

Monitoring of methylation changes in 9p21 region in patients with myelodysplastic syndromes and acute myeloid leukemia

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Epigenetic *de novo* methylation of CpG islands is an important event in malignant transformation. Two genes are frequently methylated: cyclin-dependent kinase inhibitor 2B (*CDKN2B*) and cyclin-dependent kinase inhibitor 2A (*CDKN2A*). In our study methylation of these genes was studied in 63 patients with myelodysplastic syndromes (MDS), 2 with myelodysplastic/myeloproliferative neoplasms (MDS/MPN) and 13 with acute myeloid leukemia (AML). Five patients were monitored during 5-azacytidine treatment. Twenty-six healthy donors were tested in a control group. Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) method with all associated techniques was used for detection. Aberrant methylation was present in the *CDKN2A* gene in 38% and in the *CDKN2B* gene in 77% of the patients in MDS group. The level of methylation was higher in the group of AML patients – 77% in *CDKN2A* gene and 100% in *CDKN2B* gene. In MDS patients, an aberrant methylation was associated with a tendency to disease progression towards more advanced forms according to the World Health Organization (WHO) classification and the International Prognostic Scoring System (IPSS). Significant differences in methylation level were observed between early and advanced forms of MDS in *CDKN2B* gene (P value < 0.05) but not for *CDKN2A* gene. The trend of methylation in patients treated with azacitidine was analyzed in *CDKN2B* gene and correlated with the course of the disease. Increased methylation was connected with disease progression. We concluded that the methylation level of *CDKN2B* gene might be used as a marker of leukemic transformation in MDS. Our study indicates the role of hypermethylation as an important event in the progression of MDS to AML.

Key words: DNA Methylation, CDKN2A, CDKN2B, 5-azacytidine, MS-MLPA

Epigenetic changes are important events in leukemia pathogenesis (1). DNA methylation is one of the most common alterations in leukemia. The potential role of DNA methylation as a biomarker in leukemia has not yet been proven (2). In the human genome, methylated cytosines are found in CpG dinucleotides whose palindromic nature allows for the maintenance of methylation patterns by DNA methyltransferases following semi-conservative replication of DNA. CG-rich regions also known as CpG islands are located mostly in the promoter regions of genes and are generally unmethylated in healthy tissues (3, 4). Their methylation is associated with transcriptional silencing of genes (5, 6, 7). Methylation changes belong to the most studied epigenetic events in myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML). The most frequently tested genes are *CDKN2B* gene (cyclin-dependent kinase inhibitor gene, p15^{INK4B}) and its functional homologue *CDKN2A* gene. *CDKN2A* gene gener-

ates several transcript variants which differ in their first exons (isoform 1 called p16^{INK4A} and isoform 4 called p14^{ARF}) (7, 8). Their function is to regulate cell cycle by inhibition of cyclin-dependent kinases 4 and 6, which control the progression of cells from G1 to S phase. Hypermethylation of 5'-CpG islands within the promoter regions was described as an alternative mechanism of *CDKN2A/CDKN2B* gene inactivation (7, 9). An increased hypermethylation of the *CDKN2A* and *CDKN2B* promoter regions was found during disease progression and was associated with leukemic transformation and poor prognosis (8).

Myelodysplastic syndromes are a group of clonal hematopoietic disorders. Their pathogenesis is attributed to the important role of an initial damage to hematopoietic stem cells, which ultimately leads to the formation of pathological clone and a growth advantage. This disorder is characterized by pancytopenia due to ineffective hematopoiesis and dyspla-

sia of hematopoietic cells (10, 11). The increased incidence of mutations of oncogenes stimulates proliferation and differentiation of early precursor depressants of hematopoiesis as a consequence of genome instability, leading to a gradual increase in the number of blasts at an advanced stage of disease and the transition into acute myeloid leukemia. Diagnostic criteria have been set up in two classification systems: (the French-American-British [FAB] and the World Health Organization [WHO]) and in several prognostic-scoring systems, the most widely used being the International Prognostic Scoring System (IPSS) (12). The latest revision of the WHO classification of myeloid neoplasms and acute leukemias was published in 2008 (13, 14).

