FUNCTIONAL CHARACTERIZATION OF HUMAN hMRE11 AND hMRE11-hMLH1 INTERPLAY IN DNA MISMATCH REPAIR

By

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To the faculty of Washington State University:

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FUNCTIONAL CHARACTERIZATION OF HUMAN hMRE11 AND

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Abstract

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DNA mismatch repair (MMR) is a critical pathway which can maintain genetic stability by correcting mismatched nucleotides that occur during DNA replication and recombination. Cells that are deficient in MMR display microsatellite instability (MSI) and germline mutations in various MMR genes can lead to hereditary nonpolyposis colorectal cancer (HNPCC) in humans. Our study for the first time showed that hMRE11 deficiency in HeLa cells can lead to defective 3' MMR in an *in vitro* MMR assay. Through both GFP-based MSI reporter assay and endogenous MSI marker assay, these cells were also found to display increased levels of MSI, which is comparable to that of the hMLH1 deficient cells. Together with our previous finding that hMRE11 directly interacts with hMLH1, we raised the possibility that hMRE11 might be involved in MMR through physical interaction with hMLH1, and this interaction is critical for functional MMR. Our analysis with 7 hMLH1 HNPCC mutations located within the hMRE11-interacting domain showed that 4 of them almost completely disrupt the MRE11-MLH1 interaction. We increased our study to 64 MLH1 HNPCC mutations, which contain 38 missense mutations, and the result showed that 63% (24 out of 38) of the hMLH1 missense mutations

can disrupt their interactions with hMRE11. These findings indicate that hMRE11 represents a functional protein factor in MMR pathway and disruption of hMRE11-hMLH1 could be an alternative molecular mechanism underlying the pathogenic effects of hMLH1 HNPCC mutations.

To directly study the functional impact of hMRE11-hMLH1 interplay in MMR, we blocked their interaction *in vivo* by an hMRE11 dominant negative mutant which represents the hMLH1-interacting domain. *In vitro* MMR and excision assays indicate that these cells showed dramatically decreased 3' MMR activity and 3' excision activity, suggesting that the hMRE11-hMLH1 interplay is critical for functional MMR, presumably by recruiting hMRE11 for the excision step of MMR.

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INTRODUCTION

The accurate transmission of genetic information during DNA replication is essential for cell survival. Surveillance of this transmission process is carried out by a network of proteins involved in cell-cycle regulation, DNA replication and DNA repair (Zhou and Elledge, 2000; Futaki and Liu, 2001). Defects in this protein network will lead to inability to repair DNA damage properly, and make cells more susceptible to genetic instability and tumorgenesis (Harfe and Jink-Robertson, 2000). Among different pathways in DNA damage response, DNA mismatch repair (MMR) plays a critical role in maintaining the integrity of genetic information transmitted during DNA replication. Although little is known about the mechanism of how this protein network functions, it is believed that the highly coordinated roles of the proteins in DNA repair process are partially facilitated by formation of a BRCA1-associated genome surveillance complex (BASC), which is composed of MSH2-MSH6, MLH1, ATM, BLM, RAD50-NBS1-MRE11 complex. Defects in this MMR pathway can lead to microsatellite instability (MSI) and hereditary nonpolyposis colorectal cancer (HNPCC) (Harfe and Jink-Robertson, 2000; Wang et al., 2000).

DNA Mismatch Repair System

DNA mismatch repair (MMR) is an essential surveillance system in detecting and correcting the nucleotide mismatches that occur during DNA replication. The basic features of the MMR system have been highly conserved from bacteria to mammals (Harfe and Jink-Robertson, 2000). MMR proteins were first discovered in bacteria and the *E.coli* MMR system has been completely reconstituted in vitro (Modrich and Lahue, 1996). *E.coli* MMR

system which have three key protein factors: MutS, MutL, and MutH. MutS is an ATPase which can bind as a homodimer to DNA that contains a nucleotide mismatch. MutS binding recruits another ATPase, MutL, which form a homodimer and plays a role in coupling mismatch recognition to downstream repair processes. The MutS-MutL-DNA complex then recruits MutH and stimulates its methylation-sensitive endonuclease activity. The strand discrimination between template and newly synthesized strand in bacterial MMR system is based on the unmethylated state of the nascent DNA strand. After binding to the MutS-MutL-DNA complex, the endonuclease activity of the MutH is directed to the newly synthesized, unmethylated strand in a hemimethylated GATC *dam* methylation site to make a single-strand nick (Welsh et al., 1987). Then the subsequent strand removal requires either a single-strand specific 5'-3' or 3'-5' exonuclease. For bacterial system, two 5'-3' exonucleases (exonuclease VII and the RecJ exonuclease) and two 3'-5' exonucleases (exonuclease I and X) are implicated in this single-strand exonucleolytic process, which also requires the MutU helicase activity.

Different from bacterial MMR system which utilizes a single MutS and single MutL protein, the eukaryotic MMR system have multiple MutS homologs (MSH proteins) and multiple MutL homologs (MLH proteins), but no homologs for bacterial MutH have been identified (Harfe and Jink-Robertson, 2000; Kolodner, 1996). In humans, the concerted actions among the multiple MutS and MutL homologs begin with the binding of the hMSH2-hMSH3 or hMSH2-hMSH6 heterodimers to the mismatched DNA. Here both hMSH2-hMSH3 (MutSβ) and hMSH2-hMSH6 (MutSα) complexes play a redundant role in recognizing insertion/deletion loops, whereas hMSH2-hMSH6 has an additional role in recognizing single base mismatch (Kolodner and Marsischky, 1999; Fig.1). Then a heterodimer of hMLH1-hPMS2 (or

hMLH1-hPMS1 and hMLH1-hMLH3) is recruited to the mismatch-protein complex (Kolodner and Marsischky, 1999). It is speculated that the nicks at 5' and 3' ends of Okazaki fragments might serve as the strand discrimination signal during lagging strand synthesis, while distinguishing the leading strand from its template is more problematic (Harfe and Jink-Robertson, 2000).

The MMR system plays a key role in eliminating mutational intermediates generated during DNA synthesis and thus helps to insure that DNA replication is a high-fidelity process. The most significant outcome with the MMR deficiency is genome-wide microsatellite instability (MSI) (Fishel and Wilson, 1997), which is regarded as a hallmark for defective MMR. Germline mutations of the MMR genes hMLH1, hMSH2, hMSH3, hMSH6, hPMS1 and hPMS2 appear to be responsible for most cases of hereditary non-polyposis colorectal cancer (HNPCC) (Prolla et al., 1998). Recent studies carried out with the Msh2-deficient and Msh6-deficient mouse models showed that inactivation of the MSH2 or MSH6 gene in mouse can result in MMR deficiency, cancer susceptibility and other spontaneous mutations (Prolla et al., 1998; de Wind et al., 1995; Reitmair et al., 1996; Edelmann et al., 1997). These findings indicate a direct link between the increased risk of cancer and mutations in genes involved in the DNA damage response and repair network.

Human MRE11 protein and its role in DNA damage response

Among many protein factors implicated in the process of DNA damage response, human hMRE11 represents an essential multifunctional protein that plays a role in DNA damage detection, signaling and repair (Petrini, 2000). Disruption of *MRE11* gene is embryonically

lethal in mouse and chicken, whereas null mutations of MRE11 in *S. cerevisiae* and *C. elegans* are viable but exhibited a slow growth in yeast (Zhao et al., 2000; Yamaguchi-Iwai et al., 1999; Haber, 1998; Chin and Villeneuve, 2001). Although the functional roles of hMRE11 in cellular processes are not fully understood, it is known that hRAD50-hMRE11-NBS1 complex plays essential roles in maintaining genomic stability such as processing of double strand breaks (Bressan et al., 1999; Costanzo et al., 2001), DNA damage detection and cell cycle checkpoint (Grenon et al., 2001) and maintenance of telomeric length by recruiting telomeric subunits to DNA ends (Tsukamoto et al., 2001).

Mutations in the NBS1 and MRE11 genes are responsible for the human radiation sensitivity disorders Nijmegen Breakage Syndrome (NBS) and Ataxia-Telangiectasia-like Disorder (ATLD), respectively, which are characterized by defective checkpoint response and high levels of chromosomal abnormalities. Among ATLD patients, two distinct mutations, ATLD1 and ATLD3 were identified. The ATLD1 contains a premature stop codon, truncating the protein at amino acid position 633, thus eliminating the last 76 amino acids. The ATLD3 contain a missense mutation, N117S, and encodes a full-length polypeptide. These two mutations confer a clinical phenotype virtually identical to that of Ataxia-Telangiectasia (A-T), indicating that these disorders might result from defects in a common cellular pathway. Recent study confirmed that NBS1, MRE11 and ATM function together in DNA-damage response and repair (Zhao and Elledge, 2000; Lim et al., 2000; Gatei et al., 2000; Wu et al., 2000).

hMRE11 possesses both ssDNA/dsDNA endonuclease and dsDNA 3'-5' exonuclease activity (Paull and Gellert, 2000). In the MMR process, exonuclease enzymatic activity is required to excise DNA fragment containing mismatch base or damaged nucleotides during DNA

metabolic process such as replication. However, the precise roles of the nuclease activities associated with hMRE11 complex in DNA damage and repair are elusive. It is speculated that the hMRE11 nucleases might participate in the processing of repair intermediates. Our previous study has shown that hMRE11 directly interacts with hMLH1, both *in vitro* and *in vivo* (Her et al., 2002). In addition, a number of hMLH1 HNPCC missense mutations preferentially disrupt the interactions between hMLH1 and hMRE11, suggesting that the interplay between hMLH1 and hMRE11 might play a critical role in MMR (Vo et al., 2005).

Although the nuclease activities involved in the late steps of MMR are not fully understood, there are several lines of evidence indicating the involvement of multiple nucleases in human MMR. For the bacterial system, there are four exonucleases including two 5'-3' and two 3'-5'. However, only the 5'-3' exonuclease I, hExoI, is implicated in mammalian MMR process (Alam et al., 2003). Recent study indicated that inactivation of hExoI only partially affects the MMR in both S. *cerevisiae* and mouse (Tishkoff et al., 1997; Tran et al., 1999; Wei et al., 2003), and mice lacking the functional hExoI are less likely to develop tumors when comparing with MSH2 or MLH1 knockout mice (Wei et al., 2003). This suggests that there might be functionally redundant exonucleases in mammalian MMR process. It is possible that the nuclease activities of hMRE11 and hExoI play different but overlapping roles in MMR.

Microsatellite instability (MSI) and hereditary nonpolyposis colorectal cancer (HNPCC)

The increased rate of uncorrected replication errors at simple repeat sequences is commonly known as microsatellite instability (MSI). This type of widespread genome instability was initially observed in tumors arising in hereditary nonpolyposis colorectal cancer

(HNPCC) patients (Parsons et al., 1993; Aaltonen et al., 1993; Ionov et al., 1993; Thibodeau et al., 1993) and is regarded as a hallmark for defective MMR. In addition to HNPCC, defects in MMR have been associated with sporadic colorectal, endometrial, and gastric carcinomas (Harfe and Jink-Robertson, 2000). HNPCC is the most common hereditary colon cancer, approximating 5% of all colon cancers (Lynch and Chapelle A, 1999). The majority of HNPCC and sporadic tumors that display MSI contain mutations in at least six of the MMR genes, hMLH1, hMSH2, hMSH3, hMSH6, hPMS1 and hPMS2. Among these, hMLH1 gene mutations account for 52.9% of all HNPCC cases, mutations in the hMSH2 gene account for an additional 39.5% (http://www.insight-group.org). However, the fact that only 70% of HNPCC cases carried mutations in MMR genes indicates that there might be other unidentified genes which have similar functions as MMR genes.

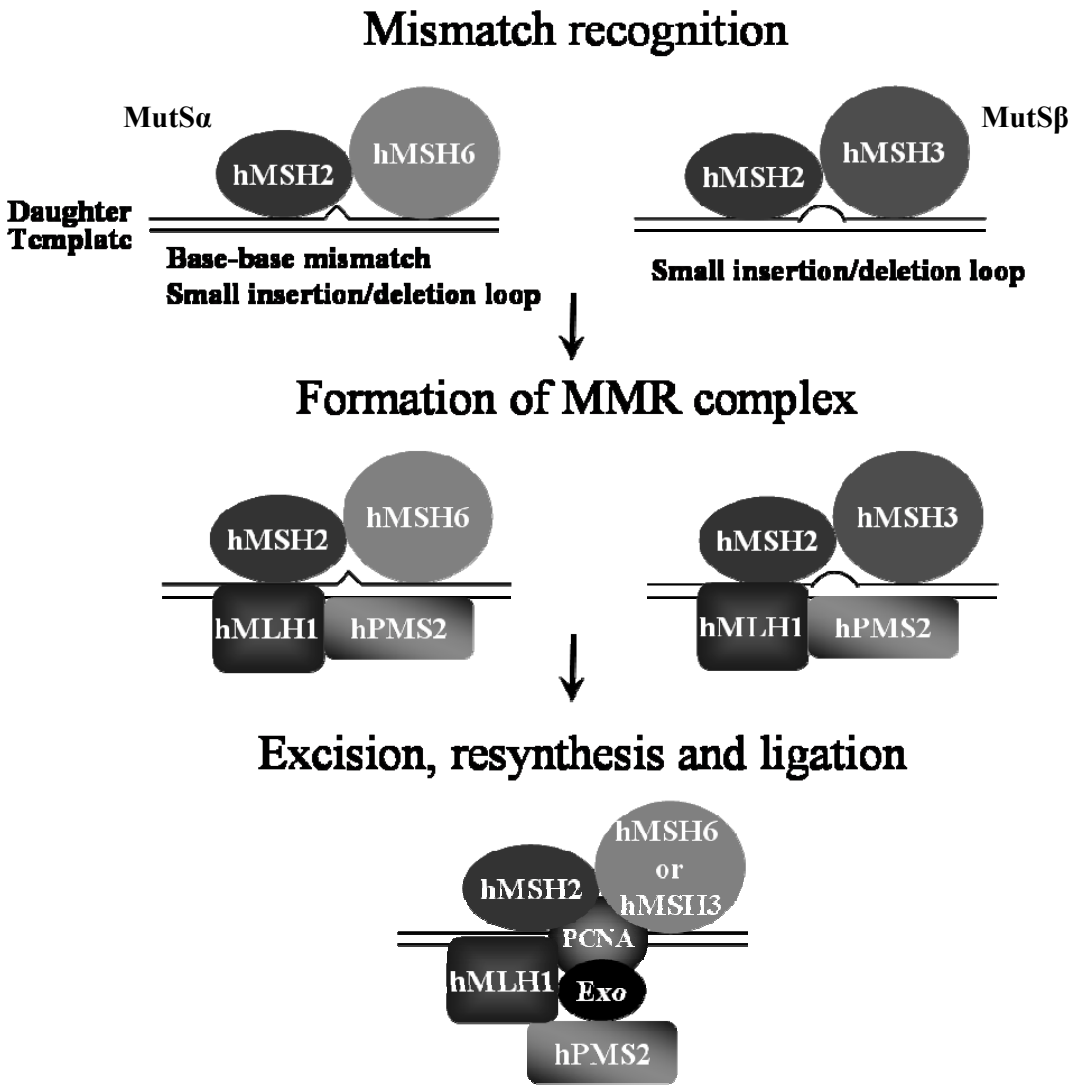
Studies of knockout mice models indicated that mutations in MMR genes predispose cells to the development of cancer. Cell lines derived from various human malignant tumors have been found to contain mutations in MMR genes (Parsons et al., 1993; Umar et al., 1994; Risinger et al., 1995; Risinger et al., 1996), and these all indicate a critical role of mismatch repair deficiency in tumorigenesis in humans. More and more evidence has shown that there are dynamic functional links among components of DNA damage response system, including MMR and hMRE11-associated proteins. Mutations that interrupt the coordination of this network of proteins will render cells susceptible to genetic instability. Therefore, a thorough understanding of the molecular mechanism of the MMR system is essential to gain a comprehensive understanding of the pathogenic effects of MMR gene mutations in cancer development.

AIMS OF STUDY

DNA mismatch repair (MMR) plays an essential role in maintaining genetic stability during DNA replication. Protein factors involved in this DNA damage response complex include major MMR proteins and hMRE11-associated protein complex. Defects in this protein network will make cells prone to mutations, genetic instability and cancer development. HNPCC is a common cancer syndrome that associates with defects in MMR genes, among which hMLH1mutations account for 52.9% and hMSH2 account for 39.5% of all the HNPCC mutations. Specifically, around 30% of mutations in hMLH1 gene are due to single amino acid substitution (Nystrom-Lahti et al., 2002). However, the molecular consequences of these missense mutations are not fully understood; it is possible that these mutations might impair the proper protein-protein or protein-DNA interactions. Therefore we need a precise understanding of the molecular mechanism of the MMR system to comprehend the pathogenic effects of MMR gene mutations in cancer development.

