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The effect of DNA mismatch repair deficiency in a MSH2 null individual

by

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ABSTRACT

The mismatch repair system (MMR) is important in repairing post-translational DNA adducts. Individuals inheriting mutations in MMR genes are at high risk of developing hereditary non-polyposis colorectal cancer (HNPCC). Recently, rare individuals with homozygous mutations in one of the MMR genes have been identified. The first MSH2 deficient individual was identified in our laboratory. Non-tumour tissue from this individual was tested for microsatellite instability (MSI) in ten genes with coding microsatellites that were previously identified to be mutated in HNPCC tumours. However, the non-tumour tissue was MSI-stable, possibly due to a difference in target genes mutated in non-colon tissue in the absence of MMR or insufficient sensitivity in the assay.

A cell line established from non-tumour lymphoblasts from the MSH2 null individual was used to demonstrate that MSH2 deficiency leads to decreased G2/M arrest following UVB radiation. The data contribute to our understanding of how MMR contributes to UVB-induced tumorigenesis.

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6-4 PP	6-4 photoproduct
6-TG	6-thioguanine
ALL	acute lymphocytic leukemia
bp	base pair
BLM	Bloom's syndrome protein
CIN	chromosomal instability
Cisplatin	*cis*-diamminedichloroplatinum(II)
CPD	cyclobutane pyrimidine dimer
EBV	Epstein Barr virus
ES	embryonic stem
GGR	global genomic repair
HNPCC	Hereditary non-polyposis colorectal cancer
IR	ionizing radiation
kDa	kilodalton
MSI	microsatellite instability
MMR	mismatch repair
MEF	mouse embryonic fibroblast
NF1	Neurofibromatosis type 1
MNNG	N-methyl-N-nitro-N-nitrosoguanidine
NER	nucleotide excision repair
O^6-meG	O^6-methylguanine
S	serine
TMZ	temozolomide
T	threonine
TCR	transcription coupled repair
Y	tyrosine
UV	ultraviolet

Chapter 1 ♦ Literature Review

Introduction

Discovering the mechanisms underlying tumorigenesis is important in developing earlier or more sensitive methods of tumour detection and enhancing anti-cancer therapy treatments. Cells become tumorigenic when mutations occur in key regulator genes and control over normal cell division is lost. Everyday our cells are subject to DNA damage that may result in mutations. DNA damage can occur through normal cellular processes such as the production of free radical oxygen species (endogenous), or the exposure of mutagenic compounds (exogenous). To combat the acquisition of these mutations and to prevent the transmission to daughter cells the cell engages in DNA repair mechanisms to repair the mutations, cell cycle arrest to assist in allowing for repair or, if the damage is too extensive, initiates self-programmed cell death (apoptosis). There are five major repair pathways that exist in the mammalian cell, nucleotide excision repair, base excision repair, non-homologous recombination repair, homologous recombination repair and mismatch repair. Each pathway is important in the repair of a specific set of DNA lesions, produced through endogenous or exogenous agents.

DNA mismatch repair

The role of the DNA mismatch repair (MMR) system is to increase DNA replication fidelity by repairing DNA mismatches and insertion/deletion loops acquired during DNA replication. This process is highly conserved from *Escherichia coli*, *Saccharomyces cerevisiae* to mammals. Cells lacking MMR demonstrate a 100-1000 fold increase in mutation frequency associated with a high degree of microsatellite instability (MSI) (Modrich & Lahue, 1996). Individuals with germline mutations in one of the MMR genes develop the cancer syndrome hereditary non-polyposis colorectal cancer (HNPCC). HNPCC will be discussed in detail later in this chapter. Four genes are isolated in the functional MMR system in *E. coli*, MutS, MutL, MutH, MutU (also known as UvrD and DNA helicase II). Orthologous genes are found in the eukaryotic system for MutS, MutL and MutU, no orthologue is identified for the MutH protein (Table 1-1).

Table 1-1: Mismatch repair proteins

E.coli	*S. cerevisiae*	Mammalian	Function in mammalian system
MutS	Msh1p	-	suspected mitochondrial DNA repair
			loop mismatch repair (complex with MSH3)
	Msh2p	MSH2	base mismatch repair (complex with MSH6)
	Msh3p	MSH3	loop mismatch repair (complex with MSH2)
	Msh4p	MSH4	Meiosis
	Msh5p	MSH5	Meiosis
	Msh6p	MSH6	base mismatch repair (complex with MSH2)
			mismatch repair (complex with MLH3 or
MutL	Mlh1p	MLH1	PMS2 or PMS1)
	Mlh2p	-	unknown
	Mlh3p	MLH3	mismatch repair (complex with MLH1)
	-	PMS1	mismatch repair (complex with MLH1)
	Pms1p	PMS2	mismatch repair (complex with MLH1)
MutH	-	-	unknown
MutU	Exonuclease I	Exonuclease I	exonuclease function

- : no orthologue identified

DNA mismatch repair mechanism

MMR is characterized most completely in *E. coli* (reviewed in (Marti *et al.*, 2002)) (Figure 1-1). MutS initially recognizes the DNA mismatch and two MutS subunits come together, forming a clamp around the DNA. ATP is exchanged for ADP and the MutS dimer undergoes a conformational change, which promotes interactions with the MutL homodimer (Allen *et al.*, 1997; Obmolova *et al.*, 2000). MutS and MutL act together to activate MutH, a latent endonuclease which binds hemimethylated dGATC sites. MutH nicks the unmethylated strand, ensuring MMR repairs the error on the newly synthesized strand. Helicase II proteins are loaded onto the DNA by interactions with MutL and unwind the DNA in proximity to the mismatch and DNA nick made by MutH (Dao & Modrich, 1998; Mechanic *et al.*, 2000). The unwounded nicked strand is then degraded by one of 4 endonucleases depending on the nick location relative to the mismatch. ExoVIII or RecJ are 5' single strand exonucleases and function when the nick is 5' to the mismatch (Cooper *et al.*, 1993). When the incision is 3' to the mismatch, ExoI and ExoX are used to degrade the DNA in a 3'-5' direction (Cooper *et al.*, 1993; Viswanathan & Lovett, 1999). DNA synthesis of the gap is filled by DNA polymerase III holoenzyme, aided by single-strand binding protein Ssb (Modrich & Lahue, 1996). The final step in MMR is the ligation of the repaired DNA strand by DNA ligase.

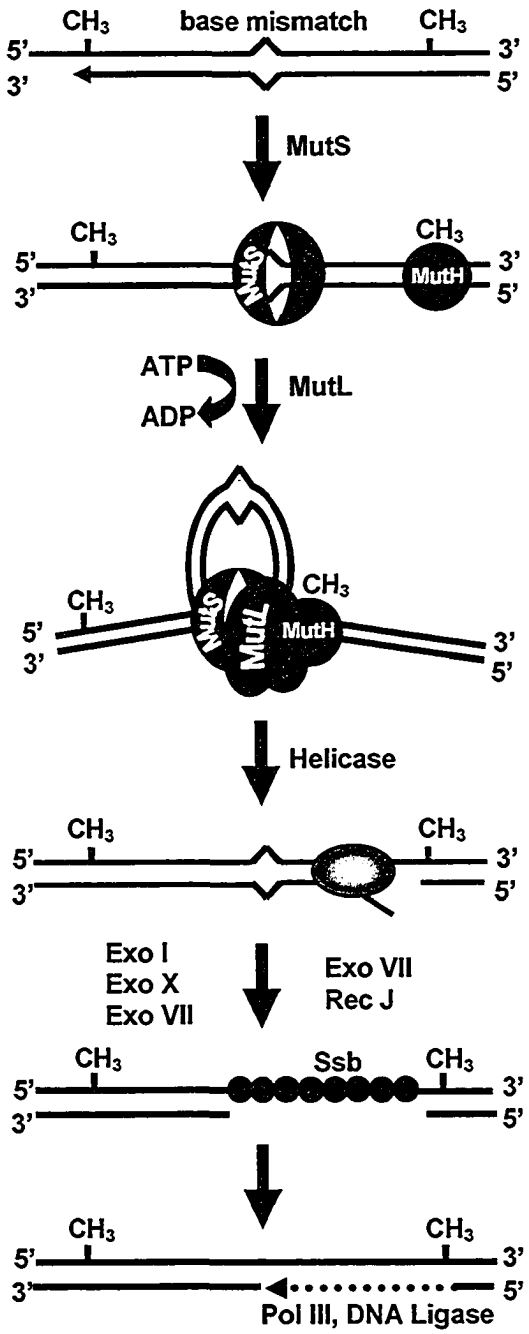

Figure 1-1: Mismatch DNA Repair in *E.coli*

DNA mismatches are recognized by the MutS dimeric protein. Hemimethylated dGATC sites are recognized and bound by MutH. ATP binding to MutS mediates the formation of the complex DNA-MutS-MutL. ATP hydrolysis activates MutH and nicks the newly synthesized strand. DNA is further processed by helicase II for DNA unwinding, and an exonuclease to degrade the DNA surrounding the mismatch. The single stranded DNA is protected by Ssb. DNA is resynthesized by polymerase II and the remaining nick is sealed by DNA ligase.(Adapted from (Marti *et al.*, 2002))

Mammalian DNA mismatch repair

The mechanisms of MMR in mammals and *E.coli* are similar, with the notable exception that MutS and MutL proteins function as homodimers in *E.coli* and as heterodimers in mammals. Repair initiates with the recognition of the base mismatch or small loop by the MutS complex (Figure 1-2). In mammals there are two complexes that form MutS heterodimers within the cell. The MSH2/MSH6 heterodimer, MutSα, is involved primarily in the recognition of single base mismatches and short insertion/deletion loops (1 nucleotide) in the DNA (Jiricny, 1998). MutSβ is comprised of the proteins MSH2 and MSH3 and is primarily involved in the recognition of larger insertion/deletion loops (2-10 nucleotides) in the DNA (Marra *et al.*, 1998; Palombo *et al.*, 1996). MutSα is found in 6-fold excess compared to MutSβ and is the primary mismatch recognition factor (Drummond *et al.*, 1997). In the absence of MutSβ, MutSα is able to compensate for the repair of larger insertion/deletion loops. However, MutSβ is unable to compensate for the loss of MutSα. Therefore, the loss of MSH2 or MSH6 has a greater effect on mutation frequency and tumorigenesis than MSH3 (Genschel *et al.*, 1998). After DNA adduct recognition, MutS recruits the MutL heterodimer to the DNA adduct. In humans there are several MutL heterodimers. MutLα is the most prevalent and is comprised of MLH1 and PMS2 (Li & Modrich, 1995). The MutLβ heterodimers play a minor role in MMR and are composed of MLH1/PMS1 or MLH1/MLH3. Strand identification is not confirmed in mammalian MMR and will be discussed later in the literature review. Following recruitment of the MutS and MutL heterodimers to the DNA adduct, EXO1, a 5' to 3' exonuclease is activated. EXO1 degrades a section of several hundred nucleotides 5' to the mismatch, eventually removing the DNA mismatch or loop. The single stranded DNA is stabilized by replication protein A, the MutS and MutL heterodimers are released from the DNA and DNA polymerase δ resynthesizes the new strand. The final step in MMR is the ligation of the nick by DNA ligase I (Dzantiev *et al.*, 2004; Genschel & Modrich, 2003).

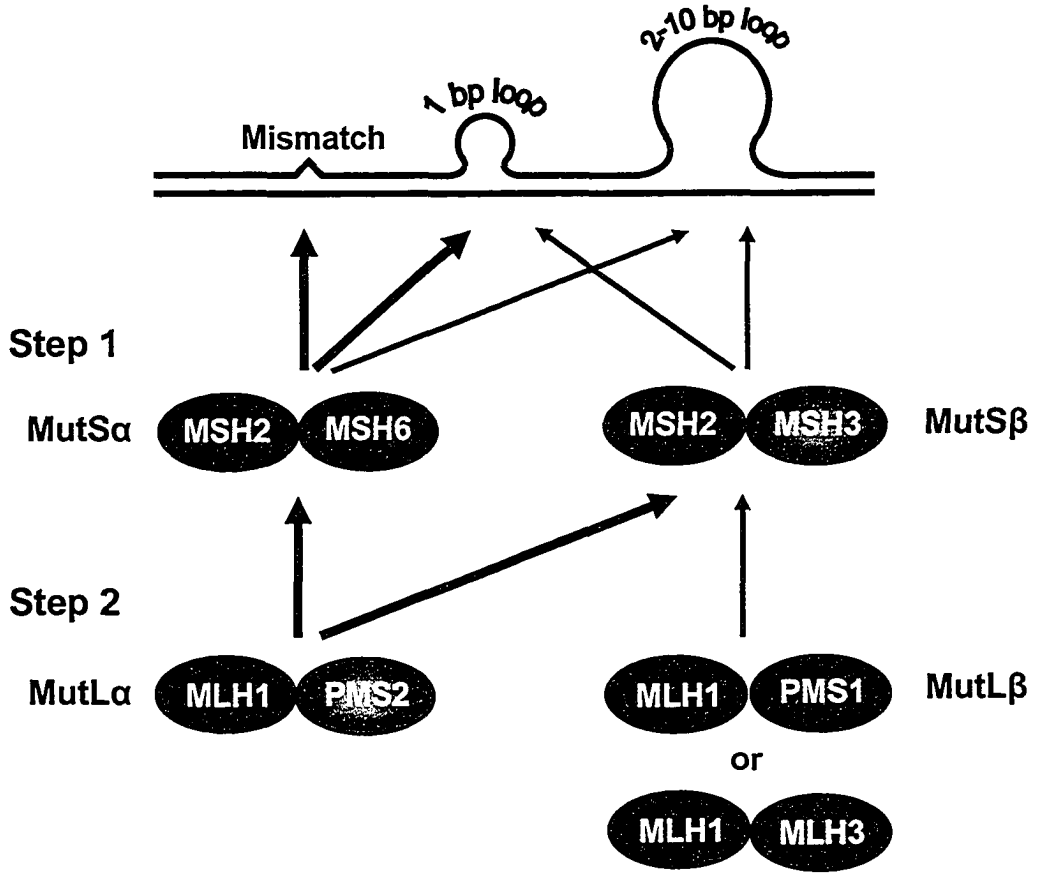

Figure 1-2: Mammalian DNA mismatch repair

Step 1: MutSα (MSH2/MSH6) heterodimer binds to DNA mismatches or 1nucleotide small loops. MutSβ (MSH2/MSH3) heterodimer binds to DNA loops up to 10 nucleotides. Step 2: MutL heterodimer binds and the DNA adduct is processed. The MutLα heterodimer, MLH1/PMS2 is the predominant MutL complex and binds both MutS dimers. The MutLβ heterodimers, MLH1/PMS1 or MLH1/MLH3 are only minor partners in the DNA mismatch repair process and only bind to MutSβ heterodimer.

MutS signal transduction

Three models have been proposed to describe how MutS transduces the DNA mismatch repair signal: 1) sliding clamp model, 2) translocation model and 3) induced fit model. In the sliding clamp model (Figure 1-3a) MutSα is proposed to act as a molecular switch regulated by ATP hydrolysis to ADP. MutSα-ADP heterodimer initially binds with high-affinity to base mismatches. The binding of mispaired DNA would produce an ADP-ATP exchange, followed by a conformational change in the MutSα complex. The ATP-bound MutSα demonstrates reduced affinity for base mismatches and is free to slide along the DNA and contact other proteins involved in the MMR machinery to complete the repair of the base mismatch (Fishel, 1998).

The translocation model (Figure 1-3b) is based on electron microscopy data displaying heteroduplexed DNA with the MutSα complex. In this model, the recognition of the mispaired base by MutSα does not require ADP, although the presence of ADP increases the specificity. Following recognition, ATP binds, and further decreases the MutSα heterodimer's affinity for the mismatch. Subsequently, bidirectional DNA movement occurs, forming a DNA loop. The MutSα complex moved along the DNA is able to interact with other MMR proteins and proceed with repair (Allen *et al.*, 1997; Blackwell *et al.*, 1998).

The third model is based on a crystal structure of MutSα bound to a mismatch. The induced fit model (Figure 1-3c) suggests that MutSα does not leave the mismatch, but directs repair events via direct protein-protein interactions with other MMR proteins (Lamers *et al.*, 2000; Obmolova *et al.*, 2000). Junop *et al* illustrated that MutSα is required to bind both ATP and mismatch DNA simultaneously in order to activate downstream MMR proteins, further supporting the induced-fit model (Junop *et al.*, 2001). Additional biochemical and structural studies must be performed to validate these models; or components there in that can be assembled to form a functional signal transduction.

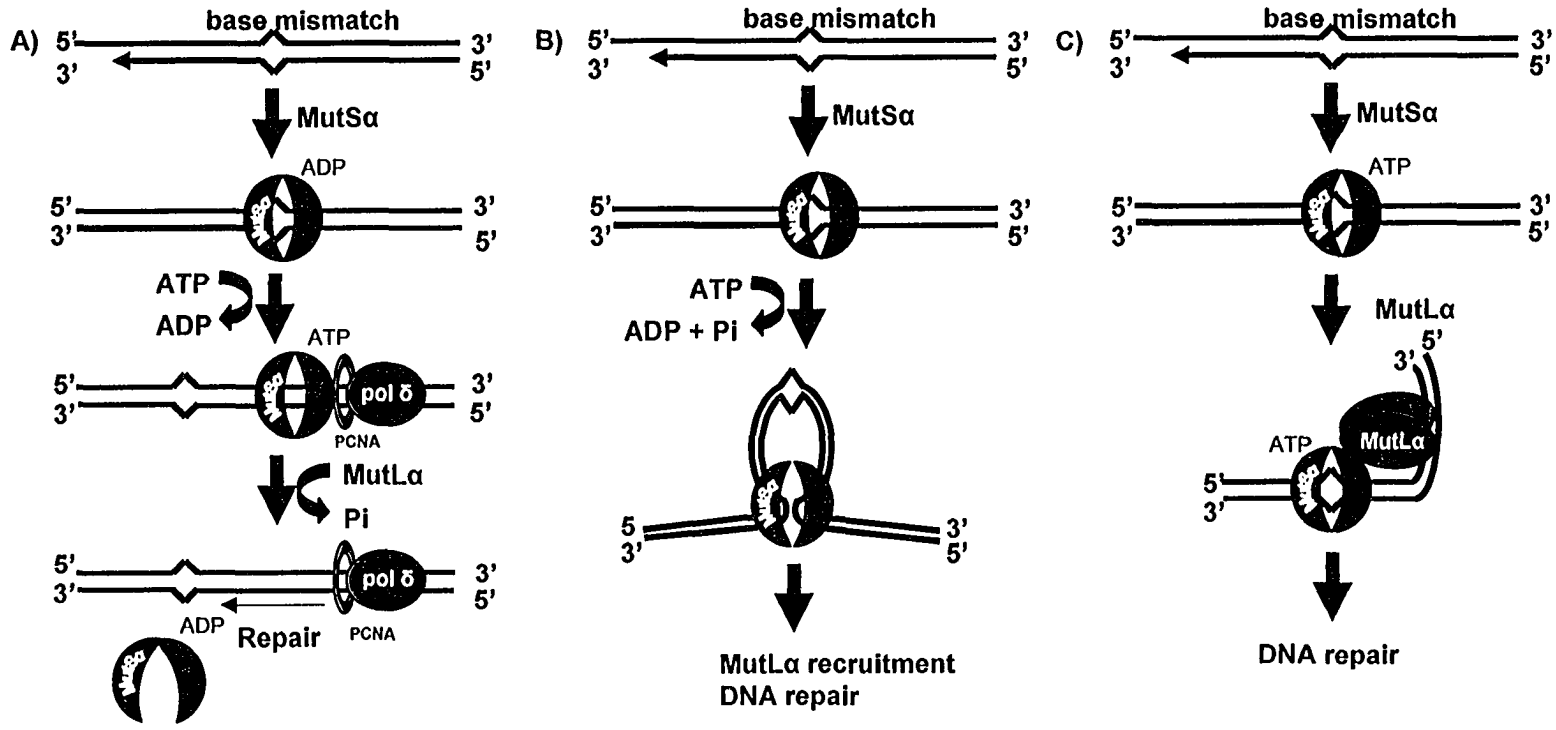

Figure 1-3: MutSα signaling models
A) Sliding clamp model: Mismatch is bound by MutSα-ADP. Following binding, ADP-ATP exchange occurs, and MutSα is able to diffuse along the DNA and contact other components of the MMR process. Upon ATP hydrolysis MutSα is released and DNA repair occurs. B) Translocation model: MutSα binds to mismatch in an ADP independent manner. Upon ATP hydrolysis the MutSα moves away from the mismatch, creating a DNA loop. Other MMR proteins are recruited and repair occurs. C) Induced fit: ATP is bound to the MutSα complex and is required for mismatch recognition. Following recognition, MutSα coordinates repair through direct protein interactions, without dissociating from the mismatch. (Adapted from (Bellacosa, 2001) & (Kunkel & Erie, 2004))

Eukaryotic strand discrimination

Strand discrimination in *E.coli* is well established due to the protein MutH, and the recognition of hemimethylated dGATC sites within the genome. In other organisms, such as humans or other mammals, the process of strand discrimination has not yet been determined. Currently, there are three models for strand discrimination in eukaryotes. The first involves the recognition of single strand breaks created during DNA synthesis. *In vitro* experiments have shown that the DNA strand with a single strand break was the predominantly repaired strand (Holmes *et al.*, 1990; Thomas *et al.*, 1991). Moreover, Pavlov *et al* demonstrate that in *S. cerevisiae* MMR heterodimers MutSα and MutSβ preferentially repair the lagging strand of replication, consistent with the hypothesis that 5' ends of Okazaki fragments present during lagging strand replication and are used for strand discrimination (Pavlov *et al.*, 2003).

