

# An Intron Splice Acceptor Polymorphism in *hMSH2* and Risk of Leukemia after Treatment with Chemotherapeutic Alkylating Agents<sup>1</sup>

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## ABSTRACT

**Purpose:** We sought to determine whether the –6 exon 13 T>C polymorphism in the DNA mismatch repair gene *hMSH2* modulates susceptibility to acute myeloid leukemia after therapy and particularly after O<sup>6</sup>-guanine alkylating chemotherapy. We also determined the extent of microsatellite instability (MSI) in therapy-related acute myeloid leukemia (t-AML) as a marker of dysfunctional DNA mismatch repair.

**Experimental Design:** Using a novel restriction fragment length polymorphism, verified by direct sequencing, we have genotyped 91 t-AML cases, 420 *de novo* acute myeloid leukemia cases, and 837 controls for the *hMSH2* –6 exon 13 polymorphism. MSI was evaluated in presentation bone marrow from 34 cases using the mononucleotide microsatellite markers BAT16, BAT25, and BAT26.

**Results:** Distribution of the *hMSH2* –6 exon 13 polymorphism was not significantly different between *de novo* acute myeloid leukemia cases and controls, with heterozygotes and homozygotes for the variant (C) allele represent-

ing 12.2 and 1.6%, respectively, of the control population. However, the variant (C) *hMSH2* allele was significantly overrepresented in t-AML cases that had previously been treated with O<sup>6</sup>-guanine alkylating agents, including cyclophosphamide and procarbazine, compared with controls (odds ratio, 4.02; 95% confidence interval, 1.40–11.37). Thirteen of 34 (38%) t-AML cases were MSI positive, and 2 of these 13 cases were homozygous for the variant (C) allele, a frequency substantially higher than in the control population.

**Conclusions:** Association of the *hMSH2* –6 exon 13 variant (C) allele with leukemia after O<sup>6</sup>-guanine alkylating agents implicates this allele in conferring a non-disabling DNA mismatch repair defect with concomitant moderate alkylation tolerance, which predisposes to the development of t-AML via the induction of DNA mismatch repair-disabling mutations and high-grade MSI. Homozygosity for the *hMSH2* variant in 2 of 13 MSI-positive t-AML cases provides some support for this model.

## INTRODUCTION

t-AML<sup>3</sup> was initially recognized >30 years ago in patients treated for multiple myeloma with melphalan (1). t-AML has since been reported subsequent to numerous primary malignancies, including Hodgkin's disease, non-Hodgkin's lymphoma, breast cancer, ovarian cancer, and testicular cancer (2–7). This devastating condition now represents up to 10% of all AML cases.

The clinical characteristics of t-AML differ according to the nature of the therapeutic exposure. After treatment with DNA topoisomerase II inhibitors such as epipodophyllotoxins, t-AML has a relatively short latency period (typically up to 2 years) often with no preleukemic phase and is associated with translocations involving the *MLL* gene at 11q23 (8). In contrast, t-AML after treatment with chemotherapeutic alkylating agents such as nitrogen mustards, nitrosoureas, platinum-based compounds, and hydrazines is characterized by a longer latency period (up to 10 years) often presenting with myelodysplasia and is associated with loss or deletions of chromosome 5 and/or 7 and p53 mutations (9, 10). These observations suggest multiple genetic mechanisms by which t-AML may arise. The prevalence of MSI suggests that dysfunctional DNA mismatch repair

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<sup>3</sup> The abbreviations used are: t-AML, therapy-related acute myeloid leukemia; AML, acute myeloid leukemia; HNPCC, hereditary non-polyposis colorectal carcinoma; MSI, microsatellite instability; t-MDS, therapy-related myelodysplastic syndrome; MRC, Medical Research Council; RFLP, restriction fragment length polymorphism; FAB, French-American-British; OR, odds ratio; 95% CI, 95% confidence interval.

may be one such mechanism of malignant transformation in t-AML (11).

The primary function of DNA mismatch repair is to correct replicative errors that escape DNA polymerase proofreading. In humans, the Mut $\alpha$  complex (composed of MSH2 and MSH6) and the Mut $\beta$  complex (MSH2 and MSH3) recognize and bind to single base mismatches and insertion/deletion loops in DNA (12, 13). Subsequent recruitment of MutL $\alpha$  (MLH1 and PMS2) facilitates degradation and resynthesis of the mispaired region in the newly synthesized daughter strand (14). Although defects in any of the proteins can impair the DNA mismatch repair system, the process is most greatly affected by abnormal MSH2 or MLH1 function (15, 16). Cells manifesting mutations as a result of dysfunctional mismatch repair are described as having a mutator phenotype. In human studies, the mutator phenotype has been demonstrated in a variety of sporadic cancers and inherited conditions, including HNPCC (17), and is associated with the presence of extensive MSI. Microsatellites are short, often polymorphic repeat sequences dispersed throughout the genome. Alterations in microsatellite length occur because of errors in replication, and instability at these sites is indicative of defects in DNA mismatch repair. MSI in hematological disorders is rare, but it has been observed in acute leukemia, including t-AML. Although Ben-Yehuda *et al.* (11) reported MSI in >90% of t-AML and t-MDS patients, subsequent studies have reported a lower incidence in t-AML (up to 50% of cases; Refs. 18–21). Generally, the reported incidence of MSI in *de novo* AML (0–30% of cases) is comparatively lower than in t-AML (18, 20, 21). Critically, abnormal MSH2 expression has been reported in microsatellite unstable AML, including t-AML (19), providing additional evidence for dysfunctional mismatch repair in these cases.

