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Methylation of the *hMLH1* promoter and its association with microsatellite instability in acute myeloid leukemia

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The *hMLH1* and *hMSH2* genes are involved in the DNA mismatch repair (MMR) pathway. Defects in either of these genes have been associated with genetic instability in a wide variety of malignancies. A molecular mechanism involved in aberrant MMR gene expression is the epigenetic silencing of transcription by promoter methylation. The importance of MMR promoter methylation in leukemia is presently unclear and we have therefore undertaken a detailed analysis of the promoter regions of *hMLH1* and *hMSH2* using the technique of bisulfite genomic sequencing. DNA from 55 patients with acute myeloid leukemia (AML) including 23 patients with therapy-related AML (t-AML) have been analyzed. Two patients with *de novo* AML demonstrated extensive methylation throughout the whole *hMLH1* region sequenced, one of whom had previously shown widespread genetic instability, measured as microsatellite instability (MSI). However methylation of *hMLH1* was not found in t-AML which has previously been associated with MSI. In addition, methylation was seen at a restricted region of the *hMLH1* promoter in both AML patients and healthy controls. The significance of this methylated region of the *hMLH1* promoter is uncertain, however, our results confirm that in some patients with AML extensive methylation of *hMLH1*, but not of *hMSH2* may occur, and as is the case in solid tumors this can be associated with the presence of a defective DNA mismatch repair pathway resulting in MSI.
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Introduction

DNA repair systems are essential for the maintenance of genetic stability. The importance of these pathways is emphasized by the large number of highly evolutionary conserved proteins. The human proteins hMLH1 and hMSH2 are two members of the mismatch repair (MMR) pathway and function to correct mismatched DNA base pairs that arise as a result of misincorporation errors during DNA replication. Particular interest was prompted in these two proteins when it was discovered that germline mutations in either one was the underlying genetic abnormality of hereditary nonpolyposis colon cancer (HNPCC).[1–4] The mutator phenotype resulting from defects in either of these genes is manifest by an elevated rate of spontaneous mutations characterized as multiple replication errors in simple repetitive DNA sequences which is functionally reflected as microsatellite instability (MSI). In addition to HNPCC, this phenotype has now been observed in the DNA from a variety of sporadic tumors.[1,5–8] A mutator phenotype has also been reported in acute myeloid leukemia (AML). Results from these studies vary but generally the incidence of MSI is low in *de novo* AML: 0–10%,[9–11] although a higher incidence has been reported in *de novo* AML in the elderly.[12] Estimates of the incidence of MSI in therapy-related AML (t-AML), which arises as a consequence of prior chemotherapy or radiotherapy, are much higher. We have found MSI in 44% of t-AML cases,[12] however, the figures in the literature vary greatly, the extremes of the estimates being 0%[11] and 94%.[13] Hence it appears that, at least in some subsets of AML, a mutator phenotype exists possibly as the result of a defective MMR system.

Although MSI has been shown to occur in a subset of patients with AML the mechanisms related to mismatch repair gene inactivation in these cases have not been fully established. Of all the MMR genes, abnormalities of *hMSH2* have been most widely investigated in AML with a mutator phenotype. Horiike *et al*[14] identified missense mutations at codon 419 of the *hMSH2* gene in two patients with therapy-related myelodysplasia. In addition, we have demonstrated LOH at the *hMSH2* loci in a subset of patients with defective hMSH2 protein expression.[15]

In solid tumors with MSI abnormalities of *hMLH1* are frequently detected but inactivation of this gene has not previously been examined in AML with a mutator phenotype. One of the most common mechanisms of inactivation of *hMLH1* in solid tumors is transcriptional silencing via promoter methylation.[16–21] A large number of genes including the MMR genes contain CpG islands within their promoters, the CpG content of these regions is increased relative to the rest of the genome and in contrast to most other CpG dinucleotides they are generally unmethylated. It has been established in a large number of these tumors that methylation of *hMLH1* correlates with transcriptional silencing demonstrated by a loss of detectable protein expression.[16,17,19–21]

