CHARACTERIZATION OF HUMAN MRE11 AND MLH1 INTERACTING DOMAINS, 
AND THE EFFECTS OF PATHOGENIC MUTATIONS ON 
PROTEIN INTERACTION 

By 

ANTHONY THIEN VO 

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Chair
CHARACTERIZATION OF hMRE11 AND hMLH1 INTERACTING DOMAINS,
AND THE EFFECTS OF PATHOGENIC MUTATIONS ON
PROTEIN INTERACTION

By Anthony Thien Vo, M.S.

Washington State University

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Chair: Chengtao Her
ABSTRACT

Hereditary Non-polyposis Colorectal Cancer (HNPCC) is largely attributed to mutations in human mismatch repair genes where currently, up to 55.6% of all incidences occur in hMLH1 gene. Our recent report on a direct protein interaction between hMLH1 and hMRE11 suggest that the interplay between these two proteins might play important roles in DNA mismatch repair and the pathogenesis of HNPCC. As an initial step to characterize the functionality of this protein interaction, we have determined the interacting domains of these two proteins. Specifically, the interacting domains were narrowed to C-terminal between amino acids 495-756 and 452-634 for hMLH1 and hMRE11 respectively. The hMRE11 interacting region is significantly overlapped with the interacting region for hPMS2. In addition, we have found that four out of seven HNPCC missense mutations (L574P, K618T, R659P, and A681T) showed a complete disruption of interaction, two mutations (Q542L and L582V) displayed partial defects, and one mutation (E578G) showed similar interaction to that of the hMLH1-hMRE11 wild type. This suggests the disruption of hMLH1-hMRE11 interaction could serve as an alternative molecular mechanism for the pathogenic effects of these mutations.

Next, we have assessed the involvement of hMRE11 in the process of MMR. Evidences of hMRE11 as an essential requirement for human MMR process were examined by an in vitro partial reconstitution MMR assay. Addition of partially purified recombinant hMRE11 protein successfully repaired base mismatch in the 3'-5' direction. Thus this lends support that hMRE11 3'-5' exonuclease activity is involved in excising DNA fragment containing mismatch base.
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Further, I would like to dedicate this thesis to my parents, whom have provided both mental and financial supports throughout my academic career.
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INTRODUCTION

An accurate and faithful transmission of genetic materials from parent to daughter cells is essential for genomic stability and mutation avoidance. Though DNA polymerase is very efficient in nucleotide incorporation, it does make mistake for every $10^{-3}$ to $10^{-6}$ base (Showalter et al. 2001). Hence, genomic surveillance is crucial for monitoring damaged DNA and mediates appropriate repair response. Once aberrant chromosome structures are detected, cell cycle progression is halted at G1/S phase (intra-S checkpoint) and/or G2/M phase until damage is repaired (Falck et al. 2002). Unchecked DNA damage would result to subsequent cellular mutation, abnormal recombination and chromosomal breakage or loss. Failure to survey, detect and repair DNA damage is clinically resulted to cancer-prone diseases such as HNPCC (Hereditary Non-polypopsis Colorectal Cancer), ataxia-telangiectasia (AT), Nijmegen breakage syndrome (NBS) and AT-like disorder (ATLD). These diseases shared common phenotypes of hypersensitivity to radio-ionization, predisposition to cancer, and immunodeficiency (Sandrine et al. 2001).

DNA damage response

Response to DNA damage involved myriad of protein complex that function in sensory and detection, transducing signals and subsequent processing damage by inducing cell cycle arrest, apoptosis, or DNA repair. Poly-ADP-ribose polymerase (PARP) and DNA dependent protein kinase (DNA-PK) have long been proposed to be DNA damage sensory protein for their ability to bind DNA and be activated by DNA DSB (Pleschke et al. 2000, Shackelford et al. 1999). In addition, study in yeast suggested four other potential candidates: Rad1, Rad9, Hus1 and Rad17, all of which do have homologs in human. Rad9, Rad1 and Hus1 (9-1-1) are PCNA related structure proteins that form a heterotrimeric DNA damage response complex. Rad17 is a
replication factor C homologous protein that associated with Rad1-Rad9-Hus1 complex that function in maintaining the damage signal until repair is completed (Bermudez et al. 2002, Zhou, et al. 2001). Recent in vitro analysis indicated that Rad17 interacted with 9-1-1 complex, through the binding of Rad17 to Rad9, in an ATP dependent manner, but did not required ATP hydrolysis.

In response to DNA damage signal, phosphoinositidekinase (PIK) related proteins, ATM (ataxia telengiectasia mutated) and ATR (ATM Rad3 related) phosphorylated and activated many downstream checkpoints effectors such as p53, Chk1, Chk2, BRCA1, Rad17, NBS1 and DNA repair proteins (Bakkenist et al. 2003, Brown et al. 2003, Wang et al. 2003).

UV radiated cells induce dimerization of pyrimidines. This poses problem for DNA transcription and replication, and is resolved by nucleotide excision repair (NER) mechanism (Costanzo et al. 2001). However, chromosomal double strand breaks (DSB) caused by ionizing radiation or reactive oxygen species is repaired by means of homologous recombination (HR) and non-homologous end joining (NHEJ), of which the latter process is more prominent in mammalian system (Costanzo et al. 2001, Critchlow et al. 1998). In HR process, the free single strand 3’ end invades the undamaged, homologous DNA duplex, using it as a template for extension. On the other hand, DSB repair via NHEJ does not require homologous template for ligation, but rather blunt ends are joined directly. This may result to insertion or deletion of sequences, which is important in V(D)J recombination as in the case of generating a diverse T-cell antigen receptors and antibodies. In NHEJ process, DNA-end binding heterodimer proteins Ku70/Ku86 bind free DNA ends, recruiting DNA protein kinase (DNA PK) and its catalytic subunits (DNA PKcs). Following sequence alignment, DNA ends are ligated by DNA ligase VI and XRCC4 (Haber et al. 1998, Hopfner et al. 2002).
MRE11-Rad50-NBS1 complex

MRE11-Rad50-NBS1 (Xrs2 in yeast) complex is a crucial component to signaling pathway for DNA damage and cellular response to DNA DSB. DNA damage activates ATM kinase activity that phosphorylates mammalian MRE11 and NBS1 protein (D’Amours et al. 2002), which in turn inactivate cdk2 and initiates S-phase check point response to delay replication (Costanzo et al. 2001, Falck et al. 2002). Failure to detect DNA damage or induce signaling process lead to chromosomal instability and generation of radio-resistant DNA synthesis (RDS) (Sandrine et al. 2001). Individuals inflicted by this genomic instability often inherited cancer prone diseases such as AT, NBS and ATLD as mentioned above.

Further, it was proposed that MRE11-Rad50-NBS1 complex involves in DNA damage sensor process. Upon inducing DSB via “ultrasoft x-ray” to human fibroplast, a homogenized distributed human MRE11 within the nucleus migrated to damaged site within 30 minutes. Also, studies with immunofluorescent showed that hMRE11-Rad50 formed distinct nuclear foci at site of DNA DSBs (Maser et al. 1997). All suggests MRE11 complex involves in early DNA damage response.

DNA Mismatch Repair (MMR)

MMR is a highly conserved process from bacterial to mammalian system. It plays a pivotal role in post-replicative repair of mistakes made by DNA polymerase, thus maintaining genomic mutation to an acceptable level. Secondly, it also function in DSB repair by recognizes mismatch recombinant intermediates; hence, promoting genomic stability by not allowing completing recombination of divergent sequences within a single genome or between organisms (Ellison et al. 2001). Thirdly, MMR is implicated to regulate cell cycle and is linked to p53-dependent apoptosis in response to DNA damage (Sandrine et al. 2001). Interestingly, two
MMR proteins, MSH4 and MSH5, appear to have no involvement in mutation avoidance but generally function in promoting and stabilizing the formation of Holliday junction (Harfe et al. 2000).

MMR proteins were first discovered in bacteria. Three major MMR proteins had been constituted in vitro: MutS, MutL, MutH. MutS is an ATPase that recognizes and binds as a homodimer to base/base mismatch or insertion/deletion loops (IDLs). MutS binding promotes DNA loop formation and protein conformational changes in presence of ATP. This binding also recruits homodimer MutL, another ATPase. MutL then sequesters MutH and activates its endonuclease activity. Strand discrimination between template and newly synthesized strand is based on the adenine-methylation on GATC sequence by dam methylase. MutL enhance ATP-hydrolysis dependant translocation of MutS along DNA in search for hemimethylated strand. MutH then binds and nicks the nascent (unmethylated) strand to enable exonuclease activity. MutL then loads MutU (HeliaseII/UvrD) onto MutH nick site. MutU then excises fragment containing mismatch. In final step, a new strand is resynthesize using template homology by single stranded binding protein (SSB), DNA Polymerase III holoenzyme and ligase(Buermeyer et al. 1999, Kolodner et al. 1999).

Unlike bacterial MMR system where the process is mediated by single MutS and MutL that homodimerized. Currently, there are 5 MutS homologs (MSH) and 4 MutL homologs (MLH) have been identified in the mammalian system but no homologs was identify for bacterial MutH (Harfe et al. 2000). In mammalian MSH proteins, MSH2 heterodimerizes with MSH6 and MSH3 to form MutS[,] and MutS[,], respectively. Here both complexes share a redundant role in recognizing IDLs. However, MutS[,] has an additional role in surveying base/base mismatch. Interestingly, MSH4 and MSH5 appear to have no mutation avoidance ability.
Though, they both function in promoting cross over events and maintaining stability of Holliday junction. In mammalian, MLH1 heterodimerizes to PMS2 and PMS1 to form MutL\[\] and MutL\[\] respectively. MutL\[\] interacts to MSH2 heterodimer complex and plays a crucial role in repairing base-base mismatch and small insertion/deletion loop (IDL). On the other hand, the role of MutL\[\] in MMR process has not yet been characterized. In addition, MLH1 heterodimerizes with MLH3 and interacts with MSH2-MSH3 complex to mediate IDLs repair (Buermeyer et al. 1999, Kolodner et al. 1999).

Inherited mutations in MMR genes greatly contribute to hereditary non-polyposis colorectal cancer (HNPCC) or Lynch syndrome. HNPCC is an autosomal dominant inherited disease. An inflicted individual displays predisposition for colorectal cancers and high risks of developing tumor in related areas of gastrointestinal and urogenital tracts (Guerrette et al. 1999). Genetical analysis of HNPCC kindreds indicates that predominant gene alteration results to the disease are amino acid substitution and small in-frame deletion. Of which, mutations in human MLH1 protein contribute the majority of the cases (56%) (Kondo et al. 1999). Moreover, 30% of all cases were due to single amino acid replacements in MLH1 protein ((Nystrom-Lahti et al., 2002).

**Aims of the study**

In this study, we aim to investigate the biochemical and functional properties of hMRE11-hMLH1 protein interaction. Specifically, we will map the interacting regions that mediate protein interaction and introduce HNPCC mutation on hMLH1 interacting domains to examine its effects, if any, on the protein interaction. Further, we would like to investigate the functionality of hMRE11 in DNA MMR, specifically regarding its 3’-5’ exonuclease activity. We speculate that this exonuclease activity is neededed to excise DNA fragment containing
mismatch on the leading strand during replication, in complementation to hExoI that would excise DNA fragment on the lagging strand. Lastly, we will attempt to map the interacting regions that mediate hMRE11 homodimerization using our current deletion constructs. Moreover, we would like to examine the effects of ATLD pathogenic mutations, N117S and truncation of aa1-634, on both hMRE11 homodimerization and hMRE11-hMLH1 interaction. In doing so, we would obtain further understanding about the molecular basis for ATLD disease.
Evidence for a direct association of hMRE11 with the Human Mismatch Repair Protein hMLH1

CHENGTAO HER *
ANTHONY T. VO
XILING WU

School of Molecular Biosciences and Center for Reproductive Biology,
PO Box 644660,
Washington State University,
Pullman, WA 99164-4660

Running title: Human hMRE11 and hMLH1.

