MGMT promoter methylation as a potential prognostic marker for acute leukemia

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Abstract

Introduction: It has been proved that genetic and epigenetic changes play a significant role in the development and progression of acute leukemia. The aim of our study was to evaluate the frequency and prognostic implications of genetic and epigenetic alterations in *p15*, *MGMT*, *DNMT3A* and *TP53* genes in acute leukemias.

Material and methods: We included in the study 59 patients with acute leukemia. Evaluation of *TP53* and *DNMT3A* mutations was performed using sequencing analysis and PCR-RFLP, respectively. Methylation status of *MGMT* and *p15* genes was evaluated using MSP and COBRA, respectively. For assessment of global DNA methylation ELISA-based kit was used.

Results: We found that overall survival was higher for ALL patients. *MGMT* promoter methylation was significantly associated with patients age at the time of diagnosis ($p = 0.03$). *TP53* and *DNMT3A* mutations were observed only in AML patients (16.67% and 8.8%, respectively). Patients with acute leukemia and *p15* promoter methylation had significantly more frequently mutated *TP53* gene ($p = 0.04$) and AML patients with *p15* promoter methylation had significantly more frequently detected global hypomethylation of DNA ($p = 0.009$). In the group of ALL patients we noted an opposite trend: only patients negative for *p15* promoter methylation were characterized by global DNA hypomethylation.

Conclusions: Our findings demonstrate that *MGMT* promoter methylation can have a considerable impact on the development of acute leukemia in older patients. *DNMT3A* and *TP53* mutations may play a significant role in AML development. However, further studies conducted in a larger cohort of patients are needed to determine its clinical utility.

Key words: acute leukemia, *MGMT*, *DNMT3A*, *p15*, *TP53*.

Introduction

Leukemias are a clinically and genetically heterogeneous group of diseases that begin in early blood-forming cells found in the bone marrow. It is a result of genetic mutations and transformation of a single early progenitor lymphoid or myeloid cell. Acute lymphoblastic leukemia (ALL) is the most common leukemia in children, accounting for up to 80%, and only for 20% of leukemias in adults [1], whereas acute myeloblastic leukemia (AML) is more frequent in patients at an older age where the median age at the time of diagnosis is about 70 years [2].

Genetics plays an increasingly important role in the classification, risk stratification, and management of acute leukemias [3]. For the past several years, an increasing number of gene mutations deregulating expression of these genes and epigenetic changes have been established in the pathogenesis of acute leukemias [4, 5]. The most commonly mutated gene in people who have cancer is the *TP53* tumor-suppressor gene [6]. The P53 protein, widely known as a transcription factor, plays an important role in the maintenance of genetic stability and preventing cancer formation. Loss of P53 function is associated with various characteristics of tumors, including genetic instability or deregulation of the cell cycle [7]. It has been reported that these mutations are associated with a poor prognosis and treatment failure in both ALL and AML [8, 9]. It has also been shown that *DNMT3A* mutations are essential for early development of acute leukemia [10, 11]. DNMT3A (DNA (cytosine-5-)-methyltransferase 3α), a member of the *de novo* methyltransferases, is closely associated with epigenetic modifications in mammalian development and diseases. More recent studies have identified that most of the recurrent somatic mutations in *DNMT3A* in AML are heterozygous [10]. The normal function of DNMT3A can be disturbed by these mutations, which subsequently results in an abnormality of epigenetic modification. In approximately 20% of AML cases mutations in the *DNMT3A* gene have been observed and correlated with a poor clinical outcome [12, 13].

There is more and more evidence that epigenetic changes play an important role in the development of cancer, including acute leukemia. DNA methylation abnormalities are common in a variety of cancers as well as in development. Global hypomethylation and hypermethylation, which repress transcription of the promoter regions of tumor suppressor genes leading to gene silencing, have been recognized as a cause of oncogenesis [14]. Numerous changes in the methylation of promoter regions of various genes have been observed in patients with acute leukemia [15, 16]. The *p15* gene, inhibitor of cyclin-dependent kinases 4/6 (CDK4/6), is among the most frequent targets of aberrant methylation in acute leukemias [17]. Hypermethylation of the DNA repair gene *MGMT* (O^6-methylguanine DNA methyltransferase) has been found in AML, but its potential prognostic value is not yet fully elucidated [18, 19].