Exon 13 of *hMSH2* is located in the most conserved region of the gene and encodes part of the DNA binding domain (22, 23). Previous studies have identified a T>C transition polymorphism at position –6 of the 3' splice acceptor site of exon 13 (22, 24). Polymorphisms within the 3' splice junction consensus sequence between +1 and –16 may alter the efficiency of RNA splicing (25). The –6 exon 13 polymorphism lies within a short poly(T) tract forming an atypical 3' splice acceptor sequence, which may weaken splice site recognition. The location of the –6 exon 13 T>C polymorphism has led to suggestions that the variant *hMSH2* sequence may result in alternatively spliced mRNA. Although this has been reported (26, 27), including transcripts lacking exon 13, there appears to be no relation with the –6 T>C exon 13 polymorphism (26, 28). Thus, the functional significance of the *hMSH2* –6 exon 13 polymorphism remains unclear.

Fishel *et al.* (22) observed heterozygosity for the –6 exon 13 transition polymorphism in the germ-line of affected individuals, but not unaffected individuals, from two chromosome 2-linked (*hMSH2*) HNPCC families, although this association remains unconfirmed. Subsequent studies have reported a significant association between heterozygosity for this polymorphism and susceptibility to sporadic colon cancer (24, 26, 28). In hematological studies, the –6 exon 13 transition polymorphism has been shown to be prevalent among human precursor T-cell lymphoblastic lymphoma cases (23). Also, the variant allele has

been significantly associated with non-Hodgkin's lymphoma (29).

The low frequency of the *hMSH2* T>C splice acceptor polymorphism has precluded analysis of any potential disease association in the homozygous state. In this study, we have determined the distribution of the *hMSH2* T>C –6 exon 13 polymorphism in a case-control series of 420 *de novo* AML cases and 837 matched unaffected controls. We have also determined genotype distribution in a case series of 91 individuals with t-AML. Where possible, MSI was also analyzed in corresponding samples as a marker of dysfunctional DNA mismatch repair and related to *hMSH2* genotype and therapeutic exposure leading to t-AML.

## MATERIALS AND METHODS

### Genotyping for the –6 Exon 13 Polymorphism

**Samples.** The *hMSH2* –6 exon 13 polymorphism has been examined in three groups of subjects; there were 91 t-AML cases, 420 *de novo* AML cases, and 837 unaffected controls (Table 1). For this study, t-AML is defined as AML after chemotherapy and/or radiotherapy diagnosed at least 2 months after the start of the initial cytotoxic therapy. All *de novo* AML cases, unaffected controls, and 24 t-AML cases were obtained as part of a United Kingdom population-based case-control study of adult acute leukemia that has been described elsewhere (30). Genotyping was performed using DNA extracted from presentation peripheral blood for all *de novo* AML cases and 24 t-AML cases. DNA extracted from presentation bone marrow was used for genotyping the remaining 67 t-AML cases. We acknowledge the remote possibility that blast contamination of peripheral blood or bone marrow samples may cause misgenotyping because of allelic loss in blast cells, which may cause a heterozygote to appear as a homozygote. However, we feel that this would be extremely unlikely for two main reasons: firstly, chromosome 2p (*hMSH2*) is rarely affected by deletion or any aberration in AML; secondly, even in patients with a high presentation blast content, we feel it is extremely unlikely that a peripheral blood or marrow sample would be completely devoid of normal constitutive DNA.

Additional DNA samples were obtained from 67 subjects with t-AML enrolled in the MRC AML trials 10, 11, and 12 (31). Eighty-nine of the 91 t-AML cases have been described in detail elsewhere (32). The remaining 2 t-AML cases were both female ages 55.4 years (FAB type M2) and 57.5 years (FAB type M5) at diagnosis of t-AML (and both had received radiotherapy only for a previous condition). Cytogenetic analysis was successful for the younger case only [50, XX, +8, +14, +21, +22 (Ref. 9)/46, XX (Ref. 1)]. All 420 *de novo* AML cases, 837 controls, and 69 of 91 t-AML cases were confirmed as United Kingdom Caucasians. Ethnicity data on the remaining 22 t-AML cases were not available. However, because the United Kingdom population is composed predominantly of Caucasians, it remains likely that most, if not all, of the remaining 22 t-AML cases were Caucasian. All 16 t-AML cases previously exposed to *O*<sup>6</sup>-guanine alkylating agents were confirmed as United Kingdom Caucasians. All subjects taking part in the Leukaemia Research Fund acute leukemia case-control study and the MRC AML trials 10, 11, and 12 gave informed consent.