*Address all correspondence and reprint requests to:
Dr. Chengtao Her at above address.
Telephone: (509) 335-7537
FAX#: (509) 335-9688
E-mail: cher@wsu.edu
ABSTRACT

In both mitotic and meiotic processes, cellular surveillance of the integrity of genetic information transmission from parental cells to their subsequent generations is carried out by a network of proteins primarily involved in cell-cycle regulation, DNA replication, DNA repair, and chromosome segregation. Within this context, the mammalian MRE11 represents an essential multifunctional protein that promotes repair of DNA double-strand breaks and plays a role in the signaling of DNA damage response. Mutations in human hMRE11 gene could contribute to the rare “AT-like” disorder. However, at present time the functional roles of hMRE11 in these cellular processes are elusive. In the current study, we provide evidence that hMRE11 interacts physically with the mismatch repair protein hMLH1 through yeast two-hybrid analysis. In addition, we show that recombinant hMRE11 and hMLH1 proteins interact when these two proteins are coexpressed in bacterial cells, and both proteins can be coimmunoprecipitated from human cell extracts. Furthermore, hMRE11 and hMLH1 display similar expression patterns when examined with a human normal/tumor DNA array. Together, these data suggest that hMRE11 and hMLH1 might act in a cooperative fashion during DNA damage detection, signaling, and repair.
1. INTRODUCTION

The accurate transmission of genetic information from a parent cell to subsequent generations is essential for survival, and is largely dependent on the integrity and the interplay of a network of protein factors involved in cell-cycle regulation, DNA replication, DNA repair, and chromosome segregation (1, 2). Cells defective for these protein factors will not respond effectively to DNA damage, and thus are more prone to mutations, genetic instability, and chromosome breakage. Recent studies have clearly established a functional link between increased risk of cancer and mutations in genes involved with the DNA damage response and repair network. Among many protein factors discovered, human hMRE11 represents an essential multifunctional protein that promotes repair of DNA double-strand breaks and plays a role in the signaling of DNA damage response (3). Disruption of MRE11 genes in mouse or chicken displays a lethal phenotype, whereas MRE11 is not required for the survival of S. cerevisiae and C. elegans cells (4-7). Although at the present time, the functional roles of hMRE11 in cellular processes are elusive, it is known that the hRAD50-hMRE11-NBS1 complex is essential for homologous recombination (HR) and non-homologous end-joining (NHEJ) repair of DNA double-strand breaks -- the most deadly forms of DNA lesions that may occur during exposure to a various of genotoxic agents such as ionizing radiation and chemotherapeutic agents, as well as during DNA replication, V(D)J recombination, and meiotic recombination (3). hMRE11-associated complexes display dynamic spatial and temporal cellular distribution and form DNA-damage induced foci at the site of DNA damage (8), suggesting that this protein complex plays an early role in DNA damage response (9, 10). Recent findings have revealed that a group of cancer predisposition and chromosome instability syndromes, including Nijmegen breakage syndrome (NBS), ataxia-telangiectasia-like disorder
(A-TLD), and ataxia telangiectasia (AT), could be attributed to defects in NBS1, hMRE11, and ATM genes, respectively (11-13). Given the similar end clinic manifestations displayed by NBS, A-TLD, and AT patients, it has long been speculated that these disorders might be resulted from defects in a common cellular process such as DNA-damage response (3). In fact, genetic and biochemical studies in recent years have demonstrated that the gene products of NBS1, hMRE11, and ATM might act together and function in DNA-damage response and repair (4, 14-16).

The maintenance of genetic integrity also requires the DNA mismatch repair (MMR) system, which defines one of the most important molecular mechanisms in the faithful transmission of genetic information during DNA replication. Eukaryotic MMR systems consist of multiple MutS and MutL homologous proteins that form higher order complexes and participate in the recognition, binding, and correction of both single-base mismatches and small loops formed by insertions or deletions in the DNA (18, 19). Mutations in human mismatch repair genes, particularly hMLH1 and hMSH2, are responsible for the pathogenesis of majority of hereditary nonpolyposis colorectal cancers (HNPCC), and inactivation of the hMLH1 gene is also a frequent observed event in sporadic tumors associated with microsatellite instability (reviewed in 20). Interestingly, components of the MMR system are also found to function in other essential cellular processes beyond the scope of post-replication repair; increasing evidence suggests that MMR proteins are linked to cell cycle checkpoint, and particularly involved in signaling apoptosis induced by DNA damages such as alkylation-derived DNA adduct, O6-methylguanine (21, 22). Moreover, MMR-deficient cells frequently confer resistance phenotypes to a wide spectrum of chemotherapeutic drugs (23). These observations highlight a potential role of MMR components in sensing certain types of DNA damage and the overall
cellular response to DNA damage repair in coupling with cell cycle regulation. This notion fits well with the observation that MLH1-deficient cells cannot undergo ATM-dependent phosphorylation of c-Abl in response to cis-platinum induced DNA damage (24). Most recently, it is found that a number of proteins involved in DNA damage repair, such as DNA mismatch repair proteins (hMSH2, hMSH6, hMLH1), ATM, BLM, and the hRAD50-hMRE11-NBS1 protein complex, resides together with the breast cancer protein BRCA1 within a protein complex of mass greater than 2 MD. Based on its composition, this complex is termed as BASC (BRCA1-associated genome surveillance complex), suggesting its potential role in the recognition and repair of aberrant DNA structures (25). However, very little is known about the stoichiometry and the dynamics of individual protein-protein interaction within the BASC complex. Recent observations that hMLH1 and BLM proteins physically interact one to the other have began to reveal the intricate and functional properties of the BASC complex (26, 27).

To obtain a better understanding of the biological processes in which hMRE11 and hMLH1 proteins involved, we now report that the multifunctional protein hMRE11 directly interacts with the mismatch repair protein hMLH1, suggesting a direct link between hMLH1 and the hRAD50-hMRE11-NBS1 protein complex.
2. MATERIALS AND METHODS

2.1. Yeast two-hybrid analysis. Human hMRE11 and the MutL homolog hMLH1 cDNA ORFs were obtained from a human testis Marathon-Ready cDNA preparation (Clontech Laboratories, Palo Alto, CA). Yeast two-hybrid analysis was carried out with the Matchmaker Two-Hybrid System (Clontech). Yeast transformants harboring both DNA-binding domain and activation domain constructs were selected on SD/-Leu-Trp medium. Positive protein-protein interactions were ascertained by the transcription activation of highly inducible GAL1 UAS driving HIS3 reporter gene in the reporter host strain AH109 (MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1_UAS-GAL1_TATA-HIS3, GAL2_UAS-GAL2_TATA-ADE2, ura3::MEL1_UAS-MEL1_TATA-lacZ).

2.2. Western blot analysis and antibodies. SDS-PAGE was performed with 4-20% gradient acrylamide at 100 volts for ~1.5 hr. Proteins that had been separated by SDS-PAGE were transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) at 15 volts for 2.5 hr with a semidry transfer apparatus (Bio-Rad Laboratories, Hercules, CA). Immunoreactive proteins were detected with the ECL Western blotting system (Amersham pharmacia biotech). Antibodies used in this study included anti-hMRE11 monoclonal 12D7 (GeneTex, Inc., San Antonio, Texas), anti-hMRE11 polyclonal (Novus Biologicals, Inc., Littleton, CO), anti-hMLH1 monoclonal (Calbiochem, San Diego, CA), anti-hMLH1 polyclonal C-20 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-β-catenin monoclonal (Transduction Laboratories, Lexington, KY), and HRP-conjugated secondary antibodies (Bio-Rad laboratories).

2.3. Co-expression of recombinant hMRE11 and hMLH1 fusion proteins. To generate recombinant proteins fused to either glutathione S-transferase (GST) or His6-tag, the hMRE11 coding sequence was cloned in-frame into pGEX-6p bacterial expression vector (Pharmacia, Piscataway, NJ), and hMLH1 coding sequence into pET-28a vector (Novagen, Madison, WI). Fusion proteins were produced in BL21(DE3)-RIL host strain (Stratagene, La Jolla, CA).
Soluble fractions of whole-cell lysate were prepared in PBS containing 1x complete EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany) with a standard sonication procedure from bacterial cells harboring expression constructs hMRE11-pGEX-6p and hMLH1-pET-28a, as well as cells harboring expression constructs hMRE11-pGEX-6p and empty pET-28a vector.

2.4. Immunoprecipitation. Co-immunoprecipitation analysis of hMRE11 and hMLH1 interaction was carried out by the use of bacterial expressed recombinant fusion proteins, as well as with HeLa cell extracts. Specifically, 3.0 ml of soluble fractions of bacterial cell lysate was precleared by incubation with 50 µl of 50% slurry of BSA-saturated Protein A/rProtein G-Agarose (GibcoBRL, Gaithersburg, MD) at 4 °C for 15 min with continuous gentle rocking, and then supernatants were recovered by centrifugation at 4 °C and incubated individually with approximately 1-2 µg of anti-hMLH1 or the control anti-β-catenin monoclonal antibody at 4 °C for 2 hours. Alternatively, in order to investigate whether the observed protein interactions could be “bridged” through DNA molecules, 1.0 ml of precleared lysate was used to incubate with 25 units of DNase (Promega, Madison, WI) at room temperature for 25 min prior to immunoprecipitation analysis. After removing aggregates from the reaction mixtures by brief centrifugation at 14,000 rpm at 4 °C, immunoprecipitates were captured with 40 µl of 50% slurry of BSA-saturated Protein A/rProtein G-Agarose. Agarose beads were then washed sequentially once each with buffer 1xPBS, RIPA (1x PBS, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS), a high-salt buffer (500 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.1% SDS, 1.0% Igepal CA-630), LiCl buffer (250 mM LiCl, 50 mM Tris-HCl, pH 8.0, 0.5% sodium deoxycholate, 1.0% Igepal CA-630), and a final wash in 1xPBS. Protein retained on agarose beads was eluted in SDS-sample buffer and subjected to Western blot analysis.

For investigation of in vivo interactions between hMRE11 and hMLH1 in human cells, immunoprecipitation of hMRE11 or hMLH1 proteins were carried out with HeLa cell extracts. HeLa-S3 cells were cultured in Joklik’s MEM containing 5% newborn calf serum, 1% non-essential amino acids, 4 mM L-glutamine, 0.2% sodium bicarbonate, 1% penicillin, 1% streptomycin, and were obtained from National Cell Culture Center (Minneapolis, MN). HeLa
cells were briefly washed twice in PBS buffer at 4 °C before resuspended in NP-40 lysis buffer containing 1x complete EDTA-free protease inhibitor cocktail. After incubation at 4 °C with gentle agitation in NP-40 lysis buffer for one hour, HeLa cell extracts were isolated by centrifugation at 18,000 rpm for 30 min at 4 °C. Cell extracts that were prepared from approximately 3.7x10⁷ HeLa cells were incubated separately with 3 µl of anti-hMRE11, or 6 µg of anti-hMLH1 C-20 polyclonal antibodies, or 5 µl of crude preimmune serum. Captured immunoprecipitates were subjected to Western analysis.

