Decreased DNA methylation in acute myeloid leukemia patients with DNMT3A mutations and prognostic implications of DNA methylation

Hana Hájková a,*, Jana Marková a, Cedrik Haškovec a, Iveta Šárová a, Ota Fuchs a, Arnošt Kostečka a, Petr Cetkovský a, Kyra Michalová b, Jiří Schwarz a

a Institute of Hematology and Blood Transfusion, Prague, Czech Republic
b General University Hospital and 1st Faculty of Medicine, Prague, Czech Republic

ABSTRACT

We examined 79 acute myeloid leukemia (AML) patients for DNA methylation of 12 tumor suppressor genes (TSG) and 24 homeobox domain (Hox) genes, and additionally for mutations in DNMT3A gene. We observed lower levels of DNA methylation (P < 0.0001) as well as smaller numbers of concurrently hypermethylated genes (P < 0.0001) in patients with DNMT3A mutations. Our study of the impact of DNA methylation on prognosis in intermediate and high risk AML patients revealed a relation between higher DNA methylation and better patients’ outcome. Lower DNA methylation was linked with higher relapse rates and an inferior overall survival.

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1. Introduction

Genetic and epigenetic changes have been established in the pathogenesis of acute myeloid leukemia (AML) [1–3]. Currently, mutations of genes involved in regulation of epigenetic processes (such as DNMT3A, TET2, EZH2, ASXL1, IDH1/2 etc.) are coming to the fore [4,5]. These mutations, together with other genetic alterations (e.g. gene translocations, deregulated gene expression etc.) affecting genes involved in epigenetic pathways, may give rise to epigenetically deregulated tumor cells. Presumably, DNA methylation signature may be influenced by the alterations of these epigenetic regulatory genes. Therefore, it will be of great importance to link information on such genetic lesions to epigenetic aberrations. Mutations in DNMT3A gene encoding the enzyme DNA methyltransferase 3A have recently been observed in approximately 20% of AML cases and correlated with a poor clinical outcome [6,7].

DNA methyltransferases (DNMTs) are a family of enzymes that catalyze the transfer of a methyl group to cytosine, forming 5-methylcytosine. DNMT3A and DNMT3B are preferentially de novo methyltransferases establishing a new pattern of DNA methylation, while the role of DNMT1 is to maintain the DNA methylation pattern during cell division [8]. DNA methylation has two prominent effects in the cell. Firstly, it stabilizes the genome by hypermethylation of repetitive sequences. Secondly, DNA methylation is involved in the process of switching on/off of genes that are regulated from promoter or another regulatory region containing CpG rich regions called CpG islands (CGI). It is reported that about 60% of human genes contains CGI in their promoter regions [9]. The importance of DNA methylation is underlined by the fact that DNMT1 and DNMT3B knockout mice displayed embryonic lethality and DNMT3A knockout mice are born alive but die within a few weeks after birth [10]. In humans, germ-line mutation in DNMT3B results in global hypomethylation and chromosomal instability, as seen in patients with ICF (immunodeficiency, centromeric instability and facial anomalies) syndrome [11,12].

The purpose of this study was to evaluate the impact of DNMT3A mutations on DNA methylation levels of selected genes. Based on previously mentioned facts, we hypothesized that mutations of the DNMT3A gene might directly influence the content of methylated cytosine compared to cells with wild-type DNMT3A. However, DNMT3A mutations have not been found to dramatically alter 5-methylcytosine content and global DNA methylation levels in AML genomes [6,7]. Therefore we applied a more specific approach focusing on selected genes and their DNA methylation levels using real-time PCR (RQ–PCR) with methylation specific primers and probe (MethylLight) and methylation specific cleavage of DNA followed by RQ–PCR to assess whether there is a difference between methylation levels of particular promoter regions in AML patients with wild-type versus mutated DNMT3A. For these purposes, we
examined 12 tumor suppressor genes (TSG) [CDKN2B, CALCA, CDH1, ESRI, SOCS1, MYOD1, DAPK1, TIMP3, ICAM1, TERT, DNMT1, and EGR1] by MethyLight. Most of these genes have been described as hypermethylated in hematological or other malignancies previously [13–16]. Additionally, we looked at the DNA methylation status of homeobox domain (Hox) genes by DNA methylation PCR array. Hox genes are involved in leukemogenesis and Yan et al. [17] have recently found hypomethylated CpG islands in the promoter region of HOXB cluster in AML patients with mutated DNMT3A.

