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ORIGINAL ARTICLE

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Promoter methylation and expression of DNA repair genes *hMLH1* and *MGMT* in acute myeloid leukemia

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Abstract Gene silencing of DNA repair genes *hMLH1* and *MGMT* caused by aberrant promoter methylation has been detected in various solid tumors. However, in acute myeloid leukemia (AML) the frequency of *hMLH1* and *MGMT* promoter methylation is not yet fully elucidated. To determine the methylation status and expression of *hMLH1* and *MGMT*, we investigated 22 AML cases by methylation-specific polymerase chain reaction (MS-PCR) and reverse transcription PCR (RT-PCR). To exclude unspecific PCR amplifications DNA sequencing was performed. *hMLH1* promoter methylation was detectable in 4 of 20 AML cases. However, DNA sequencing could only confirm a methylated *hMLH1* promoter in one case. mRNA expression was absent in one case and reduced in another. However, these cases did not display aberrant promoter methylation. In contrast, *MGMT* promoter methylation was not detectable in the investigated AML patient samples. Accordingly, *MGMT* mRNA expression was found to be normal in all but one case. Aberrant promoter methylation of *hMLH1* was detectable only in a small number of AML cases. Additionally, in two cases the promoter methylation detected by MS-PCR could not be confirmed by sequencing, clearly indicating the importance of controlling MS-PCR results by the more specific sequence analysis. Surprisingly, *hMLH1* promoter methylation was not associated with gene silencing, suggesting monoallelic methylation or promoter methylation only in a small subpopulation of malignant cells. The reduced mRNA expression in additional samples may indicate an involvement of *hMLH1* in the malignant transformation in a small subset of cases. In contrast, *MGMT* does not seem to be involved in the pathogenesis of AML.

Keywords hMLH1 · MGMT · Promoter methylation · Gene expression · Acute myeloid leukemia

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Introduction

Functional defects of the DNA repair system increase the susceptibility to neoplastic transformation. One molecular mechanism of gene inactivation of DNA repair enzymes is promoter methylation of CpG islands [22].

Promoter methylation has been described for several DNA repair enzymes such as *hMLH1* or *MGMT*. Inactivation of the *hMLH1* gene, which encodes for a mismatch repair enzyme, is associated with colorectal carcinoma [4, 17, 18], adenocarcinomas of the endometrium [7], stomach [12, 20], and ovary [21]. Similarly, silencing of the *MGMT* gene by methylation of the CpG islands within the promoter sequences has been detected in several human cancer types [3, 8, 9, 30]. The encoded O^6-methylguanine DNA methyltransferase prevents potentially mutagenic DNA alterations by removing mutagenic and cytotoxic adducts from the O^6 position of guanine [23].

In solid tumors, expression and methylation status of DNA repair genes have been intensively studied. In addition, various genes known to be gene silenced by promoter methylation have been analyzed in acute myeloid leukemia (AML). A frequent aberrant promoter methylation of the tumor suppressor gene *p15^{INK4b}*, the estrogen receptor as well as the epithelial adhesion molecule *E-cadherin* has been described by different groups [6, 16, 28]. In contrast, the role of the DNA repair system in the pathogenesis of AML has not yet been completely elucidated. Contradictory results concerning the *hMLH1* promoter methylation status have been reported. Krichevsky et al. detected aberrant *hMLH1* promoter methylation in 11 of 12 (92%) therapy-related AML patients [19], whereas Sheikhha et al. reported *hMLH1* promoter methylation in only 3.7% of the investigated AML and MDS cases [26]. Similar results were reported by Seedhouse et al. who detected extensive methylation of *hMLH1* in only 2 of 55 AML patient samples (3.6%) [25].

In contrast, the promoter methylation status and the expression of *MGMT* have not been investigated in AML so far and thus its involvement in the pathogenesis of AML is not evident.

To further elucidate the role of *hMLH1* and *MGMT* in the pathogenesis of AML, we simultaneously investigated (1) the promoter methylation status of *hMLH1* and *MGMT* and (2) the expression of the corresponding genes in 22 AML cases by methylation-specific polymerase chain reaction (MS-PCR) and reverse transcription PCR (RT-PCR). The PCR results obtained were controlled by DNA sequencing to exclude unspecific amplifications.

Materials and methods

Cell lines and culture

The colon cancer cell lines Sw48 and Lovo and the follicular lymphoma cell line Karpas 422 were obtained from the American Type Culture Collection (ATCC, Manassas, Va., USA) and maintained in cell culture according to standard conditions.