2.5. Expression profile Analysis of hMRE11 and hMLH1. To determine whether the observed interaction between hMRE11 and hMLH1 has extended physiological relevancy beyond HeLa cells, we have next attempted to investigate the tissue expression patterns of these two genes in 68 paired human normal and tumor samples, as well as in 9 cancer cell lines. Specifically, a matched human normal/tumor DNA array (Clontech) was used to determine the expression pattern of human hMRE11 and hMLH1. The probes were the human hMRE11 and hMLH1 cDNA ORFs, as well as the control probe human ubiquitin cDNA, which had been radioactively labeled with [α-³²P]dCTP by random priming performed with the Oligolabeling Kit (Pharmacia, Piscataway, NJ). Hybridization of normal/tumor array with radioactive probes was performed based on recommendations provided by the manufacturer.
3. RESULTS

One of the characteristics displayed by mismatch repair protein MLH1-deficient cells is the apparent uncoupling between sensing of cis-platinum induced DNA damage and cell cycle control mediated by an ATM kinase activity (24). This observation has raised the possibility that hMLH1 is actively involved in DNA damage response beyond its role in the correction of DNA replication errors. That leads us to test whether hMLH1 protein has a direct physical association with hMRE11 – a major component of the DNA damage and repair system. Yeast two-hybrid analyses demonstrated that hMRE11 specifically interacted with hMLH1 (Fig. 1). Yeast double transformants expressing both hMRE11-AD and hMLH1-BD displayed strong histidine prototrophy phenotype, indicating the activation of GAL1 UAS driving HIS3 reporter gene in the reporter host strain AH109 (Fig. 1A). However, neither yeast double transformants coexpressing BD and hMRE11-AD, nor BD-hMLH1 and AD conferred histidine prototrophy phenotype, confirming the specificity of the observed interaction between hMRE11 and hMLH1 (Fig. 1). This interaction was further validated with the observation that all of the yeast double transformants grew efficiently on SD/-Leu-Trp medium (Fig. 1A), eliminating the possibility of adverse effects of human hMRE11 and hMLH1 proteins on yeast growth. Qualitative Western blot analysis confirmed the expression of relevant testing proteins in AH109 double transformants (Fig. 1B).

The specificity of the interaction between hMRE11 and hMLH1 was confirmed when AD-hMRE11 failed to interact with p53 fused to the GAL4 DNA-binding domain and similarly BD-hMLH1 did not interact with T-antigen fused to the GAL4 activation domain (Fig. 1C). T-antigen and p53 represented routinely used two-hybrid controls that were known to interact with
each other in the Gal4-based assay system. Therefore, the results of yeast two-hybrid analysis suggest a direct physical interaction between human hMRE11 and hMLH1 proteins.

In order to provide further evidence that could sustain the results of yeast two-hybrid analysis, immunoprecipitation experiments were performed with the soluble fraction of the bacterial lysates containing both recombinant hMRE11 and hMLH1 proteins. Western blot analysis confirmed the expression of both fusion proteins in lysate #1 (Fig. 2A), and only recombinant GST-hMRE11 protein in lysate #2 (Fig. 2A). Immunoprecipitation was performed with anti-hMLH1 or anti-β-catenin monoclonal antibodies, and captured immunoprecipitates were analyzed by immunoblotting with anti-hMRE11 monoclonal antibody. As shown in Figure 2B, anti-hMLH1 antibody could precipitate hMRE11 from cell lysates that expressed both GST-hMRE11 and His₆-hMLH1 recombinant proteins (lane 1 in Fig. 2B), indicating a direct association existed between these two proteins. The specificity of the immunoprecipitation reaction was validated when anti-hMLH1 antibody failed to immunoprecipitate GST-hMRE11 protein in the absence of hMLH1 fusion protein (Fig. 2B, lane 2). Furthermore, the control anti-β-catenin monoclonal antibody could not immunoprecipitate GST-hMRE11 from cell lysates containing both GST-hMRE11 and His₆-hMLH1 fusion proteins (Fig. 2B, lane 3), suggesting that coimmunoprecipitation of hMRE11 from cell lysates require specific anti-hMLH1 antibody. The interaction between hMRE11 and hMLH1 was not mediated by their abilities to interact with DNA, since treatment with DNase before incubation with antibodies did not abolish the interaction between hMRE11 and hMLH1 proteins (Fig. 2C). Consistent with the results of the two-hybrid analysis, coimmunoprecipitation experiments performed with recombinant hMRE11 and hMLH1 proteins also demonstrated a direct interaction between these two proteins.
We have next analyzed *in vivo* interactions between endogenous hMRE11 and hMLH1 proteins in human cells. Western blot analysis indicated that HeLa cells expressed readily detectable hMRE11 and hMLH1 proteins, and both rabbit anti-hMLH1 and anti-hMRE11 antibodies were highly specific (Fig. 3). Therefore, HeLa cell extracts were used to perform immunoprecipitation experiments with rabbit anti-hMRE11 and anti-hMLH1 antibodies. hMLH1 protein was readily detected only in the immunoprecipitates produced by anti-hMRE11 polyclonal antibody, but not in the immunoprecipitates generated by a control non-immune rabbit anti-serum (Fig. 3A), suggesting that hMLH1 protein was associated with hMRE11 in HeLa cell extracts. In addition to the protein co-migrating with hMLH1, anti-hMRE11 immunoprecipitates contained at least two other slightly higher molecular weight proteins that were detected with anti-hMLH1 antibody (Fig. 3A). Although the exact nature of these immunoreactive proteins was unknown, it appeared that these two bands were also present in immunoprecipitates produced by the control non-immune anti-serum -- most likely caused by nonspecific immunoreactivity of the rabbit antibody. Alternatively, the higher molecular weight anti-hMLH1 reactive proteins might represent some modified forms of hMLH1 protein, which only accounted for a small fraction of hMLH1 but possessing high affinity for the binding of hMRE11. Similarly, we have also examined whether anti-hMLH1 antibody could co-immunoprecipitate hMRE11 in the same HeLa cell extracts. As shown in Figure 2B, anti-hMLH1 antibody could co-immunoprecipitate hMRE11 protein from HeLa cell extracts and immunoprecipitates produced by the control non-immune rabbit anti-serum contained no immunoreactive hMRE11 protein, demonstrating a specific association between hMRE11 and hMLH1. However, it appeared that only a small fraction of the entire hMRE11 pool co-immunoprecipitated with hMLH1 (Fig. 3B, lanes 1 and 3). Most likely, this was due to the
relatively low levels of hMLH1 expression in HeLa cells (Fig. 3A). In addition, it could also be attributable to differences in properties of the two antibodies used in the coimmunoprecipitation experiments. Taken together, two-hybrid analysis and coimmunoprecipitation experiments conducted with recombination hMRE11 and hMLH1 proteins as well as human cell extracts demonstrate that hMRE11 physically interacts with hMLH1 – not only supporting the recent observation that these two proteins are constitutive components of the BRCA1-associated genome surveillance complex (25), but providing a basis for further studies of interplay among proteins contained within the BASC protein complex as well.

We have next examined the expression profiles of hMRE11 and hMLH1 in an attempt to address whether there was a correlation between hMRE11 and hMLH1 expressions in different tissues, since similar expression patterns of both genes would be expected if the observed protein-protein interaction has common biological implications in different human tissues. To this end, we have investigated the expression profiles of these two genes using 68 pairs of matched human normal and tumor samples representing 11 different tissue types, as well as 9 cancer cell lines. Specifically, human hMRE11 and hMLH1 cDNA ORFs were used as probes to hybridize a matched human normal/tumor cDNA array (Fig. 4). Hybridization with an ubiquitin cDNA probe indicated that there was approximately equivalent hybridization signal between normal and tumor samples for the most of the matched samples examined (data not shown). It appeared that the tissue expression profiles of these two genes were similar; both genes were expressed in all tissue types including kidney, breast, prostate, uterus, ovary, cervix, colon, lung, stomach, rectum, and small intestine, as well as in 9 different cancer cell lines which displayed relatively high levels of expression as referenced with the control probe, ubiquitin cDNA (Fig. 4 and data not shown). The similar expression patterns between these two genes
were consistent with the view that the interaction between hMRE11 and hMLH1 might have a
general functional implication in all human tissues, such as playing a role in the coordination of
DNA damage repair and cell cycle control. Although the expression levels of both hMRE11 and
hMLH1 elevated in all tumor cell lines examined, there was no profound differences at the
expression levels of both genes between paired normal and tumor samples for the majority of
cases (Fig. 4).
4. DISCUSSION

The current study demonstrates that the multifunctional human hMRE11 protein specifically interacts with the mismatch repair protein hMLH1. Consistent with the notion that hMLH1 might be involved in DNA damage response beyond its apparent role in postreplicative DNA mismatch repair (24); our initial analysis performed with a yeast two-hybrid assay suggested that hMRE11 interacted with hMLH1 (Fig. 1). The direct physical interaction between hMRE11 and hMLH1 proteins was then confirmed through coimmunoprecipitation of expressed recombinant proteins (Fig. 2). Furthermore, in an attempt to address whether the observed interaction between hMRE11 and hMLH1 was biologically relevant, we have demonstrated that endogenous hMRE11 and hMLH1 proteins associated with one another in human cells (Fig. 3). Although the observation that hMRE11 and hMLH1 proteins could be coimmunoprecipitated from human cell extracts did not necessarily reflect a direct protein interaction, the results of the yeast two-hybrid analysis and in vitro coimmunoprecipitation of recombinant proteins strongly suggest that a direct physical interaction between these two proteins does occur.

It is known that the hMRE11 containing protein complex hRAD50-hMRE11-NBS1 is involved in DNA double-strand break repairs by means of both homologous recombination and non-homologous end-joining processes. In addition, this protein complex is emerging as a critical player in mediating cellular response to DNA damage. On the other hand, human hMLH1 represents a major component of the DNA mismatch repair pathway, in which hMLH1-hPMS2 complex mediates communication between the recognition of replication errors and other proteins necessary for the completion of DNA repair. In addition to DNA mismatch repair, mammalian MLH1 appears to have essential functions in meiotic homologous recombination,
and targeted mutation in the mouse Mlh1 gene cause male and female infertility due to severely disrupted meiotic recombination (28, 29). The results of our current studies demonstrate a direct link between hMRE11 and hMLH1, representing major protein components of the DNA double-strand break repair pathway and DNA mismatch repair machinery, respectively. It is conceivable that the interplay between these two DNA repair pathways can be accomplished through hMRE11-hMLH1 interaction.

The similar tissue expression patterns of hMRE11 and hMLH1 genes support the view that the interaction between hMRE11 and hMLH1 proteins might have a broad biological relevancy. Intriguingly, all tumor cell lines, but not tumor samples, display increased expression levels of both genes. Although the exact nature of this observation is unclear at present time, the involvement of both genes in DNA damage response indicate that the high expression levels of these two genes might be functionally related to the survival of cultured tumor cells frequently experiencing chromosomal instability. There are several possible functional implications for the observed interaction between hMRE11 and hMLH1 proteins in human cells. First, besides its role in mismatch repair, hMLH1 might be also involved in signaling cell cycle control proteins in response to DNA damage, which is supported by the apparent uncoupling between sensing of cis-platinum induced DNA damage and ATM-dependent c-Abl phosphorylation in hMLH1-deficient cells (24). Second, the hMRE11-hMLH1 complex might be involved in the process of recombination; it is possible that hMRE11-hMLH1 interaction serves as a guide to direct mismatch repair proteins to the site of DNA heteroduplex formed during homologous recombination repair of DNA double-stranded breaks, in addition, hMLH1 might also have a direct role in homologous recombination (28, 29). A third possibility is that the interaction between hMRE11 and hMLH1 functions as a molecular glue to stabilize the formation of a
higher-order protein complex, BASC, which serves as sensors for the surveillance of aberrant DNA structures. Obviously, the detailed cellular effects and functional significance of hMRE11-hMLH1 interaction have to be addressed in the course of future studies. Of particular pertinence to hMRE11-hMLH1 interaction in human cells, it remains to be seen whether disease-associating mutations identified in hMRE11 from patients with A-TLD and in hMLH1 of HNPCC patients disrupt interaction between these two proteins; such studies shall shed light on the molecular mechanisms by which hMRE11-associated protein complex and DNA mismatch repair system act together to maintain genetic integrity.

