar,A., Barrett,J.C., Kunkel,T.A. and Tindall,K.R. (1998) Cellular resistance and hypermutability in mismatch repair-deficient human cancer cell lines following treatment with methyl methanesulfonate. *Mutat. Res.*, **398**, 197–207.

21. Glaab,W.E., Risinger,J.I., Umar,A., Barrett,J.C., Kunkel,T.A. and Tindall,K.R. (1998) Resistance to 6-thioguanine in mismatch repair-deficient human cancer cell lines correlates with an increase in induced mutations at the *HPRT* locus. *Carcinogenesis*, **19**, 1931–1937.

22. Glaab,W.E., Tindall,K.R. and Skopek,T.R. (1999) Specificity of mutations induced by methyl methanesulfonate in mismatch repair-deficient human cancer cell lines. *Mutat. Res.*, **427**, 67–78.

23. Calmann,M.A., Nowosielska,A. and Marinus,M.G. (2005) Separation of mutation avoidance and antirecombination functions in an *Escherichia coli mutS* mutant. *Nucleic Acids Res.*, **33**, 1193–1200.

24. Vaisman,A., Varchenko,M., Umar,A., Kunkel,T.A., Risinger,J.I., Barrett,J.C., Hamilton,T.C. and Chaney,S.G. (1998) The role of hMLH1, hMSH3, and hMSH6 defects in cisplatin and oxaliplatin resistance: correlation with replicative bypass of platinum–DNA adducts. *Cancer Res.*, **58**, 3579–3585.

25. Bassett,E., Vaisman,A., Tropea,K.A., McCall,C.M., Masutani,C., Hanaoka,F. and Chaney,S.G. (2002) Frameshifts and deletions during *in vitro* translesion synthesis past Pt–DNA adducts by DNA polymerases beta and eta. *DNA Repair*, **1**, 1003–1016.

26. Hoffmann,J.-S., Pillaire,M.-J., Garcia-Estefania,D., Lapalu,S. and Villani,G. (1996) *In vitro* bypass replication of the cisplatin-d(GpG) lesion by calf thymus DNA polymerase and human immunodeficiency virus type I reverse transcriptase is highly mutagenic. *J. Biol. Chem.*, **271**, 15386–15392.

27. Bassett,E., King,N.M., Bryant,M.F., Hector,S., Pendyala,L., Chaney,S.G. and Cordeiro-Stone,M. (2004) The role of DNA polymerase eta in translesion synthesis past platinum–DNA adducts in human fibroblasts. *Cancer Res.*, **64**, 6469–6475.

28. Zhang,H., Marra,G., Jiricny,J., Maher,V.M. and McCormick,J.J. (2000) Mismatch repair is required for O$^6$-methylguanine-induced homologous recombination in human fibroblasts. *Carcinogenesis*, **21**, 1639–1646.

29. Durant,S.T., Morris,M.M., Illand,M., McKay,H.J., McCormick,C., Hirst,G.L., Borts,R.H. and Brown,R. (1998) Dependence on *RAD52* and *RAD1* for anticancer drug resistance mediated by inactivation of mismatch repair genes. *Curr. Biol.*, **9**, 51–54.

30. Vernole,P., Pepponi,R. and D'Atri,S. (2003) Role of mismatch repair in the induction of chromosomal aberrations and sister chromatid exchanges in cells treated with different chemotherapeutic agents. *Cancer Chemother. Pharmacol.*, **52**, 185–192.

31. Nowosielska,A., Calmann,M.A., Zdraveski,Z., Essigmann,J.M. and Marinus,M.G. (2004) Spontaneous and cisplatin-induced recombination in *Escherichia coli*. *DNA Repair*, **3**, 719–728.

32. Junop,M.S., Yang,W., Funchain,P., Clendenin,W. and Miller,J.H. (2003) *In vitro* and *in vivo* studies of MutS, MutL and MutH mutants: correlation of mismatch repair and DNA recombination. *DNA repair*, **2**, 387–405.

33. Stojic,L., Cejka,P. and Jiricny,J. (2005) High doses of $S_N1$ type methylating agents activate DNA damage signaling cascades that are largely independent of mismatch repair. *Cell cycle*, **4**, e37–e41.

34. Kat,A., Thilly,W.G., Fang,W.-H., Longley,M.J., Li,G.-M. and Modrich,P. (1993) An alkylation-tolerant, mutator human cell line is deficient in strand-specific mismatch repair. *Proc. Natl Acad. Sci. USA*, **90**, 6424–6428.

35. Fishel,R. (1999) Signaling mismatch repair in cancer. *Nature Med.*, **5**, 1239–1241.

36. Lamers,M.H., Perrakis,A., Enzlin,J.H., Winterwerp,H.H.K., de Wind,N. and Sixma,T.K. (2000) The crystal structure of DNA MMR protein MutS binding to a GT mismatch. *Nature*, **407**, 711–717.