The aim of the study was to determine whether the *MGMT* and *p15* promoter hypermethylation and global DNA hypomethylation, as well as *DNMT3A* and *TP53* mutations, have an impact on the development acute leukemias. Moreover, we wanted to investigate the relationship between examined molecular parameters and time at diagnosis and overall survival in patients with acute leukemia. A better understanding of the underlying genetic and epigenetic processes may lead to gaining insight into the mechanism of leukemogenesis in ALL and AML, as well as providing prognostic information and prospective healing targets.

Material and methods

Materials

Fifty-nine Caucasian patients (19 ALL: 11 male and 8 female; 36 AML: 22 male and 14 female; 4 without a precise diagnosis: 3 male and 1 female; median age: 54) involved in this study were recruited at the Department of Hematology, Medical University of Lodz, between 1998 and 2001. A detailed list of all patients' characteristics is shown in Table I. Ethics committee approval was obtained from the Institutional Review Board of the Medical University of Lodz (no. RNN/226/11/KE). Peripheral blood or bone marrow samples were obtained from the patients prior to the initiation of therapy. Genomic DNA from the peripheral blood or the bone marrow blast cells was isolated by lysis of the cells with sodium dodecyl sulfate (SDS), digestion with proteinase K at 37°C overnight followed by phenol/chloroform extraction and ethanol precipitation.

Sequencing analysis

Four genomic regions of the *TP53* gene (exons 5–8) were amplified by PCR, as described previously [20]. Sequence analysis was performed by the dideoxy termination method using the SequiTherm Excel DNA Sequencing Kit (Epicentre Technologies, Madison, WI) and fluorescent-labeled primers. Sequencing primers used were as follows: 5′-CAAGCAGTCACAGCACATGA-3′ (forward) and 5′-AACCAGCCCTGTCGTCTCT-3′ (reverse), for exon 5; 5′-CAGGCCTCTGATTCCTCACT-3′ (forward) and 5′-AGACCTCAGGCGGCTCATAG-3′ (reverse), for exon 6; 5′-ATCTCCTAGGTTGGCTCTGA-3′ (forward) and 5′-TGGCAAGTGGCTCCTGACCT-3′ (reverse), for exon 7; 5′-CTCTTTTCCTATCCTGAGTA-3′ (forward)

and 5′-CTGCTTGCTTACCTCGCTTA-3′ (reverse), for exon 8. Products of the sequencing reaction were visualized and analyzed using a LiCor automated laser fluorescence sequencer.

Methylation-specific PCR

Sodium bisulfite modification of isolated genomic DNA was performed using the CpGenome DNA kit (Chemicon International Inc. Temecula, CA) according to the manufacturer's protocol. The bisulfite-treated DNA was stored at –80°C until use. CpGenome Universal Methylated DNA (Chemicon International) was used as a methylation-positive control for the methylated alleles, and DNA from peripheral blood leukocytes was used as the control for unmethylated alleles. Methylation-specific PCR (MSP) for *MGMT* promoter methylation was performed in a two-step approach as previously reported [21]. For each PCR, methylated and unmethylated DNA was included as positive and negative controls, and water was used as a control for the PCR reaction. PCR products were separated on 3% agarose gels containing ethidium bromide. Product size of the unmethylated DNA was 91 bp and that of the methylated DNA was 83 bp.

