Evidence That a Mutation in the \textit{MLH1} 3′-Untranslated Region Confers a Mutator Phenotype and Mismatch Repair Deficiency in Patients with Relapsed Leukemia\textsuperscript{*}$^{1}$

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Defects in DNA mismatch repair (MMR) are the molecular basis of certain cancers, including hematological malignancies. The defects are often caused by mutations in coding regions of MMR genes or promoter methylation of the genes. However, in many cases, despite that a hypermutable phenotype is detected in a patient, no mutations/hypermethylations of MMR genes can be detected. We report here a novel mechanism that a mutation in the \textit{MLH1} 3′-untranslated region (3′-UTR) leads to MMR deficiency. A relapsed leukemia patient displayed microsatellite instability, but no genetic and epigenetic alterations in key MMR genes were identifiable. Instead, a 3-nucleotide (TTC) deletion in the \textit{MLH1} 3′-UTR was found in the patient’s blood sample. The mutant \textit{MLH1} 3′-UTR was found to significantly reduce the expressions of both a firefly luciferase reporter gene and an ectopic \textit{MLH1} gene in model cell lines. Consistent with these observations, a significant reduction in the steady-state level of \textit{MLH1} mRNA was observed in white blood cells of the patient. These findings suggest that the mutant \textit{MLH1} 3′-UTR can cause a severely reduced/defective MMR activity conferring leukemia relapse, likely by down-regulating \textit{MLH1} expression at the mRNA level. Although the exact mechanism by which the mutant 3′-UTR down-regulates the \textit{MLH1} mRNA is not known, our findings provide a novel marker for cancers with MMR defects.

Genetic instability is considered both a hallmark of cancer and a pre-disposing condition that can lead to cancer. Multiple cellular mechanisms exist to prevent genetic instability, including a highly complex DNA damage response network and several DNA repair pathways. DNA mismatch repair (MMR)\textsuperscript{2} is an important genome maintenance system, the primary role of which is to correct mismatches generated during DNA replication, homologous recombination, and DNA repair. The MMR system relies on the highly conserved MutS homolog (MSH) and MutL homolog (MLH) proteins for its function. In humans, at least three \textit{MSH} genes (\textit{MSH2}, \textit{MSH3}, and \textit{MSH6}) and four \textit{MLH} genes (\textit{MLH1}, \textit{MLH3}, \textit{PMS1}, and \textit{PMS2}) have been identified. The \textit{MSH} gene products form the \textit{MSH2}-\textit{MSH6} (also called MutSo) and \textit{MSH2}-\textit{MSH3} (MutS\textit{\beta}) heterodimers, whereas the \textit{MLH} gene products constitute heterodimers of \textit{MLH1}-\textit{PMS2} (MutLo), \textit{MLH1}-\textit{PMS1} (MutL\textit{\beta}), and \textit{MLH1}-\textit{MLH3} (MutL\gamma) (1). The importance of MMR in maintaining genomic stability is underscored by the fact that defects in this system are the genetic basis of certain types of hereditary and sporadic human cancers, including hereditary non-polyposis colorectal cancer (HNPCC) (1–4).

Interestingly, despite that all above \textit{MSH} and \textit{MLH} genes are implicated in MMR, almost all MMR deficient cancers are associated with alterations in \textit{MSH2} and \textit{MLH1} (2–4) characterized by either genetic mutations in these genes or hypermethylation of the \textit{MLH1} promoter, which epigenetically silences the \textit{MLH1} expression (1). The selectively targeting on \textit{MSH2} and \textit{MLH1} for alterations in HNPCC and other MMR deficient cancers appears to be consistent with the fact \textit{MSH2} or \textit{MLH1} is an obligating subunit in each of the MutS and MutL heterodimers, whereas the other \textit{MSH} and \textit{MLH} components are functionally redundant (5). Tumor cells defective in MMR display a genome-wide mutator phenotype, e.g. frequent alterations in simple repetitive DNA sequences, a phenomenon called microsatellite instability (MSI) (2–4). However, not all cancer cells with MSI have identifiable mutations or epigenetic modifications in MMR genes (6–8). For example, ~30% of MSL-positive HNPCC tumors are not associated with genetic or epigenetic alterations in any of the above MMR genes (2), suggesting that additional mechanisms are responsible for MMR deficiency in these MSI-positive tumors.

In this study, we demonstrate that a 3-nucleotide deletion in the 3′-untranslated region (3′-UTR) of \textit{MLH1} is associated with a relapsed leukemia patient displaying a mutator phenotype identical to cells defective in MMR. The mutant \textit{MLH1} 3′-UTR appears to destabilize \textit{MLH1} mRNA and reduce \textit{MLH1} expression. Although exactly how the mutant \textit{MLH1} 3′-UTR destabilizes \textit{MLH1} transcripts is unknown, the implication of this result for regulation of MMR in eukaryotic cells is discussed. Our findings here reveal a novel molecular mechanism by which the MMR system can be impaired.