Preliminary studies have been performed examining the methylation status of the *hMLH1* and *hMSH2* promoter regions in leukemia using the method of methylation-specific PCR,[22] however the results have been conflicting.[23,24] Several of the methods designed to study methylation, including methylation-specific PCR, depend upon methylated CpG dinucleotides within primer binding sites or restriction enzyme recognition sites which can be limiting. We have therefore chosen the technique of bisulfite genomic sequencing to perform a detailed methylation analysis on a large number of patients with AML, both with *de novo* and therapy-related disease, in the aim to establish the importance of this particular molecular event in AML. Unlike other methods, bisulfite genomic sequencing allows the methylation status of every cytosine residue to be examined.[25] Using this method we were able to fully characterize the methylation patterns of the CpG island associated with the *hMLH1* and *hMSH2* gene promoters in 55 AML and 25 control samples.

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Materials and methods

Samples

Blood or bone marrow samples were obtained at diagnosis from 55 patients, including 23 with t-AML. The diagnosis of AML was made using the French–American–British (FAB) criteria following conventional cytochemical and surface marker analysis. Blast cells were separated by gradient centrifugation on Histopaque (Sigma-Aldrich, Poole, UK). Control peripheral blood samples were obtained from healthy individuals. DNA was extracted from cells using QIAamp blood DNA isolation kits (Qiagen, Crawley, UK) according to the manufacturer's protocol.

Microsatellite analysis

Matched leukemic and constitutional DNA was investigated for MSI using a panel of 11 microsatellite markers as described in Das-Gupta *et al*.[12] A patient was considered to have MSI if a shift in the band pattern of the leukemic DNA was measured when comparing to reference constitutional DNA from the same patient.

Methylation analysis

DNA (1 μg) was bisulfite modified using the CpGenome modification kit (Intergen Company, Oxford, UK) according to the manufacturer's protocol. The modification procedure results in deamination of cytosine residues and their subsequent conversion to uracil, however, methylated cytosines remain unaltered. *Sss*I methyl transferase and S-adenosylmethionine (New England Biolabs, Hitchin, UK) were used to prepare methylated DNA to act as a control; 1 μg of DNA was treated with 10 units of *Sss*I methylase and 160 μM S-adenosylmethionine in the manufacturer's buffer for 4 h at 37°C. The enzyme was heat inactivated at 65°C for 20 min and the resulting methylated DNA was purified using a Wizard DNA Clean-up system (Promega, Southampton, UK). The DNA then underwent bisulfite modification as described above.

A nested PCR was performed to amplify the top strand of the promoter region of both *hMLH1* and *hMSH2* using the primers MLH1-1-4 and MSH2-1-4 respectively (Table 1). Primers were synthesised by Life Technologies (Paisley, UK). As a result of the modification process the DNA strands are no longer complementary and hence the primers are strand specific. Approximately 100 ng of modified DNA was used as a template in the first round of PCR amplification. The 50 μl reaction also consisted of 2 mM $MgCl_2$, 150 μM of each dNTP (Amersham Pharmacia Biotech, Little Chalfont, UK), 1 μM of each primer and 2 units of Amplitaq Gold (PE Applied Biosystems, Warrington, UK) in the manufacturer's buffer. Following an initial heat activation step at 95°C for 10 min amplification was performed in a PTC-100TM Programmable Thermal Controller (MJ Research, Watertown, MA, USA) using the following conditions: denaturation at 95°C for 1 min, annealing at 52°C for 1 min, extension at 72°C for 1 min for a total of 30 cycles ending with a final extension at 72°C for 10 min. The nested PCRs were performed on 4 μl of the first round PCR products using the same reagents and concentrations as above, the conditions for cycling varied only in the annealing temperature which was 57°C. The nested PCR products were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany).