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REFERENCES


FIGURE LEGENDS

Figure 1. Yeast two-hybrid analysis of hMRE11 and hMLH1 interaction. (A) Positive protein-protein interactions were ascertained by the transcription activation of highly inducible GAL1 UAS driving HIS3 reporter gene in the reporter strain AH109. Phenotype of histidine prototrophy displayed by AH109 double transformants was determined by their ability to grow on an SD/-Leu-His-Trp plate (left panel); as a control, growth potential of the same AH109 double transformants were also examined on an SD/-Leu-Trp plate (right panel). (B) Western blot analysis performed with AH109 double transformants used in the yeast two-hybrid analysis. (C) Negative controls of two-hybrid analysis performed with reporter strain AH109; histidine prototrophy phenotypes of double transformants were determined on an SD/-Leu-His-Trp plate.

Figure 2. Co-immunoprecipitation analysis of hMRE11 and hMLH1 interaction by the use of recombinant proteins. Cell lysates were collected from bacterial cells producing either recombinant GST-hMRE11 alone or together with His6-hMLH1. “Lysate #1” was prepared from bacterial cells harboring hMRE11/pGEX-6p and hMLH1/pET-28a constructs, and “lysate #2” was prepared from bacterial cells harboring hMRE11/pGEX-6p construct and empty pET-28a vector. (A) Western blot analysis of cell lysates with a-hMRE11 or a-hMLH1 antibodies. (B) Coimmunoprecipitation performed with either a-hMLH1 or control antibodies (see Materials and Methods for details). (C) Coimmunoprecipitation of hMRE11 and hMLH1 performed with DNase-treated lysates. Equal volumes of the immunoprecipitates were resolved with 4-20% SDS-PAGE, transferred onto nitrocellulose membranes, and subjected to Western blot analysis for the presence of hMRE11 fusion protein. “+” Signs were used to specify the cell lysates and antibodies used in each individual immunoprecipitation experiments.

Figure 3. Analysis of in vivo hMRE11-hMLH1 interaction in human cells. Immunoprecipitation of hMRE11 or hMLH1 proteins was carried out with HeLa cell extracts. Cell extracts were incubated separately with (A) an anti-hMLH1, and (B) an anti-hMLH1 polyclonal antibodies, or
with a control preimmune serum. Protein A/rProtein G-agarose beads were used to capture immunoprecipitates, and captured proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Retained hMLH1 and hMRE11 proteins were detected by Western blot analysis performed with an anti-hMLH1 polyclonal antibody, and an anti-hMRE11 polyclonal antibody, respectively. HeLa NP-40 lysates were also included in the Western blot analysis as a positive control.

Figure 4. Analysis of expression patterns of hMRE11 and hMLH1 in normal human tissues and matched tumor samples. Human matched tumor/normal expression array was used to analyze the expression profiles of hMRE11 and hMLH1. The probes were the human hMRE11 and hMLH1 cDNA ORFs that had been radioactively labeled with [α-32P]dCTP. (A) Expression analysis performed with hMRE11 cDNA as a probe. (B) Hybridization with an hMLH1 cDNA probe. The matched tumor/normal expression array contained SMART-amplified cDNA from 68 tumor and corresponding normal tissues from individual patients. The tissue types represented on the expression array included kidney (normal, A1 to A14, G10; matched tumor, B1 to B14, H10), breast (normal, D1 to D9; matched tumor, E1 to E9), prostate (normal, D11 to D13; matched tumor, E11 to E13), uterus (normal, G1 to G7; matched tumor, H1 to H7), ovary (normal, G10 to G12; matched tumor, H10 to H12), cervix (normal, G14; matched tumor, H14), colon (normal, J1 to J11; matched tumor, K1 to K11), lung (normal, J13 to J15; matched tumor, K13 to K15), stomach (normal, M1 to M8; matched tumor, N1 to N8), rectum (normal, M10 to M16; match tumor, N10 to N16), small intestine (normal, M18; matched tumor, N18), as well as 9 human cancer cell lines, HeLa (P1), Burkitt’s lymphoma (P2), chronic myelogenous leukemia K562 (P3), promyelocytic leukemia HL-60 (P4), melanoma G361 (P5), lung carcinoma A549 (P6), lymphoblastic leukemia MOLT-4 (P7), colorectal adenocarcinoma SW480 (P8), Brukitt’s lymphoma, Raji (P9).
Figure 1

(A)

(B)

Detected by: a-hMRE11

Detected by: a-hMLH1

(C)

SD/-Leu-Trp-His

SD/-Leu-Trp
Figure 2

(A) Lysate: #1 #2

His6-hMLH1
Western Ab:  hMLH1

GST-hMRE11
Western Ab:  hMRE11

(B) IP: Anti-MLH1
IP: Anti-β-catenin
Lysate #1
+ +
Lysate #2
+

Lysate #1

GST-hMRE11
Western Ab:  hMRE11

(C) DNase
IP:  h-MLH1
Lysate #1
+
Lysate #2
+

GST-hMRE11
Western Ab:  hMRE11
Figure 3

(A)  HeLa NP-40 Lysate  IP: Preimmune Serum  IP: a-hMLH1

Western Ab: a-hMLH1

1  2  3

hMLH1

118  85  61  50  38  kDa

(B)  HeLa NP-40 Lysate  IP: Preimmune Serum  IP: a-hMLH1

Western Ab: a-hMLH1

1  2  3

hMRE11

118  85  61  50  38  kDa
Figure 4

(A) hMRE11 ORF

(B) hMLH1 ORF
Implication of hMRE11 in MMR: Delineation of hMRE11 and hMLH1 dimerization domains and effects of HNPCC missense mutations.

Anthony Vo
Tai-Hsien Lee
Guo-Min Li
Chengtao Her*

School of Molecular Bioscience and Center for Reproductive Biology
PO Box 644660,
Washington State University,
Pullman, WA 99164-4660

Running title: Human hMRE11 and hMLH1

* Address all correspondence and reprint requests to:

Dr. Chengtao Her at above address.
Telephone: (509) 335-7537
Fax: (509) 335-9688
E-mail:
ABSTRACT

Hereditary non-polyposis colorectal cancer (HNPCC) is largely attributed to mutations in human mismatch repair genes where currently, up to 55.6% of all incidences occur in hMLH1 gene. Our recent report on a direct protein interaction between hMLH1 and hMRE11 suggest that the interplay between these two proteins might play important roles in DNA mismatch repair and the pathogenesis of HNPCC. As an initial step to characterize the functionality of this protein interaction, we have determined the interacting domains of these two proteins. Specifically, the interacting domains were narrowed to C-terminal between amino acids 495-756 and 452-634 for hMLH1 and hMRE11 respectively. The hMRE11 interacting region is significantly overlapped with the interacting region for hPMS2. In addition, we have found that four out of seven HNPCC missense mutations (L574P, K618T, R659P, and A681T) showed a complete disruption of interaction, two mutations (Q542L and L582V) displayed partial defects, and one mutation (E578G) showed similar interaction to that of the hMLH1-hMRE11 wild type. This suggests the disruption of hMLH1-hMRE11 interaction could serve as an alternative molecular mechanism for the pathogenic effects of these mutations.

Next, we have assessed the involvement of hMRE11 in the process of MMR. Evidences of hMRE11 as an essential requirement for human MMR process were examined by an in vitro partial reconstitution MMR assay. Addition of partial purified recombinant hMRE11 proteins successfully repaired base mismatch in the 3’-5’ direction. Thus this lends support that hMRE11 3’-5’ exonuclease activity is involved in excising DNA fragment containing mismatch base.
INTRODUCTION

Hereditary non-polyposis colorectal cancer (HNPCC) is a common cancer syndrome associated with defects in mismatch repair (MMR) pathway that usually lead to microsatellite instability in tumor cells. Such phenomenon has been observed in more than 70% of all HNPCC cases (Guerrette et al., 1999, Kondo et al., 2003, Nystrom-Lahti et al., 2002, Pedroni et al., 2001, Shimodaria et al., 1998). Current HNPCC database (www.nfdht.nl) registered unique predispositions with respect to MMR gene mutations from more than 650 HNPCC patients. Predisposition are often associated to germ line mutation of six DNA MMR genes, hPMS1, hPMS2, hMSH2, hMSH3, hMSH6, and hMLH1. In all, mutations in human MLH1 gene account for approximately 55.6% of all HNPCC occurrence, second by mutation in hMSH2 with an incident rate of 36.2% (Kondo et al., 2003). Further, majority of mutation arise from a single residue substitution resulted to splicing variants or deletion. Specifically, about 30% of mutation occurred in hMLH1 gene are due to single amino acid substitution (Nystrom-Lahti et al., 2002). Despite this prevelancy, the pathogenic effects of these substitutions are not fully elucidated.

hMLH1, human MutL homolog 1, encodes a of 756 amino acid protein that locates on chromosome 3p21.3 (Stewart et al., 1999). It is comprised of 19 exons where most known HNPCC mutations occurred in exon 15-16 (Wijnen et al., 1996). Upon recognition of mismatch base pairs or insertion-deletion loops (IDL), hMLH1 pairs with hPMS2 or hPMS1 to form MutL\[a\] and MutL\[b\] complex, respectively. MutL\[a\] interacts to hMSH2 heterodimer complex and plays a crucial role in reparing base-base mismatch and IDL. However, the role of MutL\[b\] in MMR process has not yet been fully characterized (Buermeyer et al., 1999, Harfe et al., 2000, Kolodner et al., 1999).
Our recent report on a direct interaction between hMRE11 with MMR protein hMLH1 provided additional information about cooperative process between DNA damage detection, signaling and repair (Her et al., 2002). This notion is further supported by the identification of BRAC1 associated genome surveillance complex (BASC) that comprises of several MMR proteins (MSH2-MSH6 heterodimer, MLH1), ATM, BLM helicase and MRE11 complex (MRE11-RAD50-NBS1) (Wang et al., 2000). hMRE11 is an essential gene that involves in many functional responses. Null mutations in C. elegans and S. cerevisiae are viable but exhibited a slow growth in yeast (Chin et al. 2001, Guerrette et al. 1999). However, deletion of MRE11 gene is embryonic lethality in chicken and mouse (Anderson et al., 2001, Yamaguchi-Iwai, et al., 1999). hMRE11 plays many crucial roles in maintaining genomic stability such as processing of double strand break (DSB) (Bressan et al. 1999, Costanzo et al. 2001), DNA damage detection and cell cycle checkpoint (Gautier et al. 2001, Grenon et al. 2001), removal of hairpin loop (Lobachev et al. 2002), and maintenance of telomeric length by recruiting telomeric subunits to DNA ends (Tsukamoto et al. 2001).

