DNA mismatch repair and cancer;
Identifying phenotypic consequences of hMLH1 mutations

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I understand my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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I. INTRODUCTION

The function of DNA mismatch repair (MMR) is to maintain genomic stability by correcting errors that occur during DNA replication and by activating cellular checkpoints to signal apoptosis in cells following certain types of DNA damage. MMR also functions to suppress non-homologous recombination (3). Cells lacking MMR exhibit high rates of spontaneous mutation, including base substitutions and insertions or deletions within repetitive DNA (known as microsatellite instability or MSI), and resistance to cell killing by several classes of chemotherapeutic DNA-damaging agents. Both prokaryotic and eukaryotic organisms have maintained MMR systems throughout evolutionary history (12,13). Mutations occurring within MMR genes, specifically MLH1, PMS2, MSH2, and MSH6, have been associated with an increase in human cancer susceptibility, including Lynch Syndrome, also known as hereditary non-polyposis colorectal cancer (HNPCC) (1,4). Lynch Syndrome is characterized by a hereditary risk of early onset (between the ages of 30-40) colorectal and endometrial cancers. Approximately 70% of cases of this disease are associated with identifiable mutations of MLH1 or MSH2 (4). Inactivation of MLH1 caused by point mutation is directly linked to reduced mismatch repair activity and increased risk of Lynch Syndrome. The disease is inherited as autosomal dominant and mutations can be highly penetrant. Individuals with these mutations can expect a lifetime risk of colorectal cancer development upwards of 90%.

Two classes of heterodimeric molecules are conserved and perform the essential functions during MMR, MutS and MutL (14,15,16,17). The MutS heterodimers, composed of the MSH2 and MSH6 proteins, or MSH2 and MSH3, functions to recognize specific replication errors. MutS proteins
bind to base-base mismatches and nucleotide loops and identify DNA exhibiting replication errors (4). MLH1 dimerizes with PMS1, PMS2, or MLH3 to form different MutL complexes, which are necessary for identification of the DNA strand containing the replication error. This process allows for the excision of the mistake-containing DNA strand by exonucleases, and resynthesis of a new strand segment by DNA polymerases as depicted in Figure 1 (3,4). For my research I focused on the MLH1/PMS2 MutL complex.

**Figure 1: Mismatch repair mechanisms.** Step 1: Initial mismatch recognized by MutS heterodimer. Step 2: Strand with replication mistake specified by MutL heterodimer. Step 3: Exonuclease activity removes replication error. Step 4: DNA polymerases resynthesize DNA strand.

The purpose of this research project was to determine the phenotypic consequences of four different mutations in the MLH1 gene (A681T, V326A, R265C, and R265H) previously identified in human cancers. Previous studies have been inconclusive as to whether these alleles are neutral polymorphisms or pathogenic mutations (2,5,6). Conclusive identification of defects in MMR activity associated with particular hMLH1 mutations should help determine the relative pathogenicity of the
mutations. Several different approaches to assess pathogenicity have been described in literature. In one approach, human cell lines (MLH1-deficient 293T cells) were transfected to express the hMLH1 variants R265C, R265H, and V326A (6). These studies indicated that each mutant was MMR competent, determined by the restoration of MMR activity in an in-vitro assay using cellular extracts of variant-expressing cells. Western Blot protein analysis of expression of these mutants indicated MLH1 and PMS2 levels similar to that of the hMLH1 wild-type control. However, studies of the same mutations in yeast cells (Saccharomyces cerevisiae) have concluded that these three mutations exhibit a moderate, but significant, decrease in MMR activity. The yeast studies detected increased mutation rates when the mutations were introduced into hybrid MLH1 genes containing portions of human MLH1 substituted for the homologous yeast MLH1 domains. The hMLH1 mutant A681T has also been tested in yeast assays but not previously in mammalian in-vitro studies. The result of the yeast assays suggested a significant decrease in MMR efficiency (2,5). The MLH1 domain map is listed in Figure 2.

