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ORIGINAL ARTICLE

Preferential loss of mismatch repair function in refractory and relapsed acute myeloid leukemia: potential contribution to AML progression

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Acute myeloid leukemia (AML) is an aggressive hematological cancer. Despite therapeutic regimens that lead to complete remission, the vast majority of patients undergo relapse. The molecular mechanisms underlying AML development and relapse remain incompletely defined. To explore whether loss of DNA mismatch repair (MMR) function is involved in AML, we screened two key MMR genes, *MSH2* and *MLH1*, for mutations and promoter hypermethylation in leukemia specimens from 53 AML patients and blood from 17 non-cancer controls. We show here that whereas no amino acid alteration or promoter hypermethylation was detected in all control samples, 18 AML patients exhibited either mutations in MMR genes or hypermethylation in the *MLH1* promoter. *In vitro* functional MMR analysis revealed that almost all the mutations analyzed resulted in loss of MMR function. MMR defects were significantly more frequent in patients with refractory or relapsed AML compared with newly diagnosed patients. These observations suggest for the first time that the loss of MMR function is associated with refractory and relapsed AML and may contribute to disease pathogenesis.

***Keywords*:** leukemia relapse, *MSH2*, *MLH1*, hypermethylation

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Introduction

Acute myeloid leukemias (AML) comprise a group of neoplastic diseases derived from the clonal expansion of myeloid precursor cells in bone marrow, blood or other tissues. Despite the fact that the majority of AML patients achieve complete remission (CR) after chemotherapy, only ~20% of patients achieve a relatively long-term disease-free survival. Most patients die of their disease due to either refractory (initial resistance to chemotherapy) or relapsed AML [1]. The molecular factors that define AML as either a chemotherapy-sensitive entity or a chemotherapy-resistant relapsed and refractory disease remain unknown.

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Like solid tumors, the development of AML is associated with various types of genetic alterations. Cytogenetic studies have revealed two major classes of karyotypes for AML patients, i.e. normal and abnormal karyotypes [2, 3]. Patients with abnormal karyotype (~55%) are characterized by chromosome changes such as translocations, inversions, insertions, deletions, trisomies, and monosomies, whereas patients with normal karyotype (~45%) contain point mutations and duplications/deletions of certain sequences in genes involved in critical cellular functions, such as signal transduction, regulation of gene expression tumor initiation and progression [2, 3]. However, the molecular mechanism(s) responsible for the genetic instability in AML are not clear.

DNA mismatch repair (MMR) plays an important role in maintaining genomic stability by correcting biosynthetic errors, blocking non-homologous recombination, and mediating DNA damage-induced cell cycle arrest and apoptosis [4-7]. It has been well documented that defects in MMR

genes, particularly the *MSH2* and *MLH1* genes, are the genetic basis for certain types of hereditary and sporadic cancers, including hereditary nonpolyposis colorectal cancer (HNPCC) [4, 6, 8]. Commonly, MMR-deficient tumors display widespread alterations in simple repetitive DNA sequences, a phenomenon also called microsatellite instability (MSI) [4, 6, 8]. Tumor cells defective in MMR are highly resistant to killing by certain chemotherapeutic drugs [7]. Genomic instability in AML has led to a search for MSI in AML patients, but the results are quite controversial. While several studies have reported MSI in AML [9-13], a study of 132 cases failed to confirm the previous observations [14]. Although reasons for the discrepancy are unclear, the use of different microsatellite markers and different stages (i.e., diagnosis and relapse) of the disease may have contributed to the differences observed. To our knowledge, none of these studies have systematically measured the loss of MMR function in AML at its individual treatment stages (i.e., diagnosis, persistence/primary refractoriness, and relapse). Therefore, it is uncertain whether the genetic instability in AML is caused by MMR-deficiency and, if so, what role MMR plays in AML pathogenesis.

Considering that most leukemia cell lines derived from relapsed patients are defective in MMR [15] and that tumor cells can acquire an MMR-deficient phenotype upon exposure to chemotherapeutic drugs [16-18], we hypothesize that a small portion of leukemic cells adopt an MMR deficient phenotype during chemotherapy, thereby leading to drug resistance and leukemia persistence and/or relapse. In this paper, we have tested this hypothesis. We have analyzed leukemia patients at different stages (diagnosis, persistence/primary refractoriness, and relapse) for mutations and promoter hypermethylation in the key MMR genes, *MSH2* and *MLH1*, and examined mutant proteins identified in these patients for MMR activity. Our results revealed that MMR deficiency is associated with all stages of AML, but the rate of the deficiency is much higher in patients with refractory and relapsed AML than in newly diagnosed patients, suggesting that the loss of MMR function may contribute to the refractory and relapsed disease.