Sequencing reactions were set up with approximately 200 ng of purified PCR product and 10 pmol primer using a Thermo Sequenase II dye terminator cycle sequencing premix kit according to the manufacturer's instructions (Amersham Pharmacia Biotech). The primers used for sequencing were MLH1-2-3 and MLH1-5-8 for *hMLH1* and MSH2-2-3 and MSH2-5-6 for *hMSH2* (Table 1); these primers generated both forward and reverse sequence for the top strand of modified DNA. The reactions were electrophoresed using an ABI 377 automated DNA sequencer (PE Applied Biosystems) as recommended by the manufacturer.

Allele-specific methylation studies

Individuals informative for the *hMLH1* –72 polymorphism relative to the transcriptional start (–93 relative to the translational start[26]) were identified by PCR amplification of genomic DNA, followed by direct sequencing. PCR reactions were performed under the conditions described above with the following changes: 1.5 mM $MgCl_2$; annealing temperature 60°C with the primers MLH1-FOR and MLH1-REV (Table 1).

Table 1 PCR primers used in the methylation analysis of *hMLH1* and *hMSH2*

Primer	*Sequence (5′–3′)*	*GenBank accession and primer coordinates*
MLH1-1	GTTTAGGATTTTTTGTTTTGTGATATTTGG	U83845 (614–643)
MLH1-2	GTAATATTTTTATGTATTGG	U83845 (697–716)
MLH1-3	CTATTAATTAAACAACTTAAATACCAATC	U83845 (1389–1417)
MLH1-4	CCTTCAACCAATCACCTCAATACC	U83845 (1480–1503)
MLH1-5	TTAGGAGTGAAGGAGG	U83845 (801–816)
MLH1-6	CCCTATACCTAATCTATC	U83845 (955–972)
MLH1-7	GGAGGTTATAAGAGTAGGG	U83845 (1088–1106)
MLH1-8	CCTCTACTAAAATAATC	U83845 (1179–1195)
MLH1-FOR	GAGACCCAGCAACCCACAGAGTTG	U83845 (1357–1380)
MLH1-REV	GCCACGAACGACATTTTGGCGCC	U17839 (10–32)
MLH1-OUT	TAACCCTTAAATAAACC	U83845 (155–171)
MLH1-IN	TACCAATTCTCAATCATCTC	U17839 (118–137)
MSH2-1	GTTTAGTGTTTGTIGGAAATTTTTTATTTGGTGG	AB006445 (4028–4061)
MSH2-2	GTAGTTGAGTAAATATAGAAAGGAG	AB006445 (4105–4129)
MSH2-3	CCACAAAACCCCIAATCCCTC	U41206 (470–490)
MSH2-4	ACTACTCACCICCCACTCTCT	U41206 (523–541)
MSH2-5	AATTAGGAGGTGAGGAGG	AB006445 (4382–4300)
MSH2-6	CCA CACCCACTAAACTATTTCCC	AB006445 (4342–4364)

Sequencing reactions were performed as described above also with the primers MLH1-FOR and MLH1-REV. DNA from individuals heterozygous for the −72 polymorphism was bisulfite modified as described above. The modified region amplified by the original primers did not contain the polymorphism site and therefore a further set of primers were designed to amplify the region containing the polymorphism. Primers for the first round of amplification were MLH1-1 and MLH1-OUT (Table 1); the nested PCRs were performed with primers MLH1-2 and MLH1-IN (Table 1).

The nested PCR products were TA cloned using a TOPO TA Cloning Kit (Invitrogen, Groningen, The Netherlands) as instructed by the manufacturer. The vector containing the *hMLH1* insert was introduced into TOP10 cells (Invitrogen) by electroporation using an output voltage of 2500V (Easyject Prima, EquiBio, Kent, UK). Following selection on antibiotic plates, individual clones were isolated and used to inoculate 2 ml LB (1 g/100 ml tryptone, 0.5 g/100 ml yeast extract, 1 g/100 ml NaCl) containing 50 μg/ml kanamycin. Following an overnight incubation with shaking at 37°C plasmid DNA was prepared from the cultures using a Perfectprep plasmid mini kit (Eppendorf, Cambridge, UK). Approximately 1 μg of plasmid DNA was used in sequence reactions with the primer MLH1-IN (Table 1).