Our previous work has demonstrated a direct physical link between hMRE11 and hMLH1, both *in vitro* and *in vivo* (Her et al., 2002). In addition, some hMLH1 HNPCC missense mutations preferentially disrupt the hMLH1- hMRE11 interaction, suggesting that the interplay between hMLH1 and hMRE11 might play a critical role in MMR. It's possible that hMLH1 could recruit hMRE11 to form a downstream complex to function in late steps in MMR such as excising the mismatched nucleotides. Hence my study was to test the hypothesis that the interplay between MMR protein hMLH1 and hMRE11-associated nuclease complex plays important roles in MMR and DNA-damage response, as well as molecular mechanism governing the pathogenic potentials of hMLH1 missense mutations in HNPCC.

Figure 1



Schematic representation of mammalian MMR process. Only major protein factors are included in the figure. "Exo" represensts ExoI and other yet to be defined exonucleases in MMR

hMRE11 deficiency leads to microsatellite instability and defective DNA mismatch repair

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ABSTRACT

DNA mismatch repair is essential in the surveillance of accurate transmission of genetic information, and defects in this pathway lead to microsatellite instability (MSI) and hereditary nonpolyposis colorectal cancer (HNPCC). Our previous study raised the possibility that hMRE11 might be involved in MMR through physical interaction with hMLH1. Here, we show that hMRE11 deficiency leads to significant increase in MSI for both mono- and dinucleotide sequences. Furthermore, RNA interference (RNAi) mediated hMRE11 knockdown in HeLa cells results in MMR deficiency. Analysis of seven HNPCC-associated hMLH1 missense mutations located within the hMRE11-interacting domain reveals that four mutations (L574P, K618T, R659P, and A681T) cause near complete disruption of the interaction between hMRE11 and hMLH1, two mutations (Q542L and L582V) cause 30% reduction of protein interaction. These findings indicate that hMRE11 represents a functional component of the MMR pathway and the disruption of hMLH1-hMRE11 interaction could be an alternative molecular explanation for hMLH1 mutations in a subset of HNPCC tumors.

INTRODUCTION

DNA mismatch repair (MMR) plays a critical role in the assurance of accurate transmission of genetic information, and MMR deficiency in humans is associated with profound genetic instability and high risk for hereditary nonpolyposis colorectal cancer (HNPCC) (Harfe and Jink-Robertson, 2000). Proteins that are involved in MMR have emerged as central components of the DNA damage response complex that presently includes major MMR proteins and the hMRE11-associated complex (Wang et al., 2000).

Studies performed with various eukaryotic systems indicate that the MMR pathway is evolutionally conserved, and the function of MMR relies on a concerted action of multiple MutS and MutL homologous proteins (Harfe and Jink-Robertson, 2000). Although biochemical studies have suggested that the 5'-3' exonuclease I (hExoI) is involved in the process of MMR in human cells (Tishkoff et al., 1998; Dzantiev et al., 2004), the inactivation of ExoI only moderately affects MMR in mouse (Wei et al., 2003). In addition, mice lacking ExoI are less likely to develop tumors in comparison to Msh2 or Mlh1 knockout mice, suggesting the existence of functionally redundant exonucleases in mammalian MMR (Wei et al., 2003).

It is presently known that the pathogenesis of HNPCC can be largely attributed to germline mutations in at least five MMR genes, in which germline mutations in the MMR gene *hMLH1* alone are responsible for over 50% of all HNPCC cases (Database of the International Collaborative Group on HNPCC, http://www.nfdht.nl). Appreciably, almost 30% of all hMLH1 mutations are scattered missense alterations causing single amino acid substitutions. Though the functional basis underlying the pathogenic effects for the majority of missense mutations is currently open-ended; these mutations could potentially alter the properties of hMLH1, which in

turn might affect the assembly of functional repair complexes.

Our previous studies have demonstrated a physical link between hMLH1 and hMRE11 (Her et al., 2002). Here, we demonstrate that hMRE11 is involved in human MMR, and HPNCC-associated hMLH1 missense mutations located within the hMRE11-interacting domain disrupt protein interaction between hMLH1 and hMRE11.

RESULTS AND DISCUSSION

To examine the role of hMRE11 in MMR, we first investigated whether hMRE11 deficiency causes MSI – a hallmark for defective MMR. To this end, we have created two MSI reporters in which the open reading frame (ORF) of the green fluorescence protein (GFP) was interrupted by the insertion of either a mononucleotide $(A)_{17}$ or dinucleotide $(CA)_{25}$ microsatellite repetitive sequences. These two out-of-frame (OF) GFP ORFs were then cloned and placed under the control of the human cytomegalovirus (CMV) immediate early promoter. Then, a series of hMRE11-deficient cell lines were generated from MMR-proficient HeLa cells using RNA interference (RNAi) technology. The levels of hMRE11 silencing were estimated in the range of 45% to 97% in this series of individual clones, of which HeLa clone #14 (HeLa 14) displayed the most significant knockdown (Fig. 1). Despite of being an essential gene, cells derived from HeLa 14 grew at similar rates with that of HeLa cells, and displayed no detectable morphological changes (Fig. 2). Immunoblotting analysis demonstrated that hMRE11 RNAi had no effects on the expression of hMLH1, hMSH2, and hRad51 proteins, indicating that hMRE11 RNAi was highly specific (Fig. 1).

Cells of HeLa 14 were then examined for MSI. The results of MSI functional assay, using pA-OF and pCA-OF, clearly indicated that hMRE11 knockdown in HeLa cells increased mononucleotide $(A)_{17}$ instability by approximately 50-fold and dinucleotide $(CA)_{25}$ instability by 22-fold (Fig. 2). Analysis of hMLH1-deficient H6 cells using the same reporters revealed that hMLH1-deficient H6 cells displayed similar levels of MSI (Fig. 3). Therefore, cells defective in hMRE11 appear to exhibit a mutator phenotype similar to that observed in hMLH1-deficient cells. In addition, human cells harboring an integrated copy of an in-frame reporter also

displayed an increase in MSI when treated with constructs encoding hMRE11 shRNAs (Fig. 4). These observations tend to suggest a critical role of hMRE11 in MMR, presumably through the interplay with hMLH1.

We then assessed the role of hMRE11 in MMR by a functional *in vitro* MMR assay. Nuclear extracts derived from HeLa 14 and its parental HeLa cells were tested for their abilities to repair heteroduplexes containing a single G-T mismatch and a strand break either 3' (3' G-T) or 5' (5' G-T) to the mismatch (Guo et al., 2004). Consistent with earlier reports (Holmes et al., 1990; Thomas et al., 1991), HeLa extracts carried out repair reactions for both substrates at comparable levels (Fig. 5). Remarkably, the repair activities for the 3' and 5' heteroduplexes in extracts of HeLa 14 were drastically different; the 3' activity was more than 50% lower than that of the control and addition of a partially purified hMRE11-enriched HeLa nuclear fraction SS1 (Fig. 6) could efficiently restore the 3' activity to the control level (Fig. 5B). Since SS1 fraction by itself cannot carry out both 5' and 3' nick directed MMR (Fig. 5A), the results of the MMR assay suggest that hMRE11 is involved in the process of MMR. Although the repair of 5' G-T was approximately 90% competent in extracts of HeLa 14 cells as compared to that of parental HeLa cells, the observed difference was not statistically significant (Fig. 5B). The preferential reduction in 3' G-T repair suggests that hMRE11 is involved in 3' nick-directed MMR, which is well reconciled with the fact that hMRE11 possesses DNA 3'-5' exonuclease activity (Moreau et al., 1999; Paull and Gellert, 1998; Trujillo et al., 1998). The observed remaining 3' G-T repair activity in HeLa 14 could be attributable to the residual hMRE11 protein and/or the involvement of other functional redundant proteins in MMR such as hExoI. It is of note that the inactivation of ExoI only caused partial reduction of MMR activities in *S. cerevisiae* and mouse (Wei et al.,

2003; Tishkoff et al., 1997; Tran et al., 1999), suggesting the existence of redundant nuclease(s) in MMR.

Our data support the notion that the functional interplay between hMLH1 and hMRE11 plays a critical role in human MMR. Despite the well-established roles of hMRE11-associated complex in DNA damage response and the repair of DNA double-strand breaks (DSBs), the role of hMRE11-associated nuclease activities in DSB repair has not been fully established (Bressan et al., 1999). Although the effect of hMRE11-knockdown on 5' G-T repair is not statistically significant, we could not eliminate the possibility that the residual hMRE11 protein in cells from HeLa 14 may be sufficient for 5' repair. This view is coherent with the observation that human hExoI could be involved in both 5' and 3' nick-initiated and mismatch-induced MMR reactions (Dzantiev et al., 2004), despite the fact that hExoI is only known to possess a 5' to 3' hydrolytic activity (Lee and Wilson, 1999; Tishkoff et al., 1997). Recent studies have revealed the presence of *hMRE11* somatic mutations in CIN cancers and MMR-deficient colorectal tumors and cancer cell lines (Wang et al., 2004; Giannini et al., 2002). Though the close correlation between an intronic $(T)_{11}$ mutation in the *hMRE11* gene and MMR deficiency has led to the speculation that *hMRE11* might represent a target in tumorigenesis driven by MMR deficiency (Giannini et al., 2002), our data suggests that hMRE11 deficiency directly results in elevated MSI and defective MMR.

To date, studies performed with HNPCC families have revealed that *hMLH1* gene mutations account for approximately 52.9% of all HNPCC cases (http://www.insight-group.org), among which about 30% of hMLH1 mutations are single amino acid substitutions. Despite this prevalence, the molecular basis underlying the pathogenic effects of these single amino acid

substitutions has not been fully elucidated. Since the interaction between hMRE11 and hMLH1 could be involved in recruiting hMRE11 during the MMR process (Her et al., 2002), it is plausible that some of the functional effects of hMLH1 mutations could result from the disruption of hMRE11-hMLH1 interaction.

Hence, to test whether the disruption of hMRE11-hMLH1 interaction represents an immediate consequence of hMLH1 mutations in HNPCC, we have determined the effects of seven hMLH1 mutations on protein interaction. The minimal interacting domains of hMLH1 and hMRE11 were first determined by yeast two-hybrid analysis performed with a series of hMLH1 and hMRE11 deletion mutants. As shown in Fig. 7A, experiments performed with LexA-BD-hMRE11 (full-length) and a series of hMLH1 VP16-AD fusion mutants demonstrated that the minimal hMRE11-interacting region was found to be located within the C-terminal portion of the hMLH1 corresponding to amino acid residues 492-756, which overlapped with hMLH1 domains implicated in mediating protein interactions to hPMS2, hMLH3, hPMS1, BLM, or hExo1 (Kondo et al., 2001; Pedrazzi et al., 2001; Schmutte et al., 2001). Similarly, the minimal region of hMRE11 that mediated interaction with hMLH1 was resolved to comprise amino acid residues 452 to 634 (Fig. 7B). The interaction between these two domains was then validated by far-Western analysis (Fig. 7C and 7D). Immobilized hMLH1 aa495-756 fragment specifically interacted with hMRE11 aa452-634 fragment either in the purified form or in the crude lysate (Fig. 7C). Vice versa, immobilized hMRE11 aa452-634 protein could specifically capture hMLH1 aa495-756 fragment (Fig. 7D). Thus, the C-terminal portion of hMLH1 interacts with a region of hMRE11 protein that resides in between two DNA binding motifs (de Jager et al., 2001).

We next investigated whether the formation of hMLH1-hMRE11 complex could be disrupted by the common HNPCC-associated hMLH1 missense mutations located within the interacting domain. Among the seven mutations studied, two (L574P, R659P) completely disrupted the interactions of hMLH1 with both hMRE11 and hPMS2 (Table 1). Whereas two (K618T, A681T) displayed differential effects on their interactions with hMRE11 and hPMS2; both point mutations abolished the interaction with hMRE11 but remained at 50% of their capacity to associate with hPMS2 (Table 1). The remaining hMLH1 mutations (Q542L, E578G, and L582V) had relatively small (less than 30%) but differential effects on hMLH1 interaction with hMRE11 and hPMS2 (Table 1). The observed defects of various hMLH1 missense mutations in protein interaction with hPMS2 are consistent with those reported previously (Kondo et al., 2003). The different effects of these hMLH1 mutations on protein interactions are most likely due to protein conformational changes caused by the amino acid sequence variations, which is supported by previous observations that most of the missense mutations did not affect the expression levels of hMLH1 variant proteins (Kondo et al., 2003).

To validate the results obtained from yeast two-hybrid analysis, we have performed far-Western assay to examine the effects of the same set of hMLH1 missense mutations on the interaction between hMLH1 and hMRE11. As shown in Fig. 8, the result of far-Western analysis was highly consistent with that of the two-hybrid assay, suggesting that the disruption of hMLH1-hMRE11 interaction could represent an alternative molecular basis underlying the functional effects of hMLH1 mutations in human cells.

Together, our results show, for the first time, that the human hMRE11 protein is involved in 3' nick-directed MMR, presumably through interaction with hMLH1. Our data

indicate that hMRE11 deficiency leads to MSI and defective MMR in human cells. Furthermore, our study suggests that the biological consequence of hMLH1 HNPCC mutations could be attributable to the uncoupling of hMRE11-hMLH1 protein complex. Obviously, a better appreciation of hMRE11-hMLH1 interplay in human cells awaits further deciphering of the precise enzymatic and/or structural roles of hMRE11 in the process of MMR. The participation of hMRE11 in MMR should predict that the dynamic interplay between MMR components and DSB repair proteins is essential for the surveillance of genomic integrity.

MATERIALS AND METHODS

Western blot analysis and antibodies

Proteins were separated on 10%SDS-PAGE and transferred to nitrocellulose membranes (Schleicher and Schuell). Antibodies used in this study included α-hMRE11 polyclonal (Novus Biologicals Inc.), α-hMRE11 12D7 (GeneTex Inc.), α-hMLH1 (Santa Cruz Biotechnology Inc.), α-hMSH2 (Calbiochem), α-RAD51 (Oncogene Research Products), α-T7 tag (Novagen). For far-Western analysis, immobilization of affinity-purified proteins on nitrocellulose membranes was carried out with Bio-Dot Microfiltration Apparatus (Bio-Rad). The procedure of far-Western analysis was essentially the same as described (Wu et al., 2000).

HeLa hMRE11-RNAi stable transfectants

HeLa cells were transfected with a mixture of pmH1P-neo (Carmell et al., 2003) based constructs targeting two regions of hMRE11 transcript at positions 400-418 (5'-GGGGCAGATGCACTTTGTG) and 1242-1260 (5'-TGGGAAACTTATCACAAAG). Transfected HeLa cells were selected with 400 μg/ml G418 (Clontech). Individual G418 resistant clones were expanded and analyzed by the use of immunoblotting techniques.

Yeast two-Hybrid analysis

cDNA fragments encoding full-length and relevant regions of hMRE11, hMLH1 and hPMS2 proteins were cloned into vectors pBTM116, pVPd, pGADT7, and pACT2. Protein interactions were ascertained with the transcription activation of *lacZ* gene in the reporter strain L40. β-galactosidase (β-gal) activities were either qualitatively monitored with X-gal filter assays or quantitatively measured by the β-gal liquid assay using *o*-nitrophenyl β-D-galactopyranoside (ONPG) as a substrate.

Microsatellite instability reporter assay

Microsatellite repetitive sequences **ATG**GC$(A)_{17}$ or **ATG**GC$(CA)_{25}$ were cloned upstream of a GFP ORF devoid of initial ATG start codon in pcDNA6 (Invitrogen) to create the MSI reporter constructs, pA-OF and pCA-OF. Cells that were transfected with pA-OF and pCA-OF were counted with a fluorescence-activated cell sorter (FACSort, Becton Dickinson), and percentage of GFP-expressing cells was analyzed with BD CellQuest Pro.

Nuclear extracts and repair assay

Nuclear extracts were prepared from parental HeLa and HeLa 14 cells as described (Holmes et al., 1990). DNA heteroduplexes used in this study contained a G-T mismatch and a strand-break either 5' (5' G-T) or 3' (3' G-T) to the mismatch (Guo et al., 2004). Mismatch repair assays were performed as described (Holmes et al., 1990).