Treatment with hypomethylating agents and inhibitors of histone deacetylases is an alternative approach to therapy of MDS patients with intermediate-2 or high-risk MDS patients who are not indicated for combination chemotherapy and allogeneic stem cell transplantation (SCT) (15, 16). 5-Azacytidine and decitabine are currently available hypomethylating agents (15, 17, 18). Hypomethylating agents are phosphorylated and incorporated into DNA (as 5-aza-dCTP) during S phase, where they covalently bind to the DNA methyltransferases (DNMTs). They are irreversibly inhibiting their function, leading to the progressive loss of methylation; this process requires that the cell go through S phase (12). Treatment with hypomethylating agents has been shown effective in almost 50% of treated patients (15).

Patients and methods

Patients. The methylation status of the *CDKN2B* and *CDKN2A* genes was studied in DNA samples isolated from bone marrow mononuclear cells (BM)-MNCs from 78 Czech patients (39 men and 39 women, median age 59 years; range, 20 – 79 years) with MDS and AML, including 44 cases of refractory cytopenia with multilineage dysplasia (RCMD), 4 cases of refractory cytopenia with multilineage dysplasia and ring sideroblasts (RCMD-RS), 7 cases of refractory anemia with excess blasts-1 (RAEB-1), 8 cases of refractory anemia with excess blasts-2 (RAEB-2), 2 cases of myelodysplastic/myeloproliferative neoplasms (MDS/MPN) and 13 cases of acute myeloid leukemia (AML). Methylation status was also analyzed in 47 DNA samples extracted from peripheral blood (PB) from 5 patients with demethylation treatment: 1 patient with MDS/MPN and 4 patients with AML treated with 5-azacytidine (Vidaza®, Celgene Corp., Summit, USA) in the dose of 75mg/m²/d for 7 days every 28 days. Patients with demethylation treatment were monitored continuously during treatment. Twenty-six DNA samples from healthy donors (HD) were used as non-malignant controls. The diagnosis and classification of the MDS were performed according to the WHO 2008 (13). The study was approved by the ethical committee of the Institute of Hematology and Blood Transfusion. All patients and control subjects signed an informed consent to participate in this study.

Table 1. List of methylation specific probes in MLPA kit ME024.

Gene	Length (bp)	Chromosomal position
<i>CDKN2A</i>	136	p14 ^{ARF} promoter
<i>CDKN2A</i>	155	p14 ^{ARF} promoter
<i>CDKN2A</i>	427	Exon 1 (p14 ^{ARF})
<i>CDKN2B</i>	130	Exon 1 (p15 ^{INK4B})
<i>CDKN2B</i>	211	Exon 1 (p15 ^{INK4B})
<i>CDKN2B</i>	220	Exon 1 (p15 ^{INK4B})
<i>CDKN2B</i>	417	p15 ^{INK4B} promoter
<i>CDKN2A</i>	171	p16 ^{INK4A} promoter
<i>CDKN2A</i>	237	Exon 2 (p16 ^{INK4A} promoter)

Isolation of DNA. Genomic DNA was extracted from bone marrow or peripheral blood mononuclear cells from patients with MDS, AML and controls. Miller's salting out method was used for isolation of DNA (19).

MS-MLPA. Samples were analyzed by MLPA kit (ME024, MRC-Holland, Amsterdam). This MLPA kit provides an easy to perform method for the detection of copy number variations and methylation changes of genes located in *CDKN2A-CDKN2B* (p15^{INK4B}-p14^{ARF}-p16^{INK4A}) region at 9p21 (20). The kit contains 43 different probes – 20 probes for the *CDKN2A-CDKN2B* genes – of which 9 are methylation specific (Table 1), 3 probes for the *MTAP* gene, 7 probes between *MTAP* and the 9p telomere region, 2 digestion control probes and 11 reference probes for data analysis. In addition, 9 control fragments are included (<http://www.mrc-holland.com>) [cit. 11-04-24]). MLPA probes for methylation quantification are similar to normal MLPA probes, except that the sequence detected by the MS-MLPA probe contains the sequence recognized by the methylation-sensitive restriction enzyme *HhaI*. Extracted genomic DNA (20–200 ng) was used, according to the instructions of the manufacturer (MRC-Holland, Amsterdam, Netherlands). Methylated hybrids of sample DNA were prevented from being digested by *HhaI*, and then amplified by PCR. Products were analyzed by capillary electrophoresis on an ABI 3500 automatic sequencer (Applied Biosystems, Foster City, CA, USA). If the DNA samples are methylated, proportional peak is visible; on the other hand, if the DNA samples are unmethylated, probes do not generate a signal.