Though we did show a direct interaction between hMRE11 and hMLH1, implicating hMRE11 as providing an essential role in MMR mechanism; however, evidences for such involvement in human MMR are still lacking. hMRE11 possesses both ssDNA/dsDNA endonuclease and dsDNA 3’-5’ exonuclease activity (Paul et al. 2000), of which the latter property is of concerned in this study. In the mismatch repair process, exonuclease enzymatic activity is required to excise DNA fragment containing mismatch base or damaged nucleotides during DNA metabolic process such as replication. Currently, ExoI is proposed as a potential mammalian exonuclease candidate because it interacts with MLH1 and possesses 5’-3’ exonuclease activity (Schmutte et al. 2001). This only offers a half solution to the problem of
DNA excision. During replication process, ExoI 5’-3’ exonuclease activity may be required to remove DNA fragment containing a mismatch on the Okazaki’s fragment. On the other hand, a 3’-5’ exonuclease may be needed to excise fragment on the leading strand. Coincidentally, hMRE11 possesses this enzymatic activity and directly interact with MMR protein hMLH1. Furthermore, hMRE11 has been shown to colocalize to nuclei foci containing proliferating cell nuclear antigen (PCNA) (Wang et al. 2000). Interestingly, PCNA has been proposed as strand discriminator in mammalian MMR process. In addition, human MMR proteins, MSH2, PMS1 and MLH1, also coimmunoprecipitated with PCNA (Gu et al. 2000). In all, evidences suggested a strong association of hMRE11 in mammalian MMR.

Evidence from scanning force microscopy study suggested a “band-aid” architectural arrangement of hMRE1 in association with Rad50 when repairing DSB (Connelly et al. 2002, Jager et al. 2001). In such conformation, two Rad50 fold intramolecularly at the Cys-X-X-Cys coiled coil domain and interact with each other. hMRE11 dimer is bounded to Rad50 complex at ATPase site. At the occurrence of DSB, accumulation of hMER11-Rad50 complexes at DNA ends tether DNA, refrain its end from tangle with replication forks and keep ends at close proximity. Subsequently, DNA ends are processed, reannealed and ligated. It has been observed that mutations resulted to low expression of hMRE11 protein production or reduction of activity level is associated with AT like disorder (AT-LD). Both pathogenic mutation forms of hMRE11, N to S mutation at residue 117 and truncation, in ATLD patients still exhibited an association with NBS1. Thus, this suggested that mutation may not completely abolished DSB repair mechanism, but rather the efficiency at which double strand break is processed may be reduced (Stewart et al. 1999). Our data from yeast two-hybrid analysis of hMRE11 homodimerization further strengthen this notion. We observed a significant reduction of binding affinity of
hMRE11 ATLD mutation to wild type, further support the hypothesis that possible defects of DSB repairing due to inefficient dimerization of hMRE11 that may lead to an inefficient pairing of DNA ends.
MATERIALS AND METHODS

Strain and plasmid constructions

Human MRE11 and MLH1 cDNAs were fused to yeast two-hybrid vectors pBTM116, pBTMd, pVPd, pGADT7, and pACT2. Yeast strain L40, hMLH1 deletion constructs in pVPd, and hPMS2 in pBTMd vector were kindly provided by Dr. Shinichi Fukushige (Kondo et al., 2001). Partial list of hMRE11 deletion constructs in pACT2 vectors were received from Dr. Patrick Concannon (Desai-Mehta et al., 2001). Deletion constructs of hMRE11 and hMLH1 were made using unique restriction endonuclease sites. Forward and reverse primers used for PCR reaction are listed below. E. coli strain Top10 (Strategene) was used to propagate all plasmids. Constructions of HNPCC mutations in truncated hMLH1 were done using overlapping PCR primers (Table 1 and 2). Two separate PCR amplifications were carried out by two sets of primers of which the inner primer contains mutation. The result of two products were mixed in equal portion for the second PCR when the outer primers were used. PCR products were digested with BamHI and EcoRI, gel purified, followed by ligation to pBTMd. All constructs are verified by DNA sequencing to eliminate possibility of other PCR mutation.

Production of recombinant proteins

Appropriated hMRE11 and hMLH1 constructs were subcloned to pET28a-His vector and transfected to E. coli. strain BL21(DE3)RIL (Strategene). Cells were cultured at 37°C till A600 reached 0.4-0.5 and induced with final IPTG concentration of 0.25 to 0.5mM at 16°C, 25°C, or 37°C. Cells were collected after 9-12 hours culture, and lysed via sonication. Clear lysate were then collected for purification using Tylon column chromatography (Clontech). Selected fraction contained recombinant protein was dialyzed against PBS to remove immidizole before
subsequent application. For generation of recombinant proteins under denatured condition, 6M guanidine was added to all relevant buffers. Proteins were renatured gradually by 4 serial incubation of 1:1 dilution in 6M guanidine-PBS with a last incubation in PBS only.

**Yeast two-hybrid analysis**

Yeast two-hybrid analysis was carried out via matchmaker two-hybrid system described earlier (Her et al. 2002). For β-galactosidase filter assay, sterile filter papers were used to lift colonies off the plates. Cell breakage was performed by 3 cycles of freeze and thaw in liquid nitrogen, placed on another filter paper presoaked with 5mL Z buffer (60mM Na₂HPO₄, 40mM Na₂HPO₄, 10mM KCl, 1mM MgSO₄, pH7.0) containing X-gal (83.5μL of 20mg/mL stock solution) and -mercaptoethanol (13.5μL of stock). Filters were then incubated at 37°C and check for color changes. Determination of positive interaction is uniformly completed within 2-4 hours time.

For β-gal liquid assay using O-nitrophenyl-1-thio-β-D-galactopyranoside (ONPG) as substrate, overnight culture of freshly transformed cells was inoculated in SD/-Leu-Trp double selection medium and refreshed in YPD medium till absorbance at A₆₀₀ reached 1.0. Next, cells from 3 aliquots of 1.5 mL culture were pelleted at 14000 rpm for 30 seconds at room temperature and washed once with Z buffer. Cells were resuspended in 0.3mL Z buffer and lysed via 3 cycles of freeze in liquid nitrogen and thaw at 37°C. For assay, 0.1mL of broken cells were added to 0.7mL of Z buffer containing 1.9μL -mercaptoethanol and 0.16mL Z buffer containing 4mg/mL ONPG. Reaction mixtures were incubated at 30°C till a yellow color was observed. Reaction was then stopped with 0.4mL of 1 M Na₂CO₃ and pelleted at 14000 rpm. Supernatants were used for measurement at A₄₂₀. β-galactosidase unit activity was calculated using following formula, 1000 x OD₄₂₀ / (time x Volume x A₆₀₀), where time is the elapsed time
of reaction, volume is the 0.1mL culture sample with dilution factor of 5, and \( A_{600} \) is the optical density reading of cell growth in YPD.

**Far Western blot analysis**

Approximately 2-3g of purified proteins in TBS was load into nitrocellulose membrane. Membrane was then immersed in 50mL denature buffer I (6M guanidine-HCl, PBS) for 10 minutes followed by an incubation of 50mL denature buffer II (6M guanidine-HCl, PBS, 1mM DTT). Membrane was washed with 5 incubations of 1:1 serial dilution of denature buffer II in PBS. Afterward, membrane was blocked with 10% milk-TBS-T (TBS, 10% milk powder, 0.3% Tween-20) for 45 minutes and probed for 1 hour with approximately 7mg/mL of purified interacting partner protein or 1:5 dilution of crude lysate in 0.25% milk TBS-T (0.25% milk powder, 0.3% Tween-20, 1mM DTT, 1mM PMSF, TBS). It was followed with 5 washed for 10 minutes each in 0.25% milk TBS-T with the second wash in 0.25% milk TBS-T containing 0.0001% glutaraldehyde. Conventional Western blot detection with appropriate antibodies will be used as described previously (Her et al. 2002).

**MMR assay**

Nuclear extraction of HeLa cell lysate fractions was done in two-step ammonium sulfate precipitation. First, HeLa S3 nuclear extract was precipitated with 35% ammonium sulfate and centrifuge to collect lysate. The lysate was then re-precipitated with addition of ammonium sulfate to a final 65% w/v. Precipitation was separated by centrifugation and supernatant was removed. Both precipitations were dialyzed against buffer A (25mM HEPES [pH7.5], 0.1mM EDTA, 2mM DTT 0.1% PMSF, 1μg of leupeptin/mL) containing 0.1M KCl, and freezed in liquid nitrogen before storage. Insoluble protein fraction from 35 and 65% ammonium sulfate were designated as FI and FII respectively. FI was then re-diluted to a final concentration of
5mg/mL in buffer B (25mM Tris [pH7.5], 10% glycerol, 0.01% NP-40, 0.1mM EDTA, 2mM DTT, 0.1% PMSF, 1μg of leupeptin/mL) containing 0.5M NaCl before loading onto ssDNA cellulose column (Sigma). Bounded proteins were eluted off column in buffer B containing 2M NaCl. Both flow through and fractions contained bounded protein, designated as SSI and SSII, were concentrated with 35% ammonium sulfate, dialyzed against buffer A containing 0.1M KCl.

For DNA substrate, a 6.4 kDa heteroduplex with 3’-CA dinucleotide insertion/deletion mispair containing strand break at 181bp 3’ to mismatch will be used for MMR assay. The mismatch substrate was constructed by hybridizing Sau96I-digested phage f1MR23 dsDNA and f1MR24 ssDNA (Ramilo et al. 2002). The actual MMR assay was carried out in a reaction mixture of 15 μL containing 100ng of mismatch DNA subtrate, 50-60μg of nuclear extract or fractioned protein, 10mM Tris-HCl (pH7.6), 5mM MgCl₂, 1.5mM ATP, and 0.1mM dNTP. Reaction mixture was incubated at 37°C for 15 min, followed by recovery via phenol extraction and ethanol precipitation. Ideally, if proper correction did occur, DNA substrate will be sensitive to XhoI digestion. Hence, addition of HindIII and Bsp106 restriction digest would yield 2 DNA fragments of 3.1kb and 3.3kb (Ramilo et al. 2002)

For reconstitution assay, hMRE11-deficient HeLa cell nuclear extract was generated by immunoprecipitation of endogenous hMRE11 protein with a-hMRE11. MMR assay was performed on hMRE11-deficient HeLa extract in comparison to wild type. Subsequently, recombinant hMRE11 protein is added to deficient HeLa extract to determine whether reconstitution of hMRE11 would rescue MMR defects.
RESULTS

Mapping of hMLH1 interacting region

Determination of hMLH1 interacting domain for hMRE11 was performed via yeast two-hybrid analysis (Materials and Methods). Results of β-galactosidase filter assay and β-galactosidase liquid assay of the yeast two-hybrid are summarized in Figure 1. It is our finding that the minimal interacting region on hMLH1 for hMRE11 located on the C-terminal between residues 492-756 such that a deletion of 104 amino acids drastically abolished the interaction. Unit of β-gal activity is relatively equivalent to that of full length hMLH1. This placed the interacting domain in the same region as hMLH1 for hMLH3, hPMS1, hPMS2, and BLM (Kondo et al. 2001, Pedrazzi et al. 2001).

Mapping of hMRE11 interacting region

We also resolved hMRE11 interacting domain for hMLH1 by using yeast-two hybrid assay (Materials and Methods). Determination of β-gal activity units and β-gal filter assay were performed in the same manner above. The interacting region was narrowed to the C-terminal from residues 452-634 (Figure 2). We attempted to further narrowing the region by making four additional constructs with deletion at N- or C-terminal. However, the interaction was completely abolished as confirmed by both β-galactosidase filter and β-galactosidase liquid assay.

Far western blot analysis of hMLH1 and hMRE11 interacting domain

To generate recombinant proteins of hMLH1 and hMRE11 for dot-blot far-Western assay, hMLH1 (495-756) was subcloned to pET28a-His, transformed in E. coli strain BL21(DE3)RIL. Cell was cultured till midlog phase and induced at 16°C overnight. Clear lysate was collected and further purified via Tylon column chromatography. Bound proteins were eluded off column and dialyzed against PBS overnight (Figure 3A, 3B). Protein production of
hMRE11 full length and variants were performed in a denatured conditioned where all relevant buffers were added with 6M guanidine-HCl (Sigma). Cells were cultured at till midlog phase, and induced overnight at 37°C. Further purification of hMRE11 proteins and variants were performed in same manner (above). Figure 3A-B depicts coomassie stained gels and western blot detection of recombinant hMRE11 proteins.