Purification of recombinant proteins.

hMRE11 and hMLH1 cDNA fragments encoding protein interaction domains were subcloned into pET-28a (Novagen), and the resulting constructs were transformed into BL21(DE3)-RIL (Stratagene) to produce recombinant T_7-hMLH1 aa495-756 and hMRE11 aa452-634 as His_6-fusion proteins. Recombinant proteins were purified with the TALON Metal Affinity Resins (Clontech).

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Table 1

Effects of various hMLH1 HNPCC missense mutations on protein-interaction

hMLH1 (495-756 LexA BD)	B-gal activity (units±SE) (Gal4 AD fusion)	
	hMRE11	hPMS2
Wildtype	128.89 ± 10.27	192.77 ± 14.00
Q542L	93.21 ± 7.84	164.55 ± 12.72
L574P	4.25 ± 0.29	3.91 ± 0.29
E578G	124.92 ± 7.29	145.01 ± 14.16
L582V	99.72 ± 8.57	191.83 ± 10.49
K618T	4.22 ± 0.44	80.45 ± 1.62
R659P	2.64 ± 0.27	3.62 ± 0.24
A681T	2.47 ± 0.32	90.23 ± 7.65
R659P/A681T	2.69 ± 0.36	3.53 ± 0.09

β-Galactosidase activity was determined with the β-gal liquid assay using ONPG as a substrate. The coding sequence for wild-type MLH1 aa495-756 and various missense mutants were fused to LexA-BD in plasmid pBTM116. R659P/A681T is a double mutant containing two independent mutations. Full-length hMRE11 and hPMS2 were fused with Gal1-AD in plasmid pGADT7. Analysis of protein interaction was carried out in reporter strain L40. The averageβ-gal activity units and standard errors (SE) were determined with at least three independent data

FIGURE LEGENDS

Figure 1. Generation of stable hMRE11-knockdown HeLa cells. Gene silencing of hMRE11 in HeLa cells was achieved by the use of small interfering RNAs (siRNAs). To generate HeLa cells stably displaying hMRE11-knockdown, two pmH1P-neo based RNAi constructs encoding short hairpin RNAs (shRNAs) were used to transfect HeLa cells. Neomycin-resistant clones were selected with 400 μg/ml G418, and 150 μg of cell extracts were analyzed by immunoblotting. HeLa, parental control; 12 and 14, two representative stable cell lines.

Figure 2. Morphology of HeLa and HeLa 14 cells. While maintained in cell culture, the hMRE11 knockdown stable HeLa cells (HeLa #14) did not display any observable growth defects when comparing to the parental HeLa cells. Furthermore, there was no apparent morphological difference between these two cell lines.

Figure 3. The effects of hMRE11-knockdown on microsatellite instability (MSI). Two out-of-frame MSI reporters, pA-OF and pCA-OF, were used to transfect an approximately equivalent number of parental and hMRE11-knockdown HeLa cells (HeLa 14), as well as the hMLH1-deficient cell line H6 as a control. Number of cells that expressed GFP was determined by FACS analysis performed with a total of 20,000 cells for HeLa and HeLa 14, as well as 10,000 of parental and transfected H6 cells.

Figure 4. Effects of hMRE11 knockdown on microsatellite instability by the use of an integrated (CA) dinucleotide MSI reporter. An in-frame MSI reporter, **<u>ATG</u>**GC$(CA)_{20}$-GFP (pCA-IF) was used to stably transfect the human colon carcinoma cell line DLD1, of which a stable clone was established following selection with 10 μg/ml Blasticidin (Invitrogen). The homogeneity of cells expended from this clone (DLD1/pCA-IF) was validated with a

fluorescence-activated cell sorter (FACS). The FACS analysis indicated that almost all cells analyzed expressed GFP, suggesting that stable integration does not affect the expression of the reporter protein GFP. The established DLD1/pCA-IF was then transiently transfected with RNAi constructs encoding either hMRE11 shRNA sh-2 or shRNA sh-3, and cells that lost GFP expression were analyzed and counted by FACS. The results indicated that treatment of DLD1/pCA-IF cells with hMRE11 shRNA sh-2 or shRNA sh-3 resulted in a 12-fold or 2-fold increase in MSI, respectively, over that of the control cells treated with an empty vector. This is consistent with our observation that hMRE11 silencing by transient transfection can only result in approximately up to 50% reduction of hMRE11 protein with the same set of shRNA constructs. It was reported that DLD1 cells displayed very low levels of dinucleotide repeat instability (Oki et al., 1999), which could account for the observed low levels of CA instability in DLD1/pCA-IF cells treated with the empty vector. The different effect between RNAi targeting constructs shRNA sh-2 and shRNA sh-3 on MSI was also reflected by the difference in the effectiveness of hMRE11 silencing by these two constructs.

Figure 5. The effects of hMRE11-knockdown on DNA mismatch repair. Fifty μg of nuclear extracts derived from parental HeLa (H) and HeLa 14 (H^i) cells, as well as 17.6 μg of a partially purified hMRE11-enriched HeLa nuclear fraction SS1 were used to perform MMR reactions with 5' G-T and 3' G-T mismatch substrates. Repaired products were distinguished from substrates by restriction digestion, and repair efficiency was scored by dividing repaired products with the total substrate DNA. (A) MMR reactions were performed as indicated. The hMRE11-enriched SS1 fraction is defective for both 3' and 5' repairs. (B) Average repair efficiencies and standard deviations (error bars) were determined from three independent data points. *p=0.003,

**p=0.007, t-Test.

Figure 6. Partial purification of an hMRE11-enriched and MMR-deficient HeLa nuclear fraction, SS1. (A) HeLa nuclear extract fractionation was performed through a two-step ammonium sulfate precipitation procedure that was essentially the same as described previously (Ramilo et al., 2002). (B) The presence of hMLH1 and hMRE11 proteins in these four fractions was analyzed by Western blot analyses performed with either rabbit anti-hMRE11 or anti-hMLH1 antibodies. Specifically, 20 μg of total protein from each fraction was used in Western blot analysis. It appeared that hMRE11 co-fractionated with hMLH1 in FI, FII, and SS1 preparations; and neither hMRE11 nor hMLH1 was contained in fraction SS2. It has been demonstrated that fractions FII, SS1, and SS2 were all required for a functional reconstitution of MMR activity *in vitro* (Ramilo et al., 2002). These results tend to support the existence of an *in vivo* association between hMLH1 and hMRE11 in human cells.

Figure 7. Interaction domain mapping for hMLH1 and hMRE11. (A) The 756-amino acid full-length hMLH1 and a series of deletion mutants were encoded in pVPd as VP16-AD fusion proteins. The average ß-gal activity units and standard errors (SE) obtained from at least three independent data points are listed. L40 double transformant harboring full-length hMRE11-BD plasmid and an empty pVPd vector displayed a background ß-gal activity reading of 2.60 $\pm$ 0.12. (B) cDNA fragments encoding the 708-amino acid full-length hMRE11 and a series of hMRE11 truncation mutants in the form of Gal4-AD fusions were used to examine protein interactions with the full-length hMLH1 in LexA-BD fusion form. The protein interaction was ascertained in the same manner as described in (A). L40 double transformant harboring full-length hMLH1-BD plasmid and an empty pGADT7 vector displayed a background ß-gal activity

reading of 4.59 ± 0.42. The two naturally occurring hMRE11 mutations R633stop and N117S were indicated by ATLD1 and ATLD3, respectively. In both (A) and (B), the black bars indicate positive protein interactions monitored by X-gal filter assay. (C) and (D) Characterization of interacting domains by far-Western analysis. The T_7 tag was removed from the construct encoding hMRE11 aa452-634 fragment. (C) Three μg of purified T_7 tagged hMLH1 aa495-756 protein and BSA were immobilized directly on the same nitrocellulose membranes, which were then probed with either purified MRE11 aa452-634 proteins or corresponding crude preparation. The α-hMRE11 and α-T_7 antibodies were used to detect the presence of hMRE11 aa452-634 and T_7-hMLH1 aa495-756 proteins respectively. (D) A reciprocal far-Western analysis while purified hMRE11 aa452-634 protein and BSA were immobilized, and then the membranes were incubated with T_7-hMLH1 aa495-756 crude lysate or purified protein. The same antibodies as in (C) were used for detection.

Figure 8. Far-Western analysis of the effects of hMLH1 HNPCC missense mutations on the hMLH1 and hMRE11 interaction. Membranes that immobilized with purified hMRE11 aa452-634 protein and BSA were probed independently with crude lysate containing wildtype and various hMLH1 mutant proteins. Conventional Western blot analysis was carried out using α-T_7 antibody to detect the captured wildtype and mutant hMLH1 aa495-756 proteins.

Figure 9. Analysis of endogenous microsatellite markers in parental HeLa and hMRE11-knockdown HeLa cells (HeLa #14). Five microsatellite loci including two mononucleotide repeats (Bat-26, Bat-25) and three dinucleotide repeats (D2S123, D17S250 and D5S346), as recommended by NCI for the detection of MSI in colorectal cancers (Boland et al., 1998), were utilized to investigate whether hMRE11-deficiency would lead to MSI in

endogenous microsatellite markers. Specifically, a PCR-based strategy was adapted; the forward PCR primers for the amplification of microsatellite loci were labeled with 6FAM, except Bat-26 in which PET labeled forward primer was used. Approximately equal amounts of genomic DNA isolated from cultured parental HeLa cells and hMRE11-knockdown HeLa cells (HeLa #14) were used as templates to perform PCR reactions. The resulting fluorescent PCR amplification products were then analyzed with an Applied Biosystems ABI 3730 Genetic Analyzer and GeneMapper Software 3.5. GeneScan 500 LIZ Size Standard was used for the fragment analysis. The results of our fragment analysis clearly indicated that, though to different extents, there were substantial variations in all five endogenous markers as judged by the appearance and/or disappearance of certain fragments (highlighted by dashed vertical lines) and alteration of fragment distribution patterns, particularly the relative peak heights (highlighted by arrows). Consistent with the fact that HeLa cells were intrinsically aneuploid, our fragment analysis revealed the existence of 7-9 fragments for all five microsatellite loci. It appeared that only one fragment from either Bat-25 or D17S250 locus displayed significant alteration, whereas alterations involved with multiple fragments were observed for Bat-26, D2S123 and D5S346 loci. It was well established that HeLa cells were both DNA mismatch repair proficient and microsatellite stable (Umar et al., 1994; Naganuma et al., 2004). Therefore, it is conceivable that the observed differences in these five microsatellite loci between parental HeLa and hMRE11-deficient HeLa cells should reflect the role of hMRE11 in the maintenance of microsatellite stability.

Figure 1

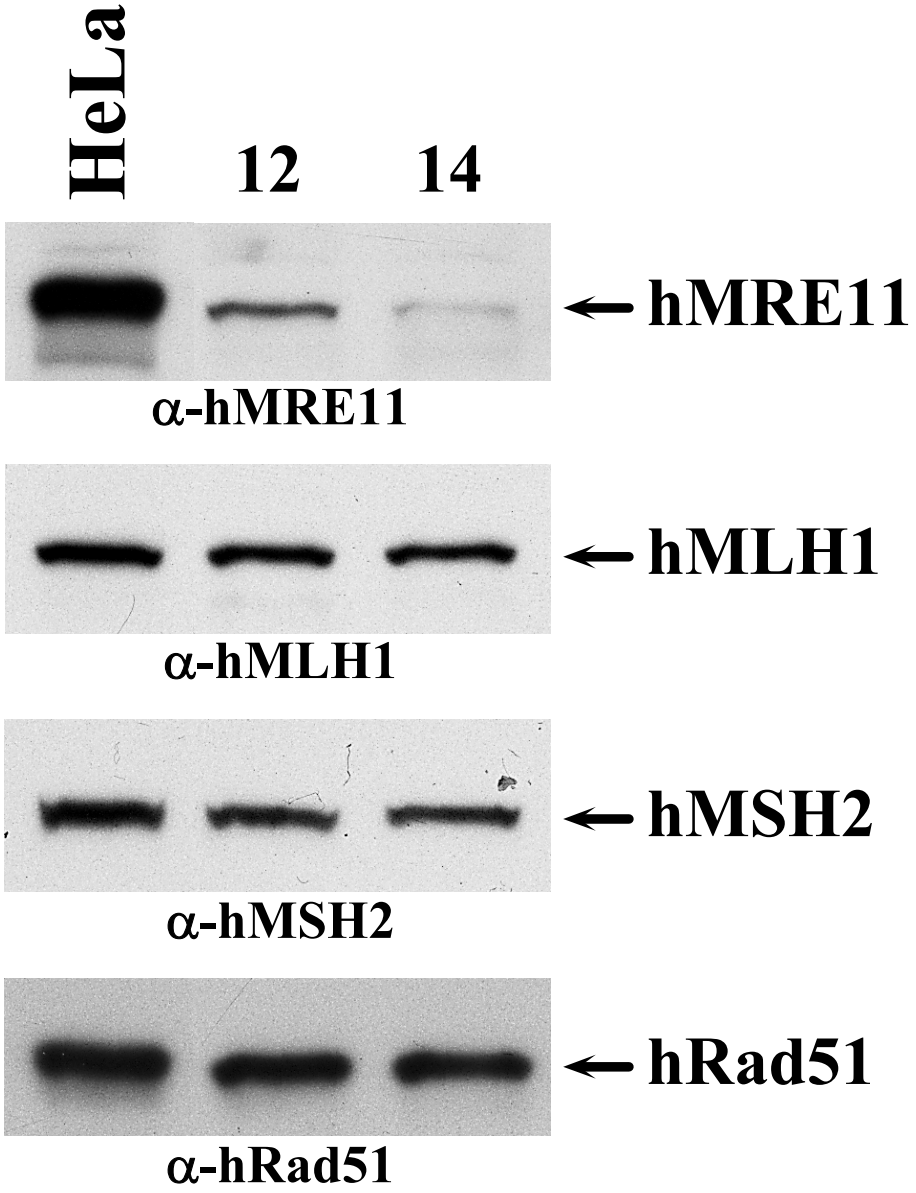
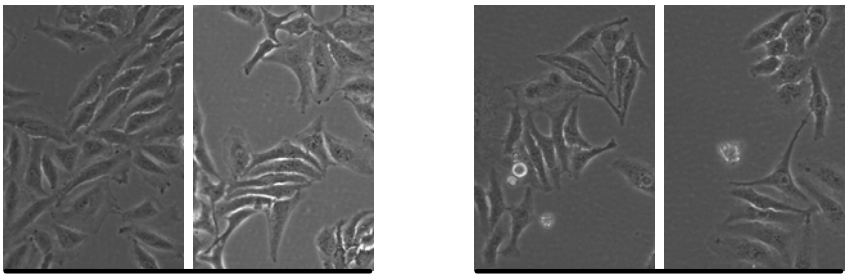