Data and statistical analysis. A MS-MLPA probe targets a single specific *HhaI* site in a CpG island; if methylation is absent for a particular CpG-site, this does not necessarily mean that the whole CpG island is unmethylated. Therefore, two approaches are possible while analyzing the results: either to evaluate every single probe or to evaluate the whole gene and average out all probes within this particular gene. We used both methods for data analysis. Data normalization and analysis were performed using the Coffalyser software developed at MRC-Holland (Jordy Coffa, MSc., MRC-Holland, Bio Informatics Research Scientist). Statistical data processing was performed using

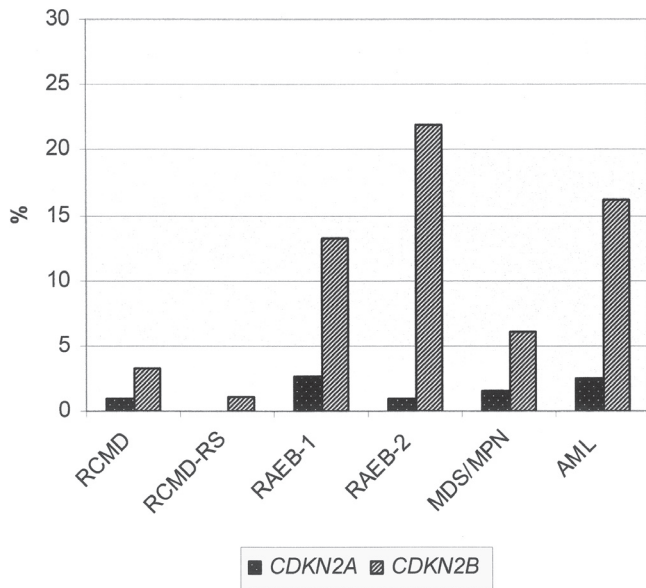


Figure 1. Methylation in each clinical subtype of myelodysplastic syndromes, MDS/MPN and AML for *CDKN2A* and *CDKN2B* genes.

The horizontal axis represents subtype and vertical axis the level of methylation in %.

RCMD, refractory cytopenia with multilineage dysplasia; RCMD-RS, refractory cytopenia with multilineage dysplasia and ring sideroblasts; RAEB-1, refractory anemia with excess blasts-1; RAEB-2, refractory anemia with excess blasts-2; MDS/MPN, myelodysplastic/myeloproliferative neoplasms; AML, acute myeloid leukemia

GraphPad software (GraphPad Software, Inc., La Jolla, CA, USA). Calculations were made with unpaired T-test with Welch's correction.

Results

Methylation analysis of the *CDKN2A* and *CDKN2B* genes. Methylation status of the *CDKN2A* and *CDKN2B* genes was examined in 9p21 regions using MS-MLPA kit in 78 samples obtained from patients with MDS or AML and in 26 samples from healthy donors. Copy number changes were analyzed in all samples. No variations – deletions or duplications – were detected either in patients' samples or in the control group. Nine MS-MLPA probes (130, 136, 155, 157, 211, 220, 237, 417 and 427 bp) were tested, all of them detect the methylation changes. Based on our experience and literature search we decided to adopt a following approach for data evaluation: average value of methylation of all used probes was used for *CDKN2A* gene (isoform 1 – p16^{INK4A} and isoform 4 – p14^{ARF}), because the values were quite comparable (for p14^{ARF} – 136 bp, 155 bp and 427 bp probes, for p16^{INK4A} – 171 bp and 237 bp probes). Four probes were included for the *CDKN2B* gene – 130 bp, 211 bp, 220 bp and 417 bp probe. The 211 bp probe was eliminated from the statistical evaluation, because there is a possibility of false positivity if insufficient *HhaI* activity is present and this probe is incompletely digested, which was also declared by the manufacturer. Methylation of *CDKN2B* gene was calculated from 130 bp, 220 bp and 417 bp probes. The percentages of methylation of genes according to diagnosis or IPSS score are presented in Table 2 and Figure 1 and in Table 3 are levels of methylation of each patient's populations and healthy donors. The results showed 38% aberrant methylation in the *CDKN2A* gene and 78% in the *CDKN2B* gene in patients with MDS and 77% in *CDKN2A* gene and 100% in *CDKN2B* gene in the group of patients with AML.