To reconfirm the interacting domain of hMLH1 for hMRE11 and vice versa, dot-blot far-Western assay was performed in addition to other assays mentioned above. This assay serves as a re-affirmation of the interaction and that this is a direct interaction rather than mediated by accessory proteins. In Figure 4A, purified hMLH1 (495-756) protein was immobilized directly onto nitrocellulose membrane and was washed to rid off any excess unbound proteins. Membrane was probed with either crude lysate extract or purified hMRE11 (452-634) protein followed by conventional western detection method with either [α]-hMRE11 or [α]-T7. For control, either BSA or purified hMLH1 (495-756) protein was loaded alone onto the membrane. It appeared that the interaction between hMLH1 (495-756) and hMRE11 (452-634) was specific, and BSA did not interact with hMRE11. Further, we verified that the antibodies used here are specific and exhibited no cross reactivity. In a reciprocal manner, purified hMRE11 (452-634) protein was immobilized onto membrane, probed with either crude lysate or purified hMLH1 (495-756) protein. BSA and purified hMRE11 (452-634) protein alone were also loaded onto same membrane to serve as controls (Figure 4B). Again, it re-validated previous probe and other assay, that purified hMLH1 (495-756) directly interacted with hMRE11 (452-634) in vitro. In a separate yeast two-hybrid assay, we were able to demonstrate that subfragment hMLH1 (500-756), a five residue less than later construct was able to interact with hMRE11 (453-634) with the same affinity as hMLH1 (495-756).
Hence, we concluded that purified recombinant hMRE11 directly interacted with hMLH1 in vitro at residues 452-634 for hMRE11 and 495-756 for hMLH1. Thus, hMLH1 shared the same interacting region for hMRE11, BLM, hPMS2, hMLH3, hPMS1, and hExol (Kondo et al. 2001, Pedrazzi et al. 2001, Schmutte et al. 2002) (Figure 5).

**Yeast three-hybrid analysis of protein interaction between hMLH1, hMRE11 and hPMS2**

Since hMRE11 and hPMS2 shared a common binding region on hMLH1 C-terminal, we were intrigued to examine the competitive binding effects of both for hMLH1 if any. A yeast three-hybrid analysis was employed. Here, pBridge vector was used to fuse full length cDNAs to Gal4-BD at multiple cloning site I (MC1), and a “bridge protein” cDNAs inserted at MCII. Interacting protein partner was cloned in pGADT7 vector. Yeast strain AH109 was used for cotransformation. Fresh grown cells were plated on conditioned media SD/-Leu-Trp or SD/-Leu-Trp-His. A positive interaction between partner proteins was confirmed by histidine prototroph in double transformed AH109 (Her et al. 2002). Various combinations of cDNAs were fused to either pBridge or pGADT7 vector (Figure 6). When hMRE11 protein was expressed as a bridge protein, interaction between hPMS2 and hMLH1 was not disrupted, suggested that hPMS2 has a higher binding affinity for hMLH1 than hMRE11 (Figure 6, lane 2 & 4). This observation was further confirmed when double transformed yeast AH109 failed to display a histidine prototroph in treatment with hPM2 expressed as a bridge protein for hMLH1 and hMRE11 (Figure 6, lane 7 & 8).

**Effects of HNPCC pathogenic mutation in hMLH1 on interaction with hMRE11 and hPMS2**

Several reported HNPCC missense mutations located within the hMLH1 interacting region for hMRE11 and hPMS2 (Guerretee et al. 1999, Kondo et al. 2003, Nystrom-Lahti et al. 2004).
Thus, in attempt to examine the functional effects of these alterations, we introduced single base mutation via site directed mutagenesis to hMLH1 (495-756) and observed their interaction with hMRE11 and hPMS2. HNPCC pathogenic mutation tested here were Q542L, L574P, E578G, L582V, K618T, R659P, and A681T. Constructs contained hMLH1 (495-756) wild type and missense mutations were inserted into LexA BD plasmid, pBTM116, and cotransformed to yeast strain L40 with either hMRE11 or hPMS2 fused in Gal4 AD plasmid, pGADT7. Fresh grown cells were used for β-galactosidase liquid assay. In general, data may be group in several categories. Missense mutation L574P and R659P exhibited a complete abolished interaction for both hMRE11 and hPMS2 whereas Q542L, E578G and L582V only showed a partial disruption (Figure 7). In the last category, hMLH1 HNPCC missense mutation K618T and A681T appeared to completely disrupt hMLH1-hMRE11 interaction but only reduced hMLH1-hPMS2 interaction to less than 50%.

To confirm the effects of HNPCC missense mutation on hMLH1-hMRE11 interaction, additional assay was carried out in vitro via dot-blot far-Western. Purified hMRE11 (452-634) protein was immobilized onto nitrocellulose membrane, renatured, probed with crude lysate containing hMLH1 (495-756) wild type and mutation, followed by conventional western detection. BSA was used as a control, loaded directly onto membrane. Results of dot-blot far-Western re-affirmed data obtained from β-galactosidase liquid assay. Several single-base mutations completely abolished the interaction such as L754P, K618T, R659P, and A681T (Figure 8). hMLH1 (495-756) contained missense mutation Q542L and L582V showed a reduction of interaction where E578G did not.
Fractionation of HeLa cell nuclear extract and partial reconstitution MMR assay

To determine the biological relevancy of hMRE1-hMLH1 interaction, we seek to examine their protein expressions in Hela cell nuclear extract. HeLa nuclear extract was fractionated by ammonium sulfate precipitation and further purified via ssDNA cellulose column. A schematic fractionation steps is diagramed in *Figure 9A*. A western blot analysis was performed to detect endogenous expression hMRE11 and hMLH1 using a-hMRE11 and a-hMLH1 respectively (*Figure 9B*). Results indicated that both hMRE11 and hMLH1 coexpressed in every fraction, thus lending further support to previous data about its direct protein interaction.

Next, we seek to examine the ability to perform MMR from hMRE11-deficient HeLa cell nuclear extract and whether partial reconstitution of recombinant protein would rescue the defects. Here, endogenous hMRE11 protein was immunoprecipitated by means of immobilized a-hMRE11. Our initial experiment suggested that the ability of hMRE11-deficient Hela extract to repair mismatch was greatly diminished in comparison to wild type (*Figure 10*). And as expected, reconstitution of recombinant hMRE11 protein partially rescued the defect. This data strongly indicated that hMRE11 is necessary for functional MMR. Further, the inherent 3’-5’ exonuclease on hMRE11 is required to excise DNA fragment containing mismatch.

hMRE11 homodimerization

It has been mentioned elsewhere that hMRE11 forms homodimerized complex. We reported here in confirmation that through yeast two-hybrid analysis and quantification of β-galactosidase activity units, the homodimerization is revalidated (*Figure 11*). Further, we attempted to map the regions that mediate homodimerization with our current availability of hMRE11 deletion constructs. However, data provided an inconclusive result. Interestingly, we observed a significant decrease in protein interaction in two hMRE11 AT-LD pathogenic
mutations.  β-galactosidase activity units in hMRE11 (N117S) and truncated hMRE11 (aa1-634) were reduced by nearly 35 and 82%, respectively, to that of the wild type (Figure 11). However, this phenomenon was not observed in protein interaction between hMRE11 AT-LD pathogenic mutations and hMLH1 (Figure 2). This may provide further insight regarding pathogenic causations and mechanisms that lead to the development of the AT-LD.
DISCUSSION

Genomic stability and faithful transmission of genetic materials from parental cells to daughter cells are under constant surveillance by large number of proteins involved in detection and repair of DNA damage. The general DNA damage response pathway includes cell cycle regulation, DNA repair or apoptosis. Failures in either detection or repair of DNA damage often results to disease state such as ataxia-telangiectasia (AT), Nijmegen breakage syndrome (NBS) and AT-like disorder (ATLD). Mutation in mismatch repair protein MLH1 is implicated in more than half of HNPCC cases, of which 30% of the gene alterations are missense mutations. Our findings of a direct protein interaction between MRE11-MLH1 raised a possibility for the involvement of hMRE11 in the MMR process. Specifically, we speculate that hMRE11 3’-5’ exonuclease is involved in MMR for its ability to excise DNA fragment containing mismatch. This notion was examined by partial reconstitution MMR assay as discuss below.

In the present study, our first aim was to examine the pathogenic mutation of ATLD on MRE11 and HNPCC on MLH1 to elucidate any significance in association to MRE11-MLH1 interaction. First, we aimed to map the interacting domains of hMLH1 for hMRE11 and hMRE11 for hMLH1. The regions were narrowed down to residues 495-756 on hMLH1 and 452-634 on hMRE11 with yeast two-hybrid analysis. The interacting domains were further validated by far-Western analysis (Figure 1, 2, 4). Coincidentally, hMRE11 interacting domain on hMLH1 located at C-terminal, sharing the same previously identified interacting regions for BLM, hPMS1, hPMS2 and hExoI. In examining hMLH1, hMRE11 and hPMS2 protein interaction, our data from yeast three-hybrid analysis suggested that hMLH1 preferentially formed complex with hPMS2 over hMRE11. Thus this indicates a competition for the same binding target on hMLH1, suggesting protein partner exchange is needed to constitute different
cell specific function. In such, specific cellular function of hMLH1 through heterodimerization may be regulated by differential expression of protein partners.

Though the roles of hMLH1-hMRE11 and hMLH1-hPMS2 have not yet fully elucidated, previously published data suggested that any disruption of both heterodimerization may affect the efficient or the MMR process itself resulting to development of HNPCC. Hence, in attempt to investigate the significance of hMLH1 HNPCC pathogenic mutations on interaction with hMRE11 and hPMS2, we examined the effects of 7 previously identified hMLH1 HNPCC mutations located within the hMRE11-interacting domain via yeast two-hybrid and far-Western analysis. Results shown that 4 mutations (L574P, K618T, R659P, A681T) completely abolished protein interaction between hMLH1 for hMRE11 (<90%), while Q542L and L582V only exhibited a partial loss (<20%). The E578G missense mutation did not appear to affect the interaction with hMRE11. These observations were further validated by dot-blot far-Western data (Figure 8). On the other hand, only two mutations (L574P, R659P) displayed total abolishment of protein interaction between hMLH1 and hPMS2, while 2 mutations (K628T, A681T) exhibited a significant disruption (<50%). The L582V alteration did not seem to affect hMLH1-hPMS2 interaction while Q542L and E578G only showed a partial loss (<30%). Comparatively, there is a differential effect of HNPCC mutations have on protein interaction with hMRE11 and hPMS2, suggesting that the disruption of hMLH1-hMRE11 interaction could serve as an alternative mechanism for the pathogenic effects of hMLH1 mutations. In all, data strongly support the notion that alteration of hMLH1-hMRE11 heterodimerization may lead to MMR defects. Consequentially, this may contribute to the susceptibility of cancer development.

In order to provide additional evidence of hMLH1-hMRE11 interaction in human cell, we examined protein expression on several fractions of HeLa cell nuclear extracts. Protein
expression detected by conventional western blot using a-hMRE11 and a-hMLH1 indicated a co-expression of both hMLH1 and hMRE11 in fraction FI, FII and SSII. However, expression of hMLH1 appeared to be less abundant than hMRE11. Taken together, co-expression of hMLH1 and hMRE11, in additional to yeast two-hybrid analysis, provide further evidence for hMLH1-hMRE11 interaction and hMRE11 direct involvement in MMR.