HeLa
12
14
hMRE11
α-hMRE11
hMLH1
α-hMLH1
hMSH2
α-hMSH2
hRad51
α-hRad51

Figure 2



HeLa **HeLa #14 (hMRE11 RNAi)**

Figure 3

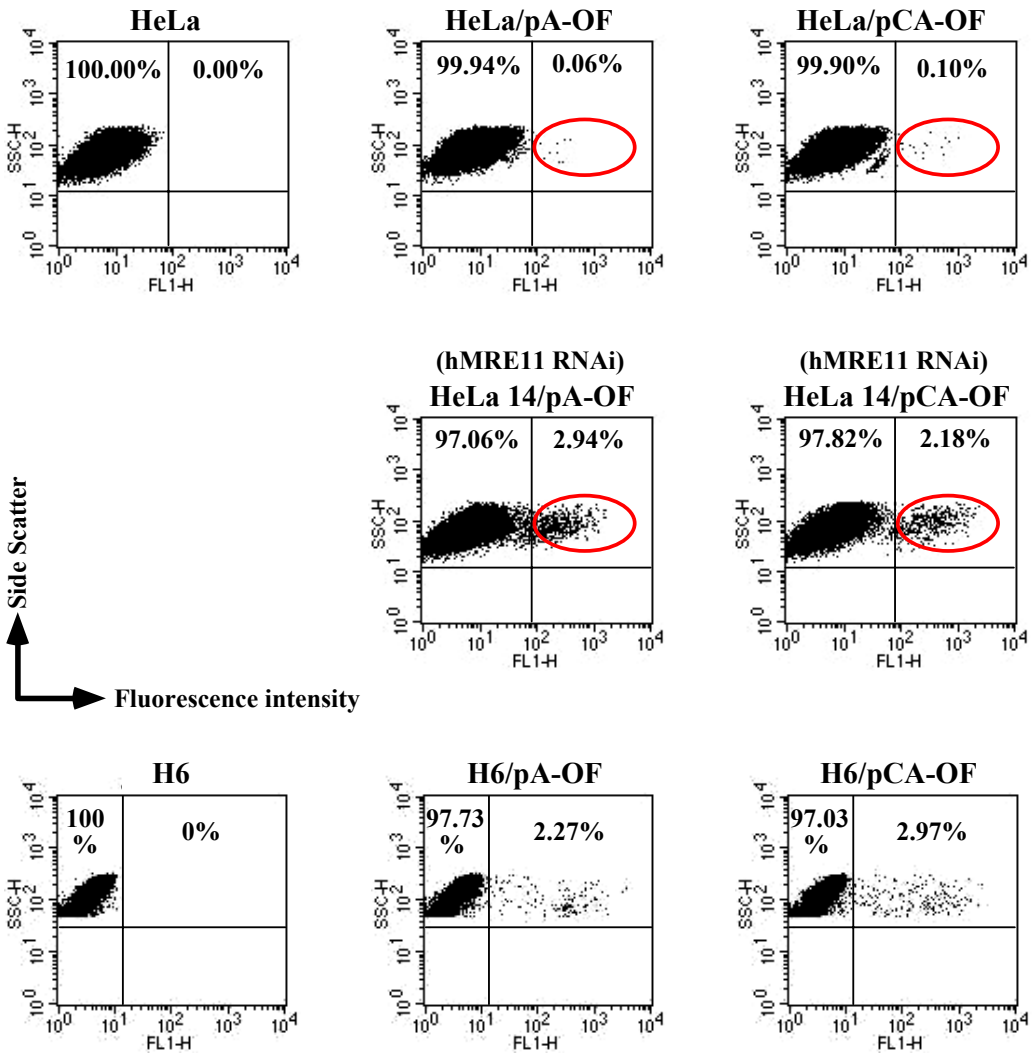

HeLa
100.00%
0.00%
HeLa/pA-OF
99.94%
0.06%
HeLa/pCA-OF
99.90%
0.10%
(hMRE11 RNAi)
HeLa 14/pA-OF
97.06%
2.94%
(hMRE11 RNAi)
HeLa 14/pCA-OF
97.82%
2.18%
Side Scatter
Fluorescence intensity
H6
100 %
0%
H6/pA-OF
97.73 %
2.27%
H6/pCA-OF
97.03 %
2.97%
SSC-H
FL1-H

Figure 4

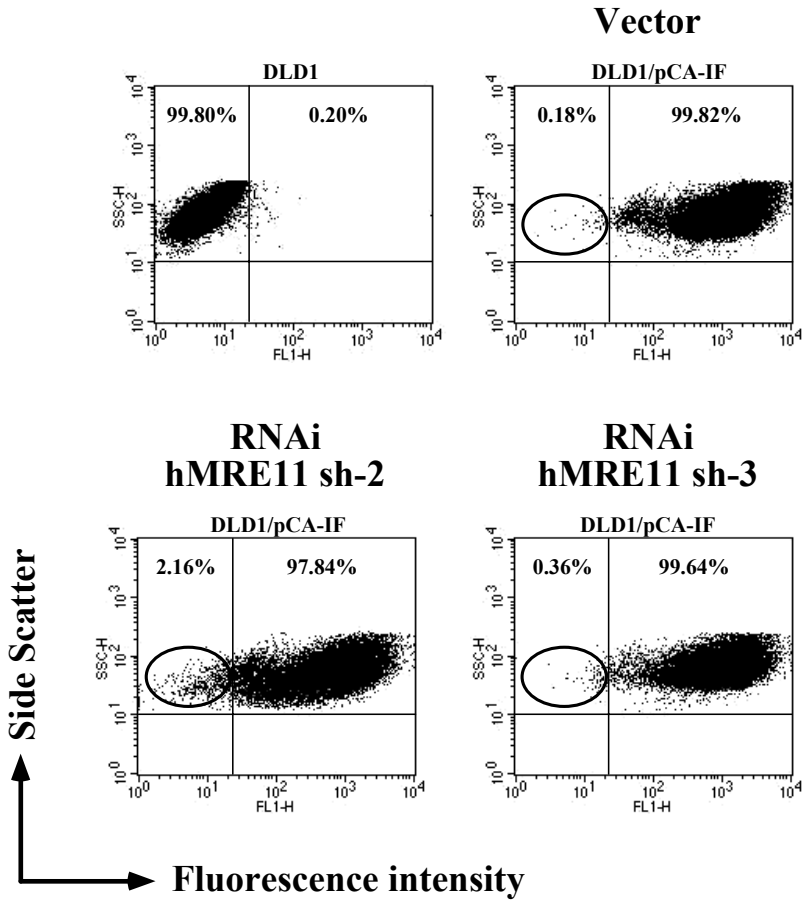

Vector
DLD1
99.80%
0.20%
DLD1/pCA-IF
0.18%
99.82%
RNAi
hMRE11 sh-2
DLD1/pCA-IF
2.16%
97.84%
RNAi
hMRE11 sh-3
DLD1/pCA-IF
0.36%
99.64%
SSC-H
FL1-H
Side Scatter
Fluorescence intensity

Figure 5

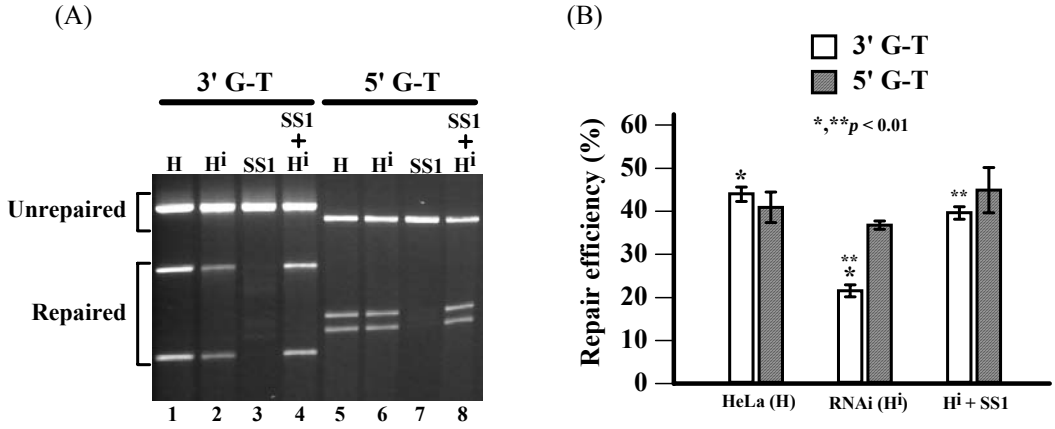

(A)
3' G-T
5' G-T
SS1 + H^i
H
H^i
SS1
Unrepaired
Repaired
1 2 3 4 5 6 7 8
(B)
3' G-T
5' G-T
*,**p < 0.01
Repair efficiency (%)
60
50
40
30
20
10
0
HeLa (H)
RNAi (H^i)
H^i + SS1

Figure 6

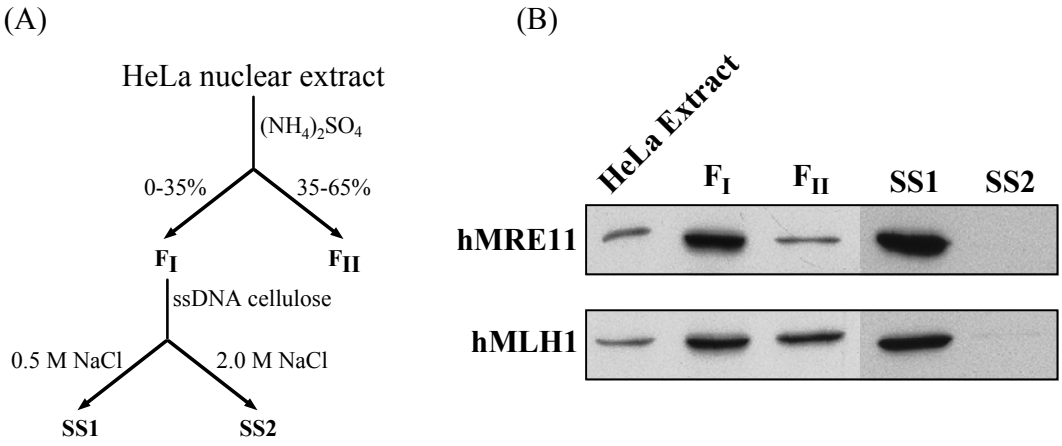

(A)
HeLa nuclear extract
$(NH_4)_2SO_4$
0-35%
35-65%
F_I
F_{II}
ssDNA cellulose
0.5 M NaCl
2.0 M NaCl
SS1
SS2
(B)
HeLa Extract
F_I
F_{II}
SS1
SS2
hMRE11
hMLH1

Figure 7

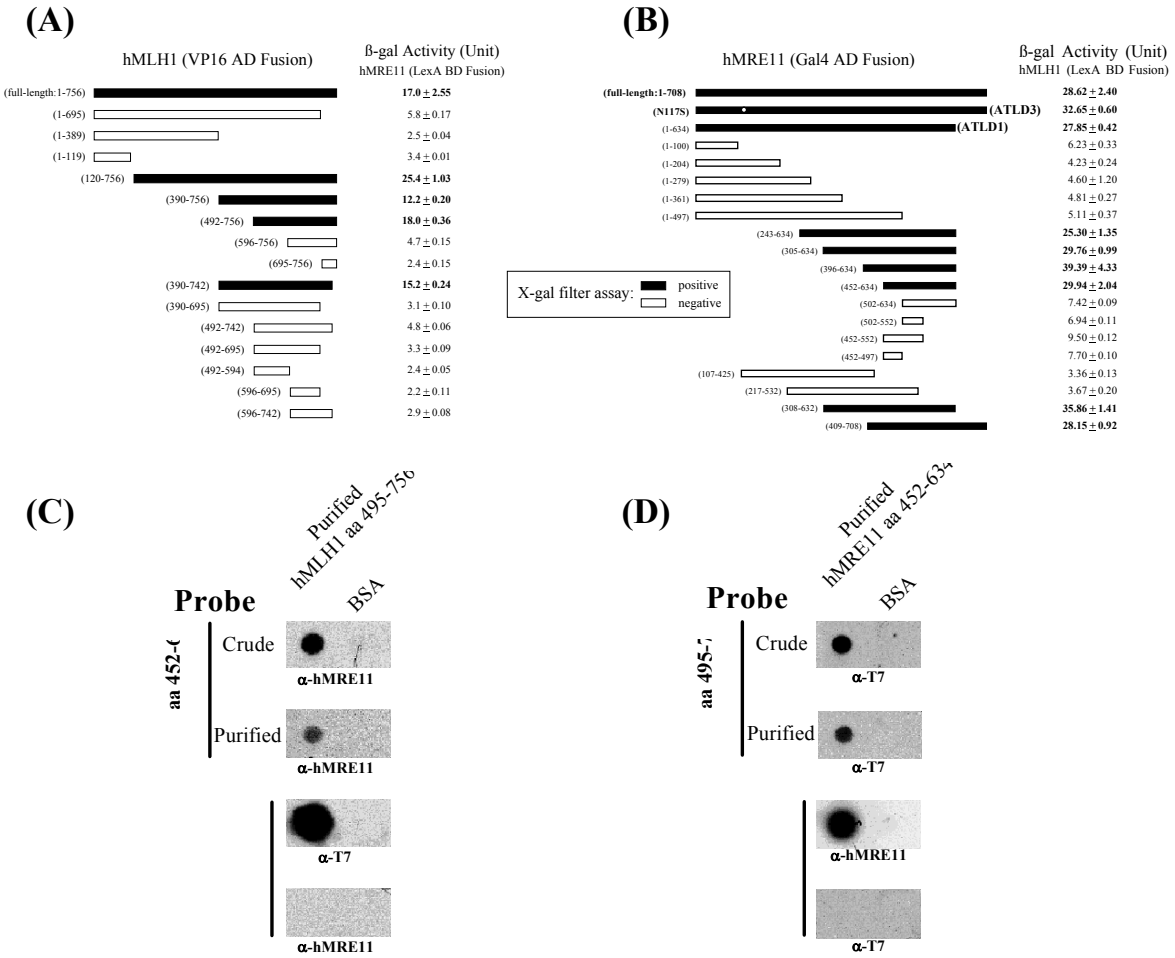

(A)
hMLH1 (VP16 AD Fusion)
ß-gal Activity (Unit)
hMRE11 (LexA BD Fusion)
(full-length:1-756) 17.0 ± 2.55
(1-695) 5.8 ± 0.17
(1-389) 2.5 ± 0.04
(1-119) 3.4 ± 0.01
(120-756) 25.4 ± 1.03
(390-756) 12.2 ± 0.20
(492-756) 18.0 ± 0.36
(596-756) 4.7 ± 0.15
(695-756) 2.4 ± 0.15
(390-742) 15.2 ± 0.24
(390-695) 3.1 ± 0.10
(492-742) 4.8 ± 0.06
(492-695) 3.3 ± 0.09
(492-594) 2.4 ± 0.05
(596-695) 2.2 ± 0.11
(596-742) 2.9 ± 0.08
(B)
hMRE11 (Gal4 AD Fusion)
ß-gal Activity (Unit)
hMLH1 (LexA BD Fusion)
(full-length:1-708) 28.62 ± 2.40
(N117S) (ATLD3) 32.65 ± 0.60
(1-634) (ATLD1) 27.85 ± 0.42
(1-100) 6.23 ± 0.33
(1-204) 4.23 ± 0.24
(1-279) 4.60 ± 1.20
(1-361) 4.81 ± 0.27
(1-497) 5.11 ± 0.37
(243-634) 25.30 ± 1.35
(305-634) 29.76 ± 0.99
(396-634) 39.39 ± 4.33
(452-634) 29.94 ± 2.04
(502-634) 7.42 ± 0.09
(502-552) 6.94 ± 0.11
(452-552) 9.50 ± 0.12
(452-497) 7.70 ± 0.10
(107-425) 3.36 ± 0.13
(217-532) 3.67 ± 0.20
(308-632) 35.86 ± 1.41
(409-708) 28.15 ± 0.92
X-gal filter assay: positive negative
(C)
Purified hMLH1 aa 495-756
BSA
Probe
Crude
Purified
α-hMRE11
α-T7
(D)
Purified hMRE11 aa 452-634
BSA
Probe
Crude
Purified
α-T7
α-hMRE11

Figure 8

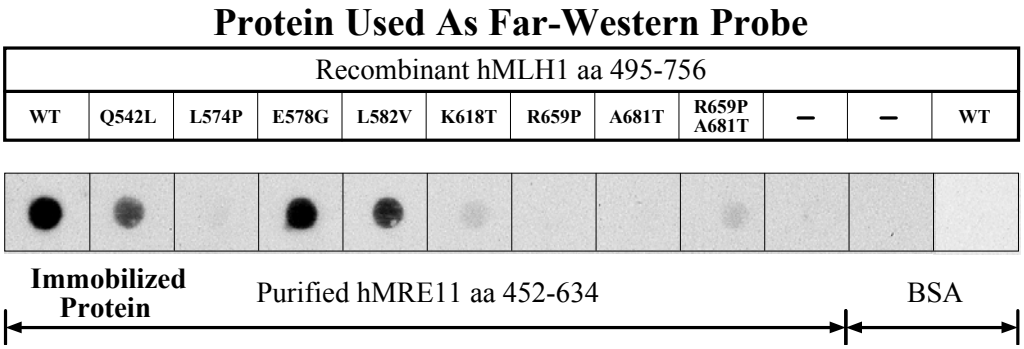
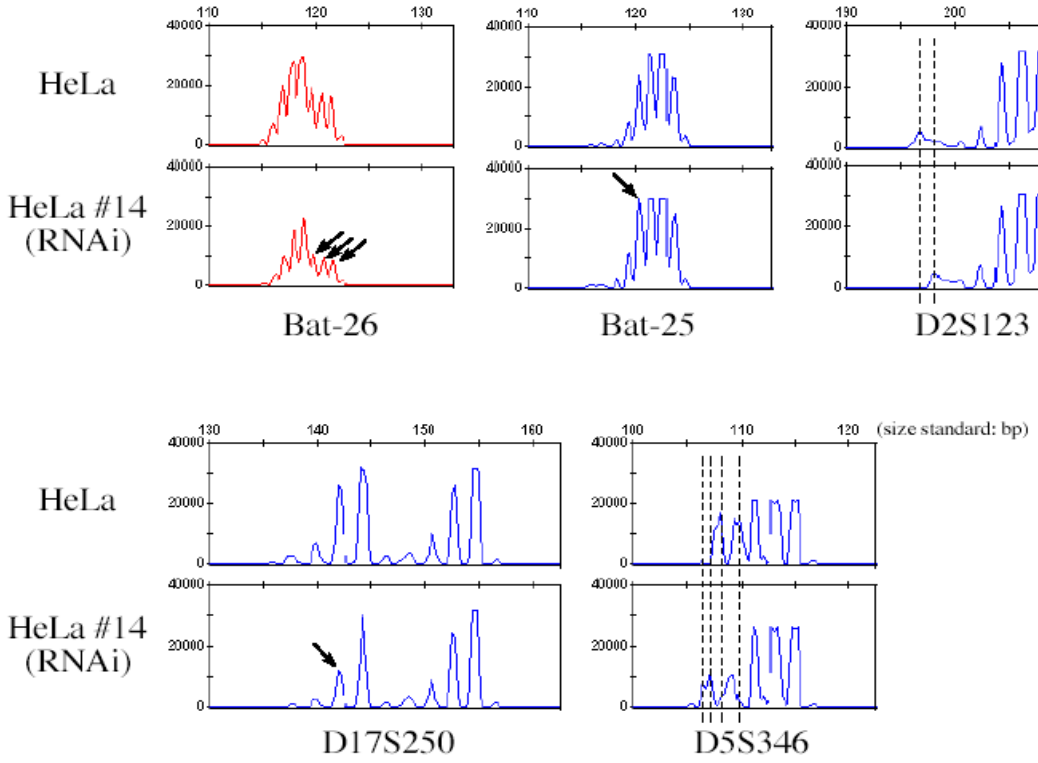