Second, the ability for MMR proteins to interact with replication enzymes via PCNA and the orientation of the replication enzymes may be another mechanism by which MMR distinguishes leading strand from lagging strand (Hsieh, 2001). The first evidence that illustrated a relationship between PCNA and MMR was a yeast two-hybrid screen, revealing PCNA interacting with MLH1 and MSH2 (Umar *et al.*, 1996). Co-immunoprecipitation experiments demonstrate that anti-MLH1 antibody is able to pulldown PCNA, MSH2 and PMS2 in HeLa cell lines in the presence of ATP (Gu *et al.*, 1998). Additional experiments by Lau *et al*, illustrated that PCNA and MSH2/MSH6 forms a stable complex with homoduplex (G/C) DNA, and heteroduplex (G/T) DNA in the presence of ATP, but in the absence of ATP MSH2/MSH6 complexed with heteroduplex (G/T) DNA is disrupted. This model suggests that MSH2/MSH6 binds to PCNA on newly replicated DNA, and is transferred from PCNA to DNA mismatches in the DNA (Lau & Kolodner, 2003).

The third mechanism proposes that the protein MED1 (also known as MBD4), a known methyl-binding protein acts to distinguish strand bias due to methylation status of DNA.

Bellacosa *et al* showed that MED1 interacts with MLH1, and in MED1 mutants lacking the methyl-CpG-binding domain, MSI was increased (Bellacosa *et al.*, 1999). Therefore, MED1 is a proposed eukaryotic orthologue of the bacterial MMR protein MutH.

The eukaryote mechanism of strand discrimination in MMR requires continued experimentation. The ability to confirm this mechanism will enhance the understanding of the MMR process and how defects in this process lead to genetic instability and tumorigenesis.

Multifunctionality of DNA mismatch repair

In addition to an established role in post-replication DNA repair, MMR proteins demonstrate other functions within the cell such as somatic and meiotic recombination and response to endogenous and exogenous DNA damaging agents. The role of eukaryotic MMR in meiosis is characterized through the creation of MMR knockout mice. *Mlh1*$^{-/-}$, *Msh4*$^{-/-}$ and *Msh5*$^{-/-}$ mice demonstrate male and female mice sterility (Baker *et al.*, 1996; de Vries *et al.*, 1999; Kneitz *et al.*, 2000). *Pms2*$^{-/-}$ mice exhibit male sterility, whereas the female mice are fertile (Baker *et al.*, 1995). In normal spermatocytes and oocytes, Mlh1 localizes to chiasmata, the point where crossing-over occurs. In *Mlh1*$^{-/-}$ mice, the chiasmata in male and female meiosis are reduced by 1-2 orders of magnitude, establishing a role for Mlh1 in chiasma formation and stabilization (Baker *et al.*, 1996). Male sterility in *Pms2*$^{-/-}$ mice is attributed to defects in chromosomal synapsis (the lining up of homologous chromosomes) and recombination during prophase I of meiosis. Similarly, *Msh4*$^{-/-}$ and *Msh5*$^{-/-}$ mice chromosomes fail to undergo normal pairing during prophase I in meiosis (de Vries *et al.*, 1999; Kneitz *et al.*, 2000). Taken together these data illustrate MSH4/MSH5 and MLH1/MLH3 promote double strand break processing to form Holliday functions reducing alternative outcomes and the preferential resolution into cross-overs rather than non-cross-overs (Nakagawa *et al.*, 1999).

MMR proteins also play a role in somatic recombination (reviewed in (Evans & Alani, 2000)). In both bacteria and yeast the loss of MMR increases homologous recombination. Nicholson *et al* created *S. cerevisiae* null mutants for various MMR proteins: Msh2p, Msh6p, Mlh1p and Pms1p. *S. cerevisiae* deficient in Msh2p displayed an increase in recombination rate for homologous substrates. In addition, Msh6 deletion strains displayed an increase in recombination rate between DNA bearing base-base mismatches or 1 nucleotide insertion loops. In comparison to the MutS homologues, MutL homologous Mlh1p and Pms1p in yeast did not confer as dramatic a phenotype, the increase in recombination rate is smaller compared to the MutS mutant strains (Nicholson *et al.*, 2000). Recent evidence by Langland *et al*, illustrated that human MLH1 interacts with the Bloom's syndrome protein (BLM). BLM protein is a helicase that unwinds duplex DNA and promotes branch migration of Holliday junctions (Langland *et al.*, 2001). This provides further evidence for the role of eukaryotic MMR in somatic recombination.

The MMR proteins demonstrate roles in other cellular responses to DNA damage other than repair. The role of MMR proteins in cell cycle arrest and apoptosis in response to endogenous and exogenous DNA damage will be furthered discussed in subsequent sections of this literature review.

Genomic instability

Types of genetic alterations leading to genetic instability

There are two general types of genetic changes within a tumour. The first is simple sequence variations that include point mutations and insertion/deletions. The second is large scale chromosomal variation, including aneuploidy (the loss or gain of an extra chromosome), chromosomal duplications, translocations and inversions.

Simple sequence variations are changes at the nucleotide level of DNA. Mutations in DNA sequence can be silent (do not affect amino acid sequence) or pathogenic. There are a variety of ways a change in the DNA sequence can cause a pathogenic affect. The

mutation can change the coding sequence to a different amino acid, interfering with the protein structure or function. The mutation can create a stop codon, producing a truncated and usually non-functional protein. The base pair mutation can be within the splice site, resulting in skipped or additional exons similarly producing a non-functional protein. In addition to base substitutions, insertion and deletions can occur. Insertion/deletions occur most commonly at repetitive sequences within the DNA called microsatellites. Microsatellite instability (MSI) is the measure of DNA level instability. To limit the amount of sequence variation the cell possesses many different repair mechanisms. Proofreading activity intrinsic to DNA polymerases is the first step in maintaining the DNA fidelity. In addition, DNA mismatch repair is involved in scanning newly synthesized DNA for base pair mismatches and the subsequent repair process. To repair DNA damage from endogenous or exogenous sources, the cell has multiple DNA repair pathways including nucleotide excision repair, base excision repair, direct reversal repair, non-homologous and homologous end joining. These pathways combine and aid in maintaining the fidelity of the genome.

Chromosomal instability (CIN) includes aneuploidy and chromosome structure changes such as inversions, translocations, duplications and deletions. Numerical chromosomal changes most likely arise from chromosomal segregation defects. Whereas, structural chromosome instability result from defects in DNA damage response, cell cycle checkpoints and/or telomere dysfunction (reviewed in (Gollin, 2005)). Mitotic-checkpoint genes are found to be mutated in multiple cancers. For example, decreased expression of *MAD2* is found in breast cancer (Li & Benezra, 1996), and mutations in *BUB1* and *BUBR1* are found in a fraction of colorectal cancers (Cahill *et al.*, 1998). Centrosome number is also important in chromosomal instability and influences chromosomal segregation during cell division. Abnormal centrosome number is detected in multiple different types of cancers including breast, lung, prostate, colon and brain (Doxsey, 1998).

Current views of genomic instability in cancer focus on the concept that tumours are either microsatellite unstable or chromosomally unstable. In MSI unstable tumours, tumour cells acquire mutations in genes required to maintain genome stability resulting in hastened acquisition of additional mutations in key cancer promoting genes. Alternatively, in CIN unstable tumours, chromosome translocations, deletions, recombinations or other alterations at the whole chromosome level can disrupt a cell sufficiently to give it oncogenic potential. Colorectal cancer is a model for the study of the contribution of CIN versus MSI phenotype in tumorigenesis. There are two different subsets of colorectal tumours, one set has the MSI phenotype and is characterized by the loss of MMR, whereas the CIN phenotype commonly involves chromosomes 5q (*APC*), 17p (*p53*) and 18q (*DCC/SMAD4*) (Gervaz *et al.*, 2004).

The mutator phenotype

The tumorigenic process is a complex and multi-step pathway with one of the key molecular events being genomic instability. Loss of genomic integrity increases the likelihood of mutations or disregulations, which inhibit tumour suppressors or activate oncogenes that contribute to tumorigenesis. Thus, limiting the mutations within the cell is paramount in preventing tumorigenesis. The cell has many different proteins involved in the regulation of DNA and chromosomes, including various "checkpoints" that occur throughout the cell cycle. The approximate spontaneous mammalian mutation rate is 1.4×10^{-10} mutations/base pair/cell generation (Loeb, 1991). This rate does not account for the number of mutations required for tumorigenesis and the thousands of mutations that are found in human cancer cells. To account for this discrepancy Loeb and Christian proposed the mutator phenotype, whereby, mutations occur in genes required to maintain the stability of the genome and this is an early event in the tumorigenic process (Loeb & Christians, 1996). The concept of the mutator phenotype explains the difference in mutation frequency between normal and cancer cells and a role for genomic instability in the tumorigenic process. Alternatively, genomic instability at the whole chromosome level, via chromosome translocations, deletions and recombination can disrupt a cell sufficiently to give it oncogenic potential.

MMR and microsatellite instability

Microsatellites are mono-, di-, tri-, quatra-, or pentanucleotide tandem repeats within the genome. Although most are commonly found in non-coding sections of the human genome, microsatellites are found within coding regions of genes such as in the genes *BAX, TGFβRII* and *PTEN*. MSI is the expansion or contraction of nucleotide repeats in the DNA due to polymerase slippage during DNA replication. In cells deficient in mismatch repair these insertion/deletion loops are not repaired leading to the expansion or contraction of the repeat in the daughter strand of DNA. Microsatellite instability is a hallmark of HNPCC tumours; over 85% of tumours display MSI-high (Pedroni *et al.*, 1999; Woerner *et al.*, 2005) compared to 17% of sporadic colorectal tumours (Pedroni *et al.*, 1999). The detection of MSI is standardized, and includes a panel of five markers; two mononucleotide (BAT25 and BAT26) and three dinucleotide (D2S123, D5S346 and D17S250) (Boland *et al.*, 1998). MSI status is classified as MSI-high, when two or more markers are unstable, MSI-low, when one marker is unstable, or MSI-stable when no markers are unstable (Boland *et al.*, 1998). In addition to this panel of markers, which are located in non-coding regions of the genome, HNPCC tumours demonstrate instability in specific secondary genes. For example, a coding mononucleotide repeat within the *TGFβRII* gene is known to be mutated in over 90% of HNPCC tumours (Markowitz *et al.*, 1995; Parsons *et al.*, 1995; Souza *et al.*, 1996b). This sequence is not generally mutated in other colorectal tumours with functional MMR nor in non-tumour tissues of HNPCC individuals; thus this secondary mutation appears to be of functional significance in colorectal tumorigenesis in the absence of MMR. The correlation between individuals that are deficient in MMR and MSI is further discussed in Chapter 3.

Hereditary non-polyposis colorectal cancer

Hereditary non-polyposis colorectal cancer syndrome (HNPCC) is an autosomal dominant disorder. Knudson hypothesized that cancer develops through the inactivation of a tumour suppressor gene by two 'hits' (Knudson, 1971). In HNPCC, the first 'hit' is an inherited germline mutation in one of the MMR genes and the second 'hit' occurs in the target tissue (colon, endometrium) and is due to somatic inactivation of the gene.

Inactivation may occur through different mechanisms such as point mutation, large deletions or hypermethylation of the promoter of *MLH1* (Herman *et al.*, 1998). Inactivation of the MMR gene leads to increased mutation frequency and acquisition of subsequent mutations in other tumour suppressor genes promoting tumorigenesis.

HNPCC incidence in the general population is 1:1000 (Umar *et al.*, 2004b). Patients diagnosed with HNPCC are predisposed to acquiring colorectal cancer with a lifetime risk estimated at 80% and a lifetime risk of 60% for endometrial cancer. In addition to these primary cancer types, patients also have an increased risk of carcinomas of the urothelium, stomach, ovary, pancreas, biliary tract, gliomas and sebaceous skin tumours (Baglioni & Genuardi, 2004). To establish HNPCC diagnosis there are four different criteria available. The original and most stringent criteria are Amsterdam I criteria. For HNPCC diagnosis, all the following criteria must be fulfilled: 1) three or more relatives diagnosed with colorectal cancer 2) of the relatives affected with cancer, one must be a first-degree relative 3) cancer must be diagnosed in two successive generations 4) one of the relatives must be diagnosed with cancer before the age of 50 years 5) familial adenomatous polyposis must have been excluded (Vasen *et al.*, 1994). In 1998 this criterion was expanded to include other types of cancer associated with HNPCC was classified as Amsterdam II HNPCC criteria (Vasen *et al.*, 1999). Due to the stringency of the Amsterdam criteria, the Bethesda criteria and revised Bethesda criteria were established in 1997 and 2003 respectively. The goal of the Bethesda criteria is to aid in the decision for whether individuals with cancer in families that do not fulfill Amsterdam criteria should undergo genetic testing (Rodriguez-Bigas *et al.*, 1997). The goals of the revised Bethesda were to identify patients who were at risk for hereditary cancer, to include a complete spectrum of colonic and extracolonic cancers and to identify *MSH2* and *MLH1* germ-line mutation carriers in patients with cancer who might or might not fulfill the Amsterdam criteria (Umar *et al.*, 2004a).

Muir-Torre and Turcot syndromes are rare variants of HNPCC, distinguished by the types of tumours patients develop. All three disorders are due to the inheritance of

mutations in one of the MMR genes. Muir-Torre patients have mutations in *MLH1* or *MSH2* (reviewed in (Hampel & Peltomaki, 2000)). Muir-Torre patients present with sweat gland or sebaceous gland tumours especially on the face and scalp. In addition to the sweat gland tumours, Muir-Torre patients develop keratoacanthomas, a type of skin tumour (reviewed in (Hampel & Peltomaki, 2000; Lucci-Cordisco *et al.*, 2003)). The second variant of HNPCC is Turcot's syndrome. Turcot patients primarily inherit mutations in *MLH1* or *PMS2* and are characterized through the manifestation of brain tumours and colorectal adenomas. Moreover, in contrast from dominantly inherited HNPCC, recessively inherited forms of Turcot syndrome have been described associated with homozygous inherited mutations in *PMS2* (De Rosa *et al.*, 2000; Hamilton *et al.*, 1995).

Genetic alterations in hereditary non-polyposis colorectal cancer

An inherited heterozygous mutation in one of the MMR genes is associated with HNPCC (Peltomaki, 2001). However, only 50% of HNPCC families meeting the Amsterdam I criteria have detectable mutations in the MMR genes (Abdel-Rahman *et al.*, 2005), suggesting that mutations in the MMR genes are not identified. Renkonen *et al* used an expression-based strategy and illustrated that 42% of mutation-negative HNPCC families have altered expression of *MLH1, MSH2* or *MSH6* (Renkonen *et al.*, 2003). Therefore, techniques must be created to detect not only sequence variation in the MMR genes but other mechanisms to inactivate MMR. Moreover, MMR proteins or other proteins involved in HNPCC are not yet identified and are likely to be associated with the HNPCC families with the mutation-negative genotype.

Mutation analysis in HNPCC families reveals that approximately 90% of HNPCC associated mutations have been identified in *MLH1* or *MSH2*. The remaining 10% of mutations are due to mutations found in MSH6 with a small percentage attributed to *PMS2* and *PMS1* (Peltomaki, 2001). Currently, there are no mutations of *MLH3* associated with HNPCC (de Jong *et al.*, 2004; Hienonen *et al.*, 2003). In contrast, Wu *et al* identified ten different *MLH3* germ-line variants that were not present in 188 normal

controls; however, the *MLH3* variants were not tested for disease segregation within the families (Wu *et al.*, 2001). Therefore, evidence indicating a role of *MLH3* in tumorigenesis of colorectal cancer must be further investigated to determine the degree of associated risk with *MLH3* mutations. However recent data demonstrates MSH3 protein is decreased in rectal carcinoma cells compared to normal tissue (Deguchi *et al.*, 2003). Moreover, *MSH3* gene expression was analyzed in bladder cancer and was decreased compared to normal epithelium. The changes in gene expression were attributed to increased methylation of the *MSH3* promoter. Therefore, the role of MSH3 in tumorigenesis and *MSH3* promoter methylation status must be further explored to determine its full role in the progression of cancers such as renal cell carcinoma, bladder cancer and potentially HNPCC.

MMR deficient individuals

Rare individuals with inherited homozygous or compound heterozygous mutations in a MMR gene have been reported. Seven families are reported with MLH1 deficiency (Table 1-2). The reports of homozygous inheritance of *MLH1* mutations in four families identified children that develop hematological, brain or gastrointestinal malignancies at a young age (1-11 years) (Gallinger *et al.*, 2004; Ricciardone *et al.*, 1999; Vilkki *et al.*, 2001; Wang *et al.*, 1999b). Mutations in *MLH1* that do not completely abrogate MLH1 function lead to a milder phenotype. Two individuals display later onset of cancer (families #1 & #7 in Table 1-2) (Hackman *et al.*, 1997; Rey *et al.*, 2004). One child with a homozygous Pro648Ser mutation developed a skin neurofibroma and café-au-lait spots but no other cancer phenotype. Repair assays demonstrated $MLH1^{P648S}$ mutant expressed in insect cells retained a low level of repair activity and ability to co-immunoprecipitate with PMS2 (Raevaara *et al.*, 2004).