Table 1 Description of controls, *de novo* AML, cases and t-AML cases genotyped for the *hMSH2* -6 exon 13 polymorphism and analyzed for MSI

	Samples genotyped for the <i>hMSH2</i> -6 exon 13 polymorphism <i>n</i> (%)					Samples analyzed for MSI <i>n</i> (%)			
	Controls	<i>de novo</i> AML	t-AML			Controls	t-AML		t-MDS North America and Finland
			All cases	Radiotherapy	Chemotherapy		United Kingdom	North America and Finland	
Total	837 (100)	420 (100)	91 (100)	40 (100)	51 (100)	29 (100)	26 (100)	8 (100)	12 (100)
Sex									
Male	442 (52.8)	227 (54.0)	36 (39.6)	11 (27.5)	25 (49.0)	19 (65.5)	10 (38.5)	1 (12.5)	0 (0)
Female	395 (47.2)	193 (46.0)	55 (60.4)	29 (72.5)	26 (51.0)	10 (34.5)	16 (61.5)	7 (87.5)	12 (100)
Age, years									
16-39	233 (27.8)	132 (31.4)	16 (17.6)		16 (31.4)	4 (13.8)	5 (19.2)	0 (0)	1 (8.3)
40-54	269 (32.1)	131 (31.2)	21 (23.1)	11 (27.5)	10 (19.6)	13 (44.8)	5 (19.2)	1 (12.5)	2 (16.7)
55 or older	335 (40.0)	157 (37.4)	54 (59.3)	29 (72.5)	25 (49.0)	12 (41.4)	16 (61.5)	7 (87.5)	9 (75)
Mean (SD)	48.8 (14.0)	47.5 (14.5)	55.3 (13.4)	61.0 (8.5)	50.8 (14.8)	52.5 (10.7)	54.8 (13.2)	60.9 (8.9)	59.2 (12.8)
DNA amplified	776 (92.7)	395 (94.0)	90 (98.9)	40 (100)	50 (98.0)	BAT25 26 (89.7) BAT26 28 (96.6) BAT16 26 (89.7)	24 (92.3) 23 (88.5) 21 (80.8)	7 (87.5) 5 (62.5) 8 (100)	10 (83.3) 11 (91.7) 11 (91.7)

***hMSH2* Genotyping by RFLP-PCR.** Forward (5'-TGTGGGAGGCTGTGGTTC-3') and reverse (5'-CTCCCATATGGGGCCTGCA-3') primers were used to amplify the *hMSH2* sequence flanking the -6 exon 13 polymorphism. The reverse primer was designed with a mispaired base at position 18 (corresponding to position -3 of exon 13) generating an artificial *PstI* restriction site upon amplification of the variant allele only (Fig. 1). PCR amplifications were performed using 0.5 units of AmpliTaq Gold (Perkin-Elmer), 10 pmol of each primer, 200  $\mu$ M of each deoxynucleotide triphosphate, and 1.5 mM MgCl<sub>2</sub> in a 20- $\mu$ l reaction mix containing 1 $\times$  PCR reaction buffer and ~50 ng of template DNA. PCR conditions were as follows: cycle 1, 15 min at 95°C; cycles 2-37, 1 min at 95°C; 1 min at 64°C, followed by 1 min at 72°C; and cycle 38, 10 min at 72°C. PCR products were digested with 20 units of *PstI* (New England Biolabs) at 37°C for 4 h. Digestion products were separated on a 3% agarose gel and visualized against a 100-bp ladder (Fig. 1).

**DNA Sequencing.** To confirm that the RFLP-PCR was discriminating between polymorphic *hMSH2* alleles, 60 samples were sequenced (14 TT homozygotes, 25 heterozygotes, and 21 variant CC homozygotes, as determined by the RFLP-PCR method). Firstly, DNA was amplified using forward (5'-GCAGCTGTGGTCTGCT-3') and reverse (5'-TTGATTTACCTCCATAT TGGG-3') *hMSH2* primers flanking the -6 exon 13 polymorphism, using the reaction condition described above at an annealing temperature of 60°C. Amplification products were then sequenced using the AmpliTaq Big Dye terminator cycle sequencing kit (Applied Biosystems). Sequencing electrophoresis was performed on the ABI Prism 377 sequencer, and results were analyzed using the ABI Prism 377 software. Of the 60 samples analyzed, 58 of the DNA sequencing results correlated with RFLP-PCR genotyping, confirming that the RFLP-PCR can discriminate between wild-type and variant alleles with a minimal error rate (3%). In both discrepant cases, heterozygotes by RFLP were subsequently sequenced as homozygotes (1 TT and 1 CC). Thus, to minimize the error frequency, all heterozy-

gotes and homozygotes for the variant (C) allele and all t-AML cases irrespective of status were genotyped at least twice. In a few samples, a third assay was performed if the first two assays gave discrepant results. Thus, the final genotyping error frequency is likely to be <3%.