PCR-RFLP

DNMT3A gene mutation (R882H; GCCGC to GCCAC) was genotyped by means of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Genomic DNA was amplified in a 20 µl volume containing 1 U of GoTaqFlexi DNA polymerase (Promega, Poland), 1 µl of template DNA, 4 µl of 5X PCR buffer, 2.0 mM $MgCl_2$, 0.3 mM dNTPs (mixture of dATP, dTTP, dCTP and dGTP), and 0.6 pmol of each primer (forward: 5′-GTGATCTGAGTGCCGGGTTG-3′ and reverse: 5′-TCTCTCCATCCTCATGTTCTTG-3′). The PCR cycling conditions were: 10 min of initial denaturation at 95°C, followed by 35 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 59°C and extension for 1 min at 72°C, followed by 7 min of final extension at 72°C. A 7 µl aliquot of PCR product was then digested with 3 U of restrictive enzyme *Sat*I (Fnu4HI) and 2 µl of 1X BufferG (Thermo Scientific) in a final volume of 20 µl. After incubation at 37°C for 16 h restriction fragments were separated in a 2% agarose gel stained with Midori Green DNA Stain (Nippon Genetics) and visualized under UV light. Positive samples showed 3 bands (289 bp, 190 bp, 114 bp) because of the loss of a restriction site of SatI caused by the mutation.

Combined bisulfite restriction analysis (COBRA)

For methylation analysis of the *p15* gene we used combined bisulfite restriction analysis

Table I. Patient characteristics

Parameter	Value
Age, median (range) [years]	54 (32.5–63.5)
Sex:	
Male	36 (61.0%)
Female	23 (39.0%)
Type of leukemia:	
ALL	19 (32.2%)
AML	36 (61.0%)
Unknown	4 (6.8%)
OS, median (25–75%) [months]:	
ALL	16.06 (6.9–103.7)
AML	9.29 (0.8–28.0)
Unknown	10.85 (6.35–11.25)
MGMT methylation:	
ALL – positive (%)	8/19 (42.1%)
AML – positive (%)	11/36 (30.6%)
Unknown – positive (%)	2/4 (50%)
NA (% of all)	0 (0%)
p15 methylation:	
ALL – positive (%)	1/17 (5.9%)
AML – positive (%)	11/33 (33.3%)
Unknown – positive (%)	2/2 (100%)
NA (% of all)	7 (11.9%)
DNMT3A mutation:	
ALL – positive (%)	0/19 (0%)
AML – positive (%)	3/34 (8.8%)
Unknown – positive (%)	0/3 (0%)
NA (% of all)	3 (5.1%)
TP53 mutation:	
ALL – positive (%)	0/19 (0%)
AML – positive (%)	6/36 (16.7%)
Unknown – positive (%)	0/4 (0%)
NA (% of all)	0 (0%)
Global DNA hypermethylation:	
ALL – positive (%)	4/12 (33.3%)
AML – positive (%)	6/23 (26.1%)
Unknown – positive (%)	1/3 (33.3%)
NA (% of all)	21 (35.6%)
Global DNA hypomethylation:	
ALL – positive (%)	8/12 (66.7%)
AML – positive (%)	17/23 (73.9%)
Unknown – positive (%)	2/3 (66.7%)
NA (% of all)	21 (35.6%)

NA – not assessed.

(COBRA). Combined bisulfite restriction analysis was estimated using the MethylEdge Bisulfite Conversion System (Promega) according to the manufacturer's instructions. The *p15* gene was amplified by PCR using TaKaRa EpiTaq HS polymerase for bisulfite treated DNA (Takara Bio Inc.) with the following primers: forward: 5′-GGAGTTTAAGGGGGTGGG-3′ and reverse: 5′-CCTAAATTACTTCTAAAAAAAAAC-3′ (Institute of Biochemistry and Biophysics Polish Academy of Sciences, PL). The amplification was carried in a 20 µl reaction mixture containing 1 µl of template DNA, 0.7 µl of 10 mM each primer, 0.6 µl 10 mM dNTPs, 1.2 µl of 10 mM $MgCl_2$, and 0.1 µl of 5 U/µl EpiTaq HS polymerase with 2 µl of 10X EpiTaq PCR Buffer. The PCR conditions were as follows: initial activation of the EpiTaq HS polymerase for 15 min at 95°C, followed by 35 cycles of 1 min denaturation at 94°C, annealing for 1 min at 53°C and extension for 1 min at 72°C and a final 10 min extension step at 72°C. After amplification, PCR products were digested with a specific *Bst*UI restriction enzyme (ABO) that digests alleles that were methylated prior to bisulfite treatment. After digestion, DNA fragments were separated in a 2.5% agarose gel stained with Midori Green DNA Stain (Nippon Genetics). Product size of the unmethylated DNA was 193 bp and the methylated DNA fragment was 115 bp.