2. Materials and methods

2.1. Patients

Bone marrow or peripheral blood samples from 79 AML patients at the time of diagnosis were collected. The median age was 53 years (19–81 years) and the female/male ratio was 45/34. Six patients had therapy-related AML, 5 had secondary AML (after MDS/MPN) and 68 had de novo AML. Patients with a favorable cytogenetic profile – t(15;17), inv(16) or t(16;16), i(8;21) – were not included in this study. Clinical characteristics of patients (including routinely examined markers FLT3/ITD, NPM1 and cytogenetic prognostic group according to Marchesi et al. [18]) are summarized in Table S1. Patients’ written consent was obtained and the study was approved by the Institutional Ethics Committee.

2.2. Sample preparation

Mononuclear cells (MNC) from peripheral blood (PB) or bone marrow (BM) at diagnosis were separated by Ficoll gradient centrifugation (Histopaque, Sigma–Aldrich, Steinheim, Germany) and then stabilized in RLT buffer (Qiagen, Hilden, Germany).

2.3. DNA/RNA isolation, bisulfite conversion and reverse transcription

DNA and RNA were extracted from peripheral blood or bone marrow mononuclear cells (PB MNC or BM MNC) using AllPrep DNA/RNA Mini Kit (Qiagen). Bisulfite conversion was performed from 1 μg DNA by EpiTect Bisulfite Kit (Qiagen) and eluted into 40 μl EB buffer. cDNA was prepared as described in Marková et al. [19].

2.4. Methylation specific qPCR (MethyLight)

Bisulfite–treated DNA (BS DNA) was amplified using methylation–specific TaqMan probe placed within the area bound by the methylation–specific primers. The principle and validation of MethyLight technology is summed up in the work of Eads et al. [20]. ACR gene, amplifying the bisulfite–converted DNA regardless of its methylation status, served as a control gene to determine a quality of BS DNA. Primer and probe sequences were taken from [21,22]. CTNN1 and EGR1 primers and probes were designed using Beacon Designer 7 (PREMIER Biosoft, Palo Alto, CA, USA) and the sequences are shown in Table S2. Levels of DNA methylation were expressed as a percentage of methylated reference (PMR). Commercially available fully methylated human DNA (Qiagen) was used as a positive reference. As a negative reference, 20 normal blood samples (MNC) were examined for each gene to set a DNA methylation cut–off defined as the value above which the DNA methylation is considered aberrant. These values varied for individual genes, but overall always resulted in PMR > 1. CDKN2B gene displayed the highest levels of basai DNA methylation and PMR > 4 were considered aberrantly methylated. TaqMan Universal Master Mix II (Applied Biosystems, Foster City, CA, USA) and manufacturer’s recommended PCR reaction conditions were used for amplification. Amplification and data analysis were carried out using the Rotor Gene 3000 thermocycler (Corbett Research, Sydney, Australia). PMR values were calculated as depicted in Ogino et al. [23].