**Statistical Analysis.** ORs and 95% CIs, adjusted for age and sex, were calculated using unconditional logistic regression (33). *hMSH2* genotype was analyzed as a trichotomous (TT, TC, CC) or dichotomous (TT, TC + CC) variable. All analyses were conducted using Stata 1999 (Stata, College Station, TX).

## MSI

**Samples.** Presentation bone marrow samples were available for 46 cases (Table 1), including 34 cases of t-AMLs and 12 cases of t-MDSs. Twenty-six of the t-AML cases were of United Kingdom origin and were part of the case series genotyped for the -6 exon 13 *hMSH2* polymorphism. The blast content in bone marrow samples from 19 of the t-AML cases was  $\geq$ 40% (median of 70%). Data regarding blast content was not available for the remaining 7 samples. Although leukemic blasts could not be separated from normal cells, a previous study shows that MSI can unambiguously be detected in samples containing low amounts of leukemic blasts (34). The remaining 8 t-AML and 12 t-MDS cases were of Finnish and North American origin and were included in prior epidemiological studies (5, 6, 10). One of the t-AML cases was male who had previously received chemotherapy for testicular cancer. The remaining t-AML and t-MDS cases were all female and had received platinum-based chemotherapy for ovarian cancer before diagnosis of t-AML or t-MDS. Data regarding blast content was available for 2 of the t-AML cases (40 and 50%) and 3 of the t-MDS cases (16, 15, and 11%) within the non-United Kingdom patient series.

Two mismatch repair-proficient (TK6 and SW480) and three mismatch repair-deficient (LoVo, MT1, and HCT116) human cell lines were used as negative and positive controls for MSI, respectively. LoVo and HCT116 have both alleles deleted