Results

Abnormal PCR-SSCP products in AML patients and control individuals

To determine wherther the loss of MMR function is associated with the development of AML, individual exons of *MSH2* (16 exons) and *MLH1* (19 exons), as well as their exon-intron boundaries and known splice sites of these two genes, were PCR-amplified using genomic DNA isolated from leukocytes of diagnostic, primary refractory, and relapsed AML patients and control individuals with no history of any malignancies. The resulting PCR products were analyzed by single-strand conformation polymorphism

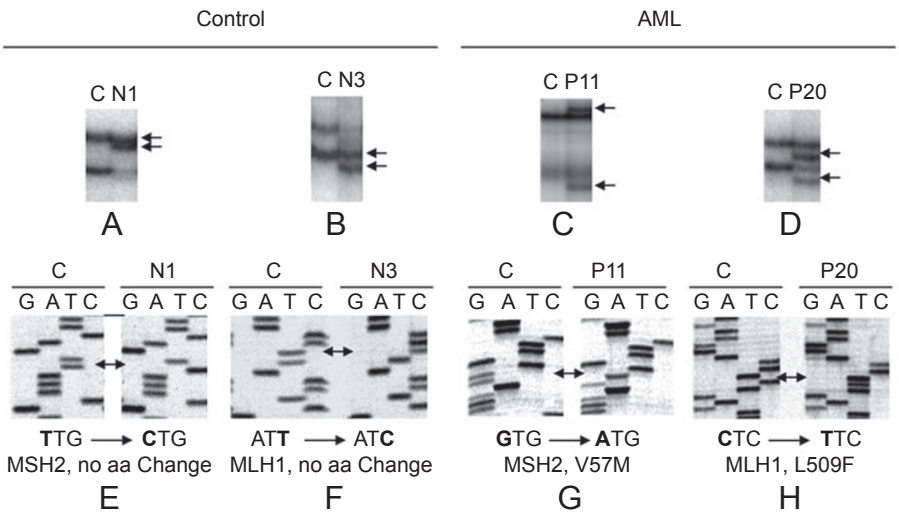

Figure 1 SSCP and sequence analyses of *MSH2* and *MLH1*. Individual exons of the *MSH2* gene and the *MLH1* gene were amplified by PCR using 50-100 ng of genomic DNA in the presence of dNTPs and [α-^{32}P]-dCTP. PCR products were fractionated in a 0.5× MED gel and were detected by a phosphor imager. Abnormal products were sequenced as described in Materials and Methods. **(A** and **B)** PCR-SSCP products of exon 11 of *MSH2* and exon 19 of *MLH1*, respectively. **(C** and **D)** PCR-SSCP products of exon 1 of *MSH2* and exon 13 of *MLH1*, respectively. **(E**-**H)** DNA sequencing analyses of the PCR products shown in **(A**-**D)**, respectively. A normal blood sample **(C)** from a healthy volunteer was used as a positive control in all cases. Arrows in SSCP analysis **(A**-**D)** point to mutant alleles, and arrows in sequencing analysis show base substitutions. The corresponding changes in codon and amino acid (aa) are indicated at the bottom of each sequencing gel.