![Figure 2. Distribution of potentially pathogenic mutations in hMLH1.](image)

Mutations identified in human cancers are indicated by vertical lines above a schematic representation of hMLH1. Approximate boundaries of various functional domains are indicated. Locations of selected variants (A681T, V326A, R265C/H) within the hMLH1 gene are identified. A681T is located in the PMS2 binding region. V326A and R265C/H are located within the N-terminal domain, necessary for ATP hydrolysis, ssDNA binding, and dimerization of the N-termini.
The goal of the current project was to determine the relative pathogenicity of particular hMLH1 gene mutants by measuring MMR activity (specifically spontaneous mutation rate and sensitivity to chemotherapeutics) in cells expressing each mutant. Gene variants are likely pathogenic if associated with measurable MMR deficiencies. I used mammalian cellular assays, specifically mouse embryonic fibroblasts (MEF), engineered to express the mutant proteins. Using MEF cellular assays provides an advantage over previous studies (using in-vitro biochemical assays or yeast model systems) in that the effects of mutation in MLH1 on both mutation rates and responses to DNA damage in living mammalian cells can be measured. Previous work done by this lab has determined that human MLH1 can complement MLH1 deficient MEF cells, reducing spontaneous mutation rates and restoring normal cellular responses to DNA damage (1). My specific hypothesis is that pathogenic mutations in MLH1 will result in measurable decreases in MMR activity detected as: i) an inability to form a stable complex with PMS2, or ii) an increased spontaneous mutation rate, or iii) decreased apoptosis and resistance to chemotherapeutic agents such as 6-Thioguanine (6-TG). Neutral polymorphisms, by contrast, should exhibit MMR behavior similar to that of wild type MLH1. These studies to identify pathogenic consequences of Hmlh1 gene variants will provide a foundation for future biochemical analyses of the mechanisms that cause MMR deficiency.
II. MATERIALS AND METHODS

Cell Culture

Mouse embryonic fibroblasts (MEF) were grown in 10% cell growth media: DMEM (Cell Gro) with 10-15% defined calf serum (Hyclone), 1x Non-essential amino acids (Cell Gro), 5 mg/ml Gentamycin Sulfate (Mediatech). Cell growth media was stored at 4°C under low light conditions. Sterile technique was required for all tissue culture to avoid outside contamination and cross-contamination of individual cell lines.

Frozen cell lines are collected in labeled cryovials (Nunc) and stored in a liquid nitrogen storage tank. Upon use, cell lines were thawed rapidly in 37°C water bath and 10% media warmed to 37°C was added drop-wise to prevent temperature shock. Cell samples were then transferred to a T75 flask containing 15 ml cell growth media. Cell cultures are grown in a 5% CO₂ incubator at 38°C. Cells were maintained as subconfluent cultures.

Cell Harvesting

Prior to harvesting, cell growth media was aspirated from the tissue culture flasks with a Pasteur pipette and vacuum suction. The cells were then rinsed once with 1x PBS (Phosphate Buffered Salt solution) to remove remaining serum, and the PBS was then aspirated from the flask. Cells were harvested with Trypsin (Sigma): Porcine Trypsin (0.5 g/L), NaCl (8 g/L), KCl (0.4 g/L), Dextrose (1 g/L), NaHCO₃ (0.58 g/L), free acid EDTA (0.2 g/L), phenol red (4 mg/L); volume of trypsin was dependant on the size of the flask (approximately 2 ml for T75). Trypsinization was limited to 1-2 minutes and flasks were cuffed to loosen the cells from the flask surface. Once detached, growth media (a
volume at least equal to or greater than the trypsin) was added to the cells to halt trypsinization. Overexposure to trypsin results in clumping of cells and less than ideal population density. The sides of the flasks were rinsed with additional media to ensure all of the cells were harvested. Cells were then transferred to a new flask for growth. Cell passage for continued growth required specific dilutions depending on the desired growth period, ranging from 1:2 to 1:50. The approximate doubling time of MEFs is 16 hours.

**Generation of Frozen Cell Stocks**

In order to maintain available stocks of each cell line for future use, it was necessary to freeze cells. Freezing media consisted of 10% DMSO (Sigma) in 10% complete media. Once cells were harvested, they were spun down in a centrifuge at 2000 rpm for four minutes. The cell media was aspirated and discarded, and the cells were resuspended in 10% freezing media. The number of cell aliquots was determined by size of the flask from which the cells were harvested; a T75 flask will yield enough cells for two 1 ml aliquots. Harvested cells were transferred to labeled cryovials for storage. The cells were stored at -80°C for short-term storage or put into a liquid nitrogen storage tank for long-term storage.