Protein Used As Far-Western Probe
Recombinant hMLH1 aa 495-756
WT
Q542L
L574P
E578G
L582V
K618T
R659P
A681T
R659P A681T
–
–
WT
Immobilized Protein
Purified hMRE11 aa 452-634
BSA

Figure 9


HeLa
HeLa #14
(RNAi)
Bat-26
Bat-25
D2S123
D17S250
D5S346
(size standard: bp)

The interaction of hMRE11 with hMLH1 is involved in DNA mismatch repair and is disrupted by hMLH1 HNPCC missense mutations

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Running title: Human hMRE11 and hMLH1

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ABSTRACT

Hereditary nonpolyposis colorectal cancer (HNPCC) is the most common form of familial colorectal cancer and germline mutations of DNA mismatch repair (MMR) genes can account for 70% of all HNPCC tumors. Among them, mutations in *hMLH1* gene are the most frequent cause of HNPCC, and one-third of them are missense mutations without clarified pathogenic explanations. Recently we demonstrated that hMRE11 is functionally involved in DNA mismatch repair, and we raised the possibility that hMRE11 might be involved in MMR through physical interaction with hMLH1. This notion is supported by our previous observation that four out of seven hMLH1 HNPCC missense mutations can preferentially disrupt their interactions with hMRE11 protein (Vo et al., 2005). In this study, we analyzed 64 HNPCC-associated hMLH1 mutations including 38 missense mutations, and it showed that 24 of them can cause significant reduction in their interactions with hMRE11, suggesting that disruption of hMLH1-hMRE11 could represent a pathogenic mechanism for hMLH1 mutations in a subset of HNPCC tumors. To further investigate the functional implications of the interaction between hMLH1 and hMRE11 *in vivo*, we have created a series of 293T cell lines expressing hMLH1, as well as two truncated forms of hMRE11 each representing a dominant negative blocker for the interaction between hMLH1 and endogenous full-length hMRE11. *In vitro* MMR assays suggested that disruption of the interaction between hMRE11 and hMLH1 could cause a significant reduction in mismatch repair, which indicated that hMRE11-hMLH1 interplay is critical for functional MMR.

INTRODUCTION

DNA mismatch repair (MMR) system is a critical mutation avoidance pathway in which the mispaired bases in DNA replication and recombination are corrected (Kolodner 1996; Modrich and Lahue, 1996). In humans, the MMR pathway requires the concerted functions of a series of MMR genes, including both MutS and MutL homologues and hMLH1, human MutL homolog 1, is a main protein factor involved. Mutations in MMR genes have been shown to increase spontaneous mutation rate and lead to cancer development. Hereditory non-polyposis colorectal cancer (HNPCC) is such a cancer syndrome that is largely attributable to MMR gene defects, which includes germ line mutations in at least 6 MMR genes, hMLH1, hMSH2, hMSH3, hMSH6, hPMS1 and hPMS2. Among them, hMLH1 and hMSH2 gene mutations can account for a majority of HNPCC cases, with an incident rate of 59.2% and 39.5%, respectively (Peltomaki and Vasen, 1997; HNPCC database http://www.insight-group.org). Many of these gene mutations lead to protein truncations which would be predicted to cause a complete loss of protein function, whereas some of the mutations only result in single amino acid substitutions that might be only partially inactivating. Presently, of the known 350 hMLH1 HNPCC mutations, one third of them are missense mutations (Han et al., 1996; Mauillon et al., 1996; Peltomaki and Vasen, 1997; Nystrom-Lahti et al., 2002). However, the mechanistic connection between hMLH1 missense mutations and their pathogenic consequences is currently elusive.

To clarify the pathogenic mechanisms of the hMLH1 HNPCC missense mutations, several *S. cerevisias* and human systems have been developed (Peltomaki 2001; Shimodaira et al., 1998; Guerrette et al., 1999; Nystrom-Lahti et al., 2002). hPMS2 is one of the central players in the MMR pathway by binding with hMLH1 following mismatch detection. In humans, it has

been found that some of the hMLH1 HNPCC mutations can disrupt the interaction between hMLH1 and hPMS2, even including mutations that reside outside the PMS2-binding domain, therefore leading to defects in DNA mismatch repair (Guerrette et al., 1999; Nystrom-Lahti et al., 2002). Another study using yeast two-hybrid analysis indicated that some hMLH1 HNPCC missense mutations can disrupt their interactions with either hPMS2 or hEXO1 (Kondo et al., 2003).

The identification of a BRCA1 associated genome surveillance complex (BASC) that included MMR proteins (hMSH2, hMSH6 and hMLH1), BLM, ATM, and the MRE11 complex (MRE11-RAD50-NBS1) seems to indicate that these protein factors might be cooperating together to function in recognition and repair of aberrant DNA structures (Wang et al., 2000). Being an essential gene in mammalian cells, *hMRE11* is involved in many functional pathways. It possesses double-stranded DNA 3'-5' exonuclease and single-stranded endonuclease activities (Moreau et al., 1999; Paull and Gellert, 1998; Trujillo et al., 1998). However, the precise roles of its nuclease activities are not fully understood. Our recent report demonstrated that human hMRE11 is directly involved in DNA mismatch repair pathway, possibly by functioning as a redundant exonuclease for hExoI. (Vo et al., 2005). Based on the previous report on the direct interaction between hMLH1 and hMRE11, and together with our recent discovery that four out of seven hMLH1 HNPCC missense mutations can preferentially disrupt their associations with hMRE11, we hypothesized that the disruption of hMLH1-hMRE11 interaction might be an alternative pathogenic mechanism for a subset of hMLH1 HNPCC mutations. (Her et al., 2002; Vo et al., 2005).

In this study we analyzed the interaction between hMRE11 and 64 hMLH1 HNPCC

variants by yeast two-hybrid assay and far-western analysis. Among those hMLH1 HNPCC variants, there are 38 missense mutations, and we also tested 12 truncating mutations caused by 7 deletions and 5 insertions, 3 nonsense mutations, 2 in-frame deletions, and 9 polymorphisms. The results indicated that 24 out of 38 hMLH1 missense mutations can disrupt their interaction with hMRE11, though to different levels. In addition, by introducing an hMRE11 dominant negative mutant together with hMLH1 protein into the 293T cells, we were also able to block the hMRE11-hMLH1 interaction in these cells, which were then submitted for *in vitro* MMR assay and excision assay. The results showed that the 3' repair and excision activity were dramatically reduced upon disruption of the hMRE11-hMLH1 interaction.

MATERIALS AND METHODS

Strain and plasmid constructions

For yeast two-hybrid analysis, cDNA encoding wild-type and various mutant hMLH1 proteins were cloned into yeast two-hybrid vector pBTM116; cDNA encoding full-length hMRE11 was fused with Gal4-AD in plasmid pGADT7. Yeast strain L40 was kindly provided by Dr. Shinichi Fukushige (Kondo et al., 2001). For Far-western analysis, cDNA fragment encoding hMRE11 aa 452-634 was subcloned into pET28a vector (Novagen Madison WI); wildtype and various HNPCC mutant hMLH1 were also cloned into pET28a vector. *E. coli* strain Top10 (Strategene) was used to propagate all plasmids.

Various hMLH1 HNPCC mutants were either obtained from Dr. Shinichi or constructed using site-directed mutagenesis method with overlapping PCR primers. To generate constructs encoding hMLH1 mutants, two rounds of PCR amplifications were performed. In the first round, two separate PCR amplifications were carried out with one PCR reaction performed with an upstream forward primer and a reverse mutagenic primer, the other PCR reaction performed by a forward mutagenic primer and a downstream reverse primer. Then the resulting two products were mixed in equal amounts for the second round of PCR amplification performed with the outer upstream and downstream primers. The final PCR products were digested with BamHI and EcoRI, gel purified, then ligated into pBTM116 or pET-28a vector. All constructs were verified by restriction digest and DNA sequencing to eliminate the possibility of other unwanted mutations.

Yeast two-hybrid analysis.

Yeast two-hybrid analysis was carried out essentially as described previously (Vo et al.,

2005). pGADT7 vector containing the full-length hMRE11, and together with the pBTMd vector containing either wildtype hMLH1 or hMLH1 HNPCC mutants, were transformed into the reporter strain L40, of which the transcription activation of *LacZ* gene could be utilized to determine protein interactions. β-gal activities were quantitatively measured by the β-gal liquid assay using O-nitrophenyl-1-thio-β-D-galactopyranoside (ONPG) as a substrate. Overnight culture of freshly transformed cells was inoculated in SD/-Leu-Trp double selection medium and refreshed in YPD medium at 30°C till the A600 reached 1.0–1.5. Then an aliquot of 1.5 ml of each culture was pelleted at 12,000 x g for 30 seconds at room temperature, washed once with Z buffer, and resuspended in 0.3 ml Z buffer. For assay, 0.1 ml of the cell suspension was disrupted with 0.1 g of glass beads using a vortex mixer, and added to 0.7 ml of Z buffer containing 1.9 µl of 2-mercaptoethanol and 0.16ml Z buffer containing 4 mg/ml ONPG. Reaction mixtures were incubated at 30 °C till a yellow color was observed. Reactions were then stopped by the addition of 400 µl of 1 M Na_2CO_3 and pelleted at 12,000 x g for 5 min. The supernatants were collected for measurement at A_{420}. The β-gal activity was calculated using the following formula: β-gal units = 1000 x A_{420} / (t x V x A_{600}); where t = time of reaction (min), and V = volume of culture used in the assay (ml).

Production and purification of recombinant proteins

cDNA fragment encoding hMRE11 aa 452-634 was cloned into pET-28a vector (Novagen, San Diego, CA, USA) and transfected into E. *coli* strain BL21 (DE3) RIL (Strategene, La Jolla, CA, USA). Cells were cultured at 37 °C till A_{600} reached 0.4-0.5 and induced with final IPTG concentration of 0.25 to 0.5 mM at 16°C, 25°C, or 37°C. Cells were collected after 9-12 hours culture, and lysed via sonication. Clear lysate was then collected for purification using

Tylon column chromatography (Clontech). Selected fraction containing recombinant protein was dialyzed against PBS to remove immidizole before subsequent application.

Far-Western blot analysis

Approximately 5μg of purified proteins were loaded onto nitrocellulose membrane by Bio-dot Microfiltration Apparatus (Bio-Rad, Hercules, CA, USA). Then membrane was blocked with 10% milk-TBS-T (TBS containing 10%powdered milk and 0.3% tween20) for 45min, and was followed by incubation with crude lysate of interacting partner protein (300ul) in 0.25% milk-TBS-T (TBS containing 0.25% powdered milk, 0.3% tween20, 1mMDTT, and 1mM PMSF) for 1 hour. Membrane was then washed 5 times for 10 min each with 0.25% milk-TBS-T (TBS containing 0.25% powdered milk and 0.3% tween20). Finally, conventional western blot analysis was carried out with appropriate antibodies. Relative interaction strength between hMLH1 variant and hMRE11 were determined by densitometry quantification.

Western blot analysis and antibodies

Proteins separated on 7.5-20% SDS-PAGE were transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) for immunoblotting analysis performed with the ECL Western blotting system (Amersham Pharmacia Biotech, Piscataway, NJ). The primary antibodies used in this study included: α-hMLH1 monoclonal (1:100 dilution, EMD Biosciences, Inc., San Diego, CA, USA), α-hMLH1 polyclonal (1:500 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) α-FLAG monoclonal (1:2000 dilution, Sigma-Aldrich Co., St. Louis, MO, USA), α-hMRE11 polyclonal (Novus Biologicals Inc., Littleton, CO, USA), α-T7 monoclonal (1:10000 dilution, Novagen, Madison, WI, USA).

Cell lines and mammalian transfection

The cDNA fragments encoding hMRE11 amino acids 1 to 634 ($hMRE11_{1-634}$) or 452 to 634 ($hMRE11_{452-634}$) were cloned into pFLAG-puro to generate corresponding mammalian expression constructs pFLAG-puro/hMRE11(1-634) or pFLAG-puro/hMRE11(452-634). cDNA encoding hMLH1 was cloned into pcDNA6 (Invitrogen) to creat a mammalian expression construct pcDNA6/hMLH1. 293T cells were first transfected with pFLAG-puro/hMRE11(1-634) or pFLAG-puro/hMRE11(452-634) separately using the calcium phosphate method. Cells were maintained in DMEM containing 10% FBS (Biomeda) and stable cell lines were selected by using 1μg/ml puromycin (A.G. Scienfitic, San Diego, CA). The positive clones were then transfected with pcDNA6(blasticidin)/hMLH1. Stable cell lines were selected by using 10 μg/ml Blasticidin. After 3 weeks, ~20 colonies were isolated and their extracts were screened by western blotting using anti-hMRE11 and anti-hMLH1 antibodies.

***In vitro* MMR assay**

Nuclear extracts were prepared as described previously (Holmes et al., 1990). DNA heteroduplexes used in this study contained an A-C mismatch and a strand break 3' (3' A-C) to the mismatch (Li and Modrich, 1995). *In vitro* mismatch repair assays were performed as described previously (Holmes et al., 1990; Ramilo et al., 2002) in a 15-μl reaction mixture which contains 100ng of heteroduplex DNA, 50 μg of nuclear extract, 10 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 1.5 mM ATP, and 0.1 mM each deoxynucleoside triphosphate. After 15 minutes' incubation at 37°C, DNA samples were recovered by phenol extraction and ethanol precipitation, and were digested with the restriction enzymes *Bse*RI and *Xho*I. Reaction products were analyzed by electrophoresis using 1% agarose gel and visualized by UV illumination in the presence of ethidium bromide.

Mismatch-provoked excision assay

This assay was carried out essentially as described previously (Holmes et al., 1990; Guo et al., 2004). The reaction is essentially identical to the mismatch repair assay except that no exogenous dNTPs is added. The 15-µl reaction mixture contains 100ng of heteroduplex DNA, 50 µg of nuclear extract, 10 mM Tris-HCl (PH 7.6), 5 mM $MgCl_2$, 110 mM KCl, 1.5 mM ATP, and no exogenous dNTP were added to the mixture to minimize DNA resynthesis. After incubation at 37°C for several times, protease K digestion was added to terminate reactions and DNA samples were recovered. To assess the amount of excision beyond the mismatch, the 3' A-C substrate was digested with the restriction enzyme *BseRI* and *HindIII*. The products were electrophoresed on 1% agarose gels, and DNA bands were visualized by UV illumination in the presence of ethidium bromide.

RESULTS

It is known that MMR deficiency can account for about 70% of all HNPCC cancer development, and more than half of all pathogenic HNPCC mutations are from hMLH1 gene alone. Although most of these hMLH1 HNPCC mutations result in premature termination of protein during translation, 30% of them only cause single amino acid substitutions, of which the mechanistic basis for the pathogenic effects is largely unknown. Based on our previous study that four out of seven hMLH1 HNPCC missense mutations can preferentially disrupt their interactions with hMRE11, we here attempted to investigate more hMLH1 HNPCC variants on their interactions with hMRE11 to demonstrate that the disruption of hMLH1-hMRE11 interplay could represent an alternative molecular basis underlying the pathogenic effects of hMLH1 in some HNPCC tumors (Vo et al., 2005). We then further linked this analysis with a functional study performed by direct blocking of the hMRE11-hMLH1 interaction, and the resulting defects in the 3' MMR activity suggested that the interaction between hMRE11 and hMLH1 plays a critical role in DNA mismatch repair.