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\textsuperscript{1}The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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\textsuperscript{2}The abbreviations used are: MMR, mismatch repair; MSH, MutS homolog; MLH, MutL homolog; HNPCC, hereditary non-polyposis colorectal cancer; MSI, microsatellite instability; AML, acute myelogenous leukemia; SSCP, PCR-based single-strand conformation polymorphism; 3′-UTR, 3′-untranslated region; WT, wild-type; MT, mutant; RT-PCR, reverse transcriptase PCR.
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EXPERIMENTAL PROCEDURES

Patient and Patient Samples—Patient AML0805 was diagnosed as acute myelogenous leukemia (AML) with cytogenetics showing a 45, XY, t(8, 21)(q22;q22), del(9)(q22q32) karyotype on August, 2005 at the University of Kentucky Hospital and treated with standard “7 + 3” cytarabine plus idarubicin in which the patient went into complete remission (including negative FISH for t(8, 21). This was followed by four courses of high dose cytarabine as postinduction therapy. However, the patient relapsed on June, 2006. Whole blood samples were obtained from the diagnostic (AML0805) and the relapsed patient (AML0606) according to an approved Institute Review Board protocol. Whole blood cells were fractionated by Ficoll-Paque (GE Healthcare) density gradient centrifugation, and the mononuclear white blood cell compartment was isolated and used to prepare genomic DNA and RNA using the FlexiGene DNA kit or QIAamp RNA blood mini kit (Qiagen), respectively, according to the manufacturer’s instructions.

Cell Culture and Transfection—HeLa-S3 and 293T cells were grown in RPMI 1640 or Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Sigma) and 100 units/ml penicillin/streptomycin (Invitrogen). Cells were maintained in a humidified atmosphere of 5% CO2 and 95% balanced air at 37 °C and transfected using FuGENE HD Transfection Reagent (Roche Applied Science) at a 3:2 ratio of transfection reagent (μl) to DNA (μg).

Single-strand Conformation Polymorphism and DNA Sequencing—Mutations in the 3′-UTR of the MLH1 gene were screened using PCR-based single-strand conformation polymorphism (SSCP) analysis and DNA sequencing, essentially as described (9, 10). PCR primers ex19F (CAAA CAGGGAGGTTTATGCACTGTGG) and ex19R (AAATAA GAAATTATGTTAAAAGCTTATGA) and ex19R (AAATAA GAAATTATGTTAAAAGCTTATGA) were used to determine MSI in mononucleotide runs in exon 2 of AML0805 and AML0606 as described (9, 10). Similarly, frameshift mutations in mononucleotide runs in exon 2 of CASPASE-5 and intron 5 of FANCD2 were investigated using the primers and conditions reported elsewhere (11) with minor modifications.

Statistical Analysis—Data from dual-luciferase assays and real-time quantitative PCR were analyzed using GraphPad Prism 4.0 software (GraphPad Software). Mean values (±S.D.) were calculated. Statistical significance was determined using Student’s t test or one-way analysis of variance, and post-test was processed using Newman-Keuls multiple comparison tests. Data were considered statistically significant when p < 0.05.

RESULTS

Identification of a Mutator Phenotype in a Relapsed Leukemia Patient—We recently investigated the impact of MMR deficiency on diagnostic and relapsed leukemia and provided compelling evidence showing that loss of MMR function is closely associated with leukemia relapse (12). Although the vast majority of the cases defective in MMR in the aforementioned study were because of either mutations in key MMR genes MSH2 and MLH1 or epigenetic modifications in the promoter region of MLH1, a patient, as described below, was found to...
carry a novel mutation that also lead to a mutator phenotype. This patient was diagnosed with AML on August 2005 and received standard chemotherapy (see “Experimental Procedures”). After achieving complete remission, the patient underwent relapse on June 2006. Blood samples collected from the patient at both the initial diagnosis and the relapse were designated as AML0805 and AML0606, respectively (12).

To determine whether the patient was associated with MMR defects, genomic DNA from blood samples of the patient was tested for MSI. Among five standard microsatellite markers examined, polymorphic PCR products were detected in a dinucleotide repeat marker ACTC between the patient’s diagnostic and relapsed blood cells (Fig. 1A), indicative of MSI (13). Instability was also examined in mononucleotide repeat markers CASPASE-5(A)10 (Fig. 1B) and FANCD2(T)10 (Fig. 1C), where a run of 10 adenines and 10 thymines were present in exon 2 of CASPASE-5 and intron 5 of FANCD2, respectively. As shown in Fig. 1 (D and E), frameshift mutations of these mononucleotide repeats were observed in the patient’s relapsed sample (see MT sequence in Fig. 1, D and E). These observations strongly suggest that the relapsed leukemia adopted a hypermutable phenotype during chemotherapy.