**Plasmid DNA Preparation**

To linearize plasmids prior to transfection, restriction digests were performed on expression plasmids containing cDNAs for the hMLH1 variants A681T, R265C, R265H, and V326A. The restriction digest solution was prepared with 20 μg of DNA, 15 μL 10X Y' Tango buffer, 4 μL (10 units p/μL) XmnI enzyme, and the remaining volume of water such that the total
solution volume was 150 µL. Digests were incubated at 37°C water bath for 1-2 hours. Samples were stored at -4°C until needed.

Samples were checked for linearization with analytical gel electrophoresis with 1% agarose mini-gels. The 30 ml gels were made of 0.3g agarose mixed with 30 ml 1X TAE (2% 50X TAE, 98% H2O). The gel solution was heated, mixed, and 1 µL of 10 mg/ml EtBr was added for visualization of DNA. Digested samples were prepared such that 250-300 ng of the DNA sample was loaded into the gel. The samples were mixed with 1.5 µL 6x buffer and a volume of water to make a final sample volume of 9-12 µL. Each sample was loaded into a separate well of the gel and gels were run for 20 minutes at 200V. Negatively charged DNA segments move with the electrical current and are separated by size. Digested DNA will move through the gel matrix slower than uncut supercoiled plasmids.

Once the plasmids had been confirmed for linearization, phenol extractions were performed to isolate the DNA from the restriction digest solution. Digest samples were combined with an equal volume of phenol (approximately 140 µL), vortexed, and centrifuged for three minutes. The aqueous top layer containing the DNA was collected into a new microtube. Back extractions were performed on the digest solution with H2O or buffer (1:2 volume). DNA was recovered by ethanol precipitation: 1/10 vol. NaOAc 3M, 5.2 pH and 2.5 vol. ethanol was added and the solutions were incubated 10-20 minutes on ice. Samples were centrifuged for 10-15 minutes at 14000 RPM. The aqueous solution was aspirated and the DNA was rinsed with 1 ml of 70% ethanol. Samples were vortexed and centrifuged for 5 minutes. The ethanol was aspirated and the supernatant was resuspended in 25 µL of 1X PBS. Samples were stored at -4°C.
Generation of MEF cell lines expressing hMLH1 variants

Linear DNA samples (10-20 μg) of MLH1 plasmid DNA for each variant was transfected into MLH1+ MEF cells. Approximately 1 x 10⁷ mouse cells were electroporated with 10-50 μg/ml of linearized DNA. Transfected cells were plated (3-5 x 10⁵ per p150 mm² dish) with 25 ml of 15% complete media. Twenty-four hours after transfection, 5 ml G418 (400 μg/ml) was added for selection against non-MLH1 (specifically the Neomycin resistance gene PDM1) expressing cells. G418-resistant colonies were isolated 10-12 days following transfection by using glass cloning rings and trypsinization. Individual colonies were cultured, and cell stocks of each cell lines A681T, R265C, R265H, and V326A were frozen for future use.

Subcloning for Stable Expression

To determine the relative expression of hMLH1 variants in individual cells in G418-resistant cultures, 100 cells of each culture were plated in p150 (30ml) dishes for colony growth. For each cell line, 10-12 colonies were isolated using glass cloning rings, and plated into 6-well dishes in 15% media. Individual subcloned-colonies were then harvested for determination of protein expression.

Whole Cell Lysates

Whole cell lysates were made for analysis of protein expression. Complete lysis buffer was made by adding a protease inhibitor (Complete Plus, EDTA-free) tablet to 2x lysis buffer: 100 mM Tris-Cl ph 6.8, 4% (w/v) SDS (Fisher), 20% (v/v) Glycerol (Acros), 200 mM DTT (Fisher). Subcloned cell lines were harvested from six-well culture plates using
trypsin. Cell samples were kept on ice during all procedural transitions. Each cell line was rinsed twice with PBS. The samples were then transferred to heat resistant microtubes and mixed with 80 μL PBS and 80 μL 2x lysis buffer with protease inhibitor. Lysates were boiled for five minutes at 100°C and stored at -80°C until use.