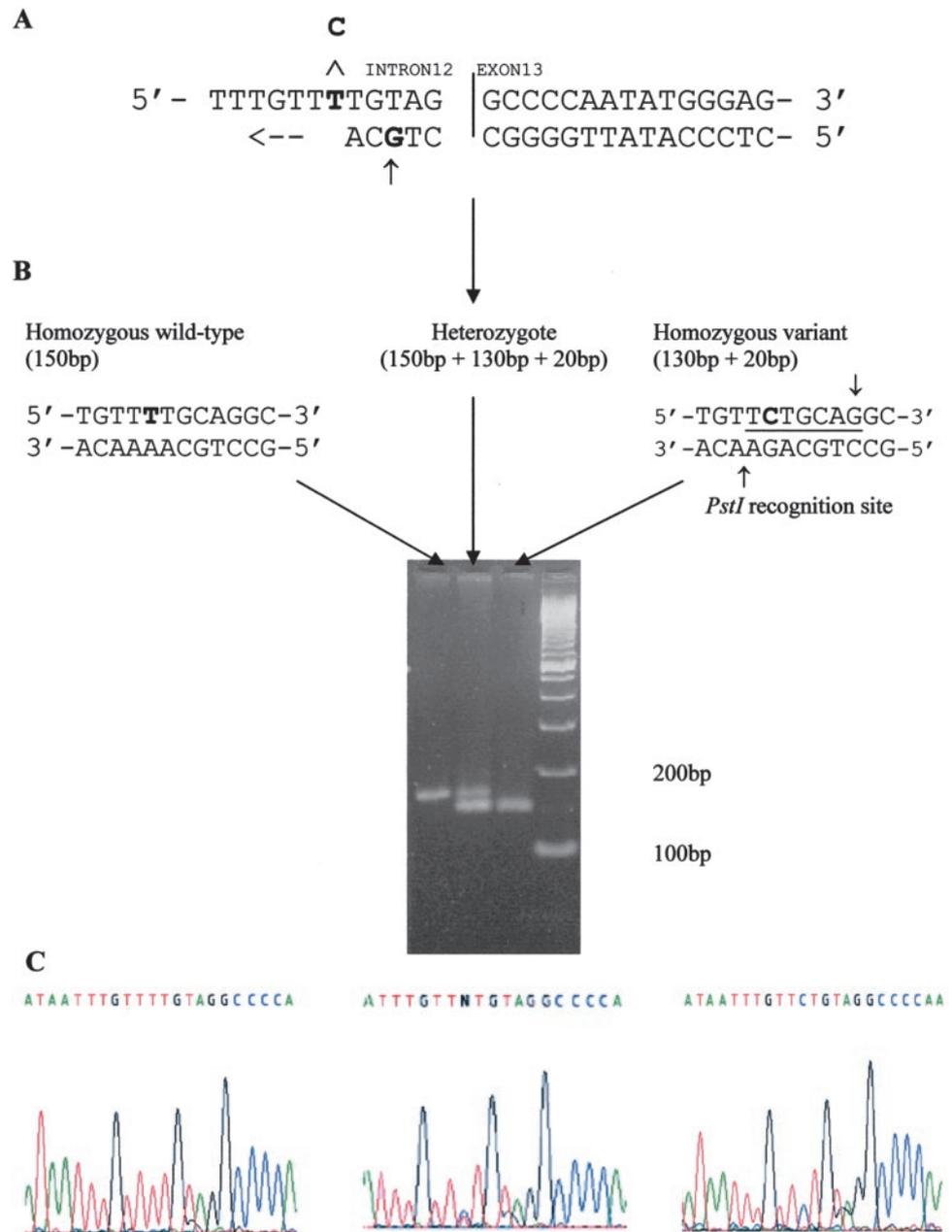


Fig. 1 Amplification of the *hMSH2* sequence flanking the -6 exon 13 (T>C) polymorphism. A, reverse primer introduces a T>C substitution at -3 bases, (B) and this forms an artificial *Pst*I restriction site in the variant allele. C, DNA sequence verification of RFLP-PCR genotype discrimination.

for *hMSH2* and *hMLH1*, respectively (35, 36), whereas MT1 is mutated in both copies of *hMSH6* (37). DNA samples extracted from the peripheral blood of 29 subjects known not to have a malignant condition were used as controls (Table 1).

**Analysis of MSI.** Primer pairs were synthesized using previously published sequences for BAT25 (chromosome 4q), BAT26 (chromosome 2p), and BAT16 (chromosome 7p) (38). One primer of each pair was 5'-end labeled with 6-carboxyfluorescein. PCR amplifications were performed using 0.5 units of TaKaRa LA TaqDNA polymerase (Takara Biomedicals, Shiga, Japan), 10 pmol of each primer, 0.4 mM of each deoxynucleotide triphosphate, and 2.5 mM MgCl<sub>2</sub>, using ~50 ng of template DNA in a 50- $\mu$ l reaction containing 1 $\times$  PCR reaction buffer. PCR conditions were as fol-

lows: cycle 1, 10 min at 94°C; cycles 2–37, 1 min at 94°C, followed by 1 min at 56°C for BAT16 or 55°C for BAT25 and BAT26 and then 1 min at 72°C; and cycle 38, 10 min at 72°C. Amplified PCR products were analyzed using an automated 373A DNA sequencer (Applied Biosystems), and results were analyzed using the Applied Biosystems Genescan software in accordance with manufacturers instructions. All three mononucleotide markers are quasimonomorphic, eliminating the requirement for constitutional DNA (38–40). MSI was diagnosed as the presence of a clearly discernable peak not present in the control population, as illustrated in Fig. 2. Normal cells could not be separated from blast cells and so a peak representing the stable constitutive alleles was always seen in the t-AML case DNA. In contrast, the mismatch

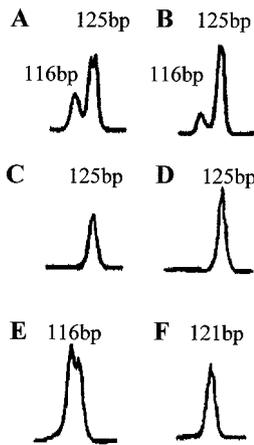


Fig. 2 Electropherograms show MSI in t-AML cases 76 (A) and 65 (B); Mismatch repair-proficient cell lines TK 6 (C) and SW480 (D); and mismatch repair deficient cell lines LoVo (E) and MT1 (F). PCR amplicon sizes are indicated and were estimated using a GeneScan-350 TAMRA size standard as a reference.

repair defective cell lines LoVo and MT1 displayed only a single unstable peak (Fig. 2). All t-AML and t-MDS cases for whom bone marrow was available were examined for MSI at all three loci.

**RESULTS**

**Distribution of the *hMSH2* –6 Exon 13 Polymorphism in *de Novo* AML and t-AML.** The *hMSH2* T>C polymorphism at the –6 position of the splice acceptor site of exon 13 was not significantly associated with *de novo* AML (Table 2), although the *de novo* AML case series has a marginally higher percentage of individuals heterozygous and homozygous for the lower frequency variant (C) allele (15.7%), compared with controls (13.8%; OR, 1.17; 95% CI, 0.83–1.64; Table 2). *hMSH2* polymorphic status was not associated with gender in either the control or *de novo* AML case series (data not shown) or with cytogenetic subgroup in the *de novo* AML case series (data not shown).