Global DNA methylation

Global DNA methylation levels of 40 cases were measured using the 5-mC DNA ELISA Kit (Zymo Research Corporation, Irvine, CA., USA) according to the manufacturer's protocol. In brief, 100 ng of unknown 5-mC DNA samples was used for analysis. Anti-5-methylcytosine monoclonal antibody (Anti-5-mC mAb) that binds specifically to methylated sites of the genome and the HRP-conjugated secondary antibody were used to detect 5-mC. Absorbance at 405–450 nm was measured using an ELISA plate reader (Tecan Infinite 200 PRO). The presence or absence of 5-mC was determined by comparing the absorbance of samples to negative (0% methylation) and positive (100% methylation) controls. A standard curve was generated by preparing 7 mixtures of the negative and positive controls (0, 5, 10, 25, 50, 75 and 100%). These were assayed in parallel with the samples. All samples were prepared in duplicate. To determine the 5-mC percentage for unknowns DNA samples the following equation, derived from the logarithmic second-order regression, was used: $\%5\text{-}5mC = e^{[(\text{absorbance} - \text{y-intercept})/\text{slope}]}$.

Statistical analysis

Statistical analysis was performed using Statistica 10.0 PL software (StatSoft, Poland). The distribution of variables was tested with the Shapiro-Wilk test and Kolmogorov-Smirnov test with Lilliefors' correction. Because of non-Gaussian distribution of all analyzed variables we used a non-parametric test and the results are shown as the median followed by the interquartile range (IQR). $P < 0.05$ was considered as significant and we used the following tests: the Mann-Whitney *U* test to verify differences between two groups; Pearson's χ^2 test and Yates's correction to verify differences in the distribution of categorical variables between groups; and the Mantel-Cox test and Kaplan-Meier curves for analysis of survival.

Results

Analysis of MGMT methylation status

We found that in patients with acute leukemia 35.59% of cases had a methylated *MGMT* promoter. Methylation of the *MGMT* promoter was detected in 42.11% of ALL samples and 30.56% of AML samples. There were no significant differences in *MGMT* methylation status between AML and ALL patients. We also found that in the total group of patients ($p = 0.03$) and ALL cases ($p = 0.04$), as well as AML patients ($p = 0.04$), methylation of *MGMT* was associated with age at the time of diagnosis (Figure 1). An example of the result of *MGMT* methylation status is shown in Figure 2.

Analysis of p15 methylation status

Methylation of the *p15* promoter was detected in 23.08% of all patients, 5.88% of ALL samples and 33.33% of AML samples. The difference between myeloid and lymphoblastic leukemia was significant ($p = 0.02$, $\chi^2 = 5.5$). There was no association between *p15* methylation status and age of diagnosis in any considered group of patients. An example of the result of *p15* methylation analysis is shown in Figure 2.

Analysis of global DNA methylation, DNMT3A and TP53 mutation

We did not find any significant correlation between global DNA methylation, *DNMT3A* or *TP53* mutation and age of diagnosis. Mutation of *TP53* was significantly ($p = 0.02$, $\chi^2 = 5.47$) more frequent in AML patients than in ALL (16.67% and 0.00% respectively). *DNMT3A* mutation was observed in 3 of all 56 (5.36%) patients, but only in AML samples (8.8%). There were no significant associations between DNA methylation, *DNMT3A* gene mutation and type of leukemia. Figure 3 shows an example of the results of *DNMT3A* (A) and *TP53* (B) mutation analysis.