Results

Microsatellite instability

The presence of microsatellite instability was assessed in 30 of the leukemia patients included in this study; constitutional DNA was not available from the remaining 25 AML patients. Although there are no internationally recognized guidelines pertaining to the analysis of MSI in AML, the majority of recommendations relating to the analysis of MSI in colorectal cancer recommend the use of at least five microsatellite markers. We used a large panel of 11 markers that were selected on the basis that they covered a wide range of chromosomal locations including sites of known tumor suppressor genes. We have defined a mutator phenotype if MSI was present at two or more of these markers. The majority of these results have previously been reported (Das-Gupta *et al*[12]) as part of a large MSI analysis in AML patients. In this study six out of the 30 AML patients tested were demonstrated to have microsatellite instability.

Mismatch repair gene methylation

To determine the methylation patterns of *hMLH1* and *hMSH2* in patients with AML we have used the method of bisulfite genomic sequencing to study the methylation status of the 56 CpG sites that constitute the entire CpG island of *hMLH1* and the 56 CpG sites that comprise the *hMSH2* CpG island. These regions include and extend upon all regions previously studied by methylation-specific PCR and enzymatic methods. Following bisulfite modification of DNA and amplification by PCR with strand-specific primers, the PCR products were sequenced in order to provide an average sequence for the population of DNA molecules. A total of 55 patients with AML were studied, 23 of these had therapy-related disease; in addition, the DNA from 25 healthy individuals was also collected to act as control samples.

Similar to other reports[17,27] no methylation was found in the promoter region of *hMSH2* in any of the 55 AML or 25 control samples. However two patient samples (AH and LW) were demonstrated to have widespread methylation within the *hMLH1* promoter, both patients had *de novo* AML but relapsed quickly following therapy. The details of these patients are shown in Table 2. The methylation was not restricted to certain sites but encompassed the entire region analyzed. Figure 1 illustrates a portion of the *hMLH1* promoter region and compares the bisulfite sequencing results from patient LW (methylated *hMLH1*) with those from control sample 1 (unmethylated *hMLH1*).

Table 2 Details of the patients who demonstrated widespread *hMLH1* methylation

Patient	*Sex*	*Age*	*FAB type*	*Cytogenetics*	*Presence of MSI*
AH	male	65	M2	46 XY, t(8;21)(q22;q22)	yes
LW	female	16	M2	46 XX	no

Common methylation events in hMLH1

In addition to the methylation found throughout the *hMLH1* promoter CpG island in two patients with AML, two isolated CpG dinucleotides at positions −241 and −248 (relative to the transcriptional start, GenBank accession number NM000249) were found to be methylated in all control samples (25/25) and all except one AML sample (54/55). It is evident from the