In this study we investigated the effects of 64 hMLH1 HNPCC mutations on their interactions with hMRE11 proteins (Table 1), by yeast two-hybrid and far-western analyses. All the mutations are scattered along all 19 exons of hMLH1, which included 38 missense mutations, 3 nonsense mutations, 12 frame-shift mutations, 2 in-frame deletion mutations (Figure 1; Table 1). Nine polymorphisms causing single amino acid substitutions, I32V, V213M/ I219V, R217C, I219V, I219L, R265H, S406N, H718, and L729V, were also included in our experiment as controls (Table 1; Kondo et al., 2003). For each hMLH1 variant, the corresponding exon number, nucleotide change, and interaction strength with PMS2 and/or ExoI were also indicated

in Table 1.

First, the relative interaction strength of hMRE11 with this series of hMLH1 variants was tested by yeast two-hybrid analysis. The cDNA sequences encoding wildtype hMLH1, or hMLH1 containing different HNPCC mutations were cloned into the LexA-BD two-hybrid vector, pBTM116, and were then cotransformed together with hMRE11/pGADT7-AD into yeast strain L40. As shown in Figure 2, the two-hybrid assay between hMRE11 and hMLH1 variants containing nonsense or frameshift mutations showed very low levels of β-gal activities, comparing with that obtained from the wildtype hMLH1. For the missense mutations, 26 out of 38 (68.4%) also display very low levels of β-gal activities, indicating that the interactions between hMRE11 and hMLH1 HNPCC missens mutations were almost completely disrupted. Moreover, these 26 missense mutations scattering along the full-length hMLH1 protein are mostly mutations that caused a polarity change in the hMLH1 protein. This suggested that the polarity change in hMLH1 HNPCC missense mutations could result in changes in its ability of hMRE11 binding. In contrast, eight out of nine hMLH1 polymorphisms showed intermediate levels of β-gal activities in comparison with wildtype hMLH1 (Figure 2).

To validate the results obtained from the yeast two-hybrid analysis, we have also performed far-western assay to examine the effects of the same set of hMLH1 HNPCC variants on the interaction with hMRE11. Purified protein encoding hMRE11 aa 452-634 (representing the hMLH1-interacting domain, Vo et al., 2005) was loaded onto nitrocellulose membrane, and then the membranes were incubated with bacterial lysates containing each of the variant hMLH1 mutant proteins. Wildtype hMLH1 was used as a positive control in this experiment, whereas BSA was used as a negative control. Conventional western blot analysis was carried out using

anti-T7 and anti-hMLH1 antibodies to detect the captured wild-type and mutant hMLH1 proteins. The result of the far-western analysis is highly consistent with that of the yeast two-hybrid analysis (Figure 2). Ten representative hMLH1 HNPCC mutations were shown in Figure 3. In order to verify that the differential interaction levels between hMRE11 and hMLH1 variants in the far-western analysis were not due to different hMLH1 expression levels, we also tested the expression of all 64 hMLH1 mutant proteins, using western-blot analysis. Anti-T7 and anti-hMLH1 antibody were used to detect wild-type and various mutant hMLH1 proteins, respectively. All proteins have relatively equivalent expression levels (Figure 4). This indicated that the different effects of these hMLH1 mutations on protein interactions with hMRE11 are most likely due to protein conformational changes caused by the amino acid sequence variations.

Taken together, our study from the yeast two-hybrid and far-western analyses for the first time indicated that most of the hMLH1 HNPCC missense mutations could affect their interactions with hMRE11 protein. Therefore, disruption of hMRE11-hMLH1 interaction might represent a common pathogenic molecular mechanism associated with some hMLH1 HNPCC mutations. This finding seems to indicate that the functional interplay between hMRE11 and hMLH1 plays a critical role during the MMR process in human cells, presumably by recruiting hMRE11 into the MMR pathway which would then function during the excision phase of MMR, with it 3'-5' exonuclease activity. To further clarify the functional involvement of hMRE11-hMLH1 interplay in DNA MMR, we next attempted to perform a functional study by directly blocking the hMRE11-hMLH1 interaction *in vivo*, and the resulting effects in DNA mismatch repair activities of the cells were investigated by an *in vitro* MMR assay.

We assumed that when an hMRE11 fragment ($hMRE11_{452-634}$) containing the hMLH1-interacting domain was introduced into the cells which contain both hMLH1 and hMRE11 proteins, it would act as a dominant negative blocker and interrupt the interaction between hMLH1 and full-length hMRE11, which will negatively affect the MMR activity. Besides the "blocker" hMRE11 fragment ($hMRE11_{452-634}$), another fragment hMRE11 ($hMRE11_{1-634}$) was also included in our study; the latter encoded hMRE11 amino acids 1 to 634, which contained the N-terminal of hMRE11 protein all the way through the hMLH1-interacting domain, including the entire exonuclease domain, and it mimics the hMRE11 mutation in ATLD1 cells (Stewart et al., 1999). The cells used here were human embryonic kidney 293T cells; they were MMR deficient due to hMLH1 gene promoter hypermethylation (Trojan et al., 2002). Therefore, to study the hMRE11-hMLH1 interplay in MMR pathway, we complemented these cells with hMLH1 besides the two truncated hMRE11 fragments. First, the mammalian expression constructs pFLAG-puro/ hMRE11 (1-634) and pFLAG-puro/ hMRE11 (452-634) were used to transfect 293T cells separately. In order to complement the hMLH1 expression in these cells, positive clones expressing the two truncated hMRE11 were then transfected with pcDNA6/hMLH1. Parental 293T cells were also transfected with pcDNA6/hMLH1 in a parallel experiment. Single clones were picked out and tested for protein expression. The established stable 293T transfectant expressing hMLH1 was designated as 293T/hMLH1, and the stable transfectants expressing hMLH1 as well as either of the two truncated hMRE11 were designated as 293T $hMRE11_{1-634}$/hMLH1 and 293T $hMRE11_{452-634}$/hMLH1, respectively. The schematic depiction of the full-length and the two truncated hMRE11 proteins were shown in Figure 5A. The western blot results of the protein expressions in the 4 generated cell lines, including hMLH1,

endogenous full-length hMRE11, and the two truncated hMRE11 (Figure 5B).

To test whether the DNA MMR activities were affected upon disruption of the hMLH1-hMRE11 interaction, nuclear extracts of parental 293T cells, 293T/hMLH1 cells, 293T $hMRE11_{1-634}$/hMLH1 cells, and 293T $hMRE11_{452-634}$/hMLH1 cells were subjected for a functional *in vitro* MMR assay. Nuclear extracts of HeLa cells were also included as a MMR proficient control. Previous finding indicated that hMRE11 deficiency in HeLa cells only preferentially affects the 3' repair, while the 5' repair was not significantly affected (Vo et al., 2005). Therefore in our study, we carried out 3' repair assay for those nuclear extracts, with a heteroduplex substrate containing a single A-C mismatch and a strand break 3' to the mismatch (Li and Modrich, 1995).

The 3' repair results were shown in Figure 6. Consistent with previous observations, HeLa cells are MMR proficient cells and their repair efficiency for the 3' A-C substrate is 27% (Lane 1, Figure 6); 293T cells were MMR deficient with a 3' directed repair efficiency of only 8 % (Lane 2, Figure 6), which is consistent with the fact that the silencing of hMLH1 expression in 293T cells is not complete (Esteller 2002). When transfected with hMLH1, designated as 293T/hMLH1, the repair activity was restored to the level of the HeLa cells, around 25% (Lane 3, Figure 6). As expected, the 3' repair activity was slightly enhanced when the $hMRE11_{1-634}$ fragment was expressed, which contains the entire functional nuclease domain of hMRE11 and hMLH1 binding region (Lane 4, Figure 6). This is well coherent with the view that the 3'-5' exonuclease activity of hMRE11 could be directly involved in the 3' directed DNA mismatch repair. Therefore, given the fact that $hMRE11_{1-634}$ contained the hMLH1-interacting domain and it was capable of mediating interaction with hMLH1, the expression of the entire functional

nuclease domain in $hMRE11_{1-634}$ could slightly contribute to the repair activity. However, the repair activity of the 3' A-C substrate was significantly reduced when $hMRE11_{452-634}$ fragment was expressed which represents the sole hMLH1-interacting domain (Lane 5, Figure 6). These results suggested that fragment $hMRE11_{452-634}$ could efficiently compete with the endogenous full-length hMRE11 to interact with hMLH1, leading to reduced MMR activity. This set of data implicated that the disruption of the hMRE11-hMLH1 interaction was correlated with the reduction of MMR activities.

To further investigate that the role of hMRE11 in the process of MMR, the nuclear extracts of the same set of cells were tested in an excision assay. The 3' nicked circular heteroduplex substrate was largely identical to the 3' A-C mismatch substrate, except that the exicision substrate contains a restriction endonuclease site (*Hind*III) immediately 5' to the A-C mismatch. Thus under conditions of no exogenous dNTPs, DNA resynthesis will be inhibited; the 3' excision in the late step of MMR could convert the *Hind*III restriction sequence from double stranded DNA to single stranded DNA, making it resistant to *Hind*III digestion. Therefore, the 3' excision activities of the cell extracts could be assessed.

The pattern of the excision assay results was quite consistent with the MMR assay results. For HeLa cells which contain both hMRE11 and hMLH1 proteins, they are proficient in the 3' excision (Lane 6, Figure 6). The parental 293T cells were deficient in 3' excision (Lane 7, Figure 6). It is known that 293T cells are hMLH1 deficient, and this would presumably affect the recruitment of hMRE11 protein into the MMR pathway, thus inhibiting its role in the 3' excision step of MMR. When hMLH1 was expressed in 293T cells with, as shown in Lane 8 (Figure 6), the excision activity was increased to about 50% of that of the HeLa cells, which

indicated that the complemented hMLH1 could efficiently recruited hMRE11 to the MMR pathway. As expected, when $hMRE11_{1-634}$ fragment was expressed, its functional nuclease domain could further enhance the 3' excision activity when compared with the 293T/hMLH1 cells (Lane 9, Figure 6). However, the expression of $hMRE11_{452-634}$ fragment could reduce the excision activity to 70% in comparison with the 293T/hMLH1 cells as shown in Lane 10 (Figure 6), indicating that the blocking of the hMRE11-hMLH1 interaction by this fragment hMRE11 dominant negative mutant could have a negative effect on 3' excision. Taken together, the excision assay indicated that the hMRE11 is functionally involved in the 3' excision of the mismatch repair substrate. Over-expression of the hMLH1-interacting domain containing $MRE11_{452-634}$ fragment in cells significantly affected the MMR process, conceivably the observed effects could be attributable to altered hMRE11-hMLH1 interplay particularly. The recruitment of hMRE11 to the MMR pathway was expected to be affected. This in turn suggests that the interaction between hMRE11 and hMLH1 is critical for functional MMR.

DISCUSSION

In the present study, we showed that the hMRE11 is functionally involved in excision of the mismatched DNA in MMR process, and the interplay between hMRE11 and hMLH1 might be responsible for recruiting hMRE11 to the MMR pathway. Analysis of the interactions between hMRE11 and various hMLH1 HNPCC mutants indicated that this interaction was greatly reduced in about 60% of the missense mutations studied, raising the possibility that the disruption of the hMRE11-hMLH1 interaction might explain certain pathogenic effects of these hMLH1 mutations in the development of HNPCC.

hMRE11 is well known as a component of the MRE11/RAD50/NBS1 (MRN) complex, which plays critical roles in repairing of DNA double strand breaks (DSBs), including homologous recombination, non-homologous end joining and so on. The MRN complex is an ATP-stimulated nuclease, and it is believed that this complex satisfied both structural and enzymatic activities that are required for processing of DSBs, with the structural activity capturing and aligning broken DNA ends, whereas the enzymatic activity processing the ends for subsequent repairs. The hMRE11 protein possessing both double-stranded DNA 3'-5' exonuclease and single-stranded endonuclease activity (Moreau et al., 1999; Paull and Gellert, 1998; Trujillo et al., 1998), but it is interesting to note that the nuclease activities associated with hMRE11 are not involved in repair of the DNA DSBs mediated by homologous recombination (Bressan et al., 1999), suggesting the possibility that its nuclease activities might be playing roles in other cellular processes such as DNA mismatch repair (MMR).

The MMR pathway includes detection of the mismatched DNA, excision of the mismatched fragment, resynthesis of new strand and ligation. In human cells, the mismatched

bases generated during DNA metabolic process are first detected by either hMSH2/hMSH6 or hMSH2/hMSH3, and then the hMLH1-hPMS2 heterodimer is recruited to the complex in an ATP dependent manner. However, the nuclease activities associated with the following excision step of MMR are largely elusive. Although currently the 5'-3' exonuclease I, hExoI is the only identified exonuclease in mammalian MMR (Tishkoff et al., 1998; Dzantiev et al., 2004), it is interesting to find that the MMR activity in mouse is not completely lost upon inactivation of hExoI (Wei et al., 2003). In addition, compared with Msh2- or Mlh1-knockout mice, ExoI-knockout mice are less likely to develop tumor, indicating that there might be functionally redundant exonucleases existed in mammalian MMR process (Wei et al., 2003). Our findings of previous and present studies implicate a likely involvement of hMRE11 in the excision step of MMR process, playing a redundant role with ExoI, and presumably through interaction with hMLH1 protein. Since there is no known enzymatic activity associated with hMLH1, it is believed that this protein probably functions by recruiting other DNA repair proteins to the MMR pathway (Mitchell et al., 2002), and hMRE11 might be one of the recruited partners. However, whether hMRE11 protein itself or the MRN complex is actually involved in the MMR process is currently unknown, since the knowndown of hMRE11 expression seemed to affect the stability of the MRN complex in HeLa cells (Zhu and Her, unpublished data).

Besides being an essential mediator in MMR pathway, hMLH1 was also found to be required for activation of c-Abl and p73 by cisplatin (Gong et al., 1999). This finding supported a role for MLH1 in DNA damage signaling. Considering that Mre11/Rad50/NBS1 complex is also a central player in the detection and processing of DNA damage (especially DSBs), it is quite plausible that the interaction between hMRE11 and hMLH1 could be also functional in the

DNA damage recognition and signaling pathway.

Previous studies with hMRE11 showed that the intronic poly$(T)_{11}$ repeat of *hMRE11* is frequently mutated and results in impaired expression of hMRE11 protein in MMR deficient cell lines or cancers (Giannini et al., 2002). The examination of 15 colorectal cancers indicated that 6 out of 15 samples have mutated MMR genes, either in MSH2 or MLH1; whereas 9 other samples have no mutations in known MMR genes. Intriguingly, mutations at the (T)11 repeat in hMRE11 gene were observed in 93% (14 out of 15) of the MMR-deficient colorectal cancers, indicating that hMRE11 poly(T)11 might also be a causative factor rather than merely a target (Giannini et al., 2002). Hence, the inactivation of hMRE11 might play certain roles in tumerigenesis of colorectal cancers. Our study further comfirmed the possibility that the hMRE11 might be functioning in the MMR pathway, thus inactivation of hMRE11 expression could in turn affect MMR and contribute to the development of cancers, especially colorectal cancer.