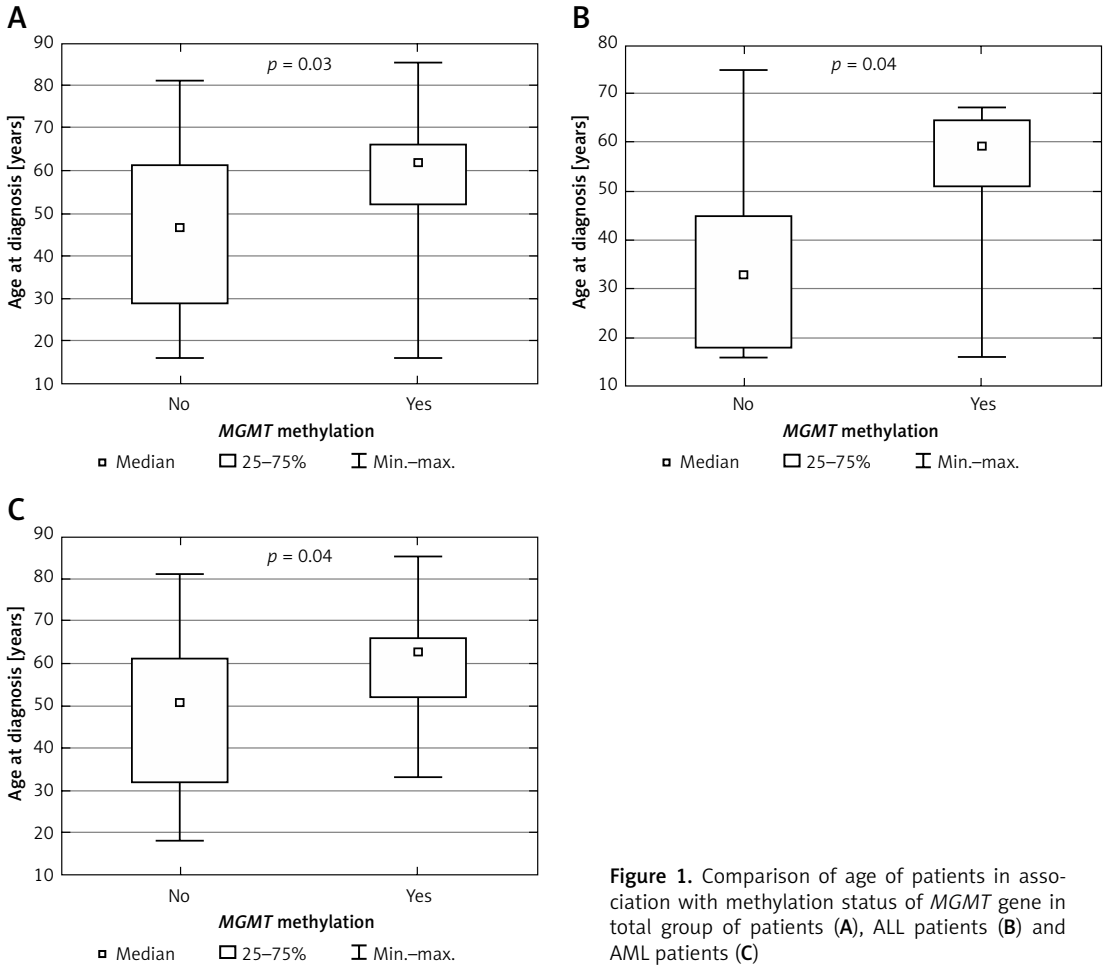

Figure 1. Comparison of age of patients in association with methylation status of *MGMT* gene in total group of patients (**A**), ALL patients (**B**) and AML patients (**C**)

Analysis of relations between examined molecular parameters

We found that patients with acute leukemia and a methylated promoter for *p15* significantly ($p = 0.04$, $\chi^2 = 4.25$) more frequently had a mutated *TP53* gene (25.00% vs. 5.00%). We also found that AML patients with a methylated promoter for *p15* significantly ($p = 0.009$, $\chi^2 = 6.76$) more frequently had global hypomethylation of DNA detected (100% vs. 61.54%). In the group of ALL patients we noted an opposite trend: only patients negative for methylation of the *p15* promoter were characterized by global DNA hypomethylation, but this difference did not reach the level of significance. These differences are shown in Figure 4. We also did not find any differences between female and male patients ($p > 0.05$).

Analysis of survival

The median overall survival in the group of patients with acute leukemia was 9.9 (16.45) months; when we consider type of leukemia it was 9.29 (27.23) months in AML and 16.06 (96.8) in ALL patients. We also assessed the influence of examined molecular parameters on OS. We did not find any correlation between examined parameters and OS of patients.