Table 2. Methylation (in %) of *CDKN2A* and *CDKN2B* genes in myelodysplastic syndromes and acute myeloid leukemia according to diagnosis and IPSS score. Brackets indicate the number of methylated/unmethylated cases.

according to diagnosis		<i>CDKN2A</i>		<i>CDKN2B</i>	according to IPSS score		<i>CDKN2A</i>		<i>CDKN2B</i>
	no.	p14 ^{ARF}	p16 ^{INK4A}			no.	p14 ^{ARF}	p16 ^{INK4A}	
RCMD	44	36% (16/28)	16% (7/37)	73% (32/12)	low-risk	14	14% (2/12)	7% (1/13)	71% (10/4)
RCMD-RS	4	0% (0/4)	0% (0/4)	50% (2/2)	intermediate-1	34	41% (14/20)	24% (8/26)	76% (26/8)
RAEB-1	7	57% (4/3)	43% (3/4)	100% (7/0)	intermediate-2	8	63% (5/3)	25% (2/8)	88% (7/1)
RAEB-2	8	50% (4/4)	13% (1/7)	100% (8/0)	high-risk	4	50% (2/2)	0% (0/4)	100% (4/0)
MDS/MPN	2	50% (1/1)	0% (0/2)	100% (2/0)					
AML	13	62% (8/5)	46% (6/7)	100% (13/0)					

Table 3. Representative data of methylation of each patient's populations for different probes and for whole genes. Levels of methylation are in % (range from 0 to 100%). 0% value is sample without methylation. HD, healthy donors. The isoform p14^{ARF} of *CDKN2A* gene included three probes with amplification product at 136, 155 and 427 bp. The isoform p16^{INK4A} of *CDKN2A* gene included two probes with amplification product at 171 and 237 bp. *CDKN2B* gene included three probes with amplification product at 130, 220 and 417 bp.

	<i>CDKN2A</i> (p14 ^{ARF})				<i>CDKN2B</i> (p15 ^{INK4B})				<i>CDKN2A</i> (p16 ^{INK4A})		
	136 bp	155 bp	427 bp	total	130 bp	220 bp	417 bp	total	171 bp	237 bp	total
RCMD	2.6%	0.7%	0.1%	1.2%	6.9%	3.7%	3.8%	4.8%	0.8%	1.2%	1.0%
RCMD-RS	0.0%	0.0%	0.0%	0.0%	2.8%	0.0%	0.0%	0.9%	0.0%	0.0%	0.0%
RAEB-1	4.7%	0.4%	1.9%	2.3%	27.7%	17.4%	6.4%	17.2%	1.9%	6.4%	4.1%
RAEB-2	3.9%	0.0%	0.0%	1.3%	31.4%	17.3%	30.1%	26.3%	0.0%	1.9%	0.9%
MDS/MPN	4.3%	0.2%	0.9%	1.8%	16.0%	0.0%	9.0%	8.3%	0.0%	0.0%	0.0%
AML	3.7%	2.3%	1.7%	2.6%	26.0%	13.5%	20.5%	20.0%	2.3%	4.5%	3.4%
Low-risk	1.5%	0.5%	0.0%	0.7%	3.3%	2.4%	2.1%	2.6%	0.0%	0.7%	0.4%
Intermediate-1	2.7%	0.8%	0.2%	1.2%	8.1%	6.1%	3.7%	6.0%	1.1%	1.3%	1.2%
Intermediate-2	3.5%	0.7%	0.8%	1.6%	23.7%	18.5%	19.6%	20.6%	0.9%	2.5%	1.7%
High-risk	2.5%	0.7%	0.3%	1.2%	14.9%	8.3%	21.4%	14.8%	0.0%	0.0%	0.0%
HD	1.1%	0.0%	0.0%	0.4%	3.2%	0.0%	1.8%	1.7%	0.0%	3.6%	1.8%

Statistical comparisons between different subtypes of MDS, AML and IPSS groups were made for each single probe and also for the gene in 9p21 region. P values for the genes are shown in Table 4.