Patients

Bone marrow (BM) and peripheral blood (PB) samples from 22 AML patients were investigated. Patients were classified into de novo and secondary AML. According to the current WHO classification, secondary AML was defined by a preceding myelodysplastic syndrome or a history of previous chemotherapy [14]. AML was further subclassified according to the French–American–British (FAB) classification [1].

MS-PCR and sequencing of the promoter region of *hMLH1* and *MGMT*

Leukemic blast cells were separated by Ficoll-Hypaque density gradient centrifugation and DNA was extracted using the QIAamp Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. DNA was subsequently treated with bisulfite (Intergen CpGenome DNA modification kit, Intergen, Purchase, N.Y., USA) resulting in the selective substitution of cytosines by uracil residues only in unmethylated but not in methylated DNA. Subsequently, the promoter regions were amplified by PCR applying primers specific for the methylated or unmethylated promoter sequences (Table 1). The applied MS-PCR primers were described previously [10, 13]. For *hMLH1* the promoter region from 1264 to 1354 was amplified applying methylation-specific primers for the methylated reaction and from 1260 to 1362 for the unmethylated sequences (according to the GenBank sequence entry U83845). This region was shown to correlate with *hMLH1* expression in colon cancer [5]. For *MGMT* previously described primer sets were applied which amplify the *MGMT* promoter from 1070 to 1150 for the methylated MS-PCR and from 1064 to 1156 for the unmethylated reaction (according to the GenBank sequence entry X61657). The PCR was performed with *Taq* polymerase (Ampli Taq Gold and Ampli Taq Polymerase, Applied Biosystems, Foster City, Calif., USA): 95°C for 10 min followed by 40 cycles (denaturation: 30 s at 95°C, annealing: 45 s at 58–62°C, extension: 45 s at 72°C) and final extension for 10 min at 72°C. The PCR products were visualized by electrophoresis on 2% agarose gel and ethidium bromide staining. PCR products were cloned into the pGEM-T Easy Vector (Promega, Madison, Wis., USA) and sequenced in both directions (BigDye sequencing system, Applied Biosystems, Foster City, Calif., USA).

Table 1 Sequences of specific primer sets

Gene	Primer	Sequence (5′–3′)
hMLH1	Unmethylated	TAA AAA TGA ATT AAT AGG AAG AGT GGA TAG TG
		AAT CTC TTC ATC CCT CCC TAA AAC A
	Methylated	AAC GAA TTA ATA GGA AGA GCG GAT AGC G
		CGT CCC TCC CTA AAA CGA CTA CCC
	mRNA	CAG CGG CCA GCT AAT GCT AT
		AAT CCT CAA AGG ACT GCA GTT
MGMT	Unmethylated	TTT GTG TTT TGA TGT TTG TAG GTT TTT GT
		AAC TCC ACA CTC TTC CAA AAA CAA AAC A
	Methylated	TTT CGA CGT TCG TAG GTT TTC GC
		GCA CTC TTC CGA AAA CGA AAC G
	mRNA	GCC GGC TCT TCA CCA TCC CG
		GCT GCA GAC CAC TCT GTG GCA CG
cABL	mRNA	GGC CAG TAG CAT CTG ACT TTG
		ATG GTA CCA GGA GTG TTT CTC C

Table 2 Patient characteristics

	n	Median age (range)	Gender (male/ female ratio)
Total group	22	67.5 (38–77)	2.1:1
De novo AML	11	62 (39–77)	1.8:1
Secondary AML	11	69 (38–77)	2.7:1

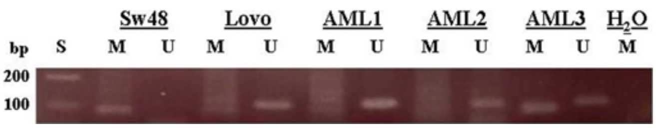

Fig. 1 MS-PCR analysis of *hMLH1* promoter status in AML (ethidium bromide-stained 2% agarose gel). *S* DNA ladder, *U* MS-PCR with primers specific for unmethylated promoter sequences, *M* MS-PCR with primers specific for methylated promoter sequences. AML patients 1 and 2 show only an unmethylated *hMLH1* promoter, whereas patient 3 displays *hMLH1* methylation

Semiquantitative RT-PCR of *hMLH1*, *MGMT*, and *cABL*

RNA from cell lines and AML patients was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Single-stranded cDNA was prepared by reverse transcription with the Gene Amp Gold RNA PCR kit (Applied Biosystems, Foster City, Calif., USA) and the corresponding protocol. The cDNA was amplified by PCR (specific primers in Table 1): 96°C for 1.5 min followed by 37 cycles (denaturation: 30 s at 94°C, annealing: 30 s at 58°C, extension: 40 s at 72°C) and final extension for 10 min at 72°C. The PCR products were visualized by electrophoresis on 2% agarose gel and ethidium bromide staining.