Table 1 Genetic and epigenetic alterations of MMR genes in AML

	Case	Region	Alteration	Protein change	MMR activity	Karyotype
Diagnosis						
MSH2	P1	E13	G**A**G→G**C**G	E698A	Proficient	Abnormal
	P2	E16	TT**T**→TT**C**	No change	Proficient[1]	Abnormal
MLH1	P3	E3	TT**T**→TT**A**	F99L	Deficient	Normal
	P4	E4	G**C**T→G**T**T	A120V	ND	Normal
	P5	E4	T**A**C→T**G**C	Y126C	ND	NA
	P6	E15	**T**TT→**C**TT	F571L	Deficient	NA
	P7	E19	CC**T**→CC**C**	No change	Proficient[1]	Abnormal
	P8	3′-UTR	'**ttc**' deletion	Less exp.	Reduced[2]	Normal
	P9	Promoter	**Methylation**	No/less exp.	Deficient[1]	Abnormal
Relapse						
MSH2	P10	E1	**G_{204}** deletion	Frameshift	Deficient[1]	Normal
	P11	E1	**G**TG→**A**TG	V57M	ND	NA
	P12	E10	**T**TT→**A**TT	F523I	Reduced	Abnormal
	P13	E10	**C**AG→**T**AG	Q510 to stop	Deficient[1]	Normal
MLH1	P14	E1	**G_{67}** deletion	Frameshift	Deficient[1]	NA
	P15	3′-UTR	'**ttc**' deletion	Less exp.	Reduced[2]	Abnormal
	P16	Promoter	**Methylation**	No/less exp.	Deficient[1]	NA
	P17	Promoter	**Methylation**	No/less exp.	Deficient[1]	Abnormal
	P18	Promoter	**Methylation**	No/less exp.	Deficient[1]	Normal
Primary refractoriness						
MSH2	P19	E10	A**A**T→A**G**T	N547S	Deficient	Normal
	P19	E10	**G**GC→**A**GC	G508S	Deficient	Normal
MLH1	P20	E13	**C**TC→**T**TC	L509F	ND	Abnormal
	P21	E19	**T**GG→**A**GG	W712R	Deficient	Normal
	P21	E19	**G**TG→**A**TG	V716M	ND	Normal
Non-cancer control						
MSH2	N1	E11	**T**TG→**C**TG	No change		
MLH1	N2	E13	TC**T**→TC**A**	No change		
	N3	E19	AT**T**→AT**C**	No change		

Only patients with genetic or epigenetic alterations are shown. Bold types indicate alterations. Underlined types are mutations tested for MMR activity (see Figure 3). ND, not determined; NA, not available. No/less exp., no protein or less protein expression.

[1]The predicted MMR phenotype based on the nature of the alteration.

[2]The mutation is associated with down regulation of *MLH1* expression (unpublished results).

(SSCP). Of 17 non-cancer control samples, three individuals (17.6%) were identified with abnormal SSCP bands, one in *MSH2* and two in *MLH1*. By contrast, as shown in Table 1, 17 (32%) of the 53 AML patients analyzed exhibited SSCP abnormalities, seven cases in *MSH2* and 10 cases in *MLH1*. Patients P19 and P21 each exhibited two abnormal SSCP products (Table 1). Representative aberrant SSCP products are shown in Figure 1 (panels A-D).

Interestingly, whereas SSCP products from AML patients contained both wild type and new alleles (see arrows in Figure 1C and 1D), SSCP products from blood samples of control individuals with abnormal bands showed only two new alleles (see arrows in Figure 1A and 1B). These results suggest that the aberrations noted in AML patients reflect either a heterozygous or a homozygous alteration in a subpopulation of the leukemic cells, whereas the abnormalities in non-cancer controls represent homozygous changes.

Mutations of MSH2 and MLH1 in AML

To determine whether the abnormal exons of *MSH2* and *MLH1* are due to DNA sequence alterations, SSCP products were reamplified and sequenced. Representative sequencing analyses are shown in Figure 1E to 1H, and specific changes in nucleotide sequences and their corresponding amino acid alterations are listed in Table 1. It was found that all abnormal SSCP bands identified, regardless of AML or non-cancer origins, were associated with a change in nucleotide sequences, with most of them being base substitutions. However, not all base substitutions lead to a change in amino acids. For example, none of the DNA sequence alterations found in the three control cases caused an amino acid substitution (Table 1 and Figure 1E and 1F), and the same was also true for SSCP abnormalities noted in patients P2 and P7 (Table 1), suggesting silent nucleotide polymorphisms in these patients. However, the remaining 17 base substitutions or nucleotide deletions in AML resulted in either protein sequence changes or reduced protein expressions (Table 1).

P10 and P14 exhibited a single base deletion in the exon 1 of *MSH2* and in the exon 1 of *MLH1* (Table 1), respectively, resulting in predicted protein sequence totally different from those of *MSH2* and *MLH1*. P13 had a nonsense mutation in exon 10 of *MSH2* predicted to encode a truncated protein. Patients P19 and P21, both of who were diagnosed with refractory AML, harbored two missense mutations in *MSH2* and *MLH1*, respectively. It is likely that single or double mutations would impair the MMR system (see functional MMR assays below). P8 and P15 had a 3-nucleotide (ttc) deletion in the 3′ un-translated region (3′-UTR) of *MLH1* (Table 1). Our recent studies revealed that this mutation is associated with significantly reduced expression of *MLH1* [19] that could substantially reduce MMR activity. Table 1 also shows mutations that are likely to reduce MMR activity, including MSH2-F523I (P12), MSH2-N547S and MSH2-G508S (P19), MLH1-F99L (P3), MLH1-F571L (P6), MLH1-L509F (P20), and MLH1-W712R (P21). It was noted that most of the mutations occurred in the carboxyl terminal regions of MSH2 and MLH1 where several important functional domains are located, including the dimerization, MutS-MutL interaction, ATP-binding/ATPase (MSH2), and EXO1 interaction domains. Therefore, these mutations should lead to a defective MMR system.