Two families are reported with MSH2 deficiencies (Table 1-3). Our laboratory described an individual with acute lymphocytic leukemia (T-cell) diagnosed at 24 months. *MSH2* and *MLH1* genes were sequenced and a novel homozygous mutation in *MSH2* was identified. The mutation, a G>A at the intron/exon boundary of exon 11 causes skipping

of exon 11 and results in stop codon in exon 12 producing a truncated MSH2 protein (Whiteside *et al.*, 2002). One patient is reported as inheriting compound heterozygous *MSH2* mutation (see family #2, Table 1-3). The affected proband died at 15 months with a T mediastinal lymphoma. The sibling died at age 4 years from a temporal glioblastoma, but genetic information was not available, therefore, the genotype of the sibling is unknown (Bougeard *et al.*, 2003). Individuals deficient in MSH2 demonstrate a similar severity of phenotype compared to MLH1 deficient patients.

Individuals deficient in MSH6 present with a milder phenotype compared to MLH1 or MSH2 individuals. This is attributed to the retention of the second MutS heterodimer, MutSβ in the MMR system (Table 1-3). Individuals from family #3 in Table 1-3 individuals develop brain or hematological malignancies at the age of onset between 5 to 8 years (Hedge MR, 2003). Menko *et al*, reported a child at the age of 10 years developing a brain tumour and subsequent development of rectal tumour at the age of 12 years. In addition to the pathogenic mutation in *MSH6* this child inherited a homozygous silent mutation in *MSH2*. Protein expression of MSH6 was determined in tumour sections and demonstrated low expression of MSH6 in the brain tumour but no MSH6 expression in the rectal tumour. Similar to other *MLH1* mutations, residual protein expression of MSH6 affects the age of onset and severity of cancer phenotype (Menko *et al.*, 2004).

Turcot syndrome is a rare variant of HNPCC, where individuals develop primary brain tumours caused by mutations in the MMR gene *PMS2*. Currently, four families have been identified with homozygous or compound heterozygous mutations in *PMS2* resulting in hematological, brain, colorectal or endometrial cancer (Table 1-4) (De Rosa *et al.*, 2000; De Vos *et al.*, 2004; Hamilton *et al.*, 1995; Trimbath *et al.*, 2001). These individuals developed cancer at a slightly older age compared to individuals lacking MLH1 and MSH2, similar to the MSH6 deficient individuals.

A subset of individuals deficient in MMR proteins develop symptoms of neurofibromatosis type 1 (NF1), with no associated family history (Tables 1-2, 1-3, 1-4). NF1 is a dominant disorder with close to 100% penetrance by the age of five years. NF1 is characterized by neurocutaneous manifestations and neurological complications such as cognitive impairment. NF1 individuals are also prone to malignancies. NF1 is caused by mutations in a tumour suppressor, neurofibromen. Neurofibromen is a GTPase-activating protein important in the regulation of p21-*ras* and the control of cell growth and differentiation (reviewed in (North, 2000)). The MMR deficient children developed café-au-lait spots, axillary freckling, lisch nodules and dermal neurofibromas. The mechanism by which MMR deficiency leads to NF1 symptoms is unknown. It has been hypothesized by Wang *et al* that neurofibromen (the protein mutated in NF1) is a mutation target or hot spot for cells lacking functional MMR (Wang *et al.*, 2003).

The phenotype of individuals completely deficient in MMR varies significantly from individuals with HNPCC. The earlier age of onset and the occurrence of brain or hematological malignancies are attributed to the complete deficiency in MMR. The development of hematological malignancies is similar to the MMR knockout mouse models. *Msh2*$^{-/-}$ and *Mlh1*$^{-/-}$ develop thymic lymphomas and have a mean survival at 5 ½ months (Baker *et al.*, 1996; de Wind *et al.*, 1995; Edelmann *et al.*, 1996; Prolla *et al.*, 1998; Reitmair *et al.*, 1995). In addition to the cancer phenotype differences, HNPCC patients do not develop symptoms of NF1. The increase of mutational frequency in MMR null individuals may result in mutation in the large neurofibromen gene early in development, predisposing individuals to NF1.

Table 1-2: *MLH1* deficient individuals

Family No.	Gene	Mutation	Patient No.	Clinical Features		Reference
				Malignancies (Age of diagnosis or * age of death)	Signs of Neurofibromatosis Type I	
1	*MLH1*	Ser44Phe [a] Ala441Thr	1	Breast cancer (35)		(Hackman *et al.*, 1997)
2	*MLH1*	Gly67Trp	1	Non-hodgkin malignant lymphoma (*2)	café-au-lait spots pseudoarthrosis tibia	(Wang *et al.*, 1999b)
			2	Acute myeloid lymphoma (6) Medulloblastoma (7)	café-au-lait spots dermal neurofibromas	
3	*MLH1*	Arg226Stop	1	Acute leukemia (2)		(Ricciardone *et al.*, 1999)
			2	Non-Hodgkin lymphoma (3)	café-au-lait spots	
			3	Atypical chronic myeloid leukemia (1)	café-au-lait spots fibromatous skin tumours	
4	*MLH1*	Exon 16 deletion	1	Glioma (*4)	café-au-lait spots axillary freckling	(Vilkki *et al.*, 2001)
5	*MLH1*	Arg687Trp	1	Duodenal cancer (11)	café-au-lait spots	(Gallinger *et al.*, 2004)
			2	Colon adenocarcinoma (9)	café-au-lait spots axillary freckling lisch nodule	
			3	Plexiform neurofibroma (tongue) (7)	café-au-lait spots 2 hairy nevi Lisch nodule	
6	*MLH1*	Pro648Ser	1	Skin neurofibroma (6)	café-au-lait spots	(Raevaara *et al.*, 2004)
7	*MLH1*	Ser269Stop	1	Colon cancer (22)		(Rey *et al.*, 2004)

[a] compound heterozygote

Table 1-3: *MSH2* & *MSH6* deficient individuals

Family No.	Gene	Mutation	Patient No.	Clinical Features		Reference
				Malignancies (Age of diagnosis or * age of death)	Signs of Neurofibromatosis Type I	
8	*MSH2*	Splice site mutation G>A at 1662-1bp	1	Acute lymphocytic leukemia (2)	café-au-lait spots	(Whiteside *et al.*, 2002)
9	*MSH2*	Deletion of exons 1-6 [a] 1bp deletion, codon 153	1	T mediastinal lymphoma (*1)		(Bougeard *et al.*, 2003)
			2	Temporal glioblastoma (*3)		
10	*MSH6*	3634insT	1	Lymphoma (5) Colorectal cancer (8)		(Hedge MR, 2003)
			2	Glioblastoma multiforme (8)	café-au-lait spots axillary freckling	
11	*MSH6* *MSH2*	3386_3388delGTG Silent 984 C>T	1	Oligodendroglioma (10) Rectal adenocarcinoma (12)	café-au-lait spots	(Menko *et al.*, 2004)

[a] compound heterozygote

Table 1-4: *PMS2* deficient individuals

Family No.	Gene	Mutation	Patient No.	Clinical Features		Reference
				Malignancies (Age of diagnosis or * age of death)	Signs of Neurofibromatosis Type I	
12	*PMS2*	Arg134Stop [a] 2184delTC	1	Glioblastoma(4) Colonic adenoma(13) Non-Hodgkin lymphoma of rectum(17)	café-au-lait spots	(De Vos *et al.*, 2004; Hamilton *et al.*, 1995)
			2	Rectal carcinoma (11) Adenomatous polyposis (14)	café-au-lait spots	
13	*PMS2*	1221delG[a] 2361delCTTC	1	Oligodendroglioma (14 & 17) Colon carcinoma (18)		(De Rosa *et al.*, 2000)
			2	Neuroblastoma (13)		
14	*PMS2*	1169ins20	1	Colorectal adenocarcinoma (16) Ovarian neuroectodermal tumour (21) Endometrial adenocarcinoma (23) Brain tumour (24)	café-au-lait spots	(Trimbath *et al.*, 2001)
			2	Anaplastic astrocytoma (7) Adenomatous polyps (20)	café-au-lait spots	
			3	Acute lymphoblastic leukemia (*4)	café-au-lait spots	
15	*PMS2*	Arg802Stop	1	B-cell Non-Hodgkin lymphoma (10)	café-au-lait spots	(De Vos *et al.*, 2004)
			2	Pineal neuroectodermal tumour (8)	café-au-lait spots	
			3	Supratentorial primitive Neuroectodermal tumour (14)	café-au-lait spots	

[a] compound heterozygote

DNA damage response

The response to endogenous or exogenous DNA damaging agents occurs through a signaling cascade (Figure 1-4). DNA damage or replication stress is recognized by sensor proteins involved in detecting DNA adducts. The signal is further passed to the transducer proteins, and finally to the effector proteins which are involved in determining the cellular fate of the damaged cell. A cell can facilitate DNA repair, by inducing cell cycle arrest, or if the resulting DNA damage is significant the cell can undergo apoptosis.

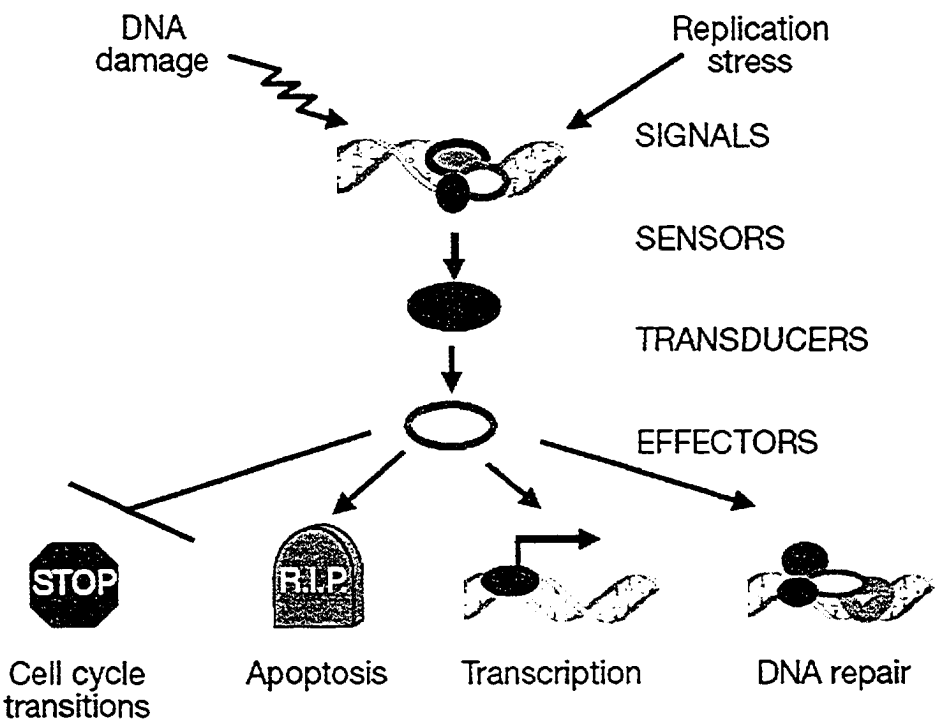

Figure 1-4: DNA damage response

Signaling cascade from DNA damage or replication stress, through the sensing of the DNA lesions, the transduction of the signal, resulting in cellular changes such as changes in transcription, apoptosis, cell cycle arrest and DNA repair (Zhou & Elledge, 2000).

The response to exogenous or endogenous DNA response is mediated through many different DNA repair pathways. Nucleotide excision repair is the prevalent pathway in repairing ultraviolet (UV) radiation induced DNA damage such as (6-4) photoproducts (6-4 PPs) and cyclobutane pyrimidine dimers (CPDs). Base excision repair is involved in the repair of single-base DNA damage caused by methylating and oxidizing agents. Two pathways are involved in the repair of DNA double and single strand breaks, non-homologous end-joining and homologous recombinational repair. DNA mismatch repair is involved in the repair of DNA adducts such as DNA damage from S_N1 methylating agents, specifically O^6-methylguanine (O^6-meG) (reviewed in (Bernstein *et al.*, 2002)). In addition to the repair of DNA adducts, many of the same proteins involved in the direct repair of the DNA adducts also signal for cell cycle checkpoints and apoptosis.

Mismatch repair and DNA damage response

MMR proteins are involved in the cellular response to endogenous or exogenous DNA damage. MMR is important in the repair of DNA lesions, affecting the signal for cell cycle checkpoints or apoptosis. MMR deficient cells demonstrate resistance to certain DNA damaging agents, illustrating a role for MMR in signaling apoptosis post-DNA damage. Point mutations in the *Msh2* and *Msh6* genes in mice have been identified that uncouple the DNA mismatch repair function and the apoptotic signaling functions of the mismatch repair proteins. Lin *et al* developed a mouse model homozygous for the G674A mutation in *Msh2* (Lin *et al.*, 2004). The $Msh2^{G674A/G674A}$ mice displayed delayed tumorigenesis compared to the $Msh2^{-/-}$ mice. DNA repair assays were performed and demonstrated that $Msh2^{G674A/G674A}$ mouse embryonic fibroblasts (MEFs) had a repair defect similar to the $Msh2^{-/-}$ mice. In contrast, $Msh2^{G674A/G674A}$ MEFs were sensitive to treatment to cisplatin, with a similar phenotype compared to $Msh2^{+/+}$ MEFs. Therefore, $Msh2^{G674A/G674A}$ mice have a repair deficiency but are able to affect apoptosis initiation following cisplatin exposure (Lin *et al.*, 2004). Furthermore, Yang *et al* developed a mouse model with a dominant missense mutation in the *Msh6* gene that results in an increased mutator phenotype compared to $Msh6^{-/-}$ mice. In addition, these mice demonstrated a high level of MSI, which is not commonly associated with mutations in

Msh6. The difference in phenotype was attributed to the interference of the mutant $Msh6^{TD}$ protein with functional Msh2/Msh3 heterodimer, affecting the repair of small and large loops. In contrast to the repair defects, $Msh6^{TD/TD}$ MEFs displayed the same sensitivity to cisplatin exposure compared to $Msh6^{+/+}$ MEFs (Yang *et al.*, 2004).

The amount of MMR protein within the cell is also important in the DNA repair and signaling functions of MMR. Cejka *et al* used human embryonic kidney cells 293T with a doxycyclin controlled *Mlh1* expression to demonstrate that 10% expression of *Mlh1* compared to normal levels was sufficient for repair activity, but not sufficient for affecting apoptosis following alkylating agent treatment (Cejka *et al.*, 2003). Therefore, the level of MMR proteins and DNA point mutations are important in separating MMR function into DNA repair and DNA damage response.

Mismatch repair is demonstrated to be involved in mediating apoptosis and cell cycle arrest in response to many different DNA damaging agents including ionizing radiation, cisplatin, alkylating agents, 6-thioguanine, anti-metabolites and ultraviolet radiation. Two models have been proposed to describe the mechanism by which mismatch repair affects apoptosis or cell cycle checkpoints following DNA damage. The "futile repair" hypothesis proposes that MMR proteins bind to the DNA lesion and, in an attempt to repeatedly repair the lesion, induce DNA double or single strand breaks. Other DNA damage sensors recognize these strand breaks and promote cell cycle arrest or apoptosis (Kaina, 1998; Karran, 2001). The second model focuses on the ability for MMR repair proteins to bind to the DNA adducts. Following recognition and an ATP-ADP exchange the MutSα complex is free to interact with other proteins, affecting the damage signal directly to proteins to transduce the signal for cell cycle checkpoint or apoptosis (Fishel, 1998).

Ionizing radiation

Ionizing radiation (IR) is important in the treatment of solid tumours in humans and induces DNA single strand and double strand breaks, DNA-protein crosslinks and

modified nucleotides (reviewed in (Meyers *et al.*, 2004a). The response of mismatch repair to IR is controversial. Data support both increased and decreased survival of MMR deficient cells following IR. Zeng *et al* demonstrated an increase in survival in Pms2, Mlh1, Msh2 deficient MEFs, supporting previous experiments performed by Fritzell *et al* (Fritzell *et al.*, 1997; Zeng *et al.*, 2000). In contrast multiple laboratories have shown a decrease of survival in MMR deficient cells post-IR. Davis *et al* found that MMR deficient cells displayed decreased survival and G2/M arrest. However it should be noted that the experiments were performed in a tumour cell line deficient in MLH1, and the control cell line was created by the re-introduction of chromosome 3 to correct the MMR deficiency (Davis *et al.*, 1998). Addition of the entire chromosome may not recapitulate the normal cellular state, as many genes, including other repair genes, would be over-expressed. Similarly, Franchitto *et al* found that following IR Msh2 deficient MEFs displayed decreased survival, increased chromosomal damage and decreased G2/M arrest compared to wildtype MEFs (Franchitto *et al.*, 2003). Variations in dosing levels and protocols may account for the differences observed however discrepancies in the published data remain unsolved.

Cisplatin

Cisplatin (*cis*-diamminedichloroplatinum(II)) and its derivatives are important chemotherapeutic drugs used for treatment of many tumours. Cisplatin induces DNA-protein crosslinks, DNA monoadducts and both interstrand and intrastrand cross-links (reviewed in (Meyers *et al.*, 2004a)). These adducts are predominantly repaired by nucleotide excision repair, although the MMR heterodimer MutSα has been shown *in vitro* to bind to the cisplatin adduct 1,2-intrastrand d(GpG) crosslink (Duckett *et al.*, 1996). Experiments performed in tumour cell lines demonstrate MMR deficiency leads to increased resistance to the cisplatin compared to MMR proficient cell lines (Aebi *et al.*, 1996; Fink *et al.*, 1996; Fink *et al.*, 1997). Similarly, experiments performed in primary Msh2 deficient MEFs demonstrate decreased ability for affecting early G2/M arrest compared to Msh2 proficient MEFs in response to cisplatin exposure (Marquez *et al.*, 2003). Moreover, Msh6 deficient MEFs display a survival advantage and reduced

apoptosis post-cisplatin treatment compared to wildtype (Yang *et al.*, 2004). In contrast, Claij *et al*, demonstrated that mouse embryonic stem (ES) cells deficient in Msh2 respond in the same way compared to Msh2 proficient embryonic stem cells (Claij & te Riele, 2004). Overall, the data presented above indicates a possible role for MMR in the cellular response to cisplatin; however, further investigation in the mechanism by which MMR influences the response to cisplatin is required.

S_N1 alkylating agents

The role of MMR in response to alkylation damage is well established and is important to consider when treating MMR deficient tumours in the clinic. S_N1 alkylating agents such as chemotherapeutics temozolomide (TMZ) and experimental reagents methylnitrosourea or *N*-methyl-*N'*- nitro-*N*-nitrosoguanidine (MNNG), cause methylation damage of DNA bases forming O^6-methylguanine (O^6-meG) as one of many adducts. O^6-meG is highly mutagenic as it mispairs with thymine instead of cytosine during DNA replication. O^6-meG DNA adducts are repaired by O^6-meG-DNA methyltransferases within the cell. However, O^6-meG mispairs are also recognized by MMR that removes the lesion. DNA polymerases reinstate the mispair, leading to new cycles of attempted repair. Cells deficient in MMR have been shown to be tolerant of alkylating agents displaying decreased apoptosis and decreased induction of G2/M arrest. The mechanism by which MMR resists the toxic effects of alkylating agents is yet to be determined. The two models, as previously described, involve futile cycles of repair with the production of double strand breaks causing cellular effects and the signaling of the damage signal to effector molecules through the MMR proteins. Levels of both apoptosis and G2/M arrest post-MNNG treatment in cells deficient for MSH2, MSH6 or MLH1 are decreased compared to wildtype controls (Adamson *et al.*, 2005; Cejka *et al.*, 2003; Cortellino *et al.*, 2003; Lutzen *et al.*, 2004; Stojic *et al.*, 2004; Toft *et al.*, 1999; Yanamadala & Ljungman, 2003; Yang *et al.*, 2004). Overall, in spite of the controversy over the mechanism by which MMR acts in response to alkylating agents, the role of MMR in the damage response is important not only in a research setting, but in the treatment of various human cancers.