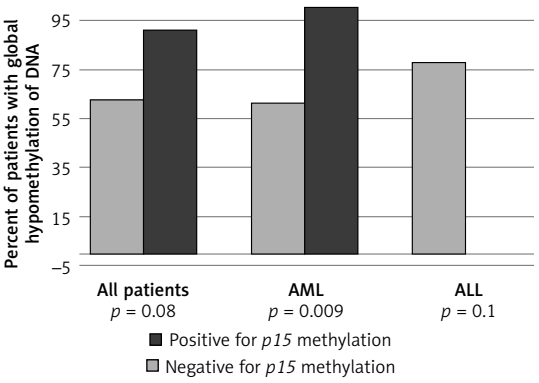

Figure 2. Differences in global DNA hypomethylation in association with type of acute leukemia and methylation of *p15*

Discussion

Genetic disorders are the basis of the malignant transformation process in acute leukemia resulting in abnormal gene expression and cellular transduction pathways and interfering with the

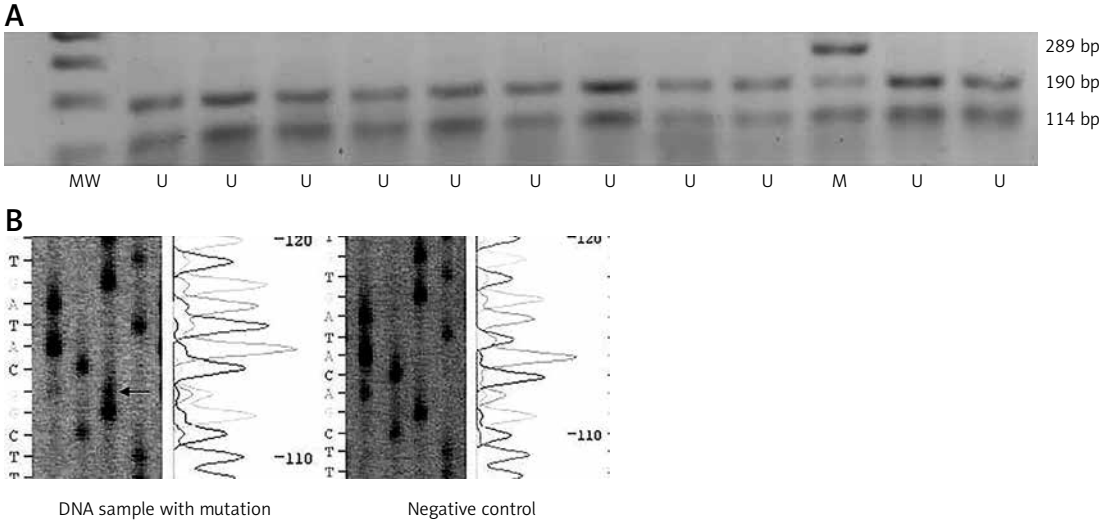

Figure 3. *DNMT3A* and *TP53* mutation in acute leukemia patients. **A** – PCR-RFLP results for the *DNMT3A* mutation. Presence of 289 bp PCR product indicates that the acute leukemia sample is positive for *DNMT3A* mutation. **B** – Example of the result of direct sequencing of a fragment of exon 6 of the *TP53* gene

M – PCR product containing mutation, U – PCR product without mutation, MW – molecular weight marker (a 100-bp marker ladder).