Methylation analysis of the *CDKN2B* gene in five patients treated with 5-azacytidine (Vidaza). We analyzed methylation status in 47 samples from five patients during the hypomethylation treatment – four patients with AML and one patient with MDS/MPN. The trend of methylation status was monitored in *CDKN2B* gene, especially in the 130 bp probe (probe 11867-L12664, the coding region of exon 1). On the basis of a previous analysis of 78 patients this probe was capable of corresponding with disease progression.

The trend of methylation changes in individual patients and the correlation with the disease progression or remission of the disease is described in detail in legend of Figure 2. The methylation changes were only subtle, in the range of 1 – 5 percent. The correlation of disease progression was particularly shown in patients P1 and P3, where the level of methylation had increased by 11% after 11th cycle of the treatment in patient P1 and by 6% after 9th cycle of treatment in patient P3.

Discussion

Silencing of proliferation-associated genes by aberrant hypermethylation is often found in neoplasia. In the present study we have shown that hypermethylation is an important event in the progression of MDS to AML. Two issues were addressed. Firstly, the intensity of methylation of *CDKN2A-CDKN2B* genes in different subtypes of MDS according to WHO classification and IPSS score has been studied. Secondly,

Table 4. Statistical comparison in 3 different groups for *CDKN2A* and *CDKN2B* genes.

Unpaired T-test with Welch's correction was used for statistical comparison.

Early versus Advanced MDS (RCMD, RCMR-RS x RAEB-1, RAEB-2)			
		P value	Statistical significance (P < 0.05)
<i>CDKN2A</i>	p14 ^{ARF}	0.3051	No
	p16 ^{INK4A}	0.1909	No
<i>CDKN2B</i>	p15 ^{INK4B}	< 0.0001	Yes
Low-risk + Intermediate-1 versus Intermediate-2 + High-risk			
		P value	Statistical significance (P < 0.05)
<i>CDKN2A</i>	p14 ^{ARF}	0.3935	No
	p16 ^{INK4A}	0.5454	No
<i>CDKN2B</i>	p15 ^{INK4B}	0.003	Yes
MDS versus AML			
		P value	Statistical significance (P < 0.05)
<i>CDKN2A</i>	p14 ^{ARF}	0.1031	No
	p16 ^{INK4A}	0.0935	No
<i>CDKN2B</i>	p15 ^{INK4B}	0.0014	Yes

the response of patients to the treatment with hypomethylation agents was evaluated.

We analyzed the intensity of methylation of *CDKN2A* and *CDKN2B* genes according to diagnosis and IPSS score. All the results were compared with the results obtained from healthy controls. The sample was considered as unmethylated, if all the probes for the gene were without methylation. Aberrant methylation was detected in 38% in *CDKN2A* gene and in 78% in *CDKN2B* gene in patients with MDS according to