6-thioguanine

6-thioguanine (6-TG) is an antimetabolite used in treatment of leukemia and as an immunosuppressant in organ transplants. 6-TG is incorporated into DNA and RNA, in DNA is methylated to form 6-methythioguanine which can mispair with thymine during replication (Glaab *et al.*, 1998). Hawn *et al* originally described a human colorectal tumour cell line deficient in MLH1 that displayed resistant to 6-TG treatment (Hawn *et al.*, 1995). These experiments have been expanded upon and work by multiple laboratories have shown that cells deficient in one of the MMR proteins (MSH2, MSH6 or MLH1) are resistant to 6-TG treatment (Berry *et al.*, 2000; Glaab *et al.*, 1998; Yamane *et al.*, 2004; Yan *et al.*, 2003; Yan *et al.*, 2004; Yang *et al.*, 2004). In addition, MMR affects the signaling of G2/M arrest post 6-TG treatment, specifically in the signaling of G2 arrest through ATR and Chk1(Hawn *et al.*, 1995; Yamane *et al.*, 2004; Yan *et al.*, 2003; Yan *et al.*, 2004).

UV radiation

Ultraviolet radiation can be classified into three different types according to wavelength (Figure 1-5). UVC (200-250nm) is a germicidal lamp and is used by several laboratories to determine the cellular response to UV radiation. Although UVC radiation carries the strongest energy, it does not play a role in skin carcinogenesis because UVC does not reach the earth's surface due to absorption by the ozone layer. UVB (280-320nm) is considered the most important type of UV radiation in skin carcinogenesis because it reaches the earth's surface with sufficient energy to cause DNA damage. UVA (320-400nm) plays a minimal part in causing direct UV-induced DNA damage, but does produce oxygen radicals in the cells that damage DNA. UVB radiation is 1000 times more efficient than UVA radiation in causing sunburn erythema. UVC and UVB radiation's harmful effects on the cell are mediated through UV-induced DNA damage including cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs) (Figure 1-6).

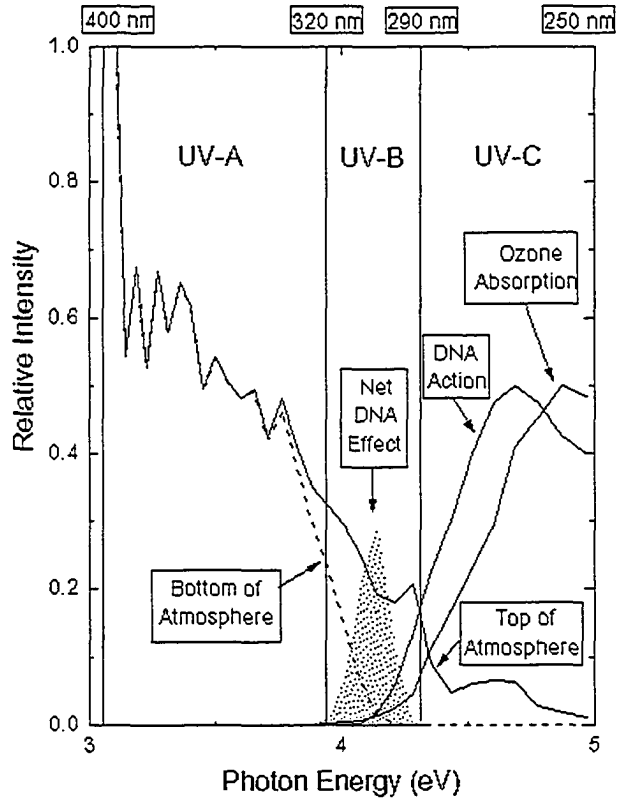

Figure 1-5: Ultraviolet light spectrum

UVC (200-250nm) has the highest energy but does not reach the earth's surface due to the ozone layer. UVB (280-320nm) is important in skin tumourigenesis and is 1000x more efficient than UVA (320-400nm) in causing skin erythema (www.phys.ksu.edu/gene/e3f3.html).

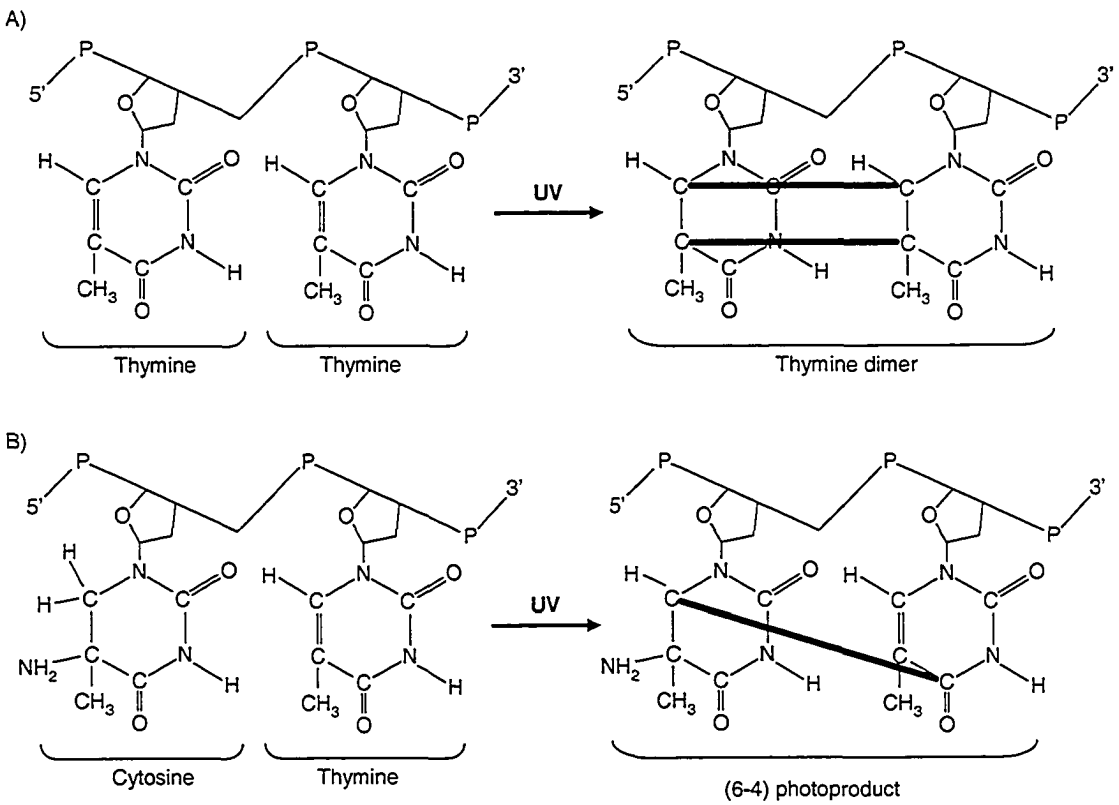

Figure 1-6: UV-induced DNA adducts

A) Formation of a cyclobutane pyrmidine dimer after UV radiation (thymine dimer illustrated). Double bond forms between C-4 and C-5 carbons of two adjacent pyrimidines TT or CC and becomes saturated to produce a four-membered ring
B) Formation of a (6-4) photoproduct after UV radiation. Single bond forms between C-4 and C-6 position of two adjacent pyrimidines, either CT or CC residues. (Adapted from (Matsumura & Ananthaswamy, 2002))

MMR and the UV damage response

UV DNA adducts within the cell are repaired by the nucleotide excision repair (NER) pathway. NER is composed of two pathways consisting of global genomic repair (GGR) and transcription-coupled repair (TCR). These pathways differ in the mode of DNA damage recognition; in GGR DNA adducts are recognized by XPC and HHR23B and in TCR DNA adducts are recognized by the stalled replication fork of RNA polymerase I and Cockayne syndrome proteins A and B.

The role for MMR in NER is complicated by conflicting studies and retracted data. The following papers presented data demonstrating that MMR proteins are involved in TCR following UV-induced DNA adducts but were retracted (Gowen *et al.*, 1998; Leadon & Avrutskaya, 1997; Leadon & Avrutskaya, 1998). In spite of the retracted data, MMR proteins have been shown to bind to the NER proteins. In *S. cerevisiae* MSH2 co-immunoprecipitates with RAD2, RAD10, RAD14 and RAD25 (mammalian homologues: ERCC5, ERCC1, XPA, ERCC3) (Bertrand *et al.*, 1998). In mammalian cells MSH2 has been demonstrated to bind to ERCC1 (Lan *et al.*, 2004). Two remaining studies suggest a direct role for MMR in NER. Mellon *et al* demonstrated that MMR deficient tumour cells lines have decreased TCR following UVC radiation (Mellon *et al.*, 1996). Similarly, Lee *et al* illustrated that LoVo and HCT116 cells had decreased levels of NER following high UVC dose >15 J/m^2 (Lee *et al.*, 2004). However, multiple studies have demonstrated that MMR proteins do not influence NER following UV radiation *in vitro* and *in vivo* (Kobayashi *et al.*, 2004; Meira *et al.*, 2002; Rochette *et al.*, 2002; Sonneveld *et al.*, 2001; Sweder *et al.*, 1996; Yoshino *et al.*, 2002). Although there is no consensus for a role of MMR proteins in NER, current data indicates that MMR does not appear to play a significant role in NER following UV radiation.

The role of MMR in response to UV-induced DNA adducts is also controversial. This is partially due to the use of differing UV wavelengths. The majority of research has been performed using UVC (200-280nm) radiation, whereas UVB (280-320nm) causes the

same UV-induced DNA adducts and is considered the more important type of UV radiation in skin tumorigenesis. MMR is not involved in the cellular survival or repair of DNA adducts following UVC radiation (Lutzen *et al.*, 2004; Rochette *et al.*, 2002; Shin-Darlak *et al.*, 2005).

In contrast, evidence demonstrates MMR is involved in the cellular response to UVB radiation. It is now accepted that the role of MMR in the response to UVB-induced DNA adducts is in a pathway other than the direct repair of the DNA adducts. Evidence suggests that MMR is involved in signaling apoptosis and/or cell cycle arrest post-UVB damage. For example, although CPDs and 6-4PPs the repair of UV-induced DNA damage is performed by NER, MutSα does bind to UV-induced DNA adducts (Wang *et al.*, 1999a). *Msh2* has been shown to be transcriptionally upregulated following UVB radiation (Scherer *et al.*, 2000). *In vivo* studies demonstrate that *Msh2* or *Msh6* deficient mice develop skin tumours at a lower cumulative UVB dose compared to to MMR proficient mice (Meira *et al.*, 2002; Meira *et al.*, 2001; Yoshino *et al.*, 2002; Young *et al.*, 2004).

Our laboratory investigates the contribution of MMR to the cellular responses following UVB radiation. Previous work in our laboratory was performed using primary MMR deficient MEFs with isogenic MMR proficient cell lines. As these cells are i) genetically matched ii) non-transformed and iii) non-tumour origin we feel our work is a good representation for the function of MMR in UV-induced DNA damage *in vivo*. Peters *et al* demonstrated that *Msh2*$^{-/-}$ MEFs treated with UVB radiation, had a decreased apoptotic response compared to *Msh2*$^{+/+}$ MEFs (Peters *et al.*, 2003). In addition, Young *et al* demonstrated that *Msh6*$^{-/-}$ MEFs treated with UVB radiation have reduced levels of apoptosis. Consistent with the *in vitro* experiments, apoptosis was decreased following UVB exposure in mouse epidermis from Msh2 or Msh6 deficient mice compared to MMR proficient mice (Young *et al.*, 2004). These data illustrate a role for MMR in the response to UVB radiation *in vitro* and *in vivo*.

Summary and Goals

MMR is important in maintaining the fidelity of DNA functioning as a post-replication repair system. Individuals who inherit a heterozygous mutation in one of the MMR genes develop the cancer syndrome HNPCC. A hallmark of HNPCC tumours is MSI. Microsatellites are found throughout the genome in both non-coding and coding regions. There are many candidate genes that have microsatellites within coding regions and are mutated in various MMR deficient tumours. Multiple candidate gene analysis has not been preformed on the rare individuals who inherit homozygous or compound heterozygous mutations of MMR genes. The first goal of my research is to determine the level of MSI in candidate genes in non-tumour DNA isolated from a MSH2 deficient individual. I hypothesize that the cells from the MSH2 deficient individual will demonstrate MSI in the candidate genes chosen for analysis, due increased mutation frequency resulting from the deficiency of MSH2 and the likelihood of mutation within specific target sequences.

In addition to an established role in post-replication DNA repair, MMR proteins have other functions within the cell affecting the DNA damage response. MMR proteins demonstrate an affect on G2/M arrest signaling following many different damaging agents, such as 6-TG, alkylating agents. MMR deficiency affects the apoptotic response to UVB radiation. The effect on G2/M arrest signaling post-UVB is not known and is the second goal of my research. I hypothesize that MSH2 deficiency will lead to decreased G2/M cell cycle arrest following UVB radiation in a human non-tumour MSH2 null cell line compared to MSH2 wildtype cell line.

Chapter 2 ♦ Materials and Methods

Candidate gene analysis

Candidate gene analysis was performed using PCR amplification and DNA sequencing on previously isolated DNA from a cell line derived from patient KM (*MSH2*$^{-/-}$), and *parents* II-1, II-2 (*MSH2*$^{+/-}$). The primer sequences and amplicon size are listed in Table 2-1. One 25μL PCR reaction included: PCR buffer (100mM Tris pH 8.4, 500mM KCl, 0.001% gelatin), 0.2mM dNTPs, 1.5mM $MgCl_2$, 0.5μL Taq (Invitrogen, Burlington, ON, Canada) 0.4mM forward primer, 0.4mM reverse primer and 3.5ng DNA. The PCR thermocycler conditions were as followed: 94°C for 2 minutes; 36 cycles of annealing temperature (see Table 2-1) for 30 seconds, 72°C for 30 seconds, 94°C for 30 seconds; concluding with 72°C for 1 minute, 4°C until stopped. PCR products were visualized on a 2% (w/v) agarose TAE gel with ethidium bromide for visualization with UV light. PCR products were extracted from the gel using the Qiagen, gel extraction kit (Qiagen Mississauga, ON, Canada) resuspended in ddH_2O and stored at 4°C until sequenced. DNA sequencing was performed in the University of Alberta, Medical Genetics sequencing facility by the technician Susan Kenney. DNA sequence analysis was performed using GeneTool v2.0 (Biotools Incorporated, Edmonton, AB, Canada).

Cell culture

JMG (*MSH2*$^{+/+}$) and KM (*MSH2*$^{-/-}$) human lymphoblastoid cell lines were cultured with RPMI 1640 plus 10% fetal bovine serum (FBS) (Gibco/Invitrogen, Burlington, ON, Canada) in 5% CO_2 at 37°C. Cells were cultured between 1 and 2 million cells per mL of media to retain quick growth times.

Table 2-1: Candidate gene primer sequences and annealing temperatures

Gene	Primer Sequences F: forward R: reverse	PCR Annealing Temperature	Product Size	Reference (for primer sequences)
BAX	F: ATCCAGGATCGAGCAGGGCG R: ACTCGCTCAGCTTCTTGGTG	55°C	93bp	(Rampino *et al.*, 1997)
β-catenin	F: AGTCACTGGCAGCAACAGTC R: TTCCGTTAGGACTCCTTCT	55°C	97bp	(Mirabelli-Primdahl *et al.*, 1999)
BCL10	F: TTAACAAGTCACAAGATGGACAGTG R: CTAGAACAGGCAAATTCAGAGAAG	57°C	290bp	(Willis *et al.*, 1999)
Caspase 5	F: CAGAGTTATGTCTTAGGTGAAGG R: CCGTTTCTACAAGAAGTACCA	55°C	140bp	(Schwartz *et al.*, 1999)
CHK1	F: AGTGACAGCTGTCAGGAGTATTCTG R: TCATATCCAAGAAATCGGTACTC	55°C	180bp	(Semba *et al.*, 2000)
E2F4	F: TGGTCCTCCTGTGTCTGGGTT R: AAGGAGGTAGAAGGGTTGG	55°C	310bp	(Souza *et al.*, 1997)
IGFRII	F: GCAGGTCTCCTGACTCAGAA R: GAAGAAGATGGCTGTGGAGC	57°C	110bp	(Souza *et al.*, 1996a)
MSH6	F: GGGTGATGGTCCTATGTGTC R CGTAATGCAAGGATGGCGT	62°C	94bp	(Yin *et al.*, 1997)
PTEN exon 7	F: ACAGAATCCATATTTCGTGTGTA R: TAATGTCTCACCAATGCCA	55°C	200bp	(Steck *et al.*, 1997)
exon 8	F: TGCAAATGTTTAACATAGGTGA R: CGTAAACACTGCTTCGAAATA	55°C	350bp	
RIZ exon 8a	F: GAGCTCAGCAAAATGTCGTC R: CAAGTCGGCCTTCTGCTTTG	55°C	120bp	(Piao *et al.*, 2000)
exon 8b	F: TCTCACATCTGCCCTTACTG R: GTGATGAGTGTCCACCTTTC	55°C	140bp	

UVB irradiation

Cells were suspended in RPMI 1640 with no FBS (Gibco/Invitrogen, Burlington, ON, Canada), and exposed to 250J/m^2 UVB (290-320nm) from a bank of six unfiltered UVB bulbs (FS20T12/UVB-BP, Light Sources Inc., Orange CT), in a culture dish with the lid on. Following UVB irradiation, RPMI with FBS was added to reach a final concentration of 10% FBS. The culture dish lids were placed on top of the sensor, an IL1700 radiometer with a SED 240/UVB-1/W detector (International Light, Newburyport, MA). The intensity of the UVB source penetrating the culture dish lid was averaged between the culture dish lid without media and a culture dish lid with RPMI no serum. Culture dish lids filtered out contaminating UVC radiation (Baross-Francis *et al.*, 1998).

Flow cytometry

Following the desired timepoints post-UVB radiation, the cells were pelleted and resuspended in freezing media (RPMI 1640, 20% FBS, 10% DMSO) for storage at -80°C. For cell cycle analysis, the cells were thawed, pelleted and washed with RPMI 1640, without FBS and resuspended in 250-500μL Vindelov propidium iodide stain [*10mM Tris pH 8.0, 10mM NaCl, 700U RNase A, 0.075mM PI, 1mM NP40*]. Cell cycle was analyzed using Becton Dickenson FACScan (Becton Dickenson, San Jose, CA), and the data was analyzed using ModFit LT, version 2.0 (Verity Software House, Topsham, ME).