Identification of a 3-Nucleotide Deletion in the 3′-UTR of MLH1 in the Relapsed Sample of the Patient—The mutator phenotype in the relapsed sample of the leukemia patient prompted us to search for alterations in key MMR genes MSH2 and MLH1 using PCR-based SSCP analysis, followed by DNA sequencing. This analysis did not reveal any mutations in exons and exon-intron junctions of either gene (data not shown). Hypermethylation analysis was also performed, but no such epigenetic modification was detected in the promoter region of MLH1 or MSH2 (data not shown). However, we did observe a deletion mutation in the MLH1 3′-UTR (Fig. 2). Two sets of primers were used to amplify the 3′-UTR and adjacent DNA sequence of MLH1. One primer set amplified exon 19 and the 3′-UTR, and the other set amplified the 3′-UTR and downstream untranscribed DNA sequences (Fig. 2A). Both primer pairs generated one set of PCR products common to AML0805 (diagnostic sample) and AML0606 (relapsed sample), and one set of PCR products unique to AML0606. These bands were designated 1–4 and 1′-3′, as shown in Fig. 2, B and C. DNA sequencing analysis revealed that bands 1–4 corresponded to the wild-type MLH1 3′-UTR sequence (Fig. 2D, left). In contrast, DNA sequencing of bands 1′ and 2′ (products of primers ex19F and ex19R) and 3′ (a product of primers 3′utrF and 3′utrR) revealed the presence of a 3-nucleotide (TTC) deletion in the 3′-UTR of MLH1 in AML0606 (Fig. 2D, center and right).

The observation of both wild-type and mutated alleles of the 3′-UTR seems to suggest a heterozygous mutation. However, based on the information from this and previous studies, we believe that the mutation is a homozygous one. First, the DNA used in this experiment was extracted from a heterogeneous population of white blood cells, which was likely to include leukemic and non-leukemic cells. Secondly, previous studies have revealed that individuals with heterozygous defects (e.g. germline mutations in HNPCC or heterozygous knockouts in mice) of an MMR gene generally possess a functional MMR system and do not display MSI (14). The identification of MSI in the patient relapsed blood sample suggests a complete loss of MMR function in a significant fraction of leukemic cells. Therefore, the detection of both the wild-type and mutant MLH1 3′-UTR is likely caused by the presence of both MMR proficient and deficient cells in the samples analyzed.

**Functional Effect of the MT 3′-UTR on Reporter Gene Expression and mRNA Stability**—To determine whether the 3-nucleotide deletion in the MLH1 3′-UTR altered expression of
upstream coding sequences, the WT or MT MLH1 3'-UTR was cloned downstream of the firefly luciferase reporter gene (Fig. 3A). The reporter gene constructs were transfected into HeLa-S3 cells, and the expression of the luciferase and the stability of the reporter transcript were quantified in transfected cells. The results showed that the reporter construct with the MT 3'-UTR reproducibly produced ~2-fold lower luciferase activity than the construct with the WT 3'-UTR (Fig. 3B). Quantification of luciferase mRNA by real-time RT-PCR also showed a statistically significant decrease ($p < 0.01$) in the stability of the firefly luciferase transcript with the MT 3'-UTR (Fig. 3C). These results clearly indicate that the 3-nucleotide deletion in the MLH1 3'-UTR has significant functional effects on reporter gene expression and stability of transcripts from an upstream reporter gene.

**Functional Effect of the MT 3'-UTR on MLH1 Expression**—The effect of the MT MLH1 3'-UTR on expression of an ectopic copy of MLH1 was also examined. For this experiment, the MLH1 gene with WT or MT 3'-UTR was cloned downstream of the cytomegalovirus promoter (P$_{Cmv}$) in the vector pcDNA3.1. MLH1 was expressed from this construct with an N-terminal His$_6$ tag (Fig. 4A). The constructs also included the gene encoding neomycin resistance and expressed the corresponding polypeptide, which conferred neomycin resistance to transfected cells. These expression constructs were transiently transfected into human kidney cell line 293T, in which the endogenous MLH1 gene was epigenetically silenced by promoter methylation (15), and the expression of ectopic MLH1 was monitored by Western blot. The neomycin resistance protein was used as an internal control. The results show that a much lower level of MLH1 was produced by the expression construct with the MT MLH1 3'-UTR (Fig. 4B). Thus, the 3-nucleotide deletion in the MLH1 3'-UTR reduces expression of either MLH1 or the luciferase reporter gene. These results suggest that the MT 3'-UTR may non-specifically decrease the stability and/or expression of co-linear RNA transcripts in cis.