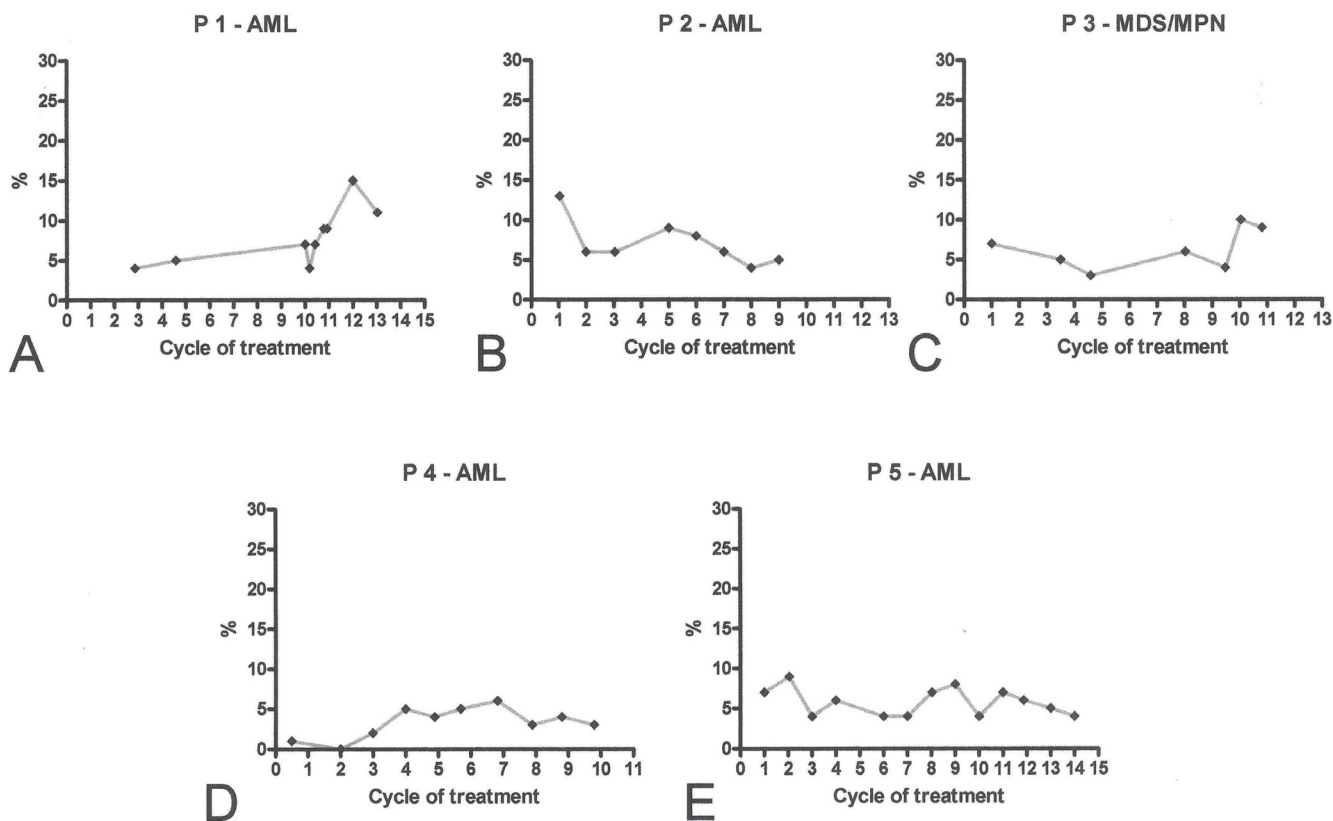


Figure 2. Monitoring of methylation in *CDKN2B* in five patients treated with 5-azacytidine.

The horizontal line represents cycles of treatment with 5-azacytidine and the vertical line represents the intensity of methylation. (A) The patient with AML achieved stable disease during the treatment with 5-azacytidine, new progression of the disease was observed after cycle 11 of the treatment. (B) Patient with AML achieved partial remission and the treatment is continuing. (C) Patient with MDS/MPN was treated because of new disease progression after allogeneic SCT, but progression of the disease was observed during the treatment after cycle 9. (D) Patient with AML achieved partial remission of the disease. (E) Patient with AML achieved stable disease but relapsed after cycle 14.

diagnosis. Hypermethylation was observed in AML patients (77% in *CDKN2A* gene and 100% in *CDKN2B* gene). The majority of other studies has focused on the detection of aberrant methylation in *CDKN2B* gene, only a few have analyzed also *CDKN2A* gene. Reports from other authors have shown aberrant methylation of *CDKN2B* gene of 50 – 80% on average in AML patients (2, 21). Agrawal et al. (2007) detected *CDKN2B* methylation in 60% (2) and Herman et al. (1997) in 88% of AML patients (21). Furthermore, there are also reports from other authors who showed that the value of methylation of *CDKN2B* gene was in the range of 40 – 60% in MDS patients (7, 8, 11, 22). Aggerholm et al. (2006) found methylation of *CDKN2B* gene in 51% of MDS patients (22). Our results are slightly different, the percentage of methylation being higher. This is probably due to the fact that the MS-MLPA method that we have used, and the very kit ME-024, contains 43 probes selectively for *CDKN2A* and *2B* gene regions. Solomon et al. (2008) detected methylation in 61% in *CDKN2B* gene and in 37% in *CDKN2A* (*p16^{INK4A}*) gene of MDS patients. They used a method of semi-nested multiplex PCR and primers located in

the CpG rich islands of the promoter at least 500 bp upstream of the transcription start site, enclosing at least one *HpaII* restriction site (8). The examined group of patients was smaller than our one; however, the results obtained in this study were very similar to our results. Uchida et al. (1997) showed that no apparent hypermethylation of *CDKN2A* (*p16^{INK4A}*) gene was detected in patients with MDS (11) while in our study we detected methylation in 38% of MDS patients. This could be caused by the different design of primers used in these two studies. Our methylation probes were designed to detect methylation in regions located nearer the gene of interest while Uchida used primers located in a different region of interest.