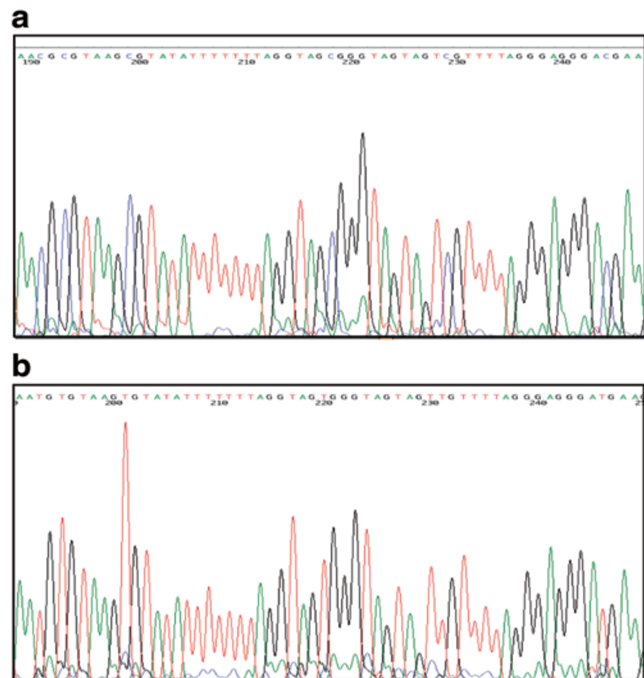

Figure 1 Bisulfite genomic sequencing of *hMLH1* promoter, region −235 to −175. A portion of the modified sequence generated using primer MLH1-7. (a) AML patient LW demonstrating methylation at all CpG dinucleotides within this region. (b) Control sample 1 illustrating complete bisulfite modification. The unmodified base sequence of *hMLH1* −233 to −175 is AACGCGCAAGCGCAT ATCCTTCTAGGTAGCGGGCAGTAGCCGCTTCAGGGAGGGACGAA.

semi-quantitative analysis shown in Figure 2 that approximately 50% of the molecules in most sample populations studied have methylated cytosines at these positions. A single AML patient had no evidence of any methylated cytosines at either of these two sites resulting in a single peak corresponding to a T residue within the modified sequence. A further AML patient apparently had only methylated residues resulting in a single C peak.

In order to establish whether the two methylated CpG dinucleotides were restricted to one allele a polymorphism at position −72 relative to the transcriptional start site (−93 relative to the translational start site[26]) within the *hMLH1* promoter was used to identify individual alleles. Samples having both A and G alleles were selected as informative cases and a region of the *hMLH1* promoter was cloned. The cloned products were then sequenced to provide methylation maps of single DNA molecules. Informative cases were studied and 10 clones of each sample were sequenced. By reference to the polymorphic A or G base at position −72, on average 50% of molecules were methylated at CpG sites −241 and −248 on the A allele and 50% on the G allele in each sample studied. This indicates that the methylation event was not allele specific (Figure 3), however, it confirms that the bisulphite sequencing is indeed semi-quantitative.

Discussion

This study has used bisulfite genomic sequencing to produce detailed methylation maps of the promoter regions of *hMLH1* and *hMSH2* comprising 672 and 515 bases, respectively. The work has demonstrated that widespread methylation of the MMR gene *hMLH1* occurs in a small number of patients with AML (2/55; 3.6%). Neither of the patients with widespread methylation had t-AML which has previously been associated with MSI, however, one patient was >60 years and we have previously reported that a proportion of these patients have MSI.[12] In this series none of the t-AML cases studied had widespread methylation of *hMLH1*, indicating that this mechanism of gene silencing does not appear relevant to the development of MSI in these cases. These results contrast with those of Krichevsky *et al*[24] who reported *hMLH1* methylation using MSP but agree with our previous studies also using MSP.[23]

Of the two patients with methylated *hMLH1* promoters, it was interesting that neither patient had poor risk cytogenetics,

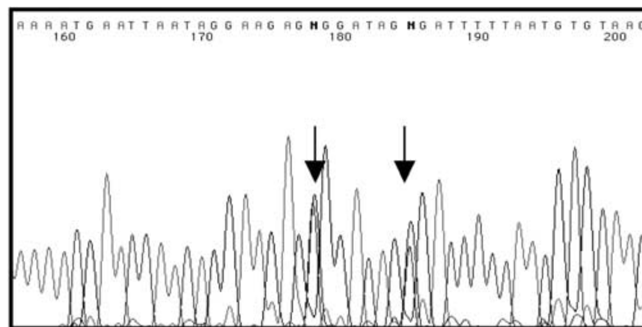

Figure 2 Bisulfite genomic sequencing of region −269 to −224 of the *hMLH1* promoter. The arrows indicate the CpG dinucleotides at positions −248 and −241 which can be seen as dual peaks in almost all DNA samples following bisulfite genomic sequencing. This suggests the presence of both methylated and unmethylated cytosine residues at these sites. The sequence was generated using primer MLH1-7.