*MSH2* is known to modulate apoptosis in response to alkylating agents, including several chemotherapeutic alkylating agents, and in particular those that alkylate the *O*<sup>6</sup> position of guanine in DNA (41, 42). The t-AML case series described in this study has a high proportion of individuals whose leukemia developed subsequent to chemotherapeutic alkylating agents (32). Individuals heterozygous or homozygous for the *hMSH2* variant allele were nonsignificantly overrepresented in the chemotherapy-related t-AML case-series, compared with controls (OR, 1.56; 95% CI, 0.75–3.21; Table 2). Sixteen of 91 t-AML cases had documented previous treatment with chemotherapeutic *O*<sup>6</sup>-guanine alkylating agents, which included procarbazine, dacarbazine, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, and cyclophosphamide. Consistent with a role in conferring susceptibility to *O*<sup>6</sup>-guanine alkylating agent-induced myeloid leukemia specifically, 6 of these 16 (37.5%) cases were carriers of at least one variant *hMSH2* allele, a frequency significantly higher than in both the control series (13.8%; OR, 4.02; 95% CI, 1.40–11.37; Table 2) and the *de novo* AML cases series (15.7%;

Table 2 Number of controls, *de novo* AML cases, and t-AML cases by *hMSH2* status, ORs and 95% CIs

	Controls n (%)		<i>de novo</i> AML n (%)		t-AML n (%)		Therapy		OR (95% CI) <sup>a</sup>	OR (95% CI) <sup>a</sup>		
	n	(%)	n	(%)	n	(%)	Radiotherapy	Chemotherapy <sup>b</sup>				
TT	669	(86.2)	333	(84.3)	77	(85.6)	37	(92.5)	1	10	(62.5)	1
TC	95	(12.2)	52	(13.2)	11	(12.2)	3	(7.5)	0.99	5	(31.3)	3.81
CC	12	(1.6)	10	(2.5)	2	(2.2)	0	0	1.52	1	(6.3)	5.55
									0.56	2.81	(0.61–13.03)	1.40
									0.56	2.81	(0.17–1.95)	1.40
TT	669	(86.2)	333	(84.3)	77	(85.6)	37	(92.5)	1	10	(62.5)	1
TC+CC	107	(13.8)	62	(15.7)	13	(14.4)	3	(7.5)	0.51	6	(37.5)	4.02
									0.51	1.56	(0.15–1.73)	1.56
									0.51	1.56	(0.75–3.21)	1.56

<sup>a</sup> ORs and 95% CIs were estimated using logistic regression adjusting for sex and age at diagnosis in comparison with controls.

<sup>b</sup> Chemotherapy alone or in combination with radiotherapy.

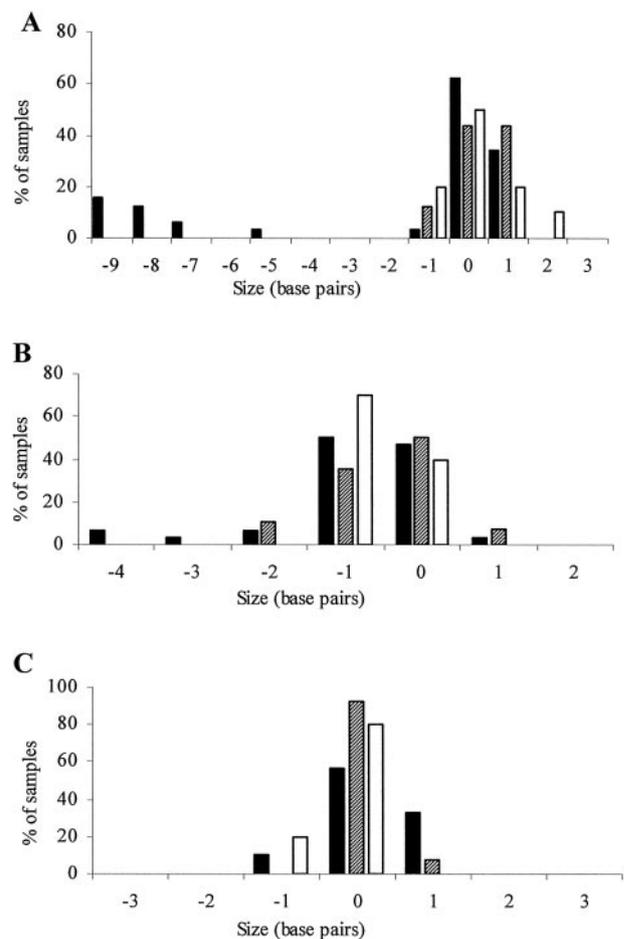
<sup>c</sup> — indicates that none of the specified subjects fall into this category.

OR, 3.10; 95% CI, 1.08–8.89). In contrast, 4 of 34 (11.8%) cases who received chemotherapy that did not include an  $O^6$ -guanine alkylating agent were carriers of at least one variant *hMSH2* allele, a frequency not significantly different from either the controls (13.8%) or the *de novo* AML case series (15.7%).

**MSI in t-AML.** To investigate whether DNA mismatch repair dysfunction may be a characteristic feature of our t-AML case series, we examined three quasimonomorphic mononucleotide microsatellite markers for instability (BAT16, BAT25, and BAT26). These markers were chosen because they have been shown to be unstable in hereditary and sporadic colon cancer and some hematological malignancies (11, 40, 43, 44). Two of these markers, BAT25 and BAT26, are recommended for MSI analysis by the International Collaboration Group for HNPCC (40). Furthermore, in a study of 44 microsatellite markers and 107 colon cancers, Halford *et al.* (45) demonstrated that instability at BAT25 and BAT26 is 99 and 98% specific, respectively, for high-grade MSI (where >30% of all markers are unstable) and is indicative of DNA mismatch repair dysfunction.