**SDS-PAGE & Western Blot**

Whole cell lysates are combined with 2x loading buffer (100 mM Tris-C1 (pH 6.8), 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol) or 6x (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (v/v) glycerol in H2O) and heated at 95°C for 5 minutes prior to SDS-PAGE. SDS-PAGE samples were loaded into 18 or 26 welled pre-cast Criterion gels (4-12% Bis-Tris) and inundated with 1x MOPS (95% H2O, 5% 10X MOPS: 0.2 M MOPS (pH 7), 20 mM sodium acetate, 10 mM EDTA (pH8)). The lysate sample size loaded was approximately 6 μL with 6 μL of loading buffer. Gels were run at 200V for 55 minutes. Proteins are separated on the gel by their molecular mass, and migration can be compared to a standard ladder. Each protein band migrates approximately to the distance: MSH6 (160 kD), PMS2 (110 kD), and MLH1 (84kD).

PVDF membranes (Immobilon) were treated with methanol and 1X transfer buffer (80% H2O, 10% methanol, 10% 10X transfer buffer: 83% glycine, 17% tris-base). Proteins were transferred to the treated membranes at 100V for 30-60 minutes. The membranes were blocked after transfer with %5 Blotto (5% powdered milk, 95% TBST). Membranes were then exposed to anti-MLH1 (1:1000), anti-PMS2 (1:1000), and anti-MSH6 (1:2500) primary antibodies in 4 mL of %5 Blotto for a minimum of 90 minutes. Residual antibody was washed from the membrane with four rinses of 0.1% TBST (1% Tween, 99% TBS (in H2O: 5.33 g/L NaCl, 0.13 g/L KCl, 2
g/L (tris-base). Secondary antibodies consist of 9 mL 5% Blotto, 0.6 μL HRP conjugate, and 1.2 μL goat-anti-mouse IgG, and were applied to PVDF membranes for 60 minutes and rewashed. Protein-bound antibodies were visualized by chemiluminescence using an imaging system (Kodak IS440) that quantifies labeled antibody signals by the intensity of representative bands. The chemiluminescent solution (50% luminol, 50% peroxide) is oxidized by the HRP conjugate. Specifically, a reaction catalyzed by HRP and peroxide yields an oxidized form of luminol that releases light. The intensity of the light signal is relative to the amount of secondary antibody bound to the specific protein of interest.

**Forward Mutation Rate Assay**

The spontaneous base substitution mutation rate in the R265H-11,12 cell lines was determined by using fluctuation analysis to measure forward mutation to ouabain resistance (12). Individual subcultures were initiated with 2000 cells each, and expanded to approximately 4-5 x 10^6 cells. Harvested cells were counted and serial dilutions were performed such that 300 cells from each cell line could be plated into two p100 dishes to determine plating efficiency. Remaining cells (0.8-1.0 x 10^6 cells p/ p150 dish) were cultured with 1 mM ouabain (Sigma) in 15% complete media for 12-14 days (re-fed after 5-7 days) depending on growth rate. Colonies were fixed and stained with a 1X ethanol and methylene blue solution, such that the cells were entirely immersed in the stain, prior to counting. The plating efficiency was determined by counting surviving cell colonies on p100 dishes relative to the number of cells plated.

The calculation of forward mutation rates based on fluctuation analyses of has been previously established by Luria and Delbruck, and
Capizzi and Jameson. Eric Bronner (Liskay Lab, OHSU 1997) produced a computational program called Chipmunk BASIC, -Rate(cb) (www.nicholson.com/rhn/basic) that performs the statistical analysis as developed by Luria/Delbruck and Capizzi/Jameson. Specifically, the program uses the total number of cells (Nt) plated (x10^6), the total number of subcultures examined (C), and the average number of ouabain resistant clones following continuous exposure (R) to determine the approximate rate of mutation per locus per cell division (a) as determined by the following equation:

\[ R = a \frac{N_t \ln(C \cdot a \cdot N_t)}{N_t} \]

The program solves for a value of a by testing a large range of numbers until a rate is found that corresponds to the other variables within the equation.