Results

Patient characteristics

In total 22 AML cases were analyzed at diagnosis. Eleven patients were diagnosed with de novo and 11 with secondary AML according to the WHO classification [14]. Three of the patients with secondary AML were treatment related (t-AML). The median age of the total group was 67.5 years (range: 38–77 years). Patient characteristics are summarized in Table 2.

Promoter methylation status and expression of *hMLH1* in AML

To evaluate the promoter status of *hMLH1*, we performed MS-PCR. The colorectal cell line Sw48, previously found

Table 3 Assessment of the *hMLH1* and *MGMT* promoter methylation status

	Methylated	Unmethylated
hMLH1 promoter		
AML cases (*n*=20)	1	19
De novo AML (*n*=11)	1	10
Secondary AML (*n*=9)	0	9
MGMT promoter		
AML cases (*n*=21)	–	21
De novo AML (*n*=11)	–	11
Secondary AML (*n*=10)	–	10

to have a hypermethylated promoter, served as positive control for the methylated PCR [17, 18], whereas bisulfite-treated DNA from Lovo cells was used as control for the unmethylated reaction [17]. We analyzed 20 of the 22 AML patient samples. In two cases only an insufficient amount of DNA was available. The investigation demonstrated a methylated *hMHL1* promoter in 4 of 20 AML cases (20%). The other 16 cases displayed only unmethylated promoter sequences (Fig. 1). To exclude false positive results, the methylated PCR products were subsequently cloned into the pGEM-T Easy Vector and DNA sequencing was performed. Sequence analysis confirmed the previously reported aberrant *hMLH1* promoter methylation of the cell line Sw48 in all clones investigated. In each AML case with promoter methylation, at least four different clones were analyzed. In two patients, the methylated *hMHL1* promoter could not be confirmed by DNA sequencing. Sequence analysis identified an unmethylated promoter sequence or other genomic sequences indicating unspecific PCR amplifica-

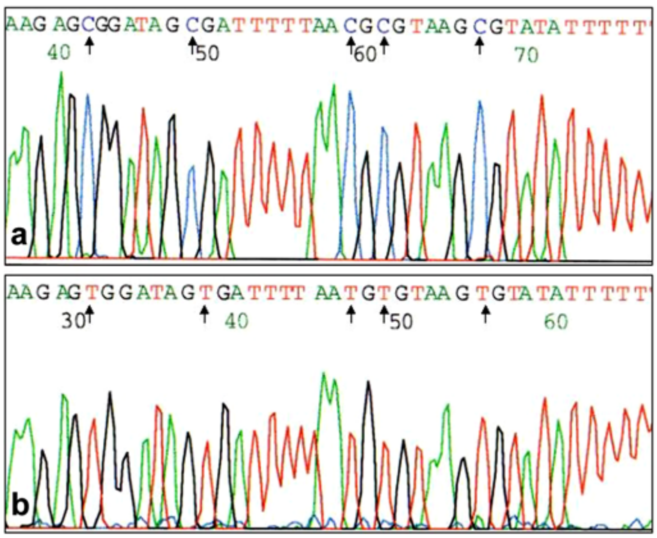

Fig. 2 **a** DNA sequence analysis of the methylated *hMLH1* promoter sequence of AML patient 3. All CpG islands are methylated (*up arrow* indicates methylated CpG island). **b** DNA sequence analysis of corresponding unmethylated *hMLH1* promoter sequence from an AML patient. All cytosines are converted to thymines indicating complete bisulfite treatment (*up arrow* indicates unmethylated CpG island)

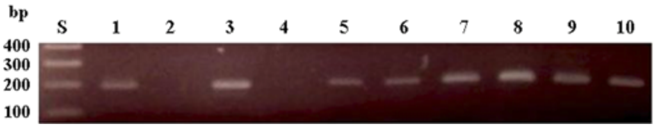

Fig. 3 RT-PCR of *hMLH1* (ethidium bromide-stained 2% agarose gel). *S* DNA ladder, *1* Lovo, *2* Sw48, *3* AML1, *4* AML2, *5* AML3, *6* AML4, *7* AML5, *8* AML6, *9* AML7, *10* AML8. AML patient 2 is devoid of *hMLH1* mRNA, whereas AML patient 3 shows a methylated *hMLH1* promoter and the other AML patients show normal *hMLH1* mRNA expression