MSI was detected in 13 of 34 (38%) t-AML cases for whom presentation bone marrow was available (Fig. 3, Table 3). In 12 of 13 MSI positive t-AML cases allelic shortening was found at BAT25 (Figs. 2 and 3). Two samples displayed instability at BAT26, one of which was also unstable at BAT25 (Table 3). Allelic shortening at BAT25 and BAT26 varied between 5 and 9 bases and 3 and 4 bases, respectively, consistent with published data (38, 39). MSI was not detected at BAT16 in any of the t-AML samples investigated. MSI was not detected at any locus in any of the 29 control DNA samples investigated or in either of the 2 DNA mismatch repair-proficient cell lines (TK6 and SW480) investigated. In contrast, MSI was detected in all three DNA mismatch repair-deficient cell lines. LoVo displayed MSI at BAT25 and BAT16. Amplification at BAT26 was not possible because of biallelic deletion. MT1 and HCT116 were both unstable at BAT25. MT1 was also unstable at BAT26. Instability was not detected at any of the three markers investigated in any of the 12 t-MDS cases.

**MSI and the *hMSH2* –6 Exon 13 Polymorphism in t-AML.** All 13 MSI-positive t-AML cases were of United Kingdom origin and were genotyped for the *hMSH2* –6 exon 13 polymorphism. Three of the 13 (23%) MSI-positive t-AML cases were carriers of at least one variant *hMSH2* allele (Table 3), and 2 of these 13 cases (15.4%) were homozygous for the variant *hMSH2* allele, a frequency considerably higher than in both the control population (1.6%) and *de novo* AML case population (2.5%). One case (case 56) had been treated with alkylating agent-based chemotherapy and radiotherapy for Hodgkin's disease (Table 3). The other case (case 68) had been treated with 5-fluorouracil and radiotherapy for colorectal cancer (Table 3). Of the remaining 21 MSI-negative t-AML cases for whom presentation bone marrow was available, 13 were of United Kingdom origin and were genotyped for the *hMSH2* –6 exon 13 polymorphism. Twelve of these 13 cases were homozygous for the common (T) allele, whereas the remaining case was heterozygous (data not shown). Thus, in the MSI-negative t-AML case population, the frequency of individuals with at least one copy of the variant *hMSH2* allele (7.7%) was not significantly different to either the control (13.8%) or the *de novo* AML case population (15.7%).



**Fig. 3** Distribution of allele sizes at mononucleotide markers BAT25, BAT26, and BAT16. Allelic sizes at BAT25 (A), BAT26 (B), and BAT16 (C) are shown for t-AML cases (■), t-MDS cases (□), and controls (▨). The most common allele in the control series is designated "0" bp. Deviations in allele size from this are indicated.

## DISCUSSION

A causal role for chemotherapeutic alkylating agents in t-AML etiology is well established. Furthermore, DNA mismatch repair status modulates apoptosis in response to alkylating agents, where loss of functional DNA mismatch repair confers tolerance to the apoptotic effects of some alkylating agents, particularly  $O^6$ -guanine alkylating agents (15, 16, 41, 42). DNA mismatch repair-defective cells have a concomitantly high rate of both spontaneous and alkylation-induced mutagenesis (42, 46). Thus, dysfunctional mismatch repair confers not only tolerance to the killing effects but also sensitivity to the mutagenic effects of alkylating agents, consistent with the acute susceptibility to alkylation-induced tumorigenesis in DNA mismatch repair-defective mice (47). Our finding that the variant *hMSH2* allele is significantly overrepresented in t-AML cases who specifically had previous treatment with chemotherapeutic  $O^6$ -guanine alkylating agents is suggestive that this allele may confer moderate alkylation tolerance with concomitant susceptibility to t-AML. However, we cannot exclude the possibility that polymorphic *hMSH2* may modulate susceptibility to the