MMR process includes sensor and detection of damage base, excision of DNA fragment, resynthesis of new daughter strand and religation. Mismatch base generated during DNA metabolic process is detected by hMSH2-hMSH6 heterodimer while hMSH2-hMSH6 and hMSH2-hMSH3 shared a redundant role of detecting small insertion/deletion loop. Subsequently, hMLH1-hPMS2 is recruited and bound to hMSH2-heterocomplex containing mismatch in an ATP dependent manner (Tomer et al. 2002). In bacterial MMR system, recruitment of MutL is necessary to activate exonuclease activity of MutH and helicase activity of UvrD (Kolodner et al. 1999). It is not known whether this model may apply to the same process in mammalian MMR. Nonetheless, no bacterial exonuclease homologs have been identified in mammals currently. However, ExoI has been proposed as a strong candidate for it possess 5’-3’ exonuclease activity and interacts with hMLH1 (Schmutte et al. 2001). Our findings of a direct protein interaction between hMRE11 and hMLH1 implicate a strong involvement of hMRE11 in MMR process. hMRE11 possesses both ssDNA/dsDNA endonuclease and ssDNA 3’-5’ exonuclease activity (Paul et al. 2000). Of which, the 3’-5’ exonuclease property may compliment ExoI 5’-3’ exonuclease activity in excising DNA fragment containing mismatch on the lagging strand during replication process. In additional to a direct interaction of hMRE11 to hMLH1, hMRE11 colocalized to nuclei foci containing proliferating cell nuclear antigents (Wang et al. 2000). PCNA was proposed as a strand
discriminator in mammalian MMR for it located on DNA primer termini and required for proper DNA MMR after excision step (Gu et al. 1998). In order to provide support for the association of hMRE11 in MMR, specifically, its 3’-5’ exonuclease property in excising DNA fragment containing mismatch, a partial reconstitution MMR assay was performed. Results indicated reconstitution of partially purified recombinant protein to the hMRE11-deficient HeLa cell extract effectively repair MMR defects. In such, a HindIII restriction site was restored so that double digestion with HindII/Bsp106 yields two predictable DNA fragments of 3.1kb and 3.3kb (Figure 10, lane 2 and 4).

Two of the ATLD pathogenic mutation being investigated here were N117S and truncation (aa1-634). We reported here that these mutations did not appear to interfere with hMRE11 binding affinity for hMLH1. Others have also reported that these mutations did not disrupt hMRE11-NBS1 interaction. However, genetical analysis of ATLD patients showed a high accumulation of chromosomal DSB. Hence, we speculate that eventhough ATLD mutations may not interfere with protein interaction, they may still reduce the efficiency of DSB repair. Here, we have made an importance observation regarding AT-LD mutational effects on hMRE11 homodimerization. Specifically, hMRE11 contained N117S mutation and truncation (aa1-634) showed a substantial loss of protein interaction to that of the wild type (<35% and <82%, respectively). However, hMRE11 AT-LD pathogenic mutation did not appear to affect hMRE11-hMLH1 interaction. Thus, the two AT-LD mutations may specifically affect DSB repair process but not necessarily influence mammalian MMR activity. Recent data from scanning force microscopy suggested hMRE11and Rad50 form a “band-aid” architectural arrangement when repairing DSB (Connelly et al. 2002, Jagger et al. 2001). The model suggested that Rad50 folded intramolecularly onto itself at the coiled coil region, in an
antiparallel manner. The coiled coil domain contained a highly conserved motif, Cys-X-X-Cys, that has been shown to mediate homodimerization. Hence, two Rad50 formed a homodimerized complex and associated with hMRE11 at the ATPase site. Upon detection of DSB due to ionizing radiation or reactive oxygen species, hMRE11-Rad50-NBS1 complexes are recruited to broken DNA ends, preventing them from tangle with the replication fork. In addition, intermolecular interactions between hMRE11-Rad50-NBS1 complexes keep DNA ends at close proximity. In rare cases where hMRE11 is hypomorphically expressed, as exhibited in AT-LD patients, protein production is often low or there is a reduction of enzymatic activity. In such, genomic instability is prevelant with high accumulation of chromosomal double strand break (Fukuda et al. 2001, Mirzoeva et al. 2003, Stewart et al. 1999). Both hMRE11 (N117S) and truncation (aa1-634) still retained binding property to NBS1, Rad50 (Stewart et al. 1999), and hMLH1 (reported here). Thus, this suggested AT-LD mutation may only affect hMRE11 homodimerization binding affinity and subsequently, the efficiency of DSB repair, but not necessarily abolished the process. This finding is in congruent with other observation that hMRE11 mutation did not drastically resulted to a completely deficient in DSB repair (Stewart et al. 1999).
ACKNOWLEDGMENT

I would like to thank BuyHun Young and Dr. Park from Dr. Chul Hee Kang lab group for all technical supports on protein purification. In addition, I would like to extend my great appreciation to Dr. Shinichi Fukushige of Tohoku University School of Medicine and Dr. Patrick Concannon of Virginia Mason Research Center in Seattle, Washington for kind gifts of hMLH1 and hMRE11 deletion constructs.
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<th>Nucleotide sequence</th>
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### Table 2.

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REFERENCES


LEGENDS

Figure 1 – Schematic diagram of hMLH1 interacting domain for hMRE11
β-gal filter assay and β-gal liquid assay were performed on yeast that had been co-transformed with various hMLH1 constructs inserted in VP16 transcriptional activation domain (AD) plasmid and hMRE11 fused in DNA-BD plasmid, pBTM116. The average and SE of at least three independent data points are listed. The β-gal activity units for transformed cells contained only hMRE11 BD plasmid and empty plasmid carrying AD domain is 2.80.

Figure 2 – Schematic diagram of hMRE11 interacting domain for hMLH1
A summary of yeast two-hybrid assay of full length hMLH1 and various hMRE11 constructs. hMLH1 cDNAs were fused to Lex-A BD plasmid, pBTM116, while hMRE11 subfragments were cloned into Gal4 AD plasmid, pGADT7. β-gal activity was performed on freshly grown cells. The average and SE of at least three independent data points are included here. The β-gal activity units for transformed cells contained only hMLH1 AD plasmid or hMRE11 BD plasmid are 0.93 and 2.78 respectively.

Figure 3 – Production of hMLH1 (495-756) recombinant proteins
(A) (B) hMRE11 full length cDNA and variants were inserted in pET28a vector and transfected in E. coli strain BL21(DE3)RIL. Cells were cultured in the same manner mention above (Materials and methods). However, cells transfected with full length hMRE11 was induced to final IPTG concentration of 0.25mM whereas other variants were induced at 0.5mM. In addition, all subsequent steps were carried out in denatured methods where 6M of guanidine was added to all relevant buffer. Figure 3A and 3B depicts coomassie stained gels of full length hMRE11 recombinant protein (3A), and hMRE11(452-634) (3B). The same protein were also used for western blot detection with α-hMRE11.
(C) hMLH1 (495-756) was inserted in pET28a-His vector and transfected to E. coli strain BL21(DE3)RIL. Cells was cultured till $A_{600}$ reached 0.4-0.5 before induction overnight with final IPTG concentration of 0.5 mM. The optimized temperature for this induction was 16°C. Clear lysate was obtained and further purified by Tylon column chromatography. Both crude lysate and fraction contained bound proteins were loaded on SDS-PAGE to performed coommassied stained gel (Figure 3A) and western blot detection with a-T7 (Figure 3B).

**Figure 4 – Far Western analysis of hMLH1 and hMRE11 interacting domains**

(A) About 3µg of purified hMLH1 (495-756) and BSA were immobilized directly onto same membrane, renatured and incubated with either crude lysate or purified hMRE11 (452-634) proteins (7mg/mL). $\alpha$-hMRE11 or $\alpha$-T7 were used to detect the presence of hMRE11 (452-634) and hMLH1 (495-756) respectively.

(B) A reciprocal far-Western blot where hMRE11 (452-634) and BSA were immobilized onto membrane, renatured and incubated with hMLH1 (495-756) crude lysate or purified protein. The same antibodies were used for conventional western blot detection.

**Figure 5 – hMLH1 interacting domains for associated proteins**

A schematic diagram of hMLH1 interacting regions for BLM, hPMS1, hPMS2, hMLH3, and hMRE11.

**Figure 6 – Yeast three-hybrid analysis of hPMS2, hMLH1, and hMRE11 protein interaction**

Either hPMS2 or hMLH1 cDNAs were fused to MCSI of Gal4-BD plasmid, pBridge vector. Various combination of hMRE11, hMLH1, and hPMS2 were inserted as a “bridge protein” to MCSII. Further, hMLH1, hPMS2 and hMRE11 cDNAs were cloned to pGADT7-AD plasmid. Yeast strain AH109 was used for double transformation. A positive protein interaction was
confirmed by histidine prototroph driven by inducible GalI-UAS His3 reporter gene in AH109 (MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, LYS2::GALI_{UAS-GALI_{TATA}}-HIS3, GAL2_{UAS-GAL2_{TATA}}-ADE2, ura3::MEL1_{UAS-MEL1_{TATA}}-lacZ).

Figure 7 – Effects of HNPCC hMLH1 pathogenic mutation on its association with hMRE11 and hPMS2

hMLH1 (495-756) wild type and missense mutation were fused to LexA-BD plasmid, pBTM116. Full length hMRE11 or hPMS2 was inserted to pGADT7-BD plasmid. Yeast strain L40 was employed for co-transformation. ß-gal liquid assay was performed as precious described (Materials and methods). Percent of interaction was calculated using ß-gal unit activities treatment group to that off the wild type where the wild type group had been normalized to give 100%.

Figure 8 – Dot-blot far-Western analysis of hMLH1 HNPCC missense mutations

Purified hMRE11 (452-634) was immobilized onto nitrocellulose membrane, washed, renatured, and probed with crude lysate contained hMLH1 (495-756) wild type or missense mutation. In general, 1mL of crude lysate was diluted in 4mL of 0.25% milk TBS-T. Conventional western analysis was carried out using ß-T7 to detect for presence of hMLH1.

Figure 9 – Western analysis of hMLH1 and hMRE11 in HeLa nuclear fractions

(A) HeLa nuclear extract was precipitated in ammonium sulfate at 35 and 65%, yielded fraction I (FI) and fraction II (FII). After diluted to a final concentration of 5mg/mL in buffer B (25mM Tris [pH7.5], 10% glycerol, 0.01% NP-40, 0.1mM EDTA, 2mM DTT, 0.1% PMSF, 1µg of leupeptin/mL) containing 0.5M NaCl, FI was further fractionated via ssDNA cellulose column chromatography. Flow through and fraction containing eluded bound proteins were designated as SSI and SSII respectively.
(B) A western analysis on all collected fractions with ∑-T7 and ∑-hMRE11 to detection the presence of hMLH1 and hMRE11 respectively.

**Figure 10 – Partial reconstitution hMRE11 recombinant protein**

hMRE11-deficient HeLa cell nuclear extract was generated by immunoprecipitation using a-hMRE11. Actual MMR assay was carried as described in *Materials & Methods*. Recombinant hMRE11 protein was reconstituted to deficient HeLa extract with the amount indicated. Proper repairing of 3’-CA dinucleotide insertion/deletion mispair in 3’-5’ direction rendered a unique restriction site where double digestion of XhoI and Bau106 yield 2 DNA fragments of 3.1kb and 3.3kb.