2.5. Methyl RQ–PCR arrays

We used Human Homebox Genes DNA Methylation PCR Array (SABiosciences, Frederick, USA) to profile DNA methylation levels of Hox genes (n = 24) (HOXA1, HOX2A, HOX4A, HOX5A, HOX6A, HOX7A, HOX9A, HOX11A3, HOX8B, HOX8B, HOXB3, HOXB4, HOXB5, HOXB6, HOXB7, HOXB8, HOXB9, HOXB13, HOX8C, HOXD3, HOXD10, HOXD11, HOXD13). This novel restriction enzyme–based technique was described by Kim et al. [24]. Briefly, genomic DNA is treated with a combination of methylation–sensitive (Ms) and/or methylation–dependent (Md) enzymes. Subsequent RQ–PCR enables comparison of Ct values between particular enzymatic reactions and thus assessment of DNA methylation levels. PCR reactions were performed using Option RQ–PCR machine (Bio–Rad, Hercules, CA, USA). Detailed description of sample preparation and PCR reaction conditions are provided in the manufacturer’s protocol. A methylation rate above “mean + 3 standard deviations” of healthy donor’s DNA methylation levels was used to set the cut–off value for aberrant DNA methylation.

2.6. Direct sequencing

Sequencing analysis was performed to detect mutations between amino acids 300 and 930 (where DNMT3A mutations occur according to Levy et al. [6]). Primer sequences and PCR reaction conditions were described previously [25]. Direct sequencing was performed on ABI Prism 310 genetic analyzer (Perkin–Elmer, Wellesley, MA, USA) using Big Dye® Terminator kit v3.1 (Applied Biosystems).

2.7. Conventional and molecular cytogenetics

Standard chromosomal preparation techniques for bone marrow cells were used. Twenty–two mitoses were analyzed using an IKAROS imaging system for karyotyping (MetaSystems, Altusseim, Germany). Chromosomal abnormalities were verified by FISH with Vysis DNA probes (Abbott Laboratories, Abbott Park, IL, USA) and mFISH/mBAND using XCyte color kits (MetaSystems), according to standard protocols. At least 20 metaphases were evaluated using an Axio–Imager Z1 microscope (Zeiss) and an IKAROS/ISIS computer analysis system (MetaSystems).

2.8. Statistical analysis

For analyses of quantitative data, medians were calculated and non-parametric two–tailed Mann–Whitney tests were performed. Kaplan–Meier curves and two–sided log–rank test were used to estimate the overall survival and to compare differences between survival curves. The relations between the parameters compared in contingency tables were analyzed using the χ2 test. All tests were conducted at a level of significance of 0.05 using GraphPad Prism4 software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. DNA methylation of 12 selected TSG by MethyLight

MethyLight assessment of 12 TSG showed the following frequencies of hypermethylation in AML patients at diagnosis (n = 79): CDKN2B (49%), CALCA (43%), SOCS1 (24%), CDH1 (22%), MYOD1 (18%), ESRI (14%), DAPK1 (5%), ICAM1 (4%), TERT (2.5%). TIMP3, CTNN1 and EGR1 were not found to be hypermethylated when compared with healthy donors’ blood samples (n = 20). Overall, 70% of AML patients had at least 1 gene hypermethylated at the time of diagnosis. We categorized DNA methylation levels for individual genes as follows: low (<15% of PMR), intermediate (≥15% and <50% of PMR) and high (≥50% of PMR). The gene displaying the highest levels of methylation was ESRI followed by CALCA and SOCS1. For cumulative DNA methylation assessment, we semi–quantitatively assigned low DNA methylation level to 1, intermediate to 2 and high to 3. Then the cumulative DNA methylation value for the respective sample was taken as the sum of DNA methylation levels for individual genes (range 0–13; median 2). ESRI methylation was most predictive for concurrent hypermethylation of other genes (P < 0.001). The median number of methylated genes was 1 (range 0–6) with 12 AML patients having 4 and more simultaneously hypermethylated genes at the same time. When available (n = 10) we compared results obtained from PB MNC to those from BM MNC and found that the resulting value was always in the same DNA methylation category (negative, low, intermediate or high). Based on this, we concluded that there was no significant difference in assessing DNA methylation from PB or BM.