Table 3 Summary of MSI-positive t-AML cases

Patient no.	Primary disorder	Presentation marrow blast content (%)	<i>hMSH2</i> status, T>C	MSI positive	Previous therapy	FAB subtype	Cytogenetics
56	Hodgkin's disease	82	CC	BAT26	Chlorambucil, prednisone, procarbazine, vinblastine, radiotherapy	M1	46, XX, der(12p) [4]/46, XX[16]
61	Hodgkin's disease	40	TT	BAT25	Unspecified chemotherapy, radiotherapy	M2	46, XX
62	NA	60	TT	BAT25	Busulphan	M1	45, XX, inv(3)(q21q26), -7
63	Breast cancer	60	TT	BAT25	5-Fluorouracil, cyclophosphamide, methotrexate, radiotherapy	M4	46, XX, inv(16)(p13q22)
64	Retinal vasculitis	NA <sup>a</sup>	TT	BAT25	Chlorambucil, radiotherapy	NOS <sup>b</sup>	45, XY, -7[30]
65	NA	80	TT	BAT25	Hydroxyurea	M5	failed
68	Colorectal cancer	NA	CC	BAT25	5-Fluorouracil, radiotherapy	M2	46, XY
70	Non-Hodgkin's lymphoma	NA	TC	BAT25	Cyclophosphamide, prednisolone, vincristine, procarbazine, radiotherapy	NOS	48, XX, +4, del(5)(q?22), -7, +8, del(12)(q?15), add(16)(p13), -17, +2mar[19]/46, XX[1]
71	NA	80	TT	BAT25	Radiotherapy	M2	46, XX
72	NA	NA	TT	BAT25	Radiotherapy	M4	46, XY, der(9)t(9;?17)(q34;q?21)[2]/46, XY[28]
73	NA	40	TT	BAT25, BAT26	Radiotherapy	M2	44-48, X,t(X;4)(q13;q12), ?(5;22)(q13;q13), dic(7;17)(q11;q11), -12, t(14;22)(q1;q13)
76	Thrombocytosis	95	TT	BAT25	Busulphan, hydroxyurea	M5	46, XY, del(4)(q21q25), der(7)t(1;7)(q10;p10), t(9;11)(p22;q23)
78	NA	NA	TT	BAT25	Radiotherapy	M1	45, XY, del(5)(q22q33), inv(9)(p13q21)c, -14, add(16)(p), -17, +mar[2]/45, XY, idem, +8, -11, add(12)(p?11), -13, +ring[3]/46, XY, idem, +ring2[7]/58, XY, +1, +2, +4, +5, del(5), +6, +8, +inv(9)c, +10, add(11)(q22or23), -13, -14, add(16), +19, +21, +22, +mar, +r[2]

<sup>a</sup> NA indicates that data for this category was not available.

<sup>b</sup> NOS indicates no specified FAB subtype.

development of primary cancer or be involved in determining response to therapy for that cancer. Either of these may affect the prevalence of the *hMSH2* polymorphism in the population at risk of subsequently developing t-AML.

The demonstration of MSI in 13 of 34 (38%) t-AML cases investigated confirms a role for compromised DNA mismatch repair in the pathogenesis of a subset of t-AML cases. These data are consistent with reports of MSI and aberrant MSH2 expression in other t-AML case series (11, 19, 21, 43). Furthermore, our data are consistent with that of Ben-Yehuda *et al.* (11), Olipitz *et al.* (43) and Gafanovich *et al.* (44), who also used the quasimonomorphic mononucleotide markers BAT25 and BAT26 and reported MSI at these markers in 30–60% of t-AML and t-MDS cases. Instability at these loci is highly predictive of extensive high-grade MSI (greater than 30% of marker instability; Ref. 45) and dysfunctional DNA mismatch repair and is very rarely seen in tumors that have low-level or no MSI (38, 39, 45). Thus, the high specificity of BAT25 and BAT26 (99 and 98%, respectively; Ref. 45) for high-grade MSI demonstrates dysfunctional mismatch repair in a significant proportion of the t-AML cases described in this study.

The high sensitivity of BAT25 and BAT26 (78 and 90%, respectively; Ref. 45) to detect high-grade MSI and the relatively poor concordance between these two markers in our study suggests that in our hands, BAT26 is underestimating MSI. As such, we cannot exclude the possibility that we have not been able to detect high-grade MSI in t-MDS, as reported by others

(43, 48), because of insensitive MSI analysis and low or undefined presentation bone marrow blast content.

Of the 13 t-AML cases with MSI, 9 had prior exposure to chemotherapy, predominantly alkylating agents and base analogues, and 4 had received radiotherapy only. Moreover, 3 cases had an apparently normal karyotype, whereas the remaining included cases with balanced and unbalanced aberrations, including 6 cases with chromosome 5 and/or 7 aberrations. Thus, the clinical and cytogenetic heterogeneity of the MSI-positive t-AML cases supports the notion of multiple pathways by which MSI may arise in t-AML and suggests a role in hematopoietic progenitor cells for DNA mismatch repair in mediating response to mechanistically diverse genotoxic agents. Consistent with this, deletion of *Msh2* in murine stem cells confers tolerance to the killing effects of chemotherapeutic alkylating agents, chemotherapeutic base analogues, and ionizing radiation (49–51). Our observations suggest a hypothesis whereby polymorphic *hMSH2* weakly attenuates DNA mismatch repair function and confers moderate tolerance to the killing effects and concomitant susceptibility to the mutagenic effects of therapy, particularly *O*<sup>6</sup>-guanine alkylating therapy. As such, individuals carrying variant *hMSH2* may be more susceptible to the induction of therapy-induced DNA mismatch repair-disabling mutations, giving rise to a true mutator phenotype and high-grade MSI. Homozygosity for the *hMSH2* variant in 2 of 13 MSI-positive t-AML cases provides some support for this model.

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