**Figure 11 – hMRE11 homodimerization**

Full length hMRE11 cDNAs and variants were inserted to either LexA-BD plasmid, pBTM116, or Gal4-AD plasmid, pGADT7. Yeast strain L40 was co-transformed and grown on SD/-Leu-Trp media. b-gal liquid assay was performed on fresh grown cells as described preciously. The average and SE were calculated based on 21 independent data points. For control, yeast was also transformed with only full length hMRE11 in pBTM116 vector alone. The b-gal activity units of the control group were 1.17.
## Figure 2

<table>
<thead>
<tr>
<th>hMRE11</th>
<th>Beta-gal Activity (Units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-708</td>
<td>28.62±2.40</td>
</tr>
<tr>
<td>1-708 (N117S)</td>
<td>32.65±0.60</td>
</tr>
<tr>
<td>1-634</td>
<td>27.85±0.42</td>
</tr>
<tr>
<td>1-100</td>
<td>6.23±0.33</td>
</tr>
<tr>
<td>1-204</td>
<td>4.23±0.24</td>
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<tr>
<td>1-279</td>
<td>4.60±1.20</td>
</tr>
<tr>
<td>1-361</td>
<td>4.81±0.27</td>
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<tr>
<td>1-497</td>
<td>5.11±0.37</td>
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<tr>
<td>243-634</td>
<td>25.30±1.35</td>
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<tr>
<td>305-634</td>
<td>29.76±0.99</td>
</tr>
<tr>
<td>396-634</td>
<td>39.39±4.33</td>
</tr>
<tr>
<td>452-634</td>
<td>29.94±2.04</td>
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<tr>
<td>502-634</td>
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<td>502-552</td>
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<tr>
<td>452-552</td>
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<td>452-497</td>
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<tr>
<td>107-425</td>
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<tr>
<td>217-532</td>
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<tr>
<td>308-632</td>
<td>35.86±1.41</td>
</tr>
<tr>
<td>409-708</td>
<td>28.15±0.92</td>
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</table>

- ■ positive X-gal filter assay
- □ negative X-gal filter assay
FIGURE 3

Expression of recombinant proteins

A

B

C

<table>
<thead>
<tr>
<th>hM.H1 (mrd95-756)</th>
<th>hM.H1 (mrd95-756)</th>
</tr>
</thead>
<tbody>
<tr>
<td>puro-control</td>
<td>puro-control</td>
</tr>
<tr>
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<td>Washed汼</td>
</tr>
<tr>
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<td>173.3</td>
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<tr>
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<td>20.5</td>
<td>20.5</td>
</tr>
<tr>
<td>16.1</td>
<td>16.1</td>
</tr>
</tbody>
</table>
## FIGURE 4

<table>
<thead>
<tr>
<th>Immobilize</th>
<th>hMLH1 (495-756) purified protein</th>
<th>BSA</th>
<th>hMRE11 (452-634) lysate</th>
<th>hMRE11 (452-634) purified protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>probe</td>
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<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

![Image](image_url)

<table>
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<th>BSA</th>
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<tbody>
<tr>
<td>probe</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

![Image](image_url)
**FIGURE 6**

Yeast Three Hybrid Analysis of hPMS2, hMLH1, and hMRE11 protein interaction

<table>
<thead>
<tr>
<th>BD-fused protein</th>
<th>Bridge protein</th>
<th>AD-fused protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hPMS2</td>
<td></td>
<td>hMLH1</td>
</tr>
<tr>
<td>2 hPMS2</td>
<td>hMRE11</td>
<td>hMLH1</td>
</tr>
<tr>
<td>3 hMLH1</td>
<td></td>
<td>hPMS2</td>
</tr>
<tr>
<td>4 hMLH1</td>
<td>hMRE11</td>
<td>hPMS2</td>
</tr>
<tr>
<td>5 hPMS2</td>
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<td>hMRE11</td>
</tr>
<tr>
<td>6 hPMS2</td>
<td>hMLH1</td>
<td>hMRE11</td>
</tr>
<tr>
<td>7 hMLH1</td>
<td></td>
<td>hMRE11</td>
</tr>
<tr>
<td>8 hMLH1</td>
<td>hPMS2</td>
<td>hMRE11</td>
</tr>
</tbody>
</table>

SD/-Leu - Trp       SD/-Leu - Trp - His
FIGURE 7

Effects of HNPCC pathogenic mutations on its association with hMRE11 and hPMS2

<table>
<thead>
<tr>
<th>hMLH1 (495-756)</th>
<th>hMRE11 (Units ± SE)</th>
<th>hPMS2 (Units ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype</td>
<td>128.89 ± 10.27</td>
<td>192.77 ± 14.00</td>
</tr>
<tr>
<td>Q542L</td>
<td>93.21 ± 7.84</td>
<td>164.55 ± 12.72</td>
</tr>
<tr>
<td>L574P</td>
<td>4.25 ± 0.29</td>
<td>3.91 ± 0.29</td>
</tr>
<tr>
<td>E578G</td>
<td>124.92 ± 7.29</td>
<td>145.01 ± 14.16</td>
</tr>
<tr>
<td>L582V</td>
<td>99.72 ± 8.57</td>
<td>191.83 ± 10.49</td>
</tr>
<tr>
<td>K618T</td>
<td>4.22 ± 0.44</td>
<td>80.45 ± 1.62</td>
</tr>
<tr>
<td>R659P</td>
<td>2.64 ± 0.27</td>
<td>3.62 ± 0.24</td>
</tr>
<tr>
<td>A681T</td>
<td>2.47 ± 0.32</td>
<td>90.23 ± 7.65</td>
</tr>
</tbody>
</table>
### FIGURE 8

<table>
<thead>
<tr>
<th>Immobilize</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
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</thead>
<tbody>
<tr>
<td>hMRE11 (452-634) purified protein</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSA</td>
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<td></td>
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<td>+</td>
</tr>
<tr>
<td>hMLH1 (aa495-756) probe</td>
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<td>Q542L</td>
<td>L574P</td>
<td>E578G</td>
<td>L582V</td>
<td>K618T</td>
<td>R659P</td>
<td>A681T</td>
<td>-</td>
<td>-</td>
<td>WT</td>
</tr>
</tbody>
</table>

**Beta-gal Activity (Units ±SE)**

<table>
<thead>
<tr>
<th></th>
<th>128.89 ±10.27</th>
<th>93.21 ±7.84</th>
<th>4.25 ±0.29</th>
<th>124.92 ±7.29</th>
<th>99.72 ±8.57</th>
<th>4.22 ±0.44</th>
<th>2.64 ±0.27</th>
<th>2.47 ±0.32</th>
</tr>
</thead>
</table>
Western analysis of hMLH1 and hMRE11 in HeLa nuclear fractions

(A) HeLa nuclear extract
   \[\text{(NH}_4\text{)}_2\text{SO}_4\]
   0-35% \hspace{1cm} 35-65%
   \text{F_I} \hspace{1cm} \text{F_{II}}
   \text{ssDNA cellulose}
   0.5 \text{ M NaCl} \hspace{1cm} 2.0 \text{ M NaCl}
   \text{SS1} \hspace{1cm} \text{SS2}

(B) HeLa Extract

<table>
<thead>
<tr>
<th></th>
<th>F_I</th>
<th>F_{II}</th>
<th>SS1</th>
<th>SS2</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMRE11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hMLH1</td>
<td></td>
<td></td>
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</table>
### FIGURE 11

<table>
<thead>
<tr>
<th>DNA-BD</th>
<th>AD</th>
<th>β-gal Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>hMRE11</td>
<td>hMRE11</td>
</tr>
<tr>
<td>2</td>
<td>hMRE11</td>
<td>hMRE11 (N117S)</td>
</tr>
<tr>
<td>3</td>
<td>hMRE11</td>
<td>hMRE11 (1-634)</td>
</tr>
</tbody>
</table>
SUMMARY

Recent studies strongly indicated that hMRE11-Rad50-NBS1 complex, in coordination by ATM, function in early DNA damager responses; thus, providing a crucial link between DNA damage sensory, detection and repair. The complex itself is implicated in many cellular functions such as creating meiotic double strand breaks (DSB), DSB repair by homologous recombination (HR) and non-homologous end joining (NHEJ), and maintenance of telomeric length (Haber et al. 1998, Paul et al. 1998). Genetical analysis of clinical patients linked mutations to cancer prone diseases such as ataxia telegiectaxia (AT), Nijmegen breakage syndrome (NBS), and AT-like disorder.

Human MRE11 possesses both endonuclease and dsDNA 3’-5’ exonuclease activity. However, to date the functional roles of its nuclease activities have yet fully elucidated. Although it has been speculated that hMRE11 nucleases might involve in processing repair intermediates. Study from S. cerevisiae nuclease-deficient mre11 mutant suggested that MRE11 nuclease activities not involved in DSB-mediated homologous recombination (Bressan et al. 1999). Hence, hMRE11 nuclease activities may participate in other cellular processes, including DNA MMR. We speculate that dsDNA 3’-5’ exonuclease is directly involved in DNA MMR in excising fragment contained mismatch. Our initial data from partial reconstitution of recombinant MRE11 protein to MRE11-deficient Hela cell extract successfully repair a G-T mismatch in the MMR assay. This suggested hMRE11 as a potential candidate, in complementary to EXOI, as mammalian exonuclease that participate in DNA repair. To further substantiate the role of hMRE11 exonuclease activity in MMR, additional experiments are underway to implement current observation. Firstly, the functional role of hMRE11 in DNA MMR is examined through in vitro inhibition of hMRE11 in Hela cells. hMRE11-deficient Hela
cell line is generated by gene silencing of hMRE11 transcription through the application of small interfering RNA (siRNA). Here, RNAi is fused with a pmH1p-neo construct and stably transformed in Hela cells to express with a short hairpin RNA (shRNAs) under G418 selection. A Hela cell line with a 80-90% reduction in hMRE11 protein expression will be used for subsequent *in vitro* MMR in comparison to wild type. This approach will demonstrate in conjunction with our previous data that hMRE11-deficient Hela cells will result in a defective MMR.

Next, to determine whether hMRE11-hMLH1 protein interaction is crucial for MMR, we propose to disrupt the association via dominant negative mutants using small peptides that competitively bind hMRE11 or hMLH1 interacting regions. The small peptide fragments are generated as His6-tagged recombinant proteins that are easily purified through affinity column chromatography. The effectiveness of dominant negative inhibitory effect is first verified by yeast three-hybrid analysis (mentioned above) before proceeding to disrupt hMRE11-hMLH1 interaction in Hela cell nuclear extract. The effects of disruption are examined by the ability of treated Hela cell lysate in repairing MMR comparing to wild type.

Human MRE11 exo- and endo-nuclease are stimulated by Rad50 whereas NBS1 only stimulates endonuclease activity. We are intrigued to examine whether association of hMLH1 does indeed activate hMRE11 exonuclease activity to excise DNA fragment containing mismatch. Schematically, a dsDNA substrate containing unique restriction site is digested to create blunt ends, purified, following with addition of recombinant hMRE11 and/or hMLH1 protein. Reaction mixtures are stopped at fixed interval, purified, follow by re-ligation and PCR amplification using two outer primers. Successful PCR amplification, intensity, and band size
are compared to wild type and controls to determine whether hMRE11 3’-5’ exonuclease activity is activated by the association with hMLH1.

In all, these and future experiments are aimed to examine the functional links between DNA damage sensory, detection and repair mechanisms. Specifically, our goals are to elucidate the molecular mechanism by which hMRE11-Rad50-NBS1 complex, in association with DNA MMR proteins, mediates genomic stability and mutation avoidance. The outcome of the research will provide new knowledge that are useful in assigning cancer susceptibility markers, diagnostic and genetic counseling, and potential novel therapeutic drug targets.
REFERENCES