Cell lysis / Quantification

Following the desired timepoints post-UVB, the cells were pelleted and frozen at -80°C. Cell pellets were resuspended in 200-400μL of lysis buffer (50mM Tris pH 7.5, 10mM $MgCl_2$, 1x protease inhibitor (Complete Mini, Roche, Laval, PQ, Canada), 1% SDS) and sonicated. Protein concentrations were determined using a detergent compatible protein assay (BioRadDC, Hercules, CA).

SDS-PAGE gel electrophoresis / Western blots

Equal amounts of protein, 40-50μg, were separated by discontinuous SDS-PAGE gels (7.5 -15%) using standard protocols and transferred to 0.45uM Immobilon-P PVDF membrane (Fisher Scientific, Pittsburgh, PA) in Tris/glycine buffer. Membranes were blocked in 4% milk in TBST (20mM Tris, 137mM NaCl, 0.001% Tween20, pH 7.6), rocked at room temperature for 1 h. Primary antibodies incubated in 5% BSA or 4% milk in TBST according to manufacturer's instructions, rocked for 1h at room temperature or 4°C overnight at the following concentrations: CDC2 (#610037, BD Bioscience, Mississauga, ON, Canada) 1:1000, CDC2phospho-Tyr15 (#9111, Cell Signaling Technology, Beverly, MA) 1:1000, CDC25C (#05-507, Upstate, Charlottesville, VA) 1:500, CDC25C phosphor-Ser216 (#9526, Cell Signaling Technology, Beverly, MA) 1:500, CHK1 (#KAM-CC111, Stressgen Bioreagents, Victoria, BC, Canada) 1:1000, p53 DO1 (#15791A, BD Biosciences, Mississauga, ON,

Canada) 1:1000, p53 phospho-Ser15 (#9284, Cell Signaling Technology, Beverly, MA) 1:1000, WEE1 (#06-972, Upstate, Charlottesville, VA) 1:1000. After washing 3 times for 5 minutes with TBST, membranes were incubated with IgG HRP-conjugated secondary antibody (anti-mouse: 1:4000, BD Biosciences, Mississauga, ON, Canada or anti-rabbit: 1:2000, Cell Signaling Technology, Beverly, MA) for 1 hour rocking at room temperature. Following, secondary antibody, membranes were washed with TBST 3 times for 5 minutes. Following washing, the blots were incubated in ECL (Amersham Bioscience, Picataway, NJ) for 1 minute or ECL Plus (Amersham Bioscience, Picataway, NJ) for 5 minutes and exposed to autoradiograph film for 10 seconds to 5 minutes. Densitometry was performed using Quantity One (Bio-Rad, Mississauga, ON, Canada). Protein levels were normalized back to loading control (amido black stain or β-actin) and a ratio calculated using normalized values comparing all timepoints to JMG (*MSH2*$^{+/+}$) untreated.

Phosphatase Assay

40ug of JMG (*MSH2*$^{+/+}$) and KM (*MSH2*$^{-/-}$) protein lysates were combined with an extra 2μL of protease inhibitor (10x protease inhibitor stock (Complete Mini, Roche, Laval, PQ, Canada)) and 20U of calf intestinal alkaline phosphatase (Sigma, Saint Louis, MO) then incubated at room temperature for 45 minutes, followed by 37°C for 45 minutes. 4x SDS-loading dye (50% glycerol, 0.25M Tris pH 6.8, 5% β-mercaptoethanol, 10% SDS 0.5% bromophenyl blue) was added to each sample for a final concentration of 1x SDS-loading dye, and run on 10% polyacrylamide gel as previously described. Protein was transferred onto 0.45uM Immobilon-P PVDF membrane (Fisher Scientific, Pittsburgh, PA) and visualization of p53 and p53 phospho-Ser15 (S15) was performed using protocols previously described.

RNA preparation / RT-PCR

One million cells were pelleted and RNA isolated following the Trizol, total RNA isolation reagent protocol (#15596-026, Invitrogen, Burlington, ON, Canada). RNA was resuspended in 25uL DEPC-treated water and stored at -80°C. Prior to cDNA

preparation, RNA was DNase treated to remove contaminating DNA from RNA sample. 1.5μg RNA was combined with 1μL DNase buffer (20mM Tris-HCL pH 8.4, 50mM KCL, 2mM $MgCl_2$), 10U DNase I, 20U RNase inhibitor (#N2515, Promega) and DEPC-H_2O up to 10μL. RNA was incubated at room temperature for 15 minutes then 1μL of 25mM EDTA was added, and incubated at 65°C for 15 minutes followed by on ice for 1 minute. cDNA was prepared using the Gibco SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Burlington, ON, Canada), using approximately 1.5ug RNA starting material, 2uL random hexamer primers, 40U RNase inhibitor (#N2515, Promega, Madison, WI) and 50U SuperScript II RT enzyme (Invitrogen, Burlington, ON, Canada). The resulting cDNA was stored at -80°C.

p53 sequencing

p53 sequencing was performed using cDNA prepared from JMG (*MSH2*$^{+/+}$) and KM (*MSH2*$^{-/-}$) cell lines. The coding region of p53 was separated into three overlapping PCR products. The following are the sets of primers used: p53cDNA-F1: GCTTTCCACGACGGTGAC, p53cDNA-R1: CTCACAACCTCCGTCATGTG, p53cDNA-F2: GGACAGCCAAGTCTGTGAC, p53cDNA-R2: AGAGGAGCTGGTGTTGTTGG, p53cDNA-F3: CTTTGAGGTGCGTGTTTGTG, p53cDNA-R3: TTCTGACGCACACCTATTGC. One 25uL PCR reaction included: PCR buffer (100mM Tris pH 8.4, 500mM KCl, 0.001% gelatin), 0.2mM dNTPs, 1.5mM $MgCl_2$, 0.5μLTaq (Invitrogen, Burlington, ON, Canada), 0.4mM forward Primer, 0.4mM reverse primer and 1.5μL cDNA. The PCR thermocycler conditions were as followed: 94°C for 4 minutes; 36 cycles of 55°C for 30 seconds, 72°C for 30 seconds, 94°C for 30 seconds; concluding with 72°C for 1 minute and 4°C until stopped. PCR products were visualized on a 2% (w/v) agarose TAE gel with ethidium bromide for visualization with UV light. PCR products were extracted from the gel using the Qiagen, gel extraction kit (Qiagen Mississauga, ON, Canada) and resuspended in ddH_2O and stored at 4°C until sequenced. DNA sequencing was performed in the University of Alberta, Medical Genetics, Sequencing Facility, by the technician Susan Kenney. DNA sequence analysis was performed using GeneTool v2.0 (Biotools Incorporated, Edmonton, AB, Canada).

Chapter 3 ♦ Genomic Instability

Introduction

The MMR system within the cell is important in maintaining the fidelity of DNA; this is attributed to the repair of post-replicative DNA mismatches and small insertion/deletions. Inheritance of a heterozygous mutation in one of the MMR genes leads to the cancer syndrome HNPCC. Somatic inactivation of the other MMR allele occurs in one of many target tissues, resulting in MMR deficiency and tumorigenesis. A hallmark of HNPCC tumours is MSI. MSI is attributed to polymerase slippage during replication, which in the absence of MMR, is left unrepaired and leads to expansion or contraction of microsatellites in DNA. The level of MSI in HNPCC tumours is high at ~90% (Lynch *et al.*, 1999) and occurs in sporadic colorectal cancer at ~15% (Boland *et al.*, 1998; Pedroni *et al.*, 1999). MSI also occurs in other sporadic tumours at varying levels depending on the tumour types tested and the markers used for testing. The finding that loss of MMR occurs in a significant percentage of sporadic cancers suggests that loss of MMR is an important contributing step towards a tumorigenic state. In sporadic tumours, inactivation of MMR genes is commonly due to somatic inactivation or epigenetic silencing. The predominant method is aberrant methylation of the *MLH1* promoter, leading to MLH1 deficiency (Kane *et al.*, 1997; Veigl *et al.*, 1998).

Microsatellites are located throughout the entire genome, predominantly in non-coding regions, although there is a subset of microsatellites within coding regions of some genes. Frameshift mutations resulting in loss of function of specific genes can affect the tumorigenic process, therefore, consistent with the multi-step process of tumour formation. The initial alteration of the MMR system results in an increased mutation frequency preferentially targeting genes with microsatellites within coding regions. There are now many different candidate genes found mutated in human tumours deficient in MMR (Table 3-1). *TGFβRII* is a receptor for *TGF-β*, an inhibitor of cell growth and important tumour suppressor commonly mutated in human cancers. Mutations in *TGFβRII* are found in over 90% of HNPCC colorectal tumours and not observed in non-

tumour tissues of HNPCC patients (Markowitz *et al.*, 1995; Parsons *et al.*, 1995). *TGFβRII* mutations are rare in colorectal tumours with functional MMR suggesting their occurrence is secondary to loss of MMR. *BAX* is a member of the BCL-2 family of apoptosis regulators and acts to promote apoptosis. Mutations in the mononucleotide repeat of *BAX* occur in approximately 50% of MSI-high colorectal cancers (Rampino *et al.*, 1997). *E2F-4* is a transcription factor that functions to regulate cell proliferation and is important in the RB/G1 cell cycle checkpoint pathway. *E2F-4* has a tri-nucleotide repeat and is frequently mutated in MMR deficient cancers (Polager *et al.*, 2002). Thus it appears that MSI, observed at repeats within various tumour suppressor genes and genes influencing cell growth and apoptosis, contributes to the tumorigenesis process in the absence of MMR.

MMR deficient individuals and MSI

Non-tumour tissue from HNPCC individuals does not demonstrate MSI as haploinsufficiency for MMR is sufficient for normal levels of repair. MSI analysis has been performed on a subset of the individuals identified with constitutive absence of MMR, on DNA isolated from non-tumour tissue and tumour tissue (Table 3-2). Small pool PCR, or diluted DNA was used to analyze six MLH1 and one PMS2 deficient individuals. Non-tumour tissue from MLH1 deficient individuals displayed levels of instability ranging from 14-47% (Gallinger *et al.*, 2004; Hackman *et al.*, 1997; Vilkki *et al.*, 2001; Wang *et al.*, 1999b) and the PMS2 deficient individual displayed one marker with a high degree of instability and two additional markers with low levels of instability (De Rosa *et al.*, 2000). One MSH2 deficient individual was tested for MSI using undiluted DNA from non-tumour lymphocytes. These results displayed stability in eight microsatellites (Whiteside *et al.*, 2002).

Tumour tissue was analyzed for MSI in eight individual deficient in MMR. Six of these individuals displayed a MSI-high phenotype in DNA isolated from tumours (De Rosa *et al.*, 2000; Gallinger *et al.*, 2004; Hamilton *et al.*, 1995; Trimbath *et al.*, 2001). One individual deficient in MSH6 had two tumours analyzed; these produced contrasting

results. The brain tumour showed MSI-stable phenotype at five markers, but was further analyzed revealing residual MSH6 protein expression. The rectal tumour displayed MSI-high phenotype and demonstrated no MSH6 protein expression. Therefore, the difference in MSI phenotype is attributed to residual MSH6 expression. These results are consistent with HNPCC tumours, deficient in MMR. In contrast, one individual deficient in MSH2 had tumour DNA tested for MSI and displayed MSI-stable phenotype in five markers (Bougeard *et al.*, 2003).

The above MSI analysis was tested using markers in non-coding regions of the genome. Extensive analysis of the various candidate genes demonstrated to be mutated in HNPCC tumours has not been performed. To date, only *TGFβRII* has been analyzed in two individuals. Non-tumour tissue of one MLH1 individual was analyzed and found to be wildtype, with no variations (Vilkki *et al.*, 2001). In contrast, tumour tissue from a PMS2 null individual was analyzed and demonstrated to have a one nucleotide contraction in one allele of the *TGFβRII* gene (De Rosa *et al.*, 2000).

Due to the relative low level of instability observed in non-tumour tissue from the MMR deficient individuals tested and lack of analysis for mutations within secondary genes I chose to sequence coding repeats in various candidate genes for instability, in one of the two known MSH2 null cases. It is important to identify subsequent mutational events when MMR is absent in order to understand the development of an oncogenic state in these rare individuals. I examined the levels of MSI at coding repeats within nine candidate genes and one mutational hotspot in *B-catenin* in the MSH2 deficient individual hypothesizing that I would observe a high level of mutation, attributed to the complete loss of MSH2 and both MutS complexes in the MMR repair pathway. The genes chosen for analysis were those previously shown to be highly mutated in HNPCC colorectal tumours. Although in this case, non-tumour DNA was being tested, I hypothesized that in the absence of MMR, such genes would be likely targets for mutation.

Table 3-1: Candidate genes with frameshift mutations in absence of MMR

Gene	Function	Repeat length	Proportion of HNPCC tumours with mutations	References
AXIN2	Wnt signaling	$(A)_6$, $(G)_7$, $(C)_6$,	11/45 (24%)	(Liu *et al.*, 2000)
BAX	Promotes apoptosis	$(G)_8$	21/41 (51%)	(Rampino *et al.*, 1997)
Caspase 5	Inflammation/apoptosis	$(A)_{10}$	62%	(Schwartz *et al.*, 1999)
CHK1	Role in G2 cell cycle checkpoint	$(A)_9$, $(A)_7$	3/17 (17.6%)	(Bertoni *et al.*, 1999)
E2F4	Cell cycle gene, family of transcription factors, involved in progression from G1 – S	$(AGC)_{13}$	11/17 (65%) 35%	(Ikeda *et al.*, 1998; Johannsdottir *et al.*, 2000)
IGFRII	Tumour suppressor	$(G)_8$	3/35 (9%)	(Souza *et al.*, 1996a)
MBD4	Methyl CpG binding protein, ability to remove thymine or uracil from mismatched CpG sites	$(A)_{10}$	10/23 (43%)	(Bader *et al.*, 1999)
MSH3	Mismatch repair protein	$(A)_8$	16/41 (39%)	(Malkhosyan *et al.*, 1996)
MSH6	Mismatch repair protein	$(C)_8$, $(T)_7$	12/40 (30%)	(Malkhosyan *et al.*, 1996)
PTEN	Tumour suppressor	$(A)_6$, $(A)_6$	6/32 (19%)	(Guanti *et al.*, 2000)
RIZ	Tumour suppressor	$(A)_8$, $(A)_9$	9/24 (37.5%) 14/51 (26%)	(Chadwick *et al.*, 2000)
TCF4	Transcription factor (Wnt signaling)	$(A)_9$	19/49 (39%)	(Duval *et al.*, 1999)
TGFβRII	Tumour suppressor	$(A)_9$	7/7 (100%) 100/111 (90%)	(Markowitz *et al.*, 1995; Parsons *et al.*, 1995)

N/A: not applicable
Adapted from (Peltomaki, 2001)

Table 3-2: MSI analysis in MMR deficient individuals

Family No.	Gene	Patient No.	Microsatellite Instability Results			Reference
			Normal tissue	Tumour tissue	*TGFβRII*	
1	*MLH1*	1	1 marker[a], 19% instability			(Hackman *et al.*, 1997)
2	*MLH1*	2	1 marker[a], 47% instability			(Wang *et al.*, 1999b)
4	*MLH1*	1	3 markers[a], 15-32% mutant alleles		Normal lymphocytes[b] no variation	(Vilkki *et al.*, 2001)
5	*MLH1*	1	2 markers[a], 14-31% instability	MSI-high[b], 3/5 markers unstable		(Gallinger *et al.*, 2004)
		2	2 markers[a], 14-31% instability	MSI-high[b], 4/5 markers unstable		
		3	2 markers[a], 14-31% instability			
8	*MSH2*	1	MSI-stable[b], 5 markers and 2 tri-nucleotide repeat genes			(Whiteside *et al.*, 2002)
9	*MSH2*	2		MSI-stable[b], 5 markers		(Bougeard *et al.*, 2003)
11	*MSH6* *MSH2*	1		Brain tumour[b], MSI-stable, 5 markers Rectal tumour[b], MSI-high, 3/5 markers unstable		(Menko *et al.*, 2004)
12	*PMS2*	1		MSI-high[b], 2/2 markers unstable		(Hamilton *et al.*, 1995)
		2		MSI present, no information given		
13	*PMS2*	1	1 marker[a] highly unstable 2 markers[a] low level of instability	MSI-high[b], 4/4 markers unstable	Tumour DNA[b], one allele contracted	(De Rosa *et al.*, 2000)
14	*PMS2*	1		MSI-high[b], 3/3 markers unstable		(Trimbath *et al.*, 2001)

[a] diluted DNA, 1-3 genomes amplified

[b] undiluted DNA, multiple genomes amplified

Results

Instability was tested at coding repeats within 9 candidate genes and a mutational hotspot in *B-catenin*, on previously isolated genomic DNA from lymphocytes obtained from a MSH2 deficient individual, KM. The lymphocytes were obtained following chemotherapy treatment for acute lymphocytic leukemia, therefore, the genomic DNA is presumed to be of non-tumour origin. Primary leukemic cells were not available for testing.

I amplified each amplicon by PCR, sequenced and analyzed the sequences for contractions or expansions of the specific exonic nucleotide repeat. The results are summarized in Table 3-3. The seven candidate genes tested; *BAX, BCL10, CHK1, IGFRII, MSH6, PTEN* and *RIZ* had mononucleotide runs ranging from six to ten repeats. No nucleotide shifts were identified in any of the repeats tested. A representative sequence illustrating the MSI analysis for *RIZ exon8b* is shown in Figure 3-1. A heterozygous contraction was detected in the mononucleotide repeat of *caspase 5*. Analysis of genomic DNA from parent II-2 indicated that the parent is heterozygous for the mutation in the nucleotide repeat. One candidate gene *E2F4* contains a tri-nucleotide repeat with 13 repeats, and consistent with the other genes containing mononucleotide repeats, no frameshift mutations were identified at the microsatellite. The final candidate analyzed was *β-catenin*. Although this gene does not contain a microsatellite in the coding region, it is demonstrated to be mutated in HNPCC tumours with a MSI positive phenotype. Exon 3 of *β-catenin* is targeted for mutation resulting in the alteration of serine/threonine phosphorylation residues (codons 41 and 45). Alteration of the phosphorylation status of β-catenin leads to stabilization and upregulation of c-Myc and cyclin-D1 (Johnson *et al.*, 2005; Mirabelli-Primdahl *et al.*, 1999). Exon 3 of *β-catenin* was sequenced and confirmed to be wildtype compared to the sequence presented in the published human genome. Contrary to my hypothesis, I did not identify any frameshift mutations at coding microsatellites, not attributed to inheritance, in any of the candidate genes tested in the MSH2 deficient individual, KM.