Promoter hypermethylation of MLH1 in AML

Promoter hypermethylation of MMR genes is a major factor leading to MMR deficiency in certain types of sporadic cancers [20, 21]. To determine wherther hypermethylation of the *MSH2 and MLH1* promoters is associated with AML, methylation-specific PCR (MSP) was performed in 21 AML cases where sufficient DNA samples are available for the methylation analysis. Of these 21 cases, P19 and P21

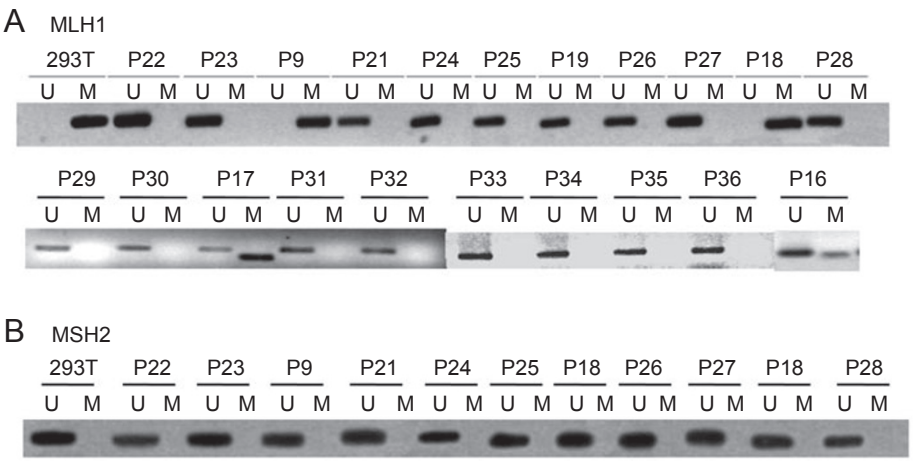

Figure 2 Analyses for hypermethylation of the *MSH2* and *MLH1* promoters. MSP was performed on bisulfite modified DNA as described in Materials and Methods. PCR products were electrophoresed on agarose gels and visualized by ethidium bromide staining. PCR products marked U and M indicates the presence of unmethylated and methylated promoter sequences, respectively. The embryonic kidney cell line 293T was used as a positive control for methylated *MLH1* promoter.

possessed altered MMR genes (Table 1), but no mutations of *MSH2* and *MLH1* were detected in the other 19 cases (data not shown).

Kidney cell line 293T was used as a positive control for MSP-PCR, as its *MLH1* promoter is known to be hypermethylated [22]. As expected, the MSP-PCR assay indeed detected hypermethylation of the *MLH1* promoter in genomic DNA from 293T cells (Figure 2A). The same analysis with material from AML patients identified hypermethylation of the *MLH1* promoter in P9, P16, P17, and P18 (Figure 2A and Table 1). These observations suggest that MMR deficiency caused by epigenetic silencing of *MLH1* is associated with AML. In contrast, hypermethylation of the *MSH2* promoter was not detected in any cases tested (Figure 2B).

MSI in patients with AML

Since MSI is a hallmark of MMR deficiency, we sought to determine MSI in patients with detected mutations in MMR genes. Given that most samples were existing specimens from the Tissue Procurement Service at the University of Kentucky Hospital, paired samples (e.g., non-cancer cells vs. leukemia cells and/or diagnosis vs. relapse) were not available for the vast majority of the patients examined, MSI analyses were only limited to four AML patients whose skin samples were also available. Of these four patients, P1, P37 and P38 were diagnostic AML, and P17 had relapsed AML. Whereas no MMR defects were identified in P37 (data not shown), P1 was found to carry a single amino acid substitution (E698A) in MSH2 (Table 1); P38 harbored a common I129V MLH1 polymorphism (data not shown); and P17 was associated with hypermethylation of the *MLH1* promoter (Figure 2 and Table 1). As expected, MSI was not detected in P37 and P38, as judged by the fact that skin samples and blood samples from the same patients displayed identical patterns for each of the six microsatellite markers tested (data not shown); instability was indeed observed in two of the six markers in P17 (Figure 3A), suggesting that a defective MMR system caused by hypermethylation-associated transcriptional silencing of *MLH1* is associated with this relapsed leukemia. Surprisingly, despite the E698A substitution in MSH2, identical microsatellite patterns of all six markers were detected in P1's skin and blood samples