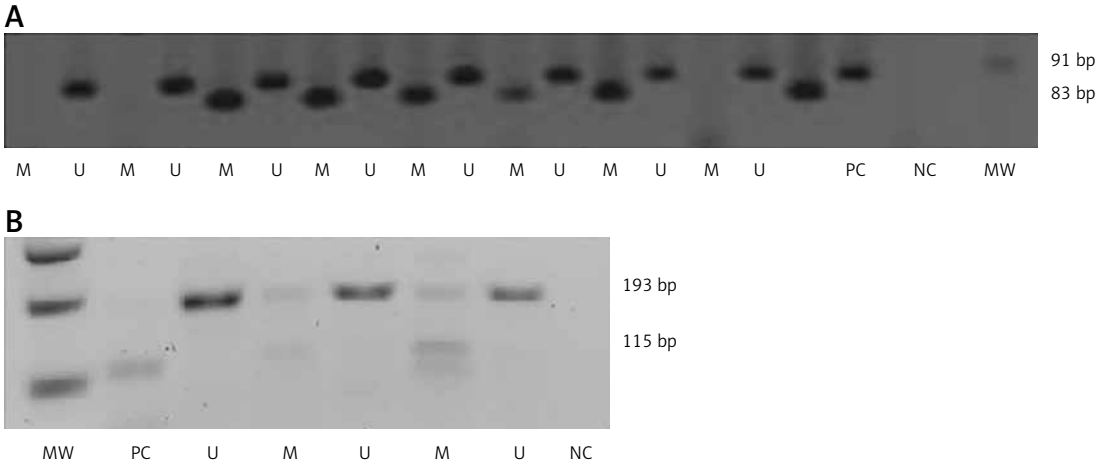

Figure 4. *MGMT* and *p15* methylation in acute leukemia patients. **A** – MSP results for the *MGMT* promoter methylation. Presence of 83-bp PCR product indicates that the acute leukemia sample is positive for *MGMT* methylation. **B** – COBRA results for *p15* methylation. Presence of 115-bp PCR product indicates that the acute leukemia sample is positive for *p15* methylation

M – methylated PCR product, U – unmethylated PCR product, NC – negative control, PC – positive control, MW – molecular weight marker (a 100-bp marker ladder).

proper course of hematopoiesis such as self-renewal, proliferation and differentiation [22]. In the present study we examined the *TP53* gene mutations that contribute to the pathogenesis of acute leukemia [23]. *TP53* is the most frequently studied gene in cancer and is well characterized in other hematological malignancies, including acute myeloid leukemia (AML) and chronic lymphocytic leukemia (CLL), but data on frequency and the prognostic impact of *TP53* mutations in ALL are limited. In our study, *TP53* mutation was not found in ALL patients. In ALL, *TP53* mutations are rather infrequent. In childhood B-ALL and pediatric T-ALL the reported prevalence is 2–5% of cases [24, 25], whilst in adult patients it is 6.4–11.1% of cases [26]. On the other hand, *TP53* mutations occur in 15.7% of ALL cases, which increases with age. Moreover, it is associated with MYC rearrangement, low hypodiploidy and a poor prognosis [27]. Other studies have shown that *TP53* mutations are frequently detected in AML with complex karyotype (AML-CK) (59–78%) [28–30] or therapy-related AML [31] but in patients without CK (2.1%) [29] and with 17p chromosomal abnormality (2.8%) [32] it occurs rather rarely. In our study, *TP53* mutation was observed in 16.67% of AML cases, which is comparable to the frequency reported by others researchers. Shih *et al.* reported

that *TP53* mutation occurs in 21% of MDS or AML patients. Moreover, patients with a *TP53* mutation or loss of the *TP53* locus had worse overall survival compared to those with wild-type *TP53* [33]. Our study shows that *TP53* mutation occurs in a significant proportion of AML cases. However, we did not find any prognostic value for this alteration, in contrast with other studies [8, 30].

In this study we also examined the *DNMT3A* mutation. DNMT3A appears to play an important role as a novel prognostic marker in adult AML [34]. Our data demonstrated that the *DNMT3A* mutation occurs in a small number of cases and only AML patients (8.8%). In other studies, *DNMT3A* mutations have been recently found in about 20% to 41% of AML patients [11, 35–37]. In contrast, *DNMT3A* mutations occur at a low frequency in myelodysplastic syndrome patients (8%) [38] and are also rare in pediatric and childhood AML [39, 40]. Ley *et al.* observed an adverse prognostic impact of the mutation for AML patients [36], and this finding is consistent with other studies [11, 34, 39]. *DNMT3A* mutation was significantly associated with advanced age and shorter OS [34, 36]. In our study we found that no mutation was detected in ALL samples. Yan *et al.* also reported that no mutations were detected in acute myeloid leukemia subtypes M1 to M3 or in acute lymphocytic leukemia [11]. However, in the study by Liu *et al.*, *DNMT3A* mutations were detected in 3 of all 57 ALL patients [41].