Table 3-3: Candidate gene results

Gene	Wildtype human repeat	Results: MSH2 deficient individual, KM
BAX	$(G)_8$	wildtype $(G)_8$
β-catenin	no microsatellite	wildtype sequence
BCL10	$(T)_7$	wildtype $(T)_7$
Caspase 5	$(A)_{10}$	KM: heterozygous $(A)_{9/10}$ Parent II-2: heterozygous $(A)_{9/10}$
CHK1	$(A)_9$ & $(A)_7$	wildtype $(A)_9$ & $(A)_7$
E2F4	$(AGC)_{13}$	wildtype $(AGC)_{13}$
IGFRII	$(G)_8$	wildtype $(G)_8$
MSH6	$(C)_8$	wildtype $(C)_8$
PTEN e7 & e8	$(A)_6$ & $(A)_6$	wildtype $(A)_6$ & $(A)_6$
RIZ	$(A)_8$ & $(A)_9$	wildtype $(A)_8$ & $(A)_9$

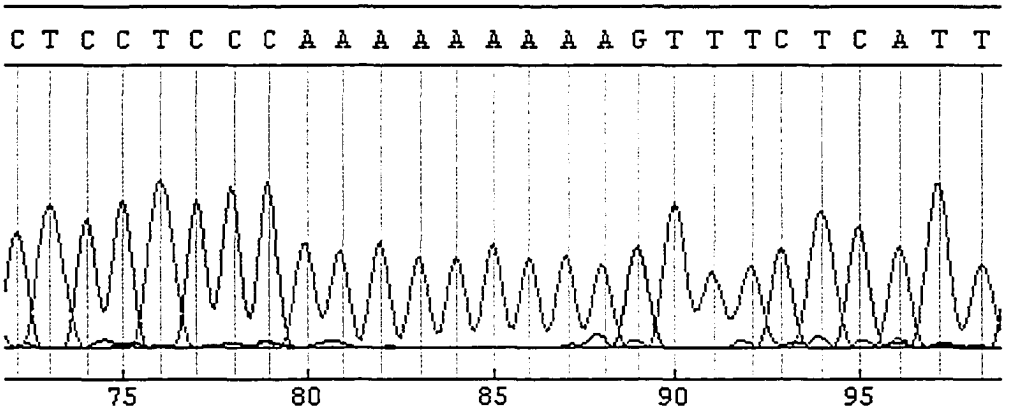

Figure 3-1: Microsatellite analysis of MSH2 deficient individual

Representative sequencing result for the candidate gene analysis. Genomic DNA from non-tumour lymphocytes sequenced for *RIZ exon 8b*, $(A)_9$ repeat illustrated.

Discussion

There are a small set of individuals who inherited compound heterozygous or homozygous mutations in DNA MMR genes and as a result, have constitutive loss of MMR (or greatly compromised repair) from conception. These individuals, depending on the severity of mutation, develop various types of cancers between the ages of 1 and 35 years. MSI analysis of these individuals has been performed revealing MSI in non-tumour tissue. These results differ from the HNPCC individuals in which normal tissue with MMR heterozygosity does not display a MSI phenotype. However, the levels of instability observed (14-47%) seem relatively low, considering that cells from these individuals have compound heterozygous or homozygous mutations in one of the MMR genes that result in a complete lack of MMR.

I examined the status of specific repetitive tracts found within ten genes previously recognized as mutational targets in HNPCC tumours, in non-tumour DNA from the MSH2 deficient individual previously characterized in our laboratory. I hypothesized that there would be a high degree of instability in the candidate genes, due to previous studies showing instability in HNPCC tumours. I saw a heterozygous contraction in the microsatellite of *caspase 5* in my analysis. To confirm a novel frameshift mutation I analyzed the parent II-2 and discovered that this individual was also heterozygous for a contraction in the microsatellite of *caspase 5*. Therefore, the frameshift mutation in the MSH2 deficient individual, KM, was inherited from parent II-2 and not a novel mutation.

Contrary to my hypothesis, I found no novel frameshift mutations within the microsatellites, in non-tumour tissue from the MSH2 deficient individual. These results are consistent with some published data. Previous analysis of the MSH2 deficient individual in our laboratory detected no MSI in eight non-coding microsatellites (Whiteside *et al.*, 2002). Moreover, data from our laboratory using an *Msh2*$^{-/-}$ mouse model demonstrated a low level of instability in five candidate genes (*Bax, Chk1, IgfrII, pTEN, Riz*). Only one out of seven thymic lymphomas from *Msh2*$^{-/-}$ mice displayed

instability, the one thymic lymphoma illustrated frameshift mutations in two candidate genes, *Igfr1I* and *pTEN* (Campbell, 2005). The other known MSH2 deficient individual was tested for MSI in tumour tissue. Bougeard *et al* demonstrated that the MSH2 deficient tumour DNA displayed a MSI-stable phenotype in a panel of five markers (Bougeard *et al.*, 2003). The lack of MSI in these samples is unexpected due to the lack of MMR in these cells, and the high mutator phenotype associated with MMR deficiency. The differences in this phenotype may be due to the sensitivity of testing for MSI or the differences in tumorigenesis between HNPCC tumours and humans or mice completely deficient in MMR.

The standard protocol for detecting MSI is to test a panel of five markers (BAT25, BAT26, D2S123, D5S346, D17S250) (Boland *et al.*, 1998). One of problems with this procedure is that MSI does not occur evenly through-out the whole genome. Different microsatellites in the genome are targeted for instability at different rates and this can vary between tissues. Therefore, the panel of five markers may be representative for some tumours, but in non-tumour tissue or tumour tissue from MSH2 deficient individuals this panel may not be representative of the MSI phenotype.

The sensitivity of detecting MSI in tissue may be one reason why I did not observe MSI in the MSH2 deficient individual. The testing protocol for MSI includes extracting tumour or non-tumour tissue and then using a PCR based assay to detect expansions or contractions of the microsatellite. The use of many cells within a tissue section for the PCR reaction may be masking the MSI phenotype. Each cell in the tissue section may not be mutated at the same microsatellite. Therefore, when testing DNA extracted from many different cells, the ability to detect a small percentage of cells mutated at one microsatellite is diminished. One change in experimental protocol used in detecting MSI, is the use of small pool PCR. Prior to the amplification the DNA is diluted one to three genomes per reaction. One problem with this protocol is successfully diluting the DNA to approximately one cell (or genome) per reaction. One reaction may have five cells, compared to twelve cells compared to one cell. Therefore, using small pool PCR

increases the sensitivity compared to undiluted DNA, but is not accurate to one cell per reaction. To improve the accuracy of small pool PCR, single cell PCR can be performed. Separating each cell and analyzing the MSI in each cell would be an accurate representation of the MSI phenotype of the tissue of interest.

The lack of MSI observed in the candidate genes in the MSH2 deficient individual and *Msh2*$^{-/-}$ mice may be attributed to the difference in tumour spectrum between HNPCC individuals and MMR deficient humans or mice. Hematological and brain malignancies are common in MMR deficient individuals and MMR deficient mice primarily develop hematological malignancies whereas loss of MMR in an HNPCC individual results in tumours of the colon, endometrium and gastrointestinal tract. The different in cancer phenotype may be one reason that the MSH2 deficient patient non-tumour DNA did not display MSI. There may be different secondary genes involved in the tumorigenesis process in this individual compared to HNPCC tumours.

Moreover because the MSI analysis was performed in non-tumour tissue, mutations in key genes may have to occur in order to increase the mutation frequency at a high enough level for detection. Previous work in non-tumour tissue was performed on the MMR deficient individuals and demonstrated a low level of MSI instability between 14-47% using a small pool PCR approach (Table 3-2). Similarly, de Wind *et al* demonstrated similar levels of instability in ES cell clones from *Msh2*$^{-/-}$ mice, at 33% and 25% for the markers D7Mit17 and M14Mit15 (de Wind *et al.*, 1995). Thymic lymphomas from *Msh2*$^{-/-}$ mice have been shown to have a greatly increased mutation frequency over the *Msh2*$^{-/-}$ non-tumour tissue and even over that of other tumours arising in the *Msh2*$^{-/-}$ background (Baross-Francis *et al.*, 1998). Therefore, MMR deficiency in non-tumour tissue may require secondary mutations to increase the mutation frequency for detection of a MSI phenotype.

Overall, although no instability was detected in the MSH2 deficient individual, the role of MSH2 is important in tumorigenesis. The sensitivity of MSI detection in non-tumour

tissue will have to increase, such as using single cell PCR, to determine the true level of MSI in non-tumour tissue of the MSH2 deficient individual.

Chapter 4 ♦ MSH2 and the UVB damage response

Introduction

MMR within the cell is a post-replicative DNA repair system responsible for the repair of DNA base mismatches and small DNA loops (up to ten nucleotides). In recent years, the role of MMR has been expanded to include the recognition of other DNA adducts caused by endogenous and exogenous DNA damaging agents. Following the recognition of the DNA adduct, MMR affects the regulation of apoptosis and G2/M cell cycle arrest. The mechanism by which MMR proteins affect apoptosis and/or cell cycle arrest is unclear. Currently there are two models: The first model, the futile repair model, involves the persistence of the DNA lesion, resulting in DNA strand breaks. For example, O^6-meG is mispaired during DNA replication with thymine. The mismatch is recognized by MMR which removes the mispaired nucleotide on the daughter strand. The O^6-meG on the template is not removed and is mispaired with thymine during the next round of DNA replication. Reinitiation of the repair of the mispair is processed by MMR. The successive rounds of repair induce DNA strand breaks, which are recognized by other DNA damage response proteins and signal downstream effects (apoptosis or cell cycle arrest) (Kaina, 1998; Karran, 2001). The second model, the direct signaling model, hypothesizes that MMR proteins recognize the DNA damage and directly signal effector proteins to induce apoptosis or cell cycle checkpoints (Fishel, 1998). MMR proteins are involved in activating apoptosis and/or cell cycle checkpoints in response to many different DNA damaging agents including cisplatin (crosslink inducer), 6-TG, 5-fluoro-2'-deoxyuridine and 5-fluorouracil (base analogues), MNNG and TMZ (alkylating agents). The cell cycle checkpoint pathway for G2/M has been the most prevalent cell cycle arrest pathway affected by MMR deficiency.

G2/M cell cycle checkpoint

The G2/M cell cycle checkpoint is a complex process of direct protein interactions, phosphorylation and dephosphorylation events that activate or inhibit protein function and protein localization. Cell cycle checkpoints are important in allowing time for DNA

repair to occur, prior to DNA replication or cell division, limiting the amount of DNA mutations passed on to daughter cells. A summary of the G2/M arrest pathway is shown in Figure 4-1.

Following genotoxic stress there are two pathways that activate G2/M arrest depending on the DNA damaging agent. The ATM-CHK2-CDC25 pathway is primarily activated following IR, whereas, the ATR-CHK1-CDC25 pathway is primarily activated following UV radiation (reviewed in (Sancar *et al.*, 2004)). These pathways are not mutually exclusive, and overlap in function depending on cellular conditions. The G2/M checkpoint is initiated by DNA damage, although the mechanism of damage recognition and transduction of the signal to downstream targets is not fully understood. MMR proteins and other proteins involved in DNA repair have been hypothesized to bridge the gap between DNA adduct and proteins such as ATM and ATR. Following the recognition of DNA damage ATM and ATR are activated via phosphorylation. ATM and ATR are serine/threonine kinases, which phosphorylate and activate target proteins, such CHK1 or CHK2 (reviewed in (Shiloh, 2003)). Once activated CHK1 and CHK2 inhibit CDC25C via phosphorylation (Matsuoka *et al.*, 1998; Sanchez *et al.*, 1997). Phosphorylation of CDC25C leads to binding of 14-3-3 proteins, and renders CDC25C catalytically less active and/or sequesters CDC25C to the cytoplasm (Graves *et al.*, 2001; Peng *et al.*, 1997).

Overall, the main driver of the G2/M cell cycle transition is the protein CDC2 and associated cyclin B1. CDC2 is phosphorylated at many different residues for different purposes. CDC2 must be phosphorylated at threonine 161 (T161) for activation, and this is mediated by Cdk-activating kinase (Solomon, 1993). CDC2 is also inhibited by phosphorylation at sites threonine 14 (T14) and tyrosine 15 (Y15). These phosphorylation events are catalyzed by proteins kinases WEE1 and MYT1 (Booher *et al.*, 1997; Parker *et al.*, 1995). WEE1 is itself activated through phosphorylation, catalyzed by CHK1. The phosphorylation of WEE1 allows for 14-3-3 binding and enhances the inhibitory kinase activity of WEE1 towards CDC2 (Lee *et al.*, 2001). The

inhibitory phosphates on CDC2 are removed by the phosphatase CDC25C, which as described previously is active when in an unphosphorylated state and inactive when phosphorylated by CHK1 or CHK2.

The tumour suppressor p53 also plays a role in the induction of G2/M arrest. Following activation of ATM/ATR, p53 is phosphorylated at multiple sites by CHK1 or CHK2 (Chehab *et al.*, 2000; Hirao *et al.*, 2000; Shieh *et al.*, 2000). p53 is stabilized and activated via phosphorylation and once activated upregulates 14-3-3 proteins (Waterman *et al.*, 1998). 14-3-3 proteins are involved in the sequestration of CDC2 and CDC25C to the cytoplasm, promoting G2/M arrest (Lopez-Girona *et al.*, 2001).

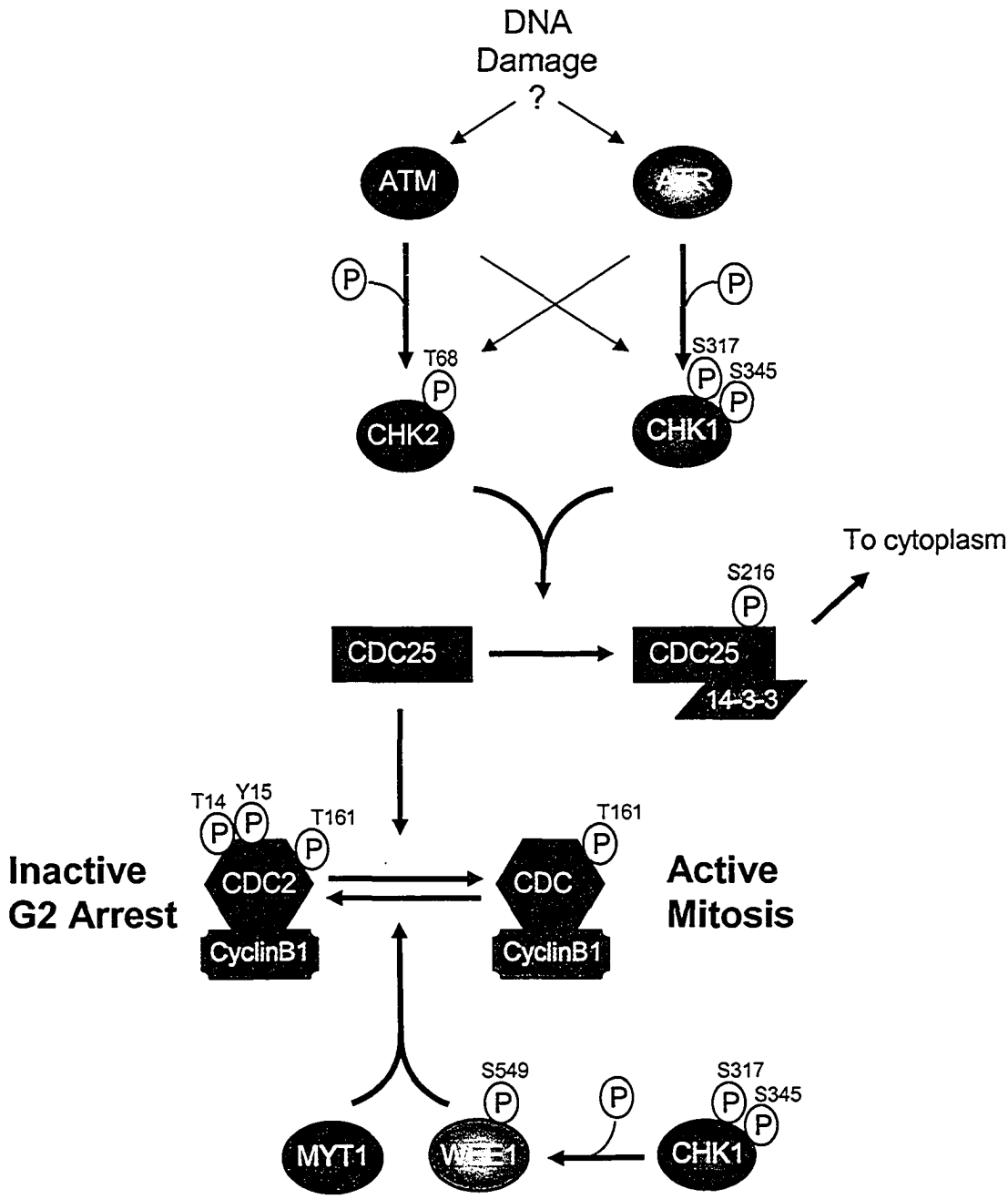

Figure 4-1: G2/M cell cycle checkpoint

In response to DNA damage, ATM and ATR are activated. Once active, ATM and ATR phosphorylate and activate CHK1 and CHK2. CHK1/2 phosphorylate and inhibit CDC25C, this promotes interaction with 14-3-3 and movement of CDC25C to the cytoplasm. Inhibiting CDC25C prevents the removal of inhibitory phosphates on CDC2 and prevents cell cycle progression into mitosis. CDC2 is phosphorylated at T14 and Y15 by MYT1 and WEE1 to induce G2/M arrest, but activated by phosphorylation at T161. Adapted from (Pietenpol & Stewart, 2002).

MMR and G2/M arrest

MMR affects the induction of G2/M arrest in response to many different DNA damaging agents; IR, cisplatin, alkylating agents and 6-TG. Cells deficient in the MMR proteins MSH2, MLH1 or PMS2 are shown to have decreased G2/M cell cycle arrest following IR, cisplatin, MNNG and 6-TG (Adamson *et al.*, 2005; Cejka *et al.*, 2003; Davis *et al.*, 1998; Fang *et al.*, 2004; Hawn *et al.*, 1995; Hirose *et al.*, 2003; Lan *et al.*, 2002; Lutzen *et al.*, 2004; Marquez *et al.*, 2003; Meyers *et al.*, 2001; Meyers *et al.*, 2004b; Stojic *et al.*, 2004; Wang & Qin, 2003; Yamane *et al.*, 2004; Yan *et al.*, 2003; Yan *et al.*, 2004; Yan *et al.*, 2001). In addition to data indicating decreased cell cycle arrest in MMR deficient cells, MMR proteins have been shown to bind to proteins involved in the G2/M arrest pathway. Wang *et al* demonstrated that MSH2 co-immunoprecipitates with ATR and ATRIP regulating the phosphorylation of CHK1 and SMC1 (Wang & Qin, 2003). The MMR protein MSH2 has been shown to interact with CHK1 and CHK2 following MNNG and ionizing radiation (Adamson *et al.*, 2005; Brown *et al.*, 2003). MLH1 is demonstrated as associating with ATM following ionizing radiation (Brown *et al.*, 2003). These results establish a role for MMR proteins in inducing cell cycle checkpoints in response to various DNA damaging agents. The role of MMR in affecting cell cycle arrest in response to UVB radiation has not been examined and is the topic of my research.

MSH2 deficient cell line

MMR deficient cell lines derived from tumours are commonly used to derive MMR function. The increased mutation frequency of these cell lines leads to the likelihood of obtaining secondary mutations that may enhance or alter the effect of MMR deficiency. Moreover, some of the experiments correct the MMR deficiency by reintroducing the chromosome which contains the MMR gene. Due to the correction with a whole chromosome, this technique may alter the effect of MMR deficiency, due to the trisomy karotype of the corrected cell line. To determine the effects of MSH2 deficiency following UVB radiation, a human, non-tumorigenic, MSH2 null cell line immortalized with Epstein Barr virus (EBV) was utilized. This cell line was established from

lymphoblasts from a patient previously identified in our laboratory to be deficient in MSH2. This KM (*MSH2*$^{-/-}$) cell line is a unique resource as it is the only known non-tumorigenic MSH2 deficient cell line. For a control cell line, the JMG (*MSH2*$^{+/+}$) cell line is used and although it is not isogenic to the KM (*MSH2*$^{-/-}$) cell line, the lymphoblastoid cell line was created in the same manner and has been shown to be MSH2 wildtype. The use of the non-tumorigenic cell line compared to cell lines derived from tumour material will more likely result in data that resembles the affect of MMR deficiency *in vivo*.