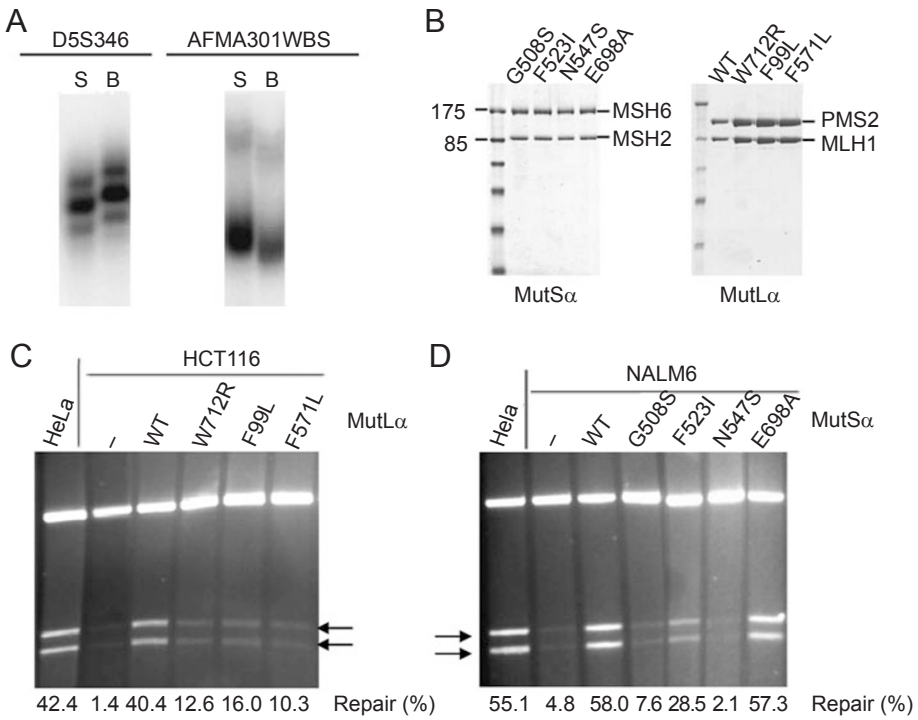

Figure 3 Microsatellite instability and MMR deficiency in AML patients. **(A)** MSI analysis. Distinct patterns were detected in dinucleotide repeat markers D5S346 and AFMA301WB5 between blood (B) and skin (S) samples derived from patient P17. **(B)** SDS PAGE of purified recombinant wild type and mutant MutSα and MutLα proteins, as indicated. **(C** and **D)** MMR assays. MMR activities of mutant MutLα and MutSα proteins corresponding to the mutations identified in AML patients were examined by their ability to restore MMR of nuclear extracts derived from the *MLH1*-deficient HCT116 cells **(C)** or the *MSH2*-deficient NALM6 cells **(D)**, respectively. Repair products (two smaller fragments) are indicated by arrows.

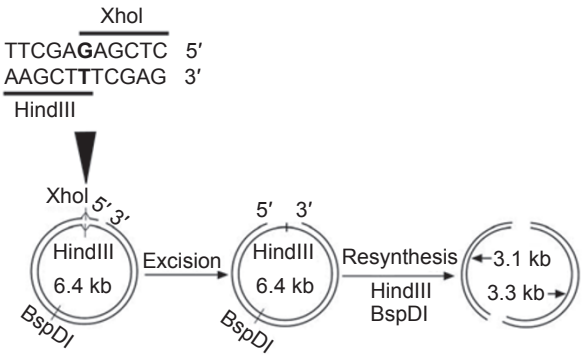

Figure 4 Diagram of DNA MMR substrate and assay.

(data not shown), indicating that MSI is not associated with this diagnostic AML and that the E698A substitution has no effect on the MMR activity.