Chemotherapeutic Resistance

MEF lines expressing R265H(-11 and -12), and control cell lines WT22 (MLH1⁺) and CMV2 (MLH1⁻) were harvested from subconfluent cultures (60-80%), counted and plated in p100 mm plates at a density of 300 cells per plate with 10 ml of 15% complete media. After 24 hours each cell line was exposed to three doses of 6-Thioguanine (Sigma) in 15% complete media (0, 0.3, 1, and 3 μM). The cell lines were grown for 8-10 days to allow for colony formation. Colonies were fixed and stained with ethanol and methylene blue prior to quantification.
III. RESULTS

A. Generation of variant-expressing cell lines

To determine the phenotypic consequences of specific mutations in hMLH1, mouse embryonic fibroblasts (MEF) were engineered to express the hMLH1 variants A681T, V326A, R265C, and R265H, by transfection and selection for integration of expression vectors. Each mutant cell line was screened for expression of MMR proteins MLH1, PMS2, and MSH6. Protein expression was compared to MLH1+/− controls for quantitative analysis.

Ai. Preparation of DNA:

Restriction endonuclease digestion with the XmnI enzyme was used to linearize hMLH1 cDNA-containing plasmids prior to transfection, in order for the DNA to be effectively integrated into the genome. Following digestion the DNA samples were analyzed by agarose gel electrophoresis.

Linear fragments migrated approximately with the 9 kB marker, as compared to supercoiled circular DNA control plasmids (WT) which typically migrate with the 4-5 kB marker fragments (Figure 5). The hMLH1 expression vector is 8.9 kB and is composed of a 6.6 kB vector with a 2.3 kB hMLH1 insert.

Phenol extraction was used to purify the plasmid DNA from the restriction digest enzymes in order to prepare for transfection. After performing the phenol extraction for each sample, I used analytical agarose gel electrophoresis to confirm sufficient recovery of linearized DNA. Samples A681T, R625H, and V326A were sufficiently recovered (Figure 4). However, recovery of R265C was somewhat lower and new digests had to
be made. The subsequent recovery rate was sufficient for transfection; Figure 5 compares R265C samples to wild-type.

Transfection of MLH1 deficient MEFs:

Mlh1-deficient mouse embryonic fibroblasts (MEF) were transfected with linearized expression plasmid for the hMLH1 variants listed above. Following transfection, the MEF cells were plated out for selection of
G418-resistant clones. The cells were grown in cell growth media containing G418 sulfate to select against any cells not containing the PDM1 (Neomycin resistance) gene present on the plasmid with the hMLH1 cDNA.

Each dish contained approximately 20-25 colonies at the time of harvesting. 10-12 cell colonies were isolated from each transfection and subcloned cell lines were grown for each hMLH1 variant.

**Analysis of hMLH1 variant protein expression:**

Western Blot analysis of whole cell protein lysates was used (Figure 6) to measure stable protein expression in each sub-cloned cell line. Specifically hMLH1 and mouse PMS2 expression was analyzed. Protein expression was quantified based on raw protein expression as well as expression relative to endogenous mouse MSH6 levels. MSH6 expression is not affected by MLH1 gene modifications and protein expression should be relatively stable in all cells. Therefore any variation in MSH6 is reflective of the amount of lysate sample loaded into the running gel. Specific bands occur at: MSH6 (160 kD), PMS2 (110 kD), and MLH1 (84 kD).

By comparing MLH1 vs. MSH6 and PMS2 vs. MSH6 of four subclones of each mutant, adjusted values of expression based on the variable amount of sample loaded were derived. Samples from four G418-resistant clones of each variant-expressing line were compared to two hMLH1-expressing MEF lines, WT-22 (relatively good expression) and WT-12 (relatively weak expression). Cell lines expressing the hMLH1 variants V362A, and R265C exhibited low overall MLH1 and PMS2 expression (Table 1). Mutant A681T was not tested due to low expression determined by previous Buermeyer lab experiments.
Figure 6. Western Blot 11/05/2004. MMR protein expression for variants (V326A, R265C, and R265H).