3.2. Hox genes DNA methylation profiling

Hox genes methylation profiling revealed the following set of differentially methylated genes (n = 12) when compared to healthy donor samples: HOX4A, HOX5A, HOX6A, HOX13, HOXB3, HOXB4, HOXB7, HOXB8, HOX8C, HOX10, HOXD11 and HOXJ3. In general, the levels of DNA methylation were lower in patients with DNMT3A mutation (see Section 3.4). The most frequently hypermethylated gene regardless of DNMT3A mutational status was HOX4A. On the contrary, the most frequently hypomethylated gene was HOXB3 within mutated DNMT3A cohort and HOX5 gene independently of
DNMT3A status. The remaining genes were hypermethylated preferentially in the DNMT3A wild-type subgroup.

3.3. DNMT3A mutational analysis

Sequencing of cDNA from PB or BM of 79 AML patients at diagnosis revealed that 32 of 79 (41%) AML patients had DNMT3A mutation. The higher DNMT3A mutation incidence (compared to published data) in our patients’ cohort is due to the preferential selection of AML patients with a higher percentage of probability of this mutation (normal karyotype and mutations in NPM1 and/or FLT3). 20 patients had a single nucleotide change at codon Arg882 (15 had Arg882His and 5 had Arg882Cys). 6 patients had another missense mutation and 3 patients displayed frameshift mutations (2 deletions and 1 insertion). 3 patients carried two different mutations at the same time.

3.4. Relationship between DNA methylation, DNMT3A mutations and other parameters

We found a strong correlation between DNA methylation and DNMT3A mutations. DNA methylation levels (P<0.001) as well as numbers of simultaneously methylated genes (P<0.001) were significantly lower in patients with mutated DNMT3A (Fig. 1). The number of cases with no methylation in any of the studied genes (zero methylation) also differed significantly (18 versus 6 in mutated versus wild-type DNMT3A, 56% versus 13% of cases respectively, P<0.001).

The same trend was obvious for Hox genes (a representative example is shown in Fig. 2). We observed lower levels of DNA methylation in patients with mutated DNMT3A even when compared to healthy donor sample (Fig. 2). There was no difference between the most frequent types of DNMT3A mutations, i.e. Arg882His/Arg882Cys, and other types of mutations, when assessing their impact on DNA methylation.

Using the same approach, we evaluated whether there was a connection between DNA methylation and age, gender, percentage of blasts (PB and BM) or cytogenetic risk group (intermediate versus high risk). DNA methylation levels or numbers of HM genes were independent of these parameters (data not shown).

3.5. DNA methylation impact on prognosis

We subdivided AML patients according to the sums of their semi-quantitatively assessed levels of DNA methylation (cumulative DNA methylation) (see Section 3.1.) – into the following groups: low (0–1) and high (2 and more) DNA methylation levels. The same was done for the number of simultaneously hypermethylated (HM) genes: low (0–2) and high (3–6) numbers of concurrently HM genes. These categories based on DNA methylation status were used for subsequent statistical analysis. Only the 6 most frequently hypermethylated genes (CDKN2B, CALCA, SOCS1, CDH1, MYOD1 and ESR1 – genes that were hypermethylated in more than 10% of patients) were involved in this analysis.

Fig. 1. Decreased cumulative DNA methylation levels (A) and numbers of hypermethylated genes (B) in AML patients with mutated (mut) vs wild-type (wt) DNMT3A.

Fig. 2. Differences between DNA methylation levels of HOX genes in a representative AML patient with wild-type DNMT3A (A), with mutated DNMT3A (B) and healthy donor sample (C); UM, unmethylated; IM, intermediately methylated; HM, hypermethylated.
PCR; all

3.5.1. Achievement of complete remission (CR) and cumulative incidence of relapse

First we analyzed the impact of DNA methylation levels and numbers of HM genes on CR rates. We did not find any correlation for either overall DNA methylation levels or individually studied genes. However, when analyzing relapse rates in the context of DNA methylation, we identified the influence of DNA methylation on a cumulative incidence of relapse (Fig. 3). Higher DNA methylation levels \( (P = 0.053) \) and numbers of HM genes \( (P = 0.012) \) were connected with the lower incidence of relapse.