It is now recognized that not only genetic but also epigenetic alterations are important in initiation and progression of both AML and ALL [42, 43]. In the present study, we examined the methylation status of *MGMT* and *p15* genes in AML and ALL patients. Reports in the literature indicate that both genes are unmethylated in normal hematopoietic cells [44–46]. Aberrant *p15* methylation has been frequently found in leukemia cell lines, childhood and adult AML and ALL [45, 47–49]. In our study, methylation of the *p15* promoter was observed in 23.08% of all patients but significantly more frequently in AML (33.3%) than ALL (5.88%). These findings are consistent with other studies. Hypermethylation of the promoter region of the *p15* gene has been detected in primary acute leukemias, with the highest frequency in AML, and it conferred an adverse prognosis [47, 50, 51]. However, we did not find any prognostic value of *p15* methylation in acute leukemias. We found that patients with acute leukemia and a methylated promoter for *p15* significantly more frequently had a mutated *TP53* gene. We also found that AML patients with a methylated promoter for *p15* significantly more frequently had global hypomethylation of DNA detected. Interestingly, in ALL patients we noted the opposite trend: only patients negative for methylation of the *p15* promoter were characterized by global DNA hypomethylation, but this difference was not statistically significant. Global hypomethylation is implicated in the development of cancer through promoting genome instability and activation or overexpression of proto-oncogenes [52]. It has been shown that promoter-specific DNA hypermethylation and global DNA hypomethylation are independently associated with the clinical outcome in both ALL and AML patients [24, 25].

MGMT is a DNA-repairing enzyme that removes methyl groups as well as larger adducts at the O(6) position of guanine [53]. Epigenetic silencing of *MGMT* expression is frequently observed in several types of cancer as a consequence of transcriptional silencing induced by hypermethylation of the CpG island of the promoter of the *MGMT* gene [54–56]. Recent studies also indicate an association between the methylation status of the *MGMT* promoter and the development of MDS [57], adult AML [58, 59] and childhood ALL [60, 61]. In our study we found methylation of the *MGMT* promoter in 35.59% of patients with acute leukemia, but there was no significant difference in *MGMT* methylation status between AML and ALL patients. Other studies have indicated that *MGMT* methylation occurs rather rarely (8–11%) [60, 61]. However, chemotherapy-induced *MGMT* methylation status differed among various AML subtypes, gender and age of patients. Changes in *MGMT* methylation status were more frequent among M4 subtype patients (50%) and were not detected in M3 or M5 subtypes [58]. In several studies, methylation of the *MGMT* gene promoter has been associated with improved prognosis in young [62] and elderly [63] patients with newly diagnosed glioblastoma and in B-DLBCL patients [64]. However, no significant correlation has been found between OS and *MGMT* promoter methylation status in colorectal adenocarcinoma patients [56]. Our findings showed that methylation of the *MGMT* gene promoter was significantly associated with the age of diagnosis and there was no correlation of methylation of the *MGMT* gene promoter as an independent prognostic factor and OS of patients. Interestingly, Kraguljac Kurtovic *et al.* showed that concomitant aberrant hypermethylation status of *p15* and *MGMT* genes in AML allows stratification of patients with AML into potentially distinct groups with different prognosis [59], but this relationship was not confirmed in our study. Moreover, Lenz *et al.* demonstrated that *MGMT* methylation does not seem to be involved in the pathogenesis of AML, because it was not detectable in the examined AML patient samples [19].

In conclusion, genetic and epigenetic abnormalities play a significant role in the pathogene-

sis of acute leukemia as well as being important prognostic and predictive factors. *MGMT* promoter methylation was found to be associated with age at diagnosis in both AML and ALL patients. It can be considered as a biomarker for risk group stratification and prognosis in acute leukemias. However, a limitation of our study is the small study group size. Therefore, further research in a larger group of patients is required to assess the clinical utility of *MGMT* methylation.

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Conflict of interest

The authors declare no conflict of interest.

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