Results

Cell cycle analysis of JMG ($MSH2^{+/+}$) and KM ($MSH2^{-/-}$) cell lines

I analyzed JMG ($MSH2^{+/+}$) and KM ($MSH2^{-/-}$) cell lines to determine the steady-state levels of cells in G1, S and G2 cell cycle phases in untreated cells. The results are shown in Figure 4-2. The cell line JMG ($MSH2^{+/+}$) has a higher percentage of cells in G2 phase compared to KM ($MSH2^{-/-}$) in resting conditions. This data was used to confirm the cell lines were in good growing conditions when analyzing cell cycle data following UVB radiation.

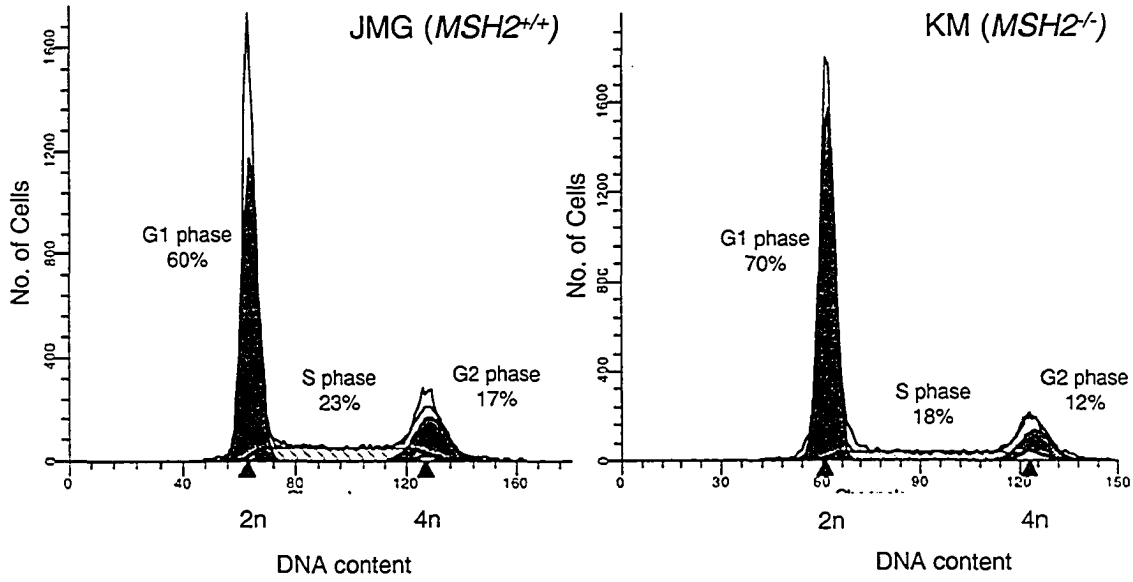

Figure 4-2: Cell cycle distribution of JMG ($MSH2^{+/+}$) and KM ($MSH2^{-/-}$) cells

Representative data comparing the number and percentage of cells in each phase of the cell cycle in untreated JMG ($MSH2^{+/+}$) and KM ($MSH2^{-/-}$) cell lines.

Role of MSH2 in G2/M arrest post-UVB radiation

I analyzed the cell cycle following UVB radiation to determine if MSH2 deficiency affects the induction of G2/M arrest. I determined that KM (*MSH2*$^{-/-}$) cells have decreased levels of G2/M arrest compared to JMG (*MSH2*$^{+/+}$) cells following 250J/m^2 UVB radiation. The results are illustrated in Figure 4-3. There is a significant difference between the percentage of cells in G2 phase, 48 and 72 hours after UVB treatment comparing JMG (*MSH2*$^{+/+}$) to KM (*MSH2*$^{-/-}$). Therefore, MSH2 deficiency leads to a partial decrease in G2/M cell cycle arrest induction following UVB radiation.

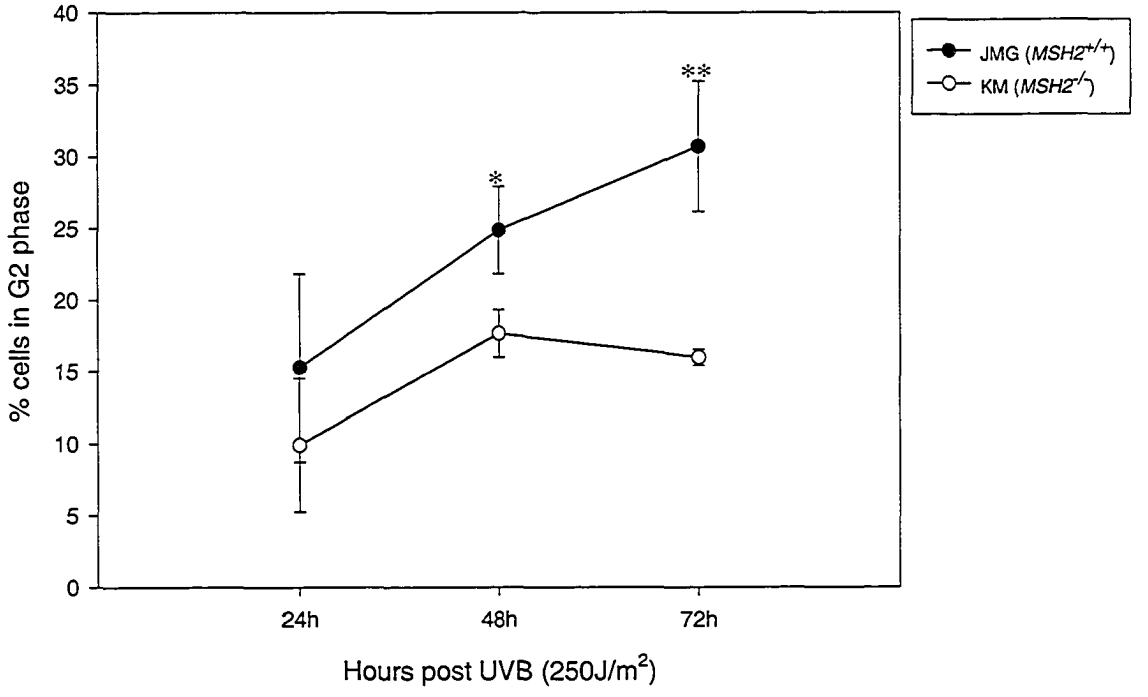

Figure 4-3: G2/M arrest in JMG (*MSH2*$^{+/+}$) and KM (*MSH2*$^{-/-}$) cells following UVB. KM (*MSH2*$^{-/-}$) cells have decreased G2/M arrest compared to JMG (*MSH2*$^{+/+}$) following 250J/m^2 UVB radiation. Data represents mean of three experiments, ± SEM. Significance of the difference between the two cell types was determined using a student *t*-test. * $p<0.05$, ** $p<0.01$.

Induction of cell cycle proteins, dependence on MSH2

To further investigate the role of MSH2 in the G2/M cell cycle checkpoint, I examined protein levels involved in this pathway. I created protein lysates from JMG (*MSH2*$^{+/+}$) and KM (*MSH2*$^{-/-}$) cells collected at various timepoints following 250 J/m^2 UVB radiation. The protein lysates were separated by SDS-PAGE gel electrophoresis and probed with the appropriate antibody. The transition between G2 phase and mitosis is controlled by the complex of CDC2 and cyclin B1. Total levels of CDC2 were analyzed and shown to be of equal levels following UVB radiation in the JMG (*MSH2*$^{+/+}$) and KM (*MSH2*$^{-/-}$) cells lines (Figure 4-4). The phosphorylation level of CDC2 was also analyzed using an antibody against CDC2 phospho-Y15. When CDC2 is phosphorylated at T14 or Y15 it is inhibited and G2/M arrest occurs. The immunohistochemistry results showed decreased amount of CDC2 phospho-Y15, 12, 24, 48 hours after UVB treatment in KM (*MSH2*$^{-/-}$) cells compared to JMG (*MSH2*$^{+/+}$) cells, consistent with the FACScan data described previously.

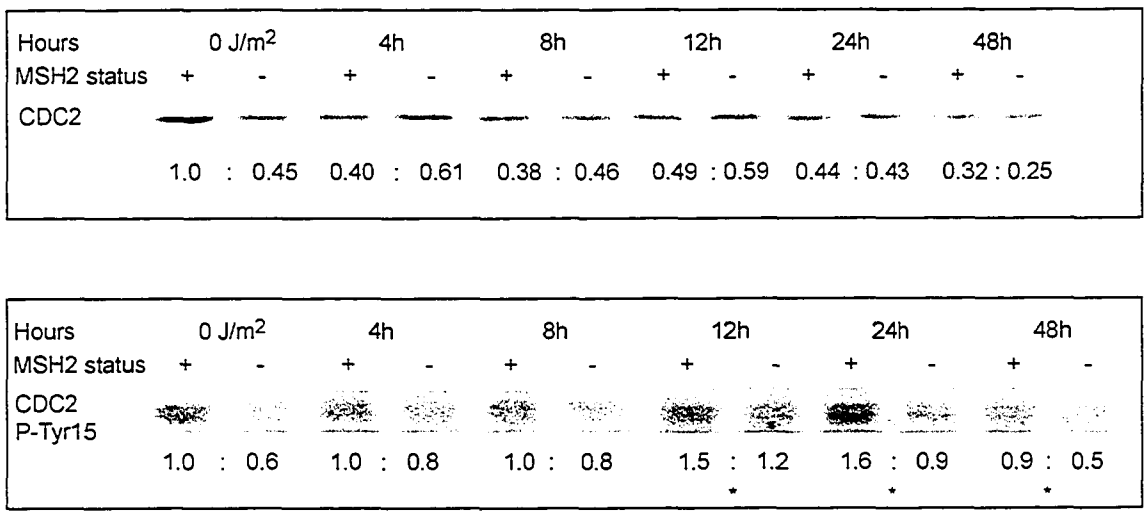

Figure 4-4: CDC2 levels following UVB radiation

Protein levels following 250 J/m^2 UVB radiation, analyzed on SDS-PAGE gels comparing JMG (*MSH2*$^{+/+}$) and KM (*MSH2*$^{-/-}$). Blots were normalized to amido black stain for loading control and compared to JMG untreated for comparison. * refers to a timepoint of interest as discussed in text.

The inhibitory phosphates on CDC2 are removed by the phosphatase CDC25C. I analyzed the levels of CDC25C and demonstrated no difference between the JMG (*MSH2*$^{+/+}$) and KM (*MSH2*$^{-/-}$) cell lines following UVB radiation (Figure 4-5). However, during G2/M arrest, CDC25C is inhibited by phosphorylation at serine 216 (S216). KM (*MSH2*$^{-/-}$) cells had decreased levels of CDC25C phospho-S216; 8, 12, 24 hours after UVB radiation, consistent with previous data.

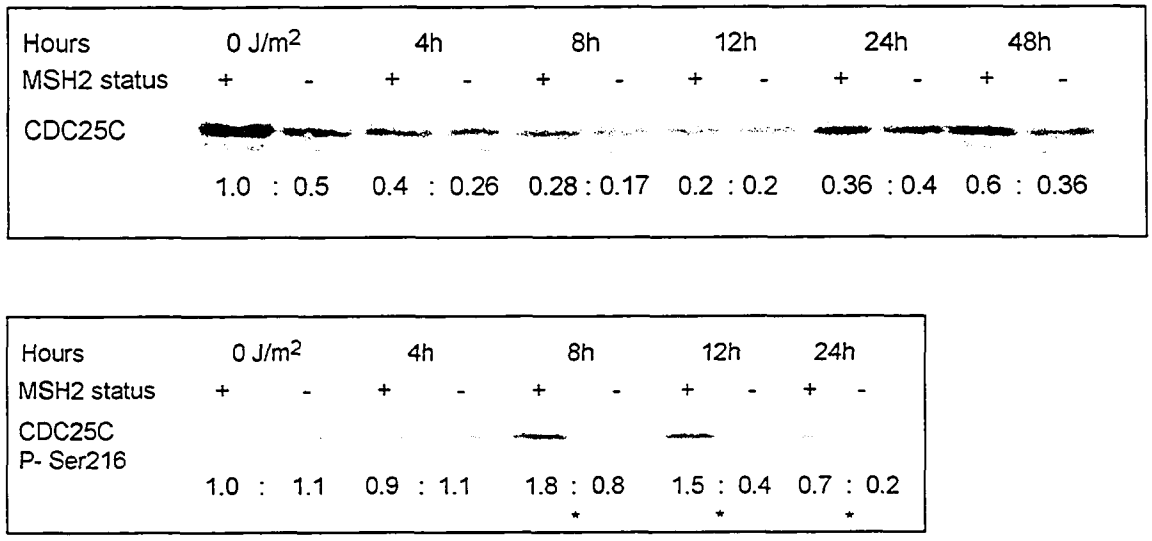

Figure 4-5: CDC25C levels following UVB radiation

Protein levels following 250 J/m^2 UVB radiation, analyzed on SDS-PAGE gels comparing JMG (*MSH2*$^{+/+}$) and KM (*MSH2*$^{-/-}$). Blots were normalized to amido black stain for loading control. * refers to a timepoint of interest discussed in text.

One of the kinases responsible for maintaining the inhibitory phosphates on CDC2 is WEE1. WEE1 levels were analyzed in JMG (*MSH2*$^{+/+}$) and KM (*MSH2*$^{-/-}$) cells following UVB radiation, and demonstrated a slight decrease in the KM (*MSH2*$^{-/-}$) cells 8, 12, 24, 48 hours after UVB exposure (Figure 4-6).

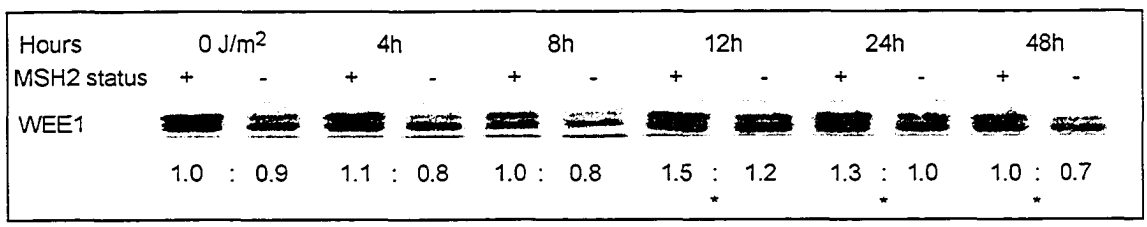

Figure 4-6: WEE1 levels following UVB radiation

Protein levels following 250 J/m^2 UVB radiation, analyzed on SDS-PAGE gels comparing JMG (*MSH2*$^{+/+}$) and KM (*MSH2*$^{-/-}$). Blots were normalized to amido black stain or β-actin for loading control. * refers to a timepoint of interest as discussed in text

The protein level of an upstream kinase in the G2/M cell cycle arrest pathway was also analyzed. CHK1 is responsible for the phosphorylation and inhibition of CDC25C and phosphorylation and activation of WEE1. Inhibition of CDC25C and activation of WEE1 lead to G2/M cell cycle arrest. The levels of CHK1 were similar in the JMG (*MSH2*$^{+/+}$) and KM (*MSH2*$^{-/-}$) cell lines following UVB radiation (Figure 4-7). However, CHK1 is activated through phosphorylation by ATM and ATR at residue serine 345 (S345). KM (*MSH2*$^{-/-}$) cells had decreased phosphorylation of CHK1 at S345, following UVB radiation as early at 4 hours post exposure and continuing to 48 hours (Figure 4-7). Therefore, KM (*MSH2*$^{-/-}$) cells have decreased activated levels of CHK1-phospho S345, leading to decreased G2/M arrest post-UVB radiation.

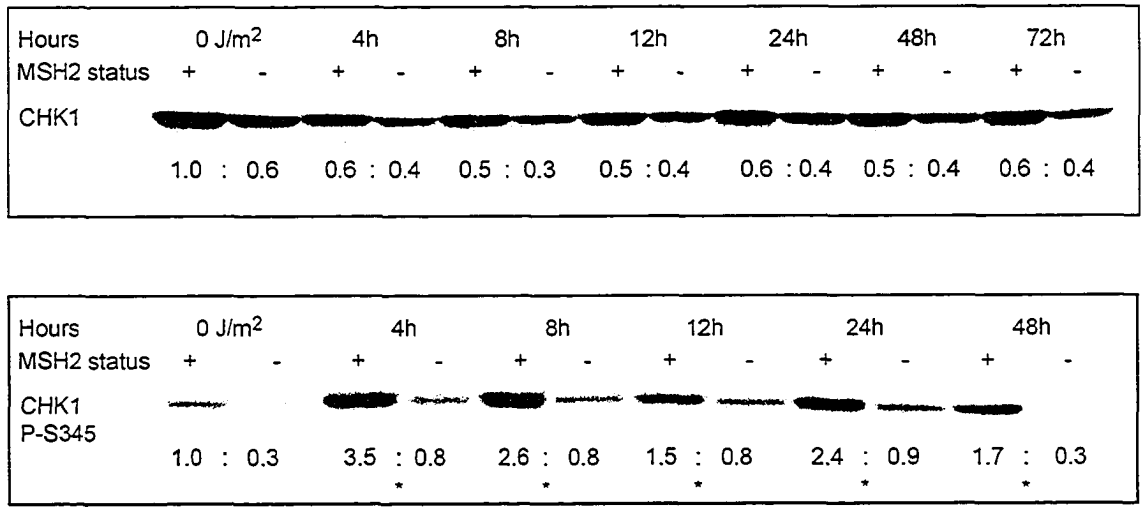

Figure 4-7: CHK1 levels following UVB radiation

Protein levels following 250 J/m^2 UVB radiation, analyzed on SDS-PAGE gels comparing JMG (*MSH2*$^{+/+}$) and KM (*MSH2*$^{-/-}$). Blots were normalized to amido black stain or β-actin for loading control. * refers to a timepoint of interest as discussed in text

KM (MSH2$^{-/-}$) cell line and p53

In addition to the previously mentioned cell cycle proteins, the tumour suppressor p53 was analyzed in JMG (*MSH2*$^{+/+}$) and KM (*MSH2*$^{-/-}$) cell lines following UVB radiation, (Figure 4-8). Total p53 was decreased in the KM (*MSH2*$^{-/-}$) cell line compared to JMG (*MSH2*$^{+/+}$) in response to UVB radiation at 12 to 48 hours after exposure. p53 phospho-S15 displayed near equal levels 8 hours after UVB radiation, but at 24 hours was greatly decreased and no continued induction of p53 phospho-S15 was observed in KM (*MSH2*$^{-/-}$) cell line.