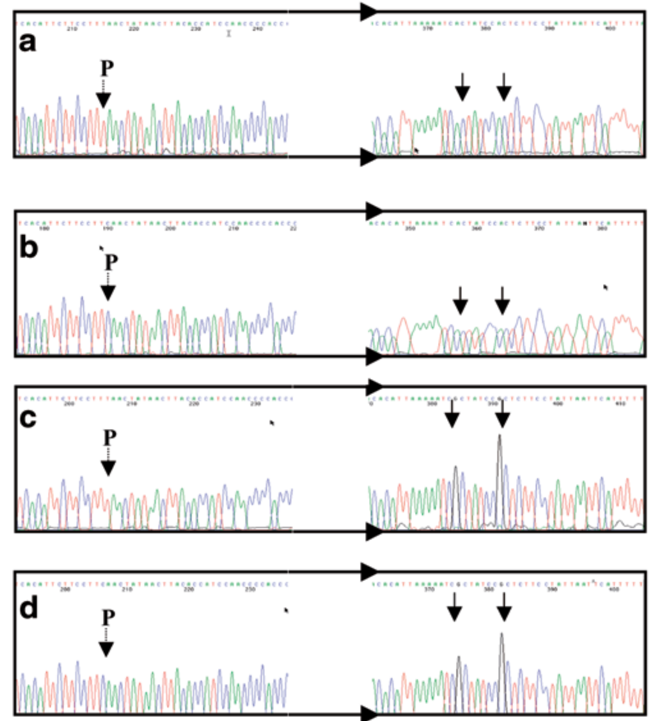

Figure 3 Bisulfite genomic sequencing results of four individual clones following the cloning of the modified *hMLH1* promoter region from control sample 4. The figure shows the reverse complement sequence of the top strand generated using primer MLH1-IN. Control sample 4 was informative for a single nucleotide polymorphism at base −72 and was therefore used for analysis. The four panels (a–d) show methylated and unmethylated patterns are present on both alleles. ↓P indicates nucleotide −72 where either a T or a C base can be seen corresponding to the A or G allele. ↓ demonstrates positions −241 and −248 where either methylated cytosines are present (seen as a G base on the complement sequence) or unmethylated cytosines which have been converted to T residues during the bisulfite modification and PCR processes (A base).

in fact, AH had favorable risk cytogenetics, although both underwent early relapses following treatment. This may indicate that although *hMLH1* methylation is a rare event it may constitute a novel and important mechanism of genetic instability in these patients. Abnormalities in MMR systems have been associated with drug resistance and we suggest this may be one explanation for the early relapse in these patients. Further work is required to examine the true clinical significance of methylated hMLH1 promoters.

We have previously reported that t-AML is associated with evidence of MSI in 44% of cases. The genetic basis for MSI in these cases remains unclear and we find no cases of methylation of *hMLH1* in our series of t-AML cases. Mutations in exon 7 (codon 419) of MSH2 have been reported in t-MDS[14] and we have found a mutation in codon 392, also in exon 7, of MSH2 in one out of 56 patients with AML (unpublished data). Other possible causes of MSI in these cases warrant further investigation, including analysis of the other genes involved in MMR and also the potential contribution of other DNA repair pathways to MSI. The widespread *hMLH1* methylation in the two AML patients described here is common to that seen in a number of sporadic solid tumors where the methylation has been demonstrated to result in transcriptional inactivation.[16,17,28,29] Therefore, although we had inadequate amounts of material to study hMLH1

expression, we can infer that the *hMLH1* gene has been silenced in these two cases with widespread methylation. Also, in common with other studies in solid tumors, no methylation of *hMSH2* was found.[17,27] Microsatellite instability studies have been performed on these samples and only one of the two cases (AH) with a methylated *hMLH1* promoter demonstrated instability.[12] It therefore appears likely that the transcriptional inactivation of *hMLH1* in LW is a late step in the disease progression in this patient. A hypermethylator phenotype called a CpG island methylator phenotype[30] has been reported in a number of sporadic malignancies, including AML.[31] It is possible that the *hMLH1* methylation in patient LW is a result of a methylation defect associated with disease progression and leading to a methylator phenotype. This is further supported by the presence of a methylated *p15* promoter previously demonstrated in this patient[32] and we suggest that the two methylated genes (*hMLH1* and *p15*) may be the result of a CpG-island methylator phenotype in patient LW. In contrast, patient AH did not have a methylated *p15* promoter[32] but did have MSI,[15] suggesting that the epigenetic inactivation of the *hMLH1* gene in this patient may have directly resulted in a mutator phenotype with associated MSI.