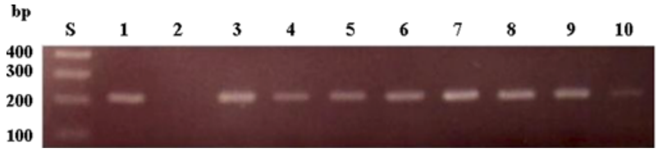

Fig. 5 RT-PCR of *MGMT* (ethidium bromide-stained 2% agarose gel). *S* DNA ladder, *1* Karpas, *2* Sw48, *3* AML1, *4* AML2, *5* AML3, *6* AML4, *7* AML5, *8* AML6, *9* AML7, *10* AML10. Patient 10 shows reduced *MGMT* expression compared to the other AML patients

tions. Thus, these patients were evaluated as being unmethylated in the *hMLH1* promoter. In contrast, in one patient with de novo AML, DNA sequencing confirmed the methylation in two of five clones, in which all investigated CpG islands were methylated (Table 3, Fig. 2a). In the fourth methylated patient, unfortunately a sufficient amount of DNA was not available for additional DNA sequencing.

The unmethylated MS-PCR amplifications were also confirmed by DNA sequencing. Unmethylated sequences were detectable in the positive cell line Lovo as well as in all patients investigated (Fig. 2b).

The expression of *hMLH1* transcript was determined by semiquantitative RT-PCR. RNA from the cell lines Lovo and Sw48 served as positive and negative control, respectively [5]. mRNA was available in 20 patients. One patient with de novo AML did not express *hMLH1* mRNA and another patient with de novo AML showed decreased mRNA levels compared to the positive control cell line. Degradation of mRNA of the corresponding patients was excluded by a *cABL*-specific RT-PCR. None of these cases showed a methylated *hMLH1* promoter. In contrast, the case with confirmed aberrant *hMLH1* promoter methylation expressed normal levels of *hMLH1* transcript (Fig. 3). Again, to exclude unspecific amplifications, direct DNA sequencing was performed and *hMLH1* mRNA sequences were confirmed.

Promoter methylation status and expression of *MGMT* in AML

The evaluation of the *MGMT* promoter methylation status was performed by MS-PCR. Similarly to *hMLH1*, bisulfite-treated DNA from the cell lines Sw48 and Lovo served as positive control for the methylated and the unmethylated MS-PCR, respectively [11]. In Sw48 only a methylated promoter was detectable, whereas Lovo and all of the AML cases (n=21, in 1 of the 22 cases only an insufficient amount of DNA was available for this analysis) showed only an unmethylated *MGMT* promoter (Table 3, Fig. 4). These results were also confirmed by cloning of PCR amplifications and subsequent DNA sequencing.

MGMT mRNA expression was determined by RT-PCR. RNA from the follicular lymphoma cell line Karpas 422, which has been shown to express *MGMT* mRNA, served as positive control and Sw48 mRNA, which is known to be devoid of *MGMT* transcript, was used as negative control [10]. Twenty AML patients were investigated for *MGMT* expression. Expression was found to be normal in all but one case of secondary AML which did not show *MGMT* mRNA (Fig. 5). The results obtained by RT-PCR were again confirmed by DNA sequencing.

Discussion

The DNA repair enzymes play an important role in maintaining genomic stability as defects in this crucial system lead to an increase of spontaneous mutagenesis. Promoter methylation of CpG islands, which has been detected in various solid tumors [4, 17, 18], is one molecular mechanism leading to specific gene silencing and subsequent loss of function of these DNA repair enzymes [22]. However, the involvement of the repair system in the pathogenesis of AML is not yet fully elucidated. So far, contradicting results especially concerning the *hMLH1* promoter methylation status have been reported [19, 25, 26]. To further determine the impact of the DNA repair system, we investigated the methylation status and the expression of two DNA repair enzymes which were selected based on previously detected alterations in solid tumors.