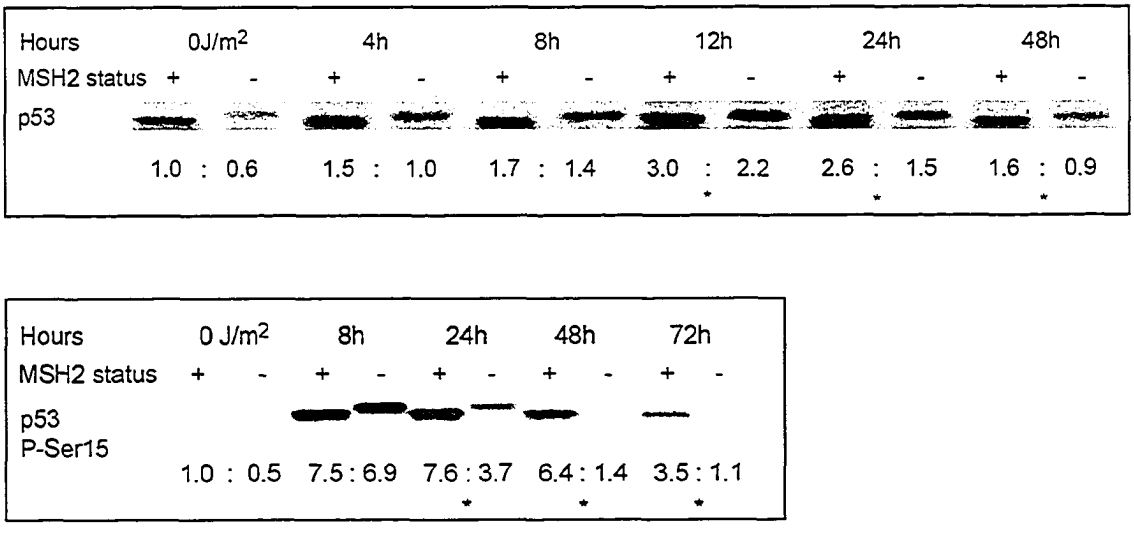

Figure 4-8: p53 and p53-phospho following UVB radiation

Protein levels following 250 J/m^2 UVB radiation, analyzed on SDS-PAGE gels comparing JMG (*MSH2*$^{+/+}$) and KM (*MSH2*$^{-/-}$). Blots were normalized to amido black stain for loading control. * refers to a timepoint of interest as discussed in text

In addition to the difference continued phosphorylation of p53 in the two cell lines a band shift of p53 was observed. To determine which cell line JMG (*MSH2*$^{+/+}$) or KM (*MSH2*$^{-/-}$) had changes in the size of p53, a western blot with other cell lines, p53 wildtype and MSH2 null cell lines was completed (Figure 4-9). This demonstrated that the KM (*MSH2*$^{-/-}$) cell line had a size increase of p53 in comparison to JMG (*MSH2*$^{+/+}$), p53 wildtype cell lines; MALME-3M, and SK-MEL5 and MSH2 deficient cell lines; Jurkat and LoVo.

To investigate the size difference in p53 further, the *p53* coding region were sequenced in JMG (*MSH2*$^{+/+}$) and KM (*MSH2*$^{-/-}$) cell lines. Sequence analysis demonstrated that both cell lines had wildtype sequence for *p53* (data not shown).

The p53 protein is extensively post-translationally modified through acetylation, phosphorylation, ubiquitination, sumoylation, ribosylation and methylation (reviewed in (Appella & Anderson, 2001; Brooks & Gu, 2003; Meek, 2004)). I hypothesized that the size increase in the KM (*MSH2*$^{-/-}$) cell line is due to an increase of post-translation modifications. The initial modification I tested was phosphorylation, due to the availability of phosphatase enzymes and fact that phosphorylation is a common post-translational modification of p53. I treated and mock treated JMG (*MSH2*$^{+/+}$) and KM (*MSH2*$^{-/-}$) cell lines with calf alkaline phosphatase and probed with a p53 antibody to determine if a phosphorylation events account for the larger p53 protein in the KM (*MSH2*$^{-/-}$) cell line. The results demonstrated that the increased size of p53 in the KM (*MSH2*$^{-/-}$) cell line was independent of phosphorylation (Figure 4-10). Further analysis of the p53 status in the KM (*MSH2*$^{-/-}$) cell line must be done to explain the cause of the increased size of p53 in KM (*MSH2*$^{-/-}$) cell line.

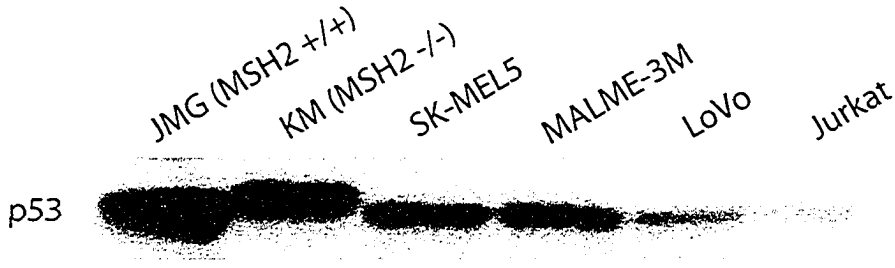

Figure 4-9: p53 comparison

Western blot comparing size of p53 in following cell lines: JMG, *MSH2*$^{+/+}$ lymphoblastoid; KM, *MSH2*$^{-/-}$ non-tumour lymphoblastoid; SK-MEL5, *p53*$^{+/+}$ melanoma; MALME-3M, *p53*$^{+/+}$ melanoma; LoVo, *MSH2*$^{-/-}$ colon tumour; Jurkat, *MSH2*$^{-/-}$ T cell leukemia.

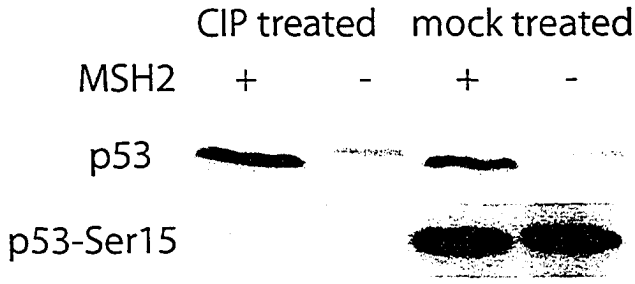

Figure 4-10: p53 phosphorylation assay

Western blots showing protein lysates treated and mock-treated with calf-intestinal phosphatase. The blot was probed with p53 to determine if dephosphorylation resolves the size different in the KM (*MSH2*$^{-/-}$) cell line. p53 phospho-S15 antibody was used as a control to confirm complete dephosphorylation of the protein lysates.

Discussion

The role of MMR in the cell is multi-functional as the proteins appear to be important in DNA repair as well as in influencing levels of apoptosis and cell cycle arrest following specific types of DNA damage. In particular, MMR is involved in inducing G2/M arrest following various DNA damaging agents such as IR, cisplatin, MNNG and 6-TG (Adamson *et al.*, 2005; Cejka *et al.*, 2003; Hawn *et al.*, 1995; Kim *et al.*, 2002; Lutzen *et al.*, 2004; Marquez *et al.*, 2003; Stojic *et al.*, 2004; Wang & Qin, 2003; Yan *et al.*, 2003; Yan *et al.*, 2004; Yan *et al.*, 2001). The role of MMR following UV radiation is less defined. The type of UV radiation used is an important distinction in the current published data when investigating the hypothesis that MMR functions to signal cellular processes such as apoptosis following UV radiation. UVB is the most physiologically significant type of UV radiation as it penetrates the ozone layer and causes DNA lesions such as CPDs and 6-4PPs (reviewed in (Matsumura & Ananthaswamy, 2002)).

I demonstrated that a human MSH2 deficient non-tumorigenic cell line had a decreased amount of G2/M arrest following UVB radiation. Since, MMR deficiency did not lead to a complete abrogation of G2/M arrest, MMR proteins are not the only proteins involved in sensing UV-induced DNA damage and inducing G2/M arrest. However, the reduction in G2/M arrest would be predicted to contribute, along with other factors such as reduced apoptosis and repair, to the development of neoplasia in the absence of MMR. As MMR is not involved specifically in the repair of UVB-induced lesions, the data supports the "signaling model" that describes the mechanism by which MMR affects G2/M arrest and apoptosis following DNA damage. I hypothesize that MMR proteins, specifically MSH2, bind to DNA lesions and recruit cell cycle proteins, such as ATR, initiating the cellular cascade for G2/M arrest. It was previously demonstrated that MSH2 co-immunoprecipitates with ATR and ATRIP, activating CHK1 following MNNG (Adamson *et al.*, 2005; Wang & Qin, 2003). MMR proteins may act similarly when inducing cell cycle arrest, irrespective of DNA damaging agent. Therefore, following UVB radiation, MSH2 may directly influence ATR and CHK1 activation, contributing to

protein complex formation necessary for ATM/ATR activation and induction of G2/M arrest. However, as MMR deficiency only partially decreases the G2/M cell cycle arrest, ATM/ATR are activated by additional DNA damage recognition proteins.

I discovered that MMR deficiency leads to decreased G2/M arrest post UVB using FACScan analysis and examining protein levels involved in signaling of G2/M cell cycle checkpoint. Two of the proteins I analyzed by immunohistochemistry had a double band when probed with a specific antibody. The first antibody I used recognizes CDC2 when phosphorylated at T15. I hypothesize that the doublet was due to a second phosphorylation event at T14 also associated with inhibition of CDC2. Therefore, the bottom band reflects Y15 phosphorylation, and the top band reflects T14 and Y15 phosphorylation together. I saw a similar pattern when evaluating WEE1 protein levels. The antibody I used detects the total amount of WEE1 within the cell. WEE1 is activated through phosphorylation at S549 by CHK1. Therefore, the top band was due to the phosphorylated, active form of WEE1 and the bottom band represents the unphosphorylated form of WEE1.

In addition to examining levels of various cell cycle proteins, I compared the levels of p53 and activated p53 phospho-S15 following UVB radiation in the JMG ($MSH2^{+/+}$) and KM ($MSH2^{-/-}$) cell lines. p53 is an important tumour suppressor and is involved in signaling apoptosis and cell cycle checkpoints following exposure to many different endogenous and exogenous DNA damaging agents. I demonstrated that KM ($MSH2^{-/-}$) cells had a decreased amount of total p53 in untreated conditions and following UVB radiation compared to JMG ($MSH2^{+/+}$) cells. p53 phospho-S15 displayed near equal levels 8 hours after UVB radiation in KM ($MSH2^{-/-}$) cells as compared to JMG ($MSH2^{+/+}$) cells, but at 24 hours the levels were greatly decreased with no continued induction of p53 phospho-S15 in the KM ($MSH2^{-/-}$) cells. Therefore MSH2 contributes to the continued induction of p53 response, but not required for the initial activation of p53 following UVB radiation.

A novel finding was observed when investigating the levels of p53 following UVB radiation in the KM (*MSH2*$^{-/-}$) cell line. A size difference was observed when comparing p53 in the JMG (*MSH2*$^{+/+}$) and KM (*MSH2*$^{-/-}$) cell lines. This was confirmed to be an abnormality in the KM (*MSH2*$^{-/-}$) cell line by comparing p53 in various p53 wildtype and other MSH2 deficient cell lines. p53 was sequenced in JMG (*MSH2*$^{+/+}$) and KM (*MSH2*$^{-/-}$) and both cell lines were shown to have no mutations, therefore, the size increase was not due to mutation or splice site variation. I hypothesized that the size increase of p53 in the KM (*MSH2*$^{-/-}$) cell line was due to an increase in post-translational modifications of p53. p53 is regulated through various modifications such as, phosphorylation, acetylation, methylation, ubiquitination, sumoylation and ribosylation. These processes regulate p53 activation, stabilization, cellular localization and degradation within the cell (reviewed in (Appella & Anderson, 2001; Brooks & Gu, 2003; Meek, 2004)). The size difference of p53 in the KM (*MSH2*$^{-/-}$) cell line according to immunohistochemistry illustrated a difference estimated to be approximately 4 kilodaltons (kDa). I analyzed the phosphorylation level of p53 in JMG (*MSH2*$^{+/+}$) and KM (*MSH2*$^{-/-}$) cell lines due to the availability of phosophatase enzymes. I demonstrated that the size increase was independent of phosphorylation because the cellular lysates were treated with calf intestinal phosphatase and the size difference of the p53 in the KM (*MSH2*$^{-/-}$) cell line did not resolve. It is unlikely that the size difference is due to increased ubiquitination or sumoylation because ubiquitin is 8 kDa and SUMO-1 is 11 kDa, which are both larger than the estimated 4 kDa size increase of p53. Therefore, the size difference in p53 in the KM (*MSH2*$^{-/-}$) cell line is likely due to an increase in the smaller covalent modifications, such as acetylation, methylation or ribosylation.

Previous data from the Andrew laboratory has demonstrated a role for Msh2 and Msh6 in the apoptotic response post-UVB. Here, I demonstrate that only cells with competent MSH2 have normal G2/M arrest post-UVB damage. Previously published data demonstrated that specific mutations in Msh6 and Msh2 decrease DNA repair function of the MMR proteins, but do not affect the apoptotic signaling following DNA damage (Lin *et al.*, 2004; Yang *et al.*, 2004). To further investigate the role of MMR proteins in the

cellular response to UV-induced DNA damage, mutation analysis can be performed to differentiate the repair function of the MMR proteins and the cell cycle checkpoint or apoptotic signaling in response to UVB radiation. The ability to determine which amino acid residues are involved in DNA repair or the response (apoptosis or cell cycle arrest) to DNA damage is important in understanding MMR's role in UVB-induced tumorigenesis.

Furthermore, the amount of MMR protein within the cell is also important in contrasting the repair function and signaling function of MMR. Cejka *et al* demonstrated that only 10% of normal levels of *Mlh1* expression was needed for DNA repair activity, but was not sufficient for inducing apoptosis following MNNG treatment (Cejka *et al.*, 2003). Therefore, in addition to mutational analysis, the levels of various MMR proteins can be analyzed to differentiate the functions of MMR proteins in response to DNA damaging agents such as UVB radiation.

Overall, MMR contributes to both the apoptotic and cell cycle checkpoint response of cells to UVB radiation. MSH2 may play an important role in preventing UV-induced skin cancer without necessarily being directly involved in the repair of UV-induced DNA adducts. Elucidating the multi-functionality of MMR is important in understanding tumorigenesis in skin and other cancers, leading to improved diagnosis, treatments and chemotherapeutics.

Chapter 5 ♦ Conclusions

MMR is important in maintaining the fidelity of DNA within the cell. MMR proteins repair DNA base mismatches and small insertion/deletions loops that occur during DNA replication. MMR also functions in the DNA damage response following various damaging agents, such as alkylating agents and 6-TG. Individuals with heterozygous mutations develop the cancer syndrome HNPCC, which presents with various types of cancer, predominantly colorectal or endometrial by the age of 50 years. There is a small cohort of individuals that have been described with homozygous or compound heterozygous mutations in one of the MMR genes. These individuals develop cancer at predominantly, a young age depending on the severity of mutation. Our laboratory described the first MSH2 deficient individual and created a cell line using non-tumour lymphoblasts for use in determining the role of MMR in the DNA damage response pathway. As most experiments to date have been performed in MMR null tumour lines that have most likely acquired a host of additional mutations that would also affect cellular responses such as apoptosis and cell cycle, this MSH2 null cell line from non-tumour material is an important resource for furthering our understanding of the roles of MMR within a cell.

Genomic instability of a MSH2 deficient individual

Deficiency in MMR leads to an increased mutation frequency and MSI. MSI is a hallmark of HNPCC tumours, and is also found in tumours from MMR deficient individuals. The level of instability in non-tumour tissue of the MMR deficient individuals is unclear. I analyzed ten genes that contain coding microsatellites that were previously shown to be mutated in HNPCC tumours. I hypothesized that there would be a high degree of instability, within non-tumour lymphoblasts isolated from the MSH2 deficient individual, due to the complete lack of MSH2. Contrary to my hypothesis I found this individual's DNA had a MSI stable phenotype in the ten candidate genes tested. I hypothesized that the lack of MSI in the ten candidate genes was either due to a

difference in genes mutated in the absence of MMR that would promote the development of hematological malignancies observed in the MMR null individuals compared to HNPCC associated tumours, or a low amount of instability that was not detected in my analysis.

Future Directions

1) Perform MSI analysis on the MSH2 deficient individual's DNA with a small pool PCR technique to increase the detection sensitivity.
2) Perform MSI analysis, using single cell technique, on KM (*MSH2*$^{-/-}$) cell line over a period of time to determine the level of MSI induced by growing time and inherent increased mutation frequency.
3) Analyze additional genes with coding microsatellites in MMR deficient individuals with hematological malignancies to determine if there are a set of genes highly mutated in hematological malignancies compared to target genes in HNPCC tumours.
4) The MSH2 deficient individual previously described by our laboratory is currently being treated for B cell lymphoma. If a sample of the B cell lymphoma can be obtained, these cells could be tested for MSI and results compared to the results of the non-tumour MSI analysis.

MSH2 and the response to UVB radiation

MMR proteins are involved in apoptosis and cell cycle checkpoint induction following many types of DNA damage. Following UVB radiation MMR proteins affect apoptosis induction *in vitro* and *in vivo*. The effect of MMR deficiency on UVB induced cell cycle arrest has not been published and was the focus of my second project. I analyzed G2/M cell cycle arrest following UVB radiation in a human, non-tumour MSH2 deficient cell line created from a previously described MSH2 deficient individual. I demonstrated that MSH2 deficiency leads to a decrease in G2/M arrest following UVB radiation. In addition, a novel result demonstrated p53 in the KM (*MSH2*$^{-/-}$) cell line to be at an increased size compared to JMG (*MSH2*$^{+/+}$) cells and other p53 wildtype cell lines as

observed using immunohistochemistry. I determined that this difference is independent of sequence variation and phosphorylation levels of p53 in KM (*MSH2*$^{-/-}$).

Future Directions

1) Create an isogenic control cell line by re-introducing MSH2 cDNA into KM (*MSH2*$^{-/-}$) cell line. Isolate stable clones with wildtype, high and low levels of MSH2 and use these cell lines to determine the effect of different MSH2 levels in the DNA damage response pathway.
2) Continue to analyze the effect of MSH2 deficiency in the UVB response, by examining the levels of ATR and ATM following UVB radiation. Determine if MSH2 interacts with ATR and ATM using co-immunoprecipitation and co-localization studies.
3) Induce a series of mutations in MSH2 to determine which amino acid residues are important in UV-induced DNA lesion binding and signaling of G2/M cell cycle arrest following DNA damage.
4) Analyze additional post-translational modifications (ubiquintination, acetylation, sumoylation, methylation, ribosylation) of p53 in the KM (*MSH2*$^{-/-}$) cell line, to determine the cause of the increased size of p53 in KM (*MSH2*$^{-/-}$) cells.
5) Perform functional analysis of the p53 isolated from KM (*MSH2*$^{-/-}$) cell line, to determine the effect of the increased size of p53.

MMR is an important system in maintaining genomic fidelity of the cell. MMR functions can be separated into two categories: the repair of post-replicative DNA lesions such as DNA mismatches and small insertion/deletion, and the recognition and signaling of endogenous or exogenous DNA damage affecting repair, apoptosis or cell cycle. My data makes use of a rare MSH2 deficient individual to further our understanding of the role of MMR after DNA damage, in particular UVB radiation. Furthermore, my characterization of the patient by assessing the levels of MSI and the unusual alteration in the key protein p53 helps add to our understanding of how a lack of MMR contributes to tumorigenesis. Finally, MMR is lost in a significant numbers of cancers of all tissue

types; therefore, the knowledge from this thesis is relevant in broadening our understanding of tumorigenesis and improved understanding of cancer genetics.

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