Other studies relating to the methylation status of *hMLH1* have used less extensive techniques such as methylation-specific PCR and PCR followed by methylation-sensitive restriction digestion. The use of bisulfite genomic sequencing in this study has led to the novel demonstration of almost universal methylation of two CpG dinucleotides within the *hMLH1* promoter in samples from both leukemic and healthy individuals. The relevance of this finding is unclear. The two methylated CpG sites (residues −241 and −248 relative to the transcriptional start site) are within the main body of the *hMLH1* promoter; they also lie at the edge of an area where methylation has been shown to be particularly important in the transcriptional silencing of this gene.[29] In addition, the residues are immediately downstream to a CCAAT-box and a PEA3 transcription factor binding site[26] (Figure 4). Recently, Deng *et al*[33] have shown the importance of this region of the promoter in the transcriptional silencing of hMLH1 via methylation because of the binding of CBF to the CCAAT box. Methylated cytosines adjacent to this box significantly reduce the ability of CBF to bind. Our identification of these two methylated residues highlights the major advantage of bisulfite sequencing; each base can be studied individually and there is no dependency on the position of the methylated residues, for example, in primer binding sites or restriction enzyme recognition sites. It is unlikely that the methylation of these two CpG dinucleotides leads to any degree of transcriptional repression due to the fact that the event is also seen in normal individuals. This highlights the importance of relevant control samples and sequencing as a tool to dissect promoter methylation profiles as universally methylated sites like these clearly have the potential to generate false positive results when less comprehensive methodologies are used. Whilst we have considered the possibility that these methylated peaks are artifactual and may be the result of an inefficient modification process we believe this not to be the case, particularly following the cloning of individual methylated and unmethylated alleles and the identification of one sample with no apparent methylation. A number of explanations can be proposed for the methylated peaks. Melki *et al*[34] suggested that methylated CpG sites may act as 'seeds' to aid methylation expansion across the CpG island. However, there is no evidence of *de novo* methylation from these sites in *hMLH1*. A further explanation is that the residues lie within a region of DNA which has a highly defined structure and as such is resistant to chemical modification during bisulfite treatment, however, the presence of unmethylated DNA molecules within the sample population make this seem unlikely. It will be interesting to see if similar events are found in promoters of other genes which are known to become methylated during malignant diseases.

In summary, we have performed a detailed methylation analysis of the *hMLH1* and *hMSH2* promoter regions on a large number of AML patients and a group of normal individuals. Widespread methylation of the *hMLH1* promoter was demonstrated in two patients with *de novo* AML. In addition, we report the novel finding of a small region of the *hMLH1* promoter which is methylated in nearly all samples studied. Whilst the relevance of this methylated region is not known, it is possible that with more detailed studies other such regions of methylation may be identified in other genes and that they will aid our understanding of methylation initiation and expansion in both malignant and other diseases.

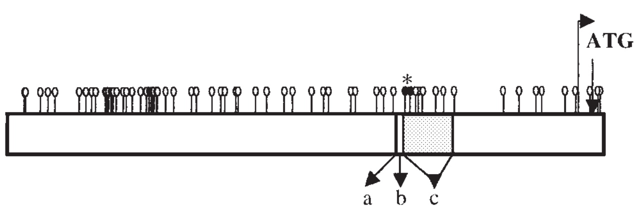

Figure 4 Representation of the CpG dinucleotides in the 5′ upstream region of *hMLH1*. The two CpG sites found to be commonly methylated are indicated along with other regions of interest. Open circle, unmethylated CpG dinucleotide; (a), CCAAT-box; (b), PEA 3; closed circle with asterisk, common methylated CpG sites; ATG translational start; (c), region shown by Deng *et al*[36] to be important in methylation associated transcriptional silencing; arrow, 5′ end of mRNA. The figure is drawn to scale.

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