In our experiments, MS-PCR detected an aberrant *hMLH1* promoter methylation in 4 of 20 cases. However,

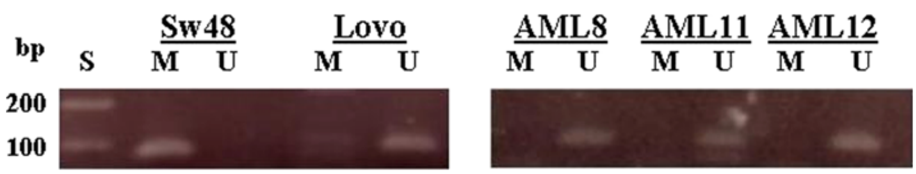

Fig. 4 MS-PCR analysis of *MGMT* promoter status in AML (ethidium bromide-stained 2% agarose gel). *S* DNA ladder, *U* MS-PCR with primers specific for unmethylated promoter sequences, *M* MS-PCR with primers specific for methylated promoter sequences. AML patients 8, 11, and 12 show only an unmethylated *MGMT* promoter

these promoter methylations could be confirmed by cloning and subsequent DNA sequencing of the amplified promoter sequences only in one of three patients. As in one patient we could not perform DNA sequencing, it is not evident whether this patient really shows *hMLH1* promoter methylation. Thus, we could detect confirmed *hMLH1* promoter methylation in just 1 of 20 (5%) AML cases. In contrast to the AML cases, methylation of the positive control cell line Sw48 could be confirmed by DNA sequencing, clearly indicating the specificity of our PCR. Thus, we conclude that despite optimized PCR conditions, unspecific amplifications may occur, especially in patient samples with potentially suboptimal quality and limited quantity of DNA. Therefore, we would like to stress the importance of DNA sequencing to confirm the obtained MS-PCR results. Although in general, MS-PCR following bisulfite treatment is an extremely sensitive method to analyze the promoter methylation status [15], its results have to confirmed by a more specific method to definitely exclude unspecific amplifications and incomplete bisulfite treatment [24, 27]. Alternatively, other assays such as combined bisulfite restriction analysis (COBRA) may be additionally applied [29].

The results of our study could not confirm previous reports published by Krichevsky et al. who detected a high incidence of *hMLH1* promoter methylation in therapy-related AML (t-AML) [19]. Unfortunately, the primer sequences of that study have not been published so far. Thus, it is not evident whether these differing results are the result of primer selection. The high incidence of *hMLH1* promoter methylation in t-AML might represent a specific event in the pathogenesis of t-AML. However, in our cohort patients with t-AML (n=3) did not show *hMLH1* promoter methylation. Alternatively, the detected *hMLH1* methylations might represent unspecific amplifications and not methylated *hMLH1* promoter sequences, as Krichevsky did not confirm his MS-PCR results by DNA sequencing. Our results are in line with two other studies, which investigated the *hMLH1* promoter methylation status either by a PCR-based restriction enzyme assay or bisulfite genomic sequencing. These studies showed *hMLH1* promoter methylation in 3.7% and 3.6%, respectively, of investigated cases [25, 26].

In addition, the analysis of *hMLH1* expression showed that the patient with methylated *hMLH1* promoter did not show decreased mRNA levels. Normally, methylation of CpG islands is associated with gene inactivation. However, the phenomenon that aberrant promoter methylation does not lead to complete gene silencing has been described previously for other genes, e.g., *p15* [2]. One explanation might be monoallelic *hMLH1* promoter methylation which does not cause complete absence of *hMLH1* expression. This has also been shown previously for *p15* [2]. Alternatively, our results might represent methylation only in a small proportion of malignant AML blasts. In addition, in two other patients with an unmethylated *hMLH1* promoter status, reduced *hMLH1* expression was detectable. This implicates the involvement of mechanisms other than methylation of CpG islands in the regulation of *hMLH1* expression. Thus, we conclude that reduced expression of *hMLH1* is detectable in a small subset of AML cases and might be involved in the pathogenesis of AML.

We also investigated the promoter methylation status and expression of *MGMT*. Silencing of *MGMT* has been detected in different solid tumors [3, 30]. In addition, *MGMT* promoter methylation has also been detected in diffuse large B-cell lymphoma [11]. However, neither promoter methylation nor expression of *MGMT* have been investigated in AML. In this analysis, only unmethylated promoter sequences of *MGMT* were detectable in 21 AML cases. Accordingly, mRNA levels were normal in all but one patient sample. Thus, *MGMT* promoter methylation is infrequent in AML.

In summary, aberrant promoter methylation of the DNA mismatch repair enzyme *hMLH1* was detectable in a small number of AML cases. As this promoter methylation was not associated with *hMLH1* gene silencing, we suggest monoallelic *hMLH1* promoter methylation or aberrant methylation of the *hMLH1* promoter only in a small proportion of malignant cells. As reduced mRNA expression was found in additional cases, we further suggest an involvement of *hMLH1* in the malignant transformation in a small subset of AML cases. In contrast, *MGMT* does not seem to be involved in the pathogenesis of AML.

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