MSH2 and MLH1 mutations identified in AML lead to MMR deficiency

To determine whether the mutations identified in AML inactivated the MMR system, selected *MSH2* (G508S, F523I, N547S, and E698A) and *MLH1* (F99L, F571L, and W721R) mutants as observed in diagnostic, relapsed, and refractory patients were generated and co-expressed with wild-type *MSH6* and *PMS2*, respectively, in the baculovirus-insect expression system [23] and their corresponding mutants MutSα and MutLα were purified (Figure 3B). The resulting MutSα and MutLα were then examined for their ability to restore MMR to nuclear extracts derived from the *MSH2*-deficient NALM6 and the *MLH1*-deficient HCT116 cells, respectively, as described [15]. The mismatched DNA substrate used and the principle of the *in vitro* assay were depicted in Figure 4 (also see Materials and Methods for description). As shown in Figure 3C, all three MutLα mutants showed little ability to complement the HCT116 extract in repair of a G-T mismatch-containing heteroduplex, indicating that the individual *MLH1* mutations identified in AML patients indeed lead to a defective MMR system. In *vitro* repair assays using MutSα mutants revealed that while the MSH2(G508S)- or MSH2(N547S)-containing MutSα failed to complement NALM6 in the repair of the G-T heteroduplex, the MSH2(F523I)-containing MutSα mutant partially restored MMR to NALM6 extracts (Figure 3D), indicative of a reduced MMR activity for this mutant. However, the MutSα with the E698A substitution in MSH2 exhibited an MMR activity comparable to that observed with wild-type MutSα (Figure 3D), suggesting that E698A is likely an MSH2 polymorphism. This result also explains why MSI was not identifiable in P1. We therefore conclude that the majority of mutations identified in AML in this study indeed lead to a defective MMR system.

Higher frequency of MMR deficiency in patients with refractory and relapsed AML

We compared the MMR status in specimens taken at diagnosis vs those in treatment failure due to refractory disease, including primary refractory cases and those in relapse. As shown in Table 2, 21.4% (6 out of 28) of samples taken at diagnosis were associated with defects in MMR, but 48% (12 out of 25) of samples taken from primary refractory or relapsed disease had lost the MMR function. It is worth noting that the difference in the frequency of MMR defect between these groups could be underestimated since paired diagnostic/refractory samples were generally not available for this study. Thus, it was unknown whether the diagnostic patients with MMR defects underwent relapse. Nevertheless, the Fisher's Exact test using the current numbers (48% in the refractory/relapse samples vs. 21.4% in the diagnostic samples) revealed that the difference in the frequency of MMR defect between these groups is significant ($P < 0.05$, Table 2). This result suggests that patients with primary refractory and relapsed AML have a much higher probability of losing the MMR function than diagnostic patients.

Correlation between patient karyotypes and MMR defects

Mutational data were analyzed for possible links with AML karyotypes. Of 39 patients with available cytogenetic information, 14 and 25 exhibited normal and abnormal karyotypes, respectively (Table 3). Among the 14 normal karyotype patients, eight (P3, P4, P8, P10, P13, P18, P19, and P21, see Table 1) of them (57%) were found to carry alterations that were proved or predicted (A120V in P4) to

Table 2 Frequency of MMR defects in diagnosis and relapse/refractory AML

Sample	Case	MMR defect				Freq. (%)	*P*-value
		MSH2	*MLH1*	Methyl[1]	Total		
Diagnosis	28	2	3	1	6	21.4	< 0.05
Relapse & refractoriness	25	4	5	3	12	48.0	
Total	53	6	8	4	18	34.0	

[1]Hypermethylation of MLH1 promoter.

Table 3 Relationship between AML karyotype and MMR defect

Karyotype	Total	MMR defect	Freq. (%)	*P*-value
Abnormal	25	5	20	<0.05
Normal	14	8	57	

inactivate the MMR system. Although eight out of the 25 patients with abnormal karyotype patients displayed alterations in MMR genes, only five (P9, P12, P15, P17, and P20, see Table 1) of them (20%) led to a defective/reduced MMR function. The difference in the rate of MMR deficiency between these two categories is statistically significant ($P = 0.023$; Table 3). We therefore conclude that AML patients with normal karyotype are associated with MMR defects. Similar analysis was also applied to patient age and gender, but correlations were not identified between MMR defects and patient age or gender (data not shown).