Table 1: Quantification of MMR protein expression based on Western blot results. a) Number of mutant subclones expressing hMLH1. b) Comparison of adjusted average values of hMLH1 expression each for mutant cell line vs. MLH1+ control.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>a) hMLH1 present</th>
<th>b) hMLH1: variant/WT22</th>
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<tr>
<td>V326A</td>
<td>4/4</td>
<td>Low (21%)</td>
</tr>
<tr>
<td>R265C</td>
<td>4/4</td>
<td>Low (20%)</td>
</tr>
<tr>
<td>R265H</td>
<td>4/4</td>
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B. Analysis of MMR activity in R265H-expressing cells

Subcloning

Following transfection of MEFs, some lines showed variable levels of MMR protein expression among individual cells. To determine if expression of R265H in two independently derived MEF lines was uniform in the cell population, protein expression was analyzed in subclones of each
cell line (R265H 11,12). The mutant expressing cell line R265H was chosen for further analysis because this variant expressed MMR proteins better than the other mutant cell lines. R265H MEFs were sub-cloned to verify the creation of a cell line with stable MLH1 expression, indicated by all cells within the population expressing MMR proteins at a similar level. Based on the amount of protein expression quantified in Figure 7, two parallel R265H cultures (11 & 12) were plated out and 10-12 colonies per cell line were isolated and cultured in parallel. Each colony was grown individually into confluent cultures and whole cell lysates were made for protein analysis.

The Western Blot, illustrated Figure 7, indicates that MLH1 signals were uniform based on the amount of sample loaded (determined by MSH6 signal). From this data we can qualitatively say that both cell lines have stable expression of MLH1 and PMS2 at levels that support the formation of the MutL complex required for MMR. Strong MLH1 and PMS2 expression indicates that MLH1 has stabilized PMS2, and the level of expression was similar to that present in known wild-type (MMR competent) cells. Although a slight fading on the membrane of the R265H-11 portion is noted, the expression of MMR proteins still remains uniform.
Figure 7. MMR protein expression of R265H-11,12 sub-clones. Controls
CMV2 (MLH1−) and WT12 (slightly MLH1 deficient) are used for comparison.
WT22 (MLH1+) control lysate is degraded.

Functional analysis of MLH1 mutation R265H

Spontaneous Mutation Rate Determination

In order to measure the forward mutation rate of mutant expressing
cell lines, I conducted a fluctuation analysis measuring forward mutation
to ouabain resistance. Exposure to ouabain causes a disruption in the
Na+/K- ATPase pump in the cell membrane which results in cell death.
Cells that have experienced point mutations in the gene for the \( \square \)-subunit
of the pump can be resistant to ouabain. Cultures of MMR deficient cells
typically produce mutants at 20 times the rate of cultures with
functional MMR. The foundations for the statistical analysis of this
experiment have been previously determined by Luria and Delbruck 1943,
Lea and Coulson 1949, and Capizzi and Jameson 1973. The fluctuation analysis involved a period of cell growth wherein a limited number of ouabain sensitive MEFs were expanded to a culture size of approximately 5x10^6 cells, after which the number of ouabain-resistant mutant cells was determined by selection in 1mM ouabain. After exposure to ouabain, resistant cells from multiple parallel cultures are identified and stained for quantitative analysis (Table 2).

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Table 2. Raw data of ouabain colony counts and quantification of efficiency plates, adjusted colony survival, and individual plate counts.

The number of colonies surviving ouabain was adjusted based on the standard colony formation rate as determined by the efficiency plates. Three efficiency plates were generated for a culture of R265H-12 separate from the fluctuation analysis. Efficiency was determined by comparing the total number of plated cells with the number of resulting colonies. The efficiency plates averaged 43 colonies per plate yielding a colony forming rate of approximately 14%. Based on an estimated plating efficiency of 14%, the mutation rate for R265H-expressing cells was calculated and compared to previously published mutation rates of MLH1-
expressing and MLH1-deficient cell lines (1). Previous studies have
indicated that hMLH1+ expressing cells demonstrate forward mutation rates
of $2 \times 10^{-7}$, whereas the rate in hMLH1- deficient cells is $6 \times 10^{-6}$. The
adjusted mutation rates of the R265H-12 mutant cells were $7.46 \times 10^{-7}$
(14% efficiency), and $4.01 \times 10^{-7}$ (35% hypothetical standard efficiency).
Both values are slightly higher than wild-type cell rates, although still
far below the rate of known mutators.