To determine the effect of the MT 3'-UTR on the stability and/or steady-state level of MLH1 mRNA in relapsed leukemia of the patient, mRNAs isolated from the patient’s relapsed (AML0606), and diagnostic (AML0805) white blood cells were analyzed for the steady-state level of MLH1 by RT-PCR. As shown in Fig. 4 (C and D), the MLH1 cDNA in AML0606 was at least 33% less abundant than in AML0805. The reduction in mRNA levels in AML0606 is statistically significant ($p < 0.05$). Nevertheless, it is worth noting that the impact of the 3'-UTR mutation on MLH1 cDNA expression could be underestimated by these data because the blood sample used for this experiment likely included a heterogenous mixture of cells with different MLH1 genotypes. These observations support a role for the MT 3'-UTR in leukemia relapse in AML0606. However, we cannot rule out the possibility that other factors also contribute to the relapse.

**DISCUSSION**

This study characterizes the MLH1 genotype in a relapsed leukemia patient, who was previously diagnosed with AML. The results indicate that leukemia relapse was associated with a
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3-nucleotide deletion in the 3′-UTR of MLH1 and that the MT MLH1 3′-UTR reduced MLH1 expression in vivo. In particular, the steady-state level of the MT MLH1 transcript in the patient’s relapsed blood (AML0606) was much lower than the steady-state level of the WT MLH1 transcript in AML0805 (Fig. 4). This is likely due to the fact that the MT MLH1 3′-UTR destabilizes mRNA transcripts in cis, as shown here by its effects on transcripts from a proximal firefly luciferase reporter gene (Fig. 2).

This paper reports the novel observation that a 3-nucleotide deletion in the MLH1 3′-UTR reduces MLH1 expression in vivo, which could in turn reduce MMR efficiency. Although there is ample precedent for 3′-UTRs regulating expression of the proximal upstream gene (16–18), to our knowledge, this is the first report of a functional regulatory role for the 3′-UTR of a human MMR gene. It will be of interest to determine the frequency at which this mutation occurs in leukemia patients treated with or without chemotherapy, in patients with other types of cancer (including HNPPC), and in healthy individuals. Although this study only reports one patient, we actually identified the same mutation in 3 out of 53 AML patients (12). Because blood samples available from the other two patients were collected during the course of their chemotherapy, it is difficult to perform similar analyses as described for patient AML0606. In addition, we have no clue if the mutation is therapy-induced or pre-existing at the diagnosis. Although these data must be interpreted with caution due to small sample number, they suggest a higher than expected frequency of this mutation in AML patients. Based on the fact that the mutation was detected in relapsed but not diagnostic cells of the AML patient, it is possible that the MT 3′-UTR could play a role in disease relapse. Because the MT MLH1 3′-UTR appears to destabilize the MLH1 transcript in white blood cells from AML0606 (Fig. 4C and D), it is predicted that these cells could be hypomorphic for MLH1 and therefore functionally deficient in MMR. This possibility is consistent with the observation that AML0606 cells are MSI-positive in one of five microsatellite markers tested and contain frameshift mutations in mononucleotide repeat sequences of CASPASE-5 and FANCD2 genes (Fig. 1), both of which have been shown to be important targets for the mutation in MSI-positive cancers (11).

The mechanism by which the MT MLH1 3′-UTR destabilizes proximal RNA transcripts is not known. Previous studies show that cis-acting DNA sequence elements in 3′-UTRs and trans-acting protein factors influence mRNA stability. For example, the AU-rich element is an extensively studied cis-acting element commonly found in eukaryotic 3′-UTRs that binds to a cognate protein, AU-rich element binding protein, and their interaction stimulates mRNA turnover (19, 20). Sequence analysis shows that the 3′-UTR of MLH1 does not contain any AU-rich element. However, secondary structure analysis (21) of the WT and MT 3′-UTR of MLH1 showed that the TTC-deletion identified in this study lies in a large stem-loop structure. The deletion of 3-nucleotide in the region reduced the size of stem-loop and created a new small stem-loop (supplemental Fig. 1). The impact of altering the size of this stem-loop and loop numbers on MLH1 mRNA structure, stability and/or functionally deficient in MMR. This possibility is consistent with the observation that AML0606 cells are MSI-positive in one of five microsatellite markers tested and contain frameshift mutations in mononucleotide repeat sequences of CASPASE-5 and FANCD2 genes (Fig. 1), both of which have been shown to be important targets for the mutation in MSI-positive cancers (11).
mutation down-regulates MLH1 expression at both the mRNA and protein levels remains to be investigated.

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