Discussion

While the importance of the MMR system in preventing carcinogenesis has been well established in solid cancers, including HNPCC and sporadic colorectal cancer, the involvement of the repair system in human hematological malignancies, especially AML, is less well defined. We provide evidence here that like HNPCC and sporadic colorectal cancer, a significant fraction (>30%; see Table 2) of AML patients exhibited mutations in key MMR genes, *MSH2* and *MLH1*, or hypermethylation of the *MLH1* promoter, suggesting that loss of MMR function is associated with the development of AML.

Interestingly, we identified a close association of MMR defects with the normal karyotype (Table 3). This result correlates well with the finding in HNPCC, the classical MMR-deficient cancer syndrome [24]. HNPCC tumors display instability in microsatellite and other DNA sequences [6, 8, 25], but no chromosome instability [26]. Likewise, MMR deficiency in AML is also closely linked to patients with the normal karyotype. These AML patients are classified as the intermediate risk group, characterized by point mutations, small duplications, or deletions in genes such as *FLT3*, *NPM1*, *MLL*, and *CEBPA* [2, 3]. These types of mutations in the intermediate risk group of AML are typical phenotypes observed in cells defective in MMR [2, 3].

In all AML cases where abnormal SSCP products were detected, we observed both wild type and mutated alleles of the targeted amplicon (Figure 1). This phenomenon can be simply interpreted as heterozygous mutations. It is also possible that the phenomenon reflects a mixture of MMR proficient and defective leukemic cells. Based on the information from this and previous studies, we favor the latter assumption. First, DNA samples used in this study were isolated from all leukocytes likely to contain both MMR deficient and proficient cells. Secondly, studies in HNPCC and mice have revealed that individuals with heterozygous defects (germline mutations in HNPCC or heterozygous knockout in mice) of an MMR gene generally possess a functional MMR system and do not display MSI [27]. Thus, the identification of MSI in AML (Figure 3A and Refs. [9-13]) suggests a complete loss of the MMR function in a significant fraction of leukemic cells in AML patients. Therefore, we believe that the observation of both wild type and mutant alleles in *MSH2* and *MLH1* amplicons is not due to heterozygous mutation of the genes, but the presence of both MMR proficient and deficient leukemic cells in the samples analyzed.

It is well documented that although ~80% of adult patients diagnosed with AML achieve a CR after intensive chemotherapy, more than 70% of these patients eventually relapse [28]. The molecular mechanism underlying AML relapse is not fully understood. However, increasing evidence suggests that leukemia relapse may be related to minimal residual disease (MRD), a small fraction (below the threshold of morphological detection) of leukemic cells persisting within leukemia patients after achieving CR. Since high levels of MRD are significantly associated with a high frequency of relapse and a short duration of survival, MRD has been considered an important risk factor for leukemia relapse [29, 30]. It has been postulated that MRD cells adopt a drug-resistant phenotype during the course of chemotherapy and have the potential to form a regrowing leukemic population, thereby leading to leukemia relapse [31]. Interestingly, the drug resistant phenotype of MRD cells is similar to that of tumor cells defective in MMR [7].

Previous studies have established the following concepts: (i) cells defective in MMR are highly resistant to a number of chemotherapeutic drugs and other chemicals, including methylators, cisplatin, 6-thioguanine, 5-fluorouracil, and environmental carcinogens [7, 32-34]; (ii) cells can acquire MMR deficiency with continuous exposure to therapeutic drugs [16-18]. The primary treatment for AML is chemotherapy and a complete remission usually requires extensive treatment [1]. Under these conditions, the vast majority of leukemic cells are killed, but a small number of cells may become resistant, possibly due to the pre-existing and/or drug-induced mutator phenotype, e.g., defect in MMR, and these cells could eventually be the MRD cells. Although it remains to be determined if these MMR deficient cells represent MRD cells, deficiency in MMR renders these cells resistant to drug-induced apoptosis [7]. Given the importance of the MMR system in maintaining genomic stability, proliferation of these MMR-deficient

cells will lead to hypermutations that favor uncontrolled expansions of the hypermutable cells. Therefore, it is possible that leukemia relapse is originated from leukemic cells defective in MMR that have survived the therapeutic treatments. This also applies to refractory patients, who exhibit an earlier resistant phenotype. In support of this hypothesis, a significantly higher rate of MMR deficiency in samples from relapsed/refractory AML was observed compared to those from diagnostic AML (Table 2). This number may be underestimated given the fact that SSCP analysis cannot identify all mutations, and that other MMR genes, e.g., *MSH6*, *PMS2*, and *EXO1*, whose defects also lead to loss of MMR function and genomic instability, were not analyzed in this study. Therefore, the loss of MMR function is significantly correlated with treatment failure (primary refractory and relapsed AML) in AML patients, particularly those with normal karyotypes. Since MMR-deficient cancer cells are resistant to certain chemotherapeutic drugs and that the current AML protocol achieves the highest success in complete remission when combined with chemotherapy, understanding how the MMR system sensitizes cellular responses to drug treatments is likely to contribute importantly to more promising treatments for AML.