**Cytotoxicity Resistance**

Chemotherapeutic resistance was tested by cell survival following
exposure to 6-thioguanine, as in previous studies (1,19). MMR deficient
cell lines have been shown to exhibit a significant decrease in
cytotoxicity upon exposure to chemotherapeutic drugs. Cytotoxicity is
determined by the ability of MMR proteins to signal apoptosis after
chemotherapeutic exposure. A direct correlation between lowered MLH1
expression and decreased apoptosis signaling following exposure to 6-TG
has been made. This is of particular interest because some cancers
caused by hMLH1 mutations can demonstrate a decreased response to
chemotherapeutic treatment. In order to determine the effect of the
R265H mutation on the ability of MMR to trigger apoptosis following
exposure to chemotherapeutic drugs, I measured cell survival of R265H-
expressing cells following exposure to 6-TG using a colony forming assay.
Cell lines, including a MLH1+ control (WT-22), a hMLH1- control (CMV2),
and one hMLH1 mutant represented by two independent subcloned lines
(R265H 11,12) were tested for cytotoxic resistance by exposing the cells
to varying dosages of 6-TG over an eight day period. Surviving colonies
were counted and compared to the original number of plated cells (300) to
derive a plating efficiency. The mean efficiency for the 0 dose plates
was used as a standard and represented 100% survival. The standard deviation for each efficiency set was determined and used to calculate the normal error. The survival data for each cell line was compiled and plotted on a logarithmic scale (Figure 8).

All of the cell lines exhibited significant sensitivity to 6-TG such that nearly complete cell death occurred at the 3 μM dosage. The relative survival rates are illustrated in Figure 8 and the raw data is compiled in Table 3. From these results it appears that the cell lines expressing the hMLH1 mutant R265H exhibit survival levels similar to that of CMV2 (MLH1-') which was greater than that of WT-22 (MLH1'). The differences were not apparent with the 0.3 and 1.0 μM doses.

![6-TG-Induced Cytotoxicity in MEFs (A)](image)

![6-TG-Induced Cytotoxicity in MEFs (B)](image)

**Figure 8.** Relative survival rates of R265H-11,12 compared to positive control WT-22, and negative controls CMV2 and WT-12. (A) Logarithmic scale. (B) Linear scale.
Table 3. Survival rates of R265H-12 colonies under varying levels of 6-TG exposure. Percentage is calculated by setting zero dose plates as 100% survival.

<table>
<thead>
<tr>
<th>Dose</th>
<th>WT-22</th>
<th>CMV2</th>
<th>R265H11</th>
<th>R265H12</th>
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</thead>
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<tr>
<td>0</td>
<td>100.00</td>
<td>100.00</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.3</td>
<td>20.70</td>
<td>74.32</td>
<td>144.21</td>
<td>46.14</td>
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<td>8.90</td>
<td>37.67</td>
<td>41.05</td>
<td>24.44</td>
</tr>
<tr>
<td>3</td>
<td>0.62</td>
<td>3.42</td>
<td>7.37</td>
<td>1.11</td>
</tr>
</tbody>
</table>
IV. DISCUSSION

Pathogenicity of A681T, R265C, and V326A

In order to determine the pathogenicity of the hMLH1 mutants A681T, R265C, and V326A, I created four mutant-expressing MEF cell lines through stable transfection. MMR protein expression was quantified by Western Blot analysis. Mutants A618T, R265C, and V326A exhibited relatively low MLH1 and PMS2 expression compared to wild-type expressing cells. Due to time constraints, further analysis of these mutants was not possible.

The results of my research, and other Buermeyer lab results (specifically A681T, unpublished), suggest that hMLH1 mutants A681T and V326A are expressed poorly compared to wild-type MLH1 due to instability in MLH1. These results suggest that these particular mutations cause a destabilization of the MLH1 protein. This destabilization can alter its ability to form the MutL dimer with PMS2 and therefore decrease MMR efficiency. Thus, the results are consistent with previous studies that suggest these mutations are pathogenic. It should be noted, however, that only four subclones for each of these mutants were tested for protein expression and protein comparison of these three mutants was only conducted once. More definitive research would require additional subcloning to verify stable or unstable expression of MMR proteins.