The level of methylation was increasing in accordance with IPSS score (from low-risk to intermediate-2-risk patients), except for the high-risk group. In this group the level of methylation was 14.8% in *CDKN2B* gene, however, in the intermediate-2-risk group the level was 20.6%. This result is most likely due to the small number of patients (only four) in the high-risk group and this could be the source of bias. Other studies detected an increase of methylation intensity continu-

ally from low-risk to high-risk (7, 11) and we suppose that we would observe the same trend if more patients had been included. These results support our current understanding of the mechanism of methylation and its role in neoplasia, as it was proposed by Herman et al. (1996, 1997) who showed that aberrant DNA methylation is associated with inactivation of tumor suppressor gene (p15^{INK4B}, p16^{INK4A}) (21, 23) and therefore with disease progression.

Vasikova et al. (2010) compared gene expression profiles of CD34+ cells of MDS patients and healthy controls using HumanRef-8 v2 Expression Bead Chips from Illumina. They observed expression differences between patients and controls. The expression of genes involved in cell cycle and DNA repair was significantly lower in advanced MDS patients, what might indicate altered and inappropriate reaction of the cell towards mutagenic agents during the disease progression. On the other hand, the level of expression of protooncogenes, angiogenic, anti-apoptotic genes and genes of signal transduction was increased. They have concluded, that overexpression of these genes might be important for a malignant clone expansion in MDS patients (24). Twenty-two of our patients were included in their study. The link between our study and the study of Vasikova et al. (2010) is that level of methylation and expression of several transcription factors is closely related. The level of methylation, which was detected in our study, showed increasing trend in our patients (at RCMD 4.8%, at RAEB-1 17.2% and RAEB-2 26.3%). Concurrently, there was detected firm adherence in trend of downregulation of transcription factors E2F1 and E2F2 from the early to advanced forms of MDS (at RCMD -1.1x, at RAEB-1 -1.7x and RAEB-2 -2.4x than healthy controls).

Our study focused on monitoring the intensity of methylation in patients treated with the hypomethylation agent 5-azacytidine. Based on the results of our previous analysis of seventy-eight samples, we decided to monitor the methylation status only in the *CDKN2B* gene, especially in 130 bp probe. This probe is located in exon 1a, whose *HhaI* site is located 33 base pairs before the first methionine. Other probes coding for *CDKN2B* gene are the 220 bp probe, whose *HhaI* site is located 100 base pairs before the first methionine, or the 417 bp probe located 155 bp before the exon 1a. The most consistent results were obtained for the 130 bp probe and we speculate that this probe might be located in an important area, the closest to the coding region of the *CDKN2B* gene. We suppose that this region might play an important role in the regulation of the *CDKN2B* gene. However, further experiments are needed.

In the group of patients treated with 5-azacytidine, the trend of methylation partly correlated with disease progression, but the methylation changes were only subtle. In our study, two from five patients achieved partial remission while the three remaining patients progressed. The results of other studies are fairly consistent, showing about 40% of patients responding to treatment with hypomethylating agents (25, 26).

Allogeneic SCT is the only treatment modality in MDS, but is available only for selected patients depending mainly on

the amount of the bone marrow blasts, comorbidities, suitable donor and other criteria (15, 18). For patients who are not indicated for combination therapy and allogeneic SCT or do not have a suitable donor for SCT, 5-azacytidine represents a new treatment option. Results from the literature show that treatment with 5-azacytidine may be effective in patients with intermediate-2 and high-risk MDS because of prolongation of overall survival (15, 18, 27). 5-Azacytidine has been observed to produce better responses in patients with unfavourable cytogenetics and was effective in reducing the risk of disease relapse or in the treatment AML relapse after allogeneic SCT (15, 18, 28, 29). There are also reports supporting the feasibility and effectiveness of 5-azacytidine treatment in patients with lower-risk MDS (16). 5-Azacytidine has been also used as a first line treatment in patients with de novo acute myeloid leukemia similarly as several patients in our cohort.

In summary, aberrant methylation in the CpG islands was present frequently in the *CDKN2B* gene and increase of methylation was accompanied by disease progression. Thus, the methylation level of this gene might be used as a marker of leukemic transformation in MDS (1). Our study indicates an important role of hypermethylation in the progression of MDS to AML.

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