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Table 1 Summary of 64 hMLH1 variants

hMLH1 HNPCC variants	Exon	Nucleotide change	SIFT predication	HNPCC association	Interaction		MMR defects	Reference
					hEXO1	hPMS2		
I25F[a]	1	A→T	+	yes	ND	ND	yes	Weber et al., 1999
125T	1	T→C	+	yes	ND	ND		Norbury et al. (unpublished)
P28L[a]	1	C→T	+	yes	-	-		Wehner et al., 1997
I32V	1	A→G			+ +	+ +		Kondo et al., 2003
M35R	1	T→G	+	yes	-	-		Tannergard., 1995
S44F	2	C→T	+	yes	-	-	yes	Bronner et al., 1994
Q62K	2	C→A		yes	ND	ND	yes	Wijnen et al., 1997
N64S[a]	2	A→G	+	yes	ND	ND		Wijnen et al., 1997
G67R	2	G→A	+	yes	-	-	yes	Tannergard et al., 1995
I68N	2	T→A	+	yes	-	-	yes	Tannergard et al., 1995
C77Y[a]	3	G→A			-	-	yes	Muller-Koch et al., 2001
E102K	4	G→A	+	yes	ND	ND		Dela Chapelle et al., 2001 (unpublished)
I107R	4	T→G	+	yes	-	-	yes	Nystrom-Lahti et al., 1996
T117M	4	C→T	+	yes	-	-	yes	Liu et al., 1996
T117R	4	C→G	+	yes	-	-		Buerstedde et al., 1995
A128P[a]	5	G→C	+	yes	-	-		Pensotti et al., 1997
V185G	7	T→G	+	yes	-	-	yes	Kohonen et al., 1996
E199Q	7	G→C			ND	ND		Wahlberg et al., 1999
V213M[b]	8	G→A		yes	+ +	+ +		Fidalgo et al., 2000
R217C[b]	8	C→T		yes	+ +	+ +		Miyaki et al., 1995
I219V[b]	8	A→G			+ +	+ +		Liu et al., 1995
I219L[b]	8	A→C			+ +	+ +		Kondo et al 2003
G244D	9	G→A		yes	-	-	yes	Pensotti et al., 1997
I262 del	9	Del ATC	+	yes	ND	ND		Hunter et al., 1998
R265C	10	C→T	+	yes	ND	ND		Wahlberg et al., 1999
R265H[b]	10	G→A	+		+ +	+ +	yes	Viel et al., 1997
V326A[a]	11	T→C	+	yes	+ +	+ +		Liu et al., 1996
S406N[b]	12	G→A		yes	+ +	+ +		Genuardi et al., 1999
A492T	13	G→A		yes	+ +	+ +		Moslein et al., 1996
497ins1bp	13	Ins C		yes	ND	ND		Moslein et al., 1996
497del1bp	13	Del G			ND	ND		Weber and Rodriguezbigas 1996 (unpublished)
V506A[a]	13	T→C		yes	+ +	+		Liu et al., 1996
519ins1bp	13	Ins T		yes	ND	ND		Kolodner et al., 1995
E523D[a]	14	G→T			ND	ND		Myriad et al (unpublished)
541del1bp	14	Del C		yes	ND	ND		Friedl et al., 1996

								(unpublished)
Q542L	14	A→T	+	yes	+ +	+		Han et al., 1995
L549P[a]	14	T→C	+	yes	ND	ND		Han et al., 1996
N551T	14		+	yes	ND	ND		Wang et al., 1997
L574P[a]	15	T→C	+	yes	ND	ND		Han et al., 1995
E578G	16	A→G		yes	+ +	+		Tannergard et al., 1995
L582V[a]	16	C→G	+	yes	+ +	+ +		Han et al., 1995
586ins1bp	16	Ins C			ND	ND		Han et al., 1995
590del4bp	16	Del TAGA		yes	ND	ND		Moslein et al., 1996
593del2bp	16	Del AG		yes	ND	ND		Wijnen et al., 1996
K616del	16	Del AAG		yes	ND	ND		Miyaki et al., 1995
K618A	16	A→G		yes	ND	ND		Mauillon et al., 1996
K618T	16	A→C		yes	+ +	-		Han et al., 1995
K618del	16	Del AAG			ND	ND		Myriad et al (unpublished)
633del	17	Del G			ND	ND		Buerstedde et al., 1995
								Deffen baugh et al
L653R	17	T→G	+		ND	ND		(unpublished)
P654L[a]	17	C→T	+	yes	ND	ND		Norbury et al (unpublished)
R659P	17	G→C	+	yes	-	-	yes	Nystrom-Lahti et al., 1996
R659X	17	C→T			ND	ND		Nystrom-Lahti et al., 1996
								Bressac –de Pailllerets
E663G	17	A→G		yes	ND	ND		(unpublished)
L676R	18	T→G	+		ND	ND		Myriad et al (unpublished)
A681T	18	G→A	+	yes	+	-		Froggatt et al., 1996
W712X	19	G→A		yes	ND	ND		Liu et al., 1996
W714X	19	G→A		yes	ND	ND		Hunter et al., 1998
V716M[b]	19	G→A		yes	+ +	+ +		Hunter et al., 1998
								Bressac de paillerets
H718Y[b]	19	C→T	+	yes	+ +	+ +		(unpublished)
726del4bp	19	Del CACA		yes	ND	ND		Papadopoulos et al., 1994
L729V	19	C→G			+ +	+ +		Kondo et al., 2003
733ins4bp	19	Ins AAAC		yes	ND	ND		Risinger et al., 1996
756ins4bp	19	Ins TGTT		yes	ND	ND		Papadopoulos et al., 1994

Note. All information was obtained from www.expasy.org and www.insight-group.org

MLH1 HNPCC variants: [a]: no change in polarity; [b]: polymorphism.
SIFT prediction: the "+" means the amino acid change is affecting protein function, based on the prediction from the program SIFT (Sorting Intolerant From Tolerant). Information was obtained from http://blocks.fhcrc.org/sift/SIFT.html (Ng 2003).
Interaction: the interaction strength with hEXOI or/and hPMS2. All information was obtained from the reference. "++": normal interaction; "+": reduced interaction; "-": no interaction; "ND": not done.
Reference: reference that first identified the particular hMLH1 HNPCC variant.

FIGURE LEGEND

Figure 1. hMLH1 protein and defined functional domains. Three functional domains of hMLH1, ATPase domain, central region and C-terminal interaction domain are indicated. The short vertical lines within the "rectangle" represent 38 hMLH1 HNPCC missense mutations included in our study; the first row of diamonds below the rectangle represent 15 hMLH1 HNPCC truncation mutations and the lower two rows of diamonds represent hMLH1 HNPCC in-frame deletion mutations and polymorphisms, respectively. The bars above the rectangle represent the hMLH1's relative interaction domains with hPMS2, hMLH3, hPMS1, hMRE11 and BLM.

Figure 2. Effects of 64 hMLH1 variants on their interactions with hMRE11. (A) Results of yeast two-hybrid analysis. cDNA encoding wild-type and various mutant hMLH1 proteins were cloned into pBTM116; cDNA encoding full-length hMRE11 was fused with Gal4-AD in plasmid pGADT7. The average β-gal activity units and standard errors (error bar) were determined with three independent data points. (B) Results of far western blot analysis. Relative interaction strength between hMLH1 variant and hMRE11 were determined by densitometry quantification, with 100% for interaction strength between wildtypes.

Figure 3. Far-western blot analysis of 10 representative hMLH1 HNPCC mutants on their interactions with hMRE11. Membranes were immobilized with purified hMRE11 aa 452-634 protein (represents the hMLH1 interacting domain) and BSA, then probed independently with crude lysate containing wild-type and various hMLH1 mutants proteins. Western blot was carried out to detect the captured wild-type and mutant hMLH1 proteins, respectively.

Figure 4. Expression levels of wild-type and 64 variant hMLH1 proteins. Bacterial lysates

containing each of the variant hMLH1 proteins (used in far-western analysis) were examined for hMLH1 expression levels. Anti-T7 and anti-hMLH1 antibody were used to detect wild-type and mutant hMLH1 proteins.

Figure 5. Expression of both full-length and truncated hMRE11 proteins as well as hMLH1 proteins. (A) Schematic depiction of the full-length and two truncated hMRE11 proteins, $hMRE11_{1-634}$ and $hMRE11_{452-634}$. (B) Western blots showing the protein expressions. The hMLH1 expression in the three cell lines were examined by α-hMLH1, and they all have similar levels of hMLH1; full length as well as two truncated hMRE11 proteins were examined by α-hMRE11 antibody. Since the two truncated hMRE11 fragments were also FLAG tagged, they could also be tested by α-FLAG antibody.

Figure 6. DNA mismatch repair assay and excision assay. Nuclear extracts derived from the indicated 5 cell lines were used to perform MMR reactions. 293T cells were deficient in 3' repair, but the repair was restored to the cells when complemented with hMLH1 protein. The expression of $hMRE11_{1-634}$, containing the entire functional nuclease domain of hMRE11, could slightly increase the MMR activity, whereas the expression of $hMRE11_{452-634}$, representing the hMLH1-interacting domain, can function as a dominant negative blocker for the interaction between endogenous hMRE11 and hMLH1. The 3' excision assay was carried essentially identical to the MMR assay, expect that no exogenous dNTP was added to the reaction. 293T cells were completely defective in 3' excision, while the complemented hMLH1 could restore some of the excision activity. The expression of the fragment $hMRE11_{1-634}$ could enhance the excision activity comparing with the 293T/hMLH1 cells; while the fragment $hMRE11_{452-634}$ could reduce the excision efficiency to about two thirds of that of the 293T/hMLH1 cells.

Figure 1

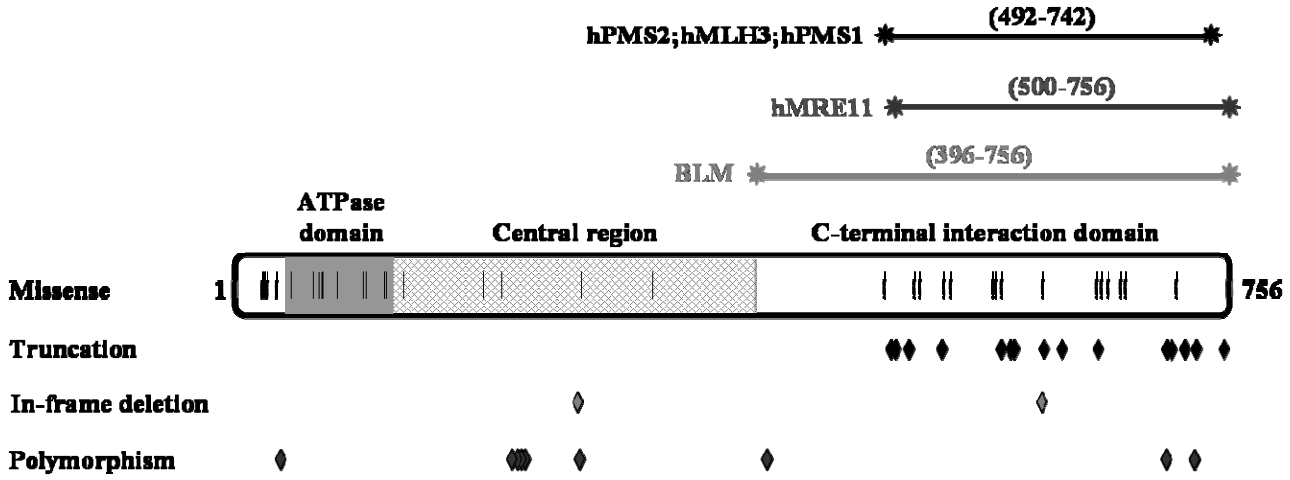

hPMS2;hMLH3;hPMS1
(492-742)
hMRE11
(500-756)
BLM
(396-756)
ATPase domain
Central region
C-terminal interaction domain
Missense
1
756
Truncation
In-frame deletion
Polymorphism

Figure 2

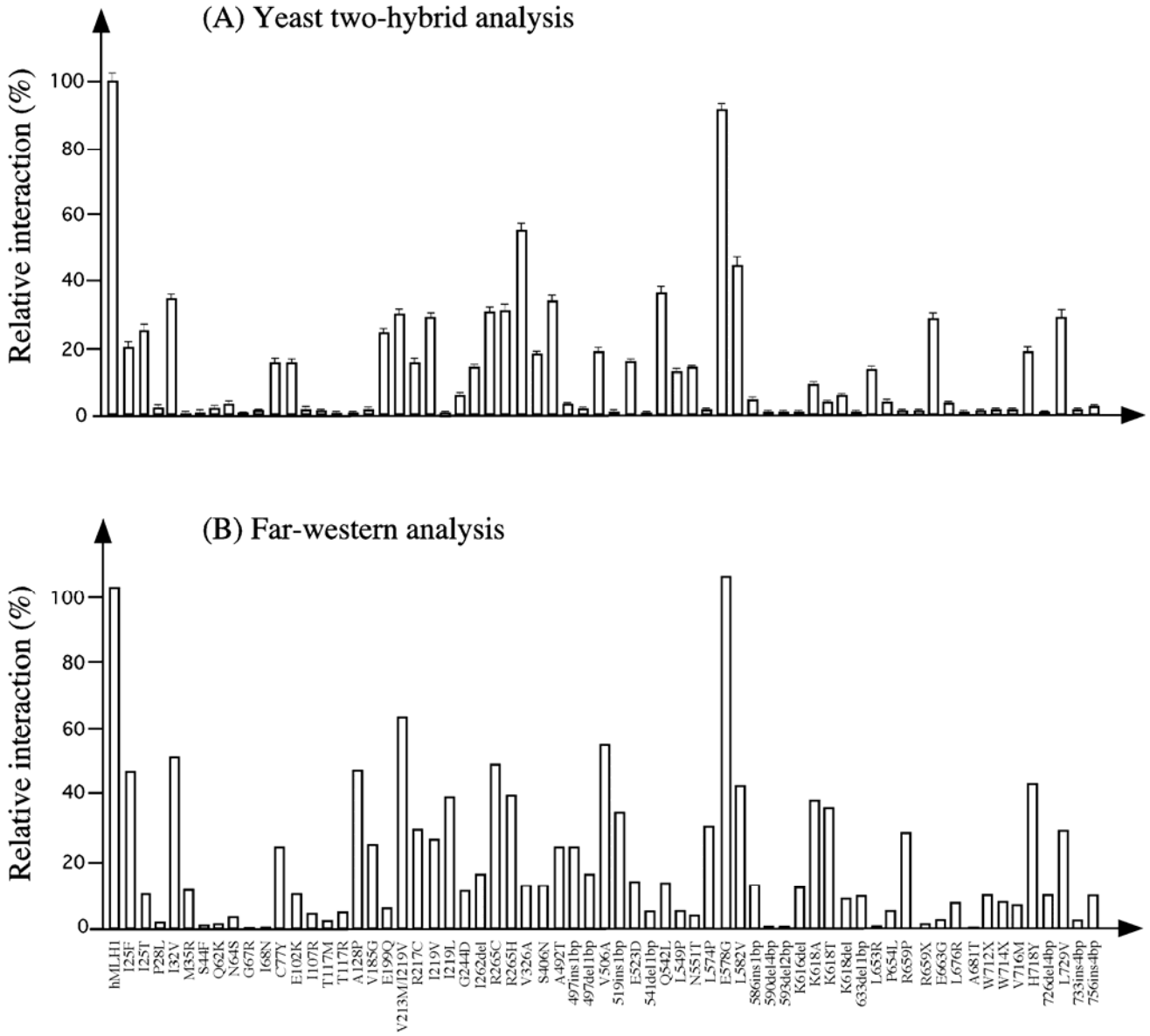

(A) Yeast two-hybrid analysis
Relative interaction (%)
100
80
60
40
20
0
(B) Far-western analysis
Relative interaction (%)
100
80
60
40
20
0
hMLH1
I25F
I25T
P28L
I32V
M35R
S44F
Q62K
N64S
G67R
I68N
C77Y
E102K
I107R
T117M
T117R
A128P
V185G
E199Q
V213M I219V
R217C
I219V
I219L
G244D
I262del
R265C
R265H
V326A
S406N
A492T
497ins1bp
497del1bp
V506A
519ins1bp
E523D
541del1bp
Q542L
L549P
N551T
L574P
E578G
L582V
586ins1bp
590del4bp
593del2bp
K616del
K618A
K618T
K618del
633del1bp
L653R
P654L
R659P
R659X
E663G
L676R
A681T
W712X
W714X
V716M
H718Y
726del4bp
L729V
733ins4bp
756ins4bp