Pathogenicity of R265H

In addition to protein expression analysis, which was similar to that of the MLH1 wild-type control, the mutant R265H was tested for an increase (compared to wild-type) in spontaneous base substitution mutation rate by fluctuation analysis. The results of this assay
provided a forward mutation rate of $4-7 \times 10^7$, which is slightly higher than MLH1+ cell lines ($\leq 2 \times 10^7$). This mutant was also tested for chemotherapeutic drug resistance and cytotoxicity following exposure to 6-TG. Data from this experiment shows that R265H expressing mutant cell lines exhibit decreased cytotoxicity, similar to that of MLH1- cells (CMV2).

Based on protein quantification alone, R265H would seem to have sufficient MLH1 and PMS2 expression for the formation of the MutL complex and therefore functional MMR. However, the cytotoxicity and fluctuation analysis assays provide further insight into the MMR efficiency of this mutant. The R265H-12 mutant cell line exhibits an increased forward mutation rate compared to MMR functional cell lines. This suggests that this cell line has decreased MMR efficiency compared to wild-type. There were, however, some technical difficulties experienced during this experiment. The efficiency plates of the ouabain assay were not run concurrently with the ouabain test plates; ideally all cell lines should be grown under the exact same conditions. The overall plating efficiency was lower than average (14% vs. 35%-40%). If the test plates grew at the same rate, then the number of surviving colonies may be below average. This could cause the forward mutation rate for each mutant to appear lower than the actual rate. If this is the case, then the mutation rate of R265H may be closer to the known mutator rate of $6 \times 10^{-6}$.

Furthermore, only the plates from one subclone (R265H-12) were able to be counted. The plates from R265H-11 showed an excessive amount of background cell spotting, and could not be counted with any confidence. The spotting may have been a result of clumped cells being plated for ouabain exposure. Clumping may have occurred because of over-trypsinization while harvesting, or overly confluent cells being passed
from previous harvests. This experiment should be repeated with at least two concurrently exposed cell lines.

Following treatment with 6-TG the hMLH1 mutant expressing cell line exhibited a level of cytotoxicity similar to that of the MLH1+ control (CMV2). The results of this assay further indicate a reduction in MMR activity in the R265H cell line. The differentiation between cell lines is not very large (approximately 30% at 0.3 mM and 1 mM doses), but the experiment was successful with no known technical difficulties. The calculated survival rates for the 3 µM dose exhibit the highest level of error because these plates had the fewest number of colonies for all five of the cell lines. It may be helpful for future experiments to initially plate more cells, perhaps 500 or more, to increase the amount of variation in survival between each cell line. The result of this assay indicates a decrease in MMR activity in the R265H mutant cell line represented by a lower rate of cytotoxicity, similar to that of MLH1+ cells.

Together, the results of the ouabain fluctuation assay and 6-TG cytotoxicity assay indicate that the mutant R265H has decreased MMR capability. This data also supports previous yeast studies and categorizes this mutant as having a moderate decrease in MMR efficiency. Therefore I can conclude that these hMLH1 mutations are pathogenic. The significance of these results is limited, however, since each assay was only performed once.

The applied genetic applications of this research are significant and related to the risk of development of Lynch Syndrome. As previously mentioned, the mutations A681T, R265C, R265H, and V326A have previously been identified in human colon cancers and likely pathogenic. Some mutations with only a moderate decrease in MMR, such as R265H, may also
result in an elevated risk of cancer development, however such risk is likely to be lower than full loss-of-function alleles. These individuals would likely benefit from regular colon screens and healthy lifestyle modifications designed to limit the development of cancer. Furthermore, the knowledge that a particular mutation is resistant to chemotherapeutic drugs is essential for effective treatment following cancer development. Future work with these mutants should include further measurements of the stability of R265C and V326A. Repeated ouabain and 6-TG assays should be performed on R265C, R265H, and V326A for definitive analysis of MMR activity. The Buermeyer lab is already collaborating with an X-Ray crystallography lab to determine the structure of the MLH1 and PMS2 proteins. This research would be extremely useful in analyzing the interaction domains of the MutL complex, and the effect of specific protein modifications arising from hMLH1 mutations. Finally, transgenic mice could be developed to quantify the cancer risk associated with these hMLH1 mutants by monitoring the development of colorectal cancers.