Materials and Methods

Blood samples from AML patients and normal control

Following IRB approval, peripheral blood samples from 53 patients with diagnostic, refractory, or relapsed AML and 17 control individuals with no history of leukemia and other cancers were collected either from the University of Kentucky Hospital or from the existing specimens of the Tissue Procurement Service Center of the hospital. Samples selected contained no therapy-related leukemia patients. Among the AML patients, there were 40 male and 13 female. The age of the youngest patient was 1 and the oldest was 91. Using criteria described by Cheson *et al*. [35], samples were defined as (1) diagnostic; (2) primary refractory (also called persistent) – samples taken after induction therapy from patients with evidence of disease (blast count ≥ 5% or blasts with Auer rods, abnormal karyotype/FISH or with an aberrant leukemic immunophenotype); (3) relapsed – samples with disease taken after diagnosis from patients who were at least temporarily devoid of disease as defined above. The samples were evaluated in a blinded manner in this study. Peripheral blood cells collected from patients and controls were fractionated by Ficoll-Paque (Pharmacia Biotech) density gradient centrifugation and the white blood cells were isolated and used for genomic DNA preparations employing QIAamp Blood Kit (Qiagen, Valencia, CA, USA) as instructed by the manufacturer.

Mutation detection

Mutations in the *MSH2* and *MLH1* genes were screened using PCR-based single-strand conformation polymorphism (SSCP) analysis combined with DNA sequencing. PCR primers were designed to amplify 200-350 bp fragments of individual exons and exon-intron junctions of *MSH2* and *MLH1*. These procedures were performed essentially as described previously [36]. PCR products were excised from SSCP gels, amplified by PCR, and sequenced.

Methylation-specific PCR

DNA methylation in *MSH2* and *MLH1* promoter regions was determined by MSP. MSP distinguishes methylated from unmethylated alleles based on sequence changes produced by sodium bisulfite modification, which converts unmethylated cytosine but not methylated cytosine to uracil. PCR primers were designed to anneal to methylated or unmethylated DNA and selectively amplify the methylated or unmethylated target DNA. MSP-PCR was performed essentially as previously described [37].

Microsatellite instability assay

Six microsatellite markers (D3S1298, D5S346, D17S250, D3S1611, D11S614, and AFMA301WB5) were used to determine MSI in AML patients whose both blood samples and non-cancer tissues were available. PCR sense primers were end-labeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase (USB Corp., Cleveland, OH, USA) prior to their inclusion in PCR. Final products were analyzed by electrophoresis on 6% denaturing polyacrylamide gels and, detected by autoradiography.

Heteroduplex preparation and MMR assay

The DNA heteroduplex used in this study was a 6.4-kb circular molecule containing a G-T mismatch and a strand break 128 bp 5′ to the mismatch (Figure 4). The DNA substrate was constructed utilizing DNA derived from f1MR phage series [38]. The mismatch was located in the overlapping recognition sequence of two restriction endonucleases so that the DNA substrate is resistant to digestion by both endonucleases. However, the nick-directed MMR and subsequent repair DNA synthesis render the DNA substrate sensitive to one of the restriction enzymes, which can be used to score the repair of the mismatch (Figure 4). Unless otherwise specified, MMR assays were performed in a 15-μl reaction containing 50 μg of *MSH2*- or *MLH1*-deficient nuclear extract, 24 fmol (100 ng) heteroduplex DNA, 10 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 1.5 mM ATP, and 0.1 mM each of the four dNTPs, in the presence or absence of 100 ng of MutSα or MutLα as described [15]. After incubation at 37 °C for 15 min, DNA samples were recovered by phenol extraction and ethanol precipitation and double-digested with *Bsp*DI/*Hin*dIII. Reaction products were separated on a 1% agarose gel and visualized by UV-illumination in the presence of ethidium bromide.

Statistical analysis

χ^2 and Fisher's exact tests were used for analysis of statistical significance. $P < 0.05$ was designated as significant.

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