Figure 3

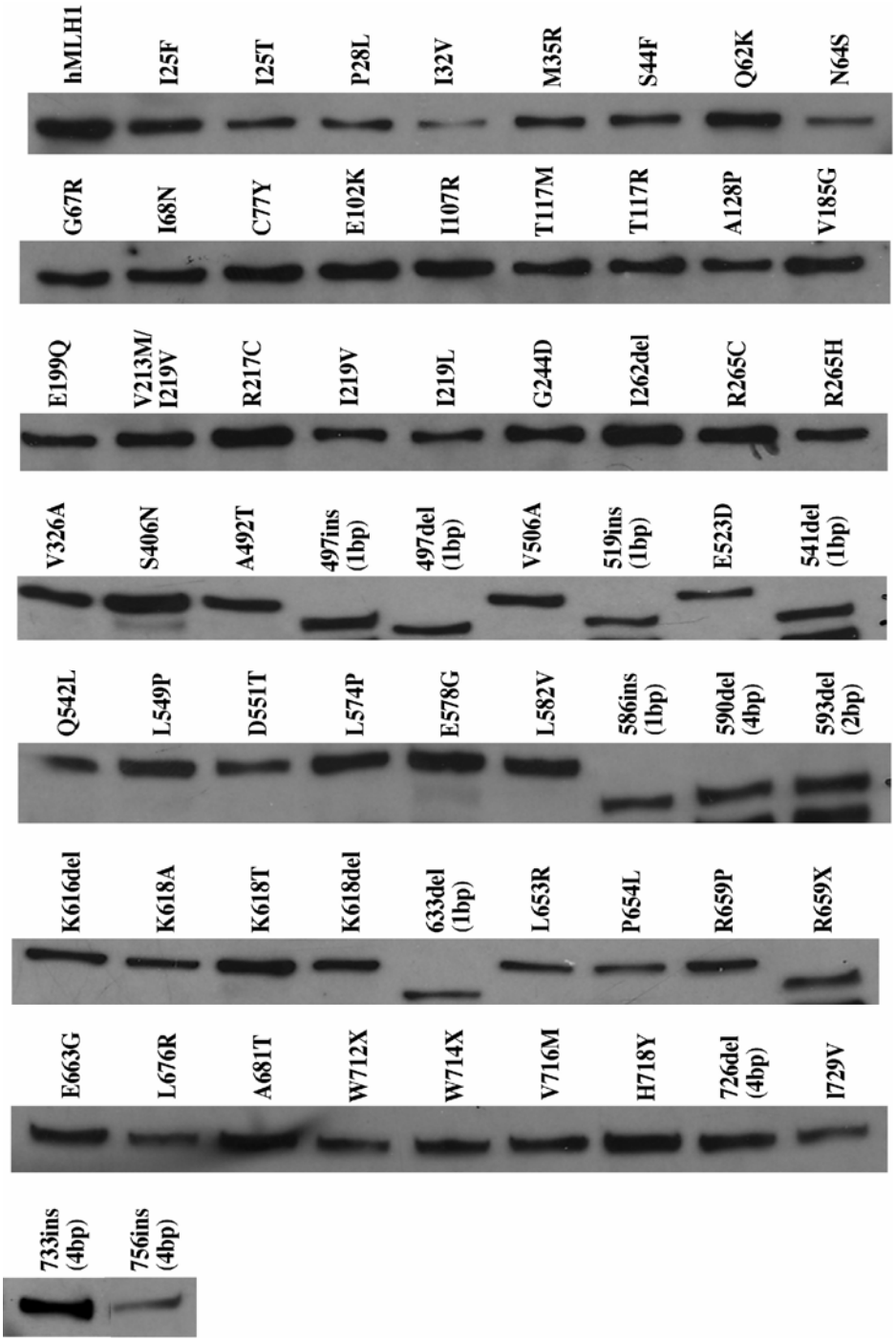

hMLH1
I25F
I25T
P28L
I32V
M35R
S44F
Q62K
N64S
G67R
I68N
C77Y
E102K
I107R
T117M
T117R
A128P
V185G
E199Q
V213M/ I219V
R217C
I219V
I219L
G244D
I262del
R265C
R265H
V326A
S406N
A492T
497ins (1bp)
497del (1bp)
V506A
519ins (1bp)
E523D
541del (1bp)
Q542L
L549P
D551T
L574P
E578G
L582V
586ins (1bp)
590del (4bp)
593del (2bp)
K616del
K618A
K618T
K618del
633del (1bp)
L653R
P654L
R659P
R659X
E663G
L676R
A681T
W712X
W714X
V716M
H718Y
726del (4bp)
I729V
733ins (4bp)
756ins (4bp)

Figure 4

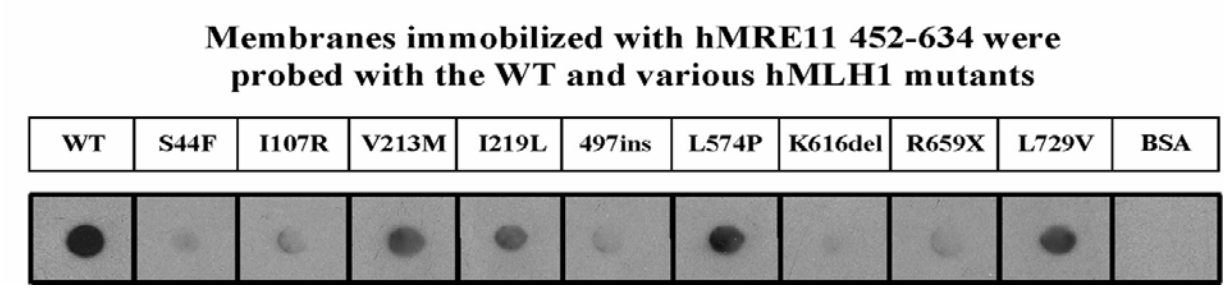

Membranes immobilized with hMRE11 452-634 were probed with the WT and various hMLH1 mutants
WT
S44F
I107R
V213M
I219L
497ins
L574P
K616del
R659X
L729V
BSA

Figure 5

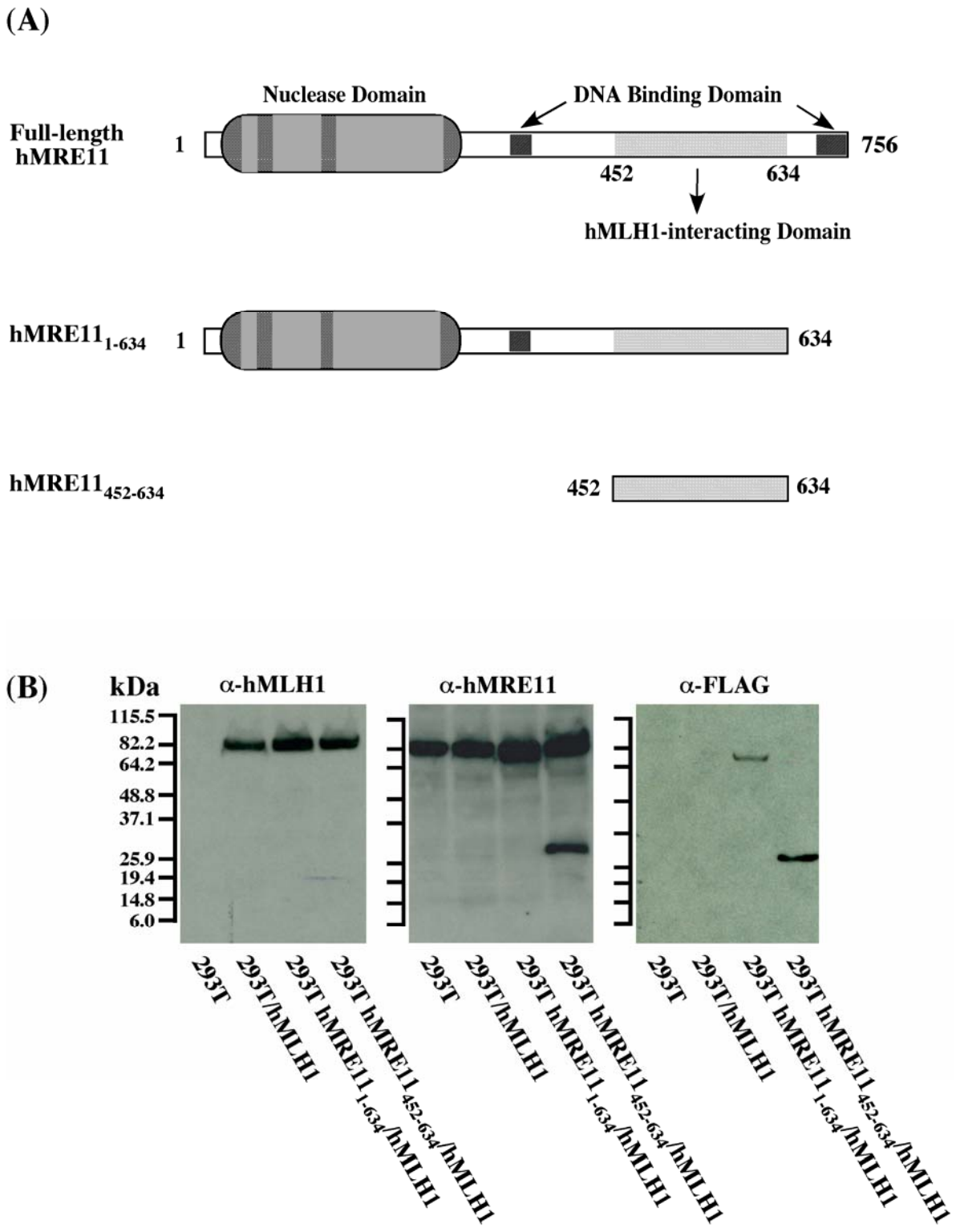
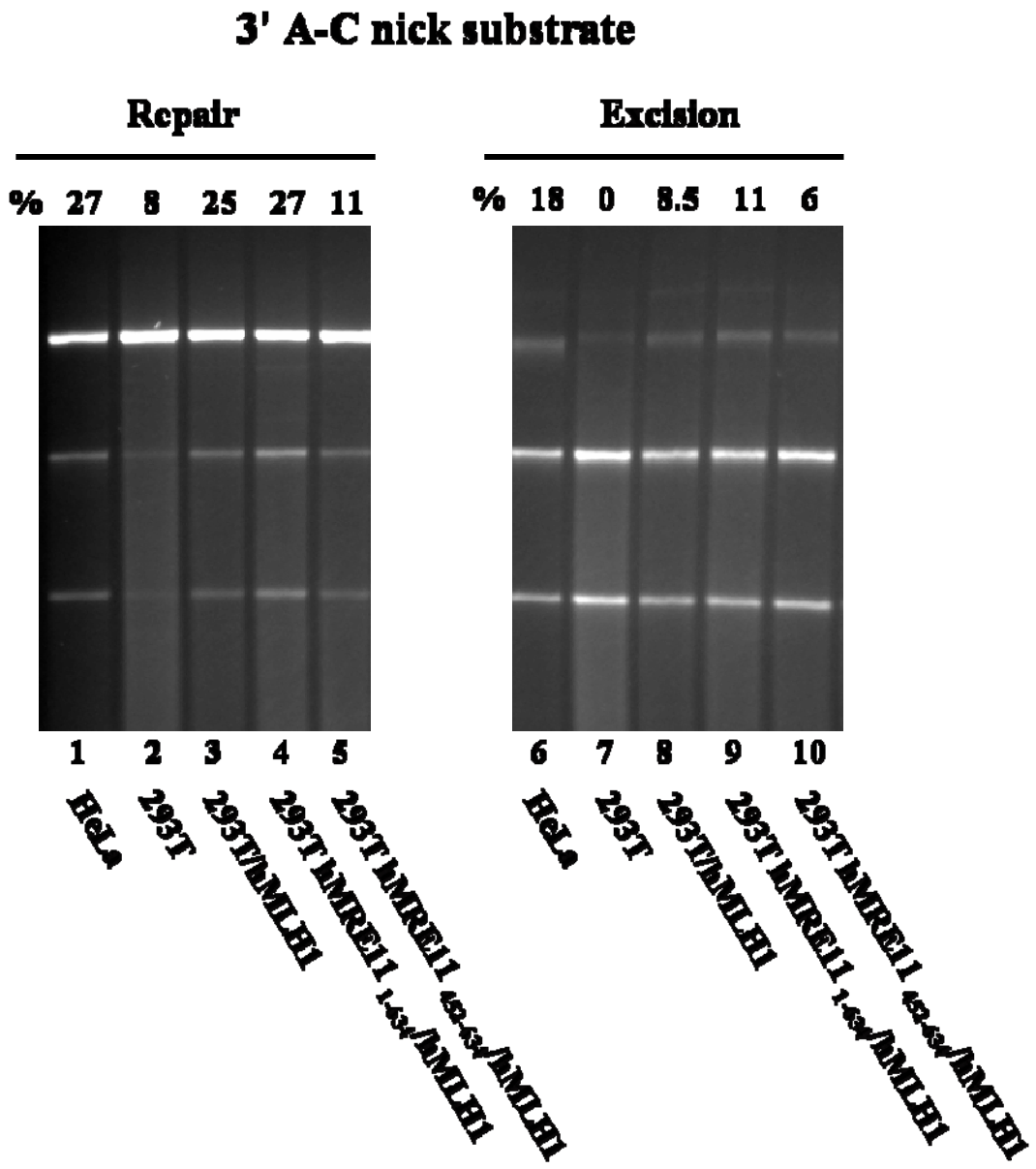

(A)
Nuclease Domain
DNA Binding Domain
Full-length hMRE11
1
756
452
634
hMLH1-interacting Domain
hMRE11 1-634
1
634
hMRE11 452-634
452
634
(B)
kDa
α-hMLH1
α-hMRE11
α-FLAG
115.5
82.2
64.2
48.8
37.1
25.9
19.4
14.8
6.0
293T
293T/hMLH1
293T hMRE11 1-634/hMLH1
293T hMRE11 452-634/hMLH1
293T
293T/hMLH1
293T hMRE11 1-634/hMLH1
293T hMRE11 452-634/hMLH1
293T
293T/hMLH1
293T hMRE11 1-634/hMLH1
293T hMRE11 452-634/hMLH1

Figure 6


3' A-C nick substrate
Repair
Excision
% 27 8 25 27 11
% 18 0 8.5 11 6
1 2 3 4 5
6 7 8 9 10
HeLa
293T
293T/hMLH1
293T hMRE11 1-434/hMLH1
293T hMRE11 43-434/hMLH1
HeLa
293T
293T/hMLH1
293T hMRE11 1-434/hMLH1
293T hMRE11 43-434/hMLH1

SUMMARY

Human hMRE11 protein is an essential nuclease of the MRE11/RAD50/NBS1 (MRN) complex which mainly functions in promoting repair of DNA double-strand breaks, signaling DNA damage response as well as meiotic recombination and telomere maintenance. However, the exact function of dsDNA 3'-5' exonuclease activity associated with hMRE11 has not been fully elucidated. Study from *S.cerevisiae* nuclease-deficient *mre11* mutant suggested that MRE11 nuclease activities were not involved in DSB-mediated homologous recombination (Bressan et al., 1999), raising the possibility that the hMER11 nuclease activities may participate in other cellular processes, such as mismatch repair (MMR). Our study for the first time identified hMRE11 as a functional protein factor involved in MMR pathway. Here, hMRE11-deficient HeLa cell line was generated by gene silencing of hMRE11 transcription through the application of small interfering RNA (siRNA). The resulting cell line (HeLa14) had 97% reduction in hMRE11 expression was then subjected for *in vitro* MMR assay and in vivo microsatellite instability (MSI) assay, using either two GFP-based MSI reporters or five endogenous MSI markers. The results indicated that hMRE11 deficiency in HeLa cells render those cells defective MMR activity, especially the 3' repair, and these cells display increased level of microsatellite instability, comparable to those MMR-deficient cell lines. This suggested hMRE11 as a potential candidate, in addition to ExoI, as a mammalian exonuclease that participate in the excision step of MMR process. In the future study, purified hMRE11 protein will be produced using the baculovirus system and added to the hMRE11-dificient HeLa cell extract, then in vitro MMR assay will be carried out again to test whether the complemented hMRE11 could rescue the defective MMR activity.

We also demonstrated that the interplay between hMRE11 and hMLH1, an essential protein in MMR process, was critical for functional MMR. Several initial observations led us to this hypothesis, which included that hMRE11 directly interacts with hMLH1 (Her et al., 2002), hMRE11 and hMLH1 coexist in a large protein complex involved in DNA damage response (Wang et al., 2000), and both hMRE11 and hMLH1 colocalize with PCNA at replication forks (Maser et al., 2001; Kleczkowska et al., 2001). Our study of 64 hMLH1 HNPCC mutants indicated that a large portion of the hMLH1 missense mutations disrupted their interactions with hMRE11, which could serve as an alternative explanation for the pathogenic potentials of those HNPCC tumors. We further tested the impact of hMRE11-hMLH1 interplay in MMR process by blocking their interaction *in vivo*. An hMRE11 dominant negative mutant was introduced to the cells which could competitively bind hMLH1, and *in vitro* MMR assay showed that the MMR activity was greatly reduced upon the disruption of hMRE11-hMLH1 interaction.

Taken together, the results of our experiments provide a solid foundation towards to a better understanding of the molecular mechanisms underlying the hMRE11 protein and the hMRE11-hMLH1 interplay in the process of DNA mismatch repair. It also provided us an in-depth comprehension with regard to the molecular mechanisms of the pathogenic effects of s subset of hMLH1 missense mutations in HNPCC tumors. The outcome of this research will provide us new knowledge which are valuable in assigning new diagnostic cancer markers and potential novel therapeutic drug targets.

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