3.5.2. Survival analysis

We correlated DNA methylation of individual genes and overall survival (OS). The most informative gene was SOCS1, which was connected with better OS when hypermethylated in its promoter region \( (P = 0.04) \) (Fig. 4A). The same trend was obvious for the remaining genes \( (e.g. CDKN2B, P = 0.06, \text{Fig. 4B}) \).

Further, we compared OS for patients with low and high levels of cumulative DNA methylation as well as for low and high numbers of concurrently methylated genes (Fig. 4C and D). Our results suggest that patients with lower levels of DNA methylation \( (P = 0.0274) \) or with smaller numbers of hypermethylated genes \( (P = 0.012) \) have a worse OS compared to those with higher DNA methylation at multiple loci.

4. Discussion

DNA methylation as a crucial epigenetic mechanism is involved in regulating chromatin state, its remodeling and subsequent gene expression. DNMT3A is an enzyme catalyzing de novo addition of methyl \( (\text{CH}_3\text{-}) \) group to the cytosine base. Generally, cancer cells are characterized by site-specific hypermethylation of tumor suppressor genes that leads to their silencing. This abnormal hypermethylation is thought to be a consequence of altered DNA methyltransferase activity. Presumably, de novo DNA methyltransferases (as DNMT3A) may be responsible for the onset of hypermethylator phenotype in many cancers, including leukemia. Recently, mutations in DNMT3A have been discovered in AML patients and have been shown to be connected with shorter overall survival and/or disease-free survival \([6,7,25]\). Our assumption was that if a leukemic clone has a DNMT3A mutation, there may be a block in one of the possible mechanisms of acquiring hypermethylation. In other words, there will be a tendency to hypomethylation when compared to wild-type DNMT3A leukemic cells. Therefore, we examined the impact of DNMT3A mutation on DNA methylation levels of selected tumor suppressor genes \( (n = 10) \) as well as Hox genes \( (n = 24) \).

The present study indicates that there is a significant link between DNMT3A mutational status and DNA methylation state in AML patients within an intermediate and high risk group. Firstly, we identified AML patients with mutated DNMT3A to have lower cumulative DNA methylation levels of selected tumor suppressor genes as well as Hox genes. Secondly, they have less concurrently hypermethylated genes compared to the patients with wild-type DNMT3A. To the best of our knowledge, this is the first report showing such a clear correlation. None of the previous studies has addressed this issue by using the highly sensitive approach of methylation specific real-time PCR (MethyLight). Thol et al. did not reveal differential methylation levels in DNMT3A mutated versus wild-type cohorts \([7]\). Ley et al. found that genomes with or without DNMT3A mutations displayed virtually identical 5-methylcytosine content, but when using a more specific approach of MeDIP-chip analysis, they detected 182 genomic regions hypomethylated within the DNMT3A-mutated subgroup \([6]\). Finally, another work by Yan et al. also using MeDIP-chip analysis showed the presence of hypomethylated CpG islands in the HOXB cluster \([17]\). This finding is consistent with our results showing that AML patients with DNMT3A mutation have lower levels of DNA methylation in Hox
genes. Interestingly, Hox DNA methylation levels within DNMT3A-mutated AML patients were even lower than those observed in healthy donor samples (Fig. 2). Not only numbers and levels of hypermethylated genes were decreased among patients with mutated DNMT3A but also numbers of cases with so called zero methylation (i.e. none of the studied genes was methylated) were significantly lower. This supports the theory about the functional relation between the absence of hypermethylation phenotype and mutation in DNMT3A.

Furthermore, we focused on the impact of DNA methylation on prognosis. Based on DNMT3A mutation negative influence on the outcome [6,7,25], our presumption was that DNA hypomethylation rather than DNA hypermethylation of promoter regions of studied genes could be connected with unfavorable outcome. The prognostic relevance of DNMT3A mutational status has been reported in detail for larger intermediate-risk AML patients’ cohort in our previous study [25]. In the current study, we performed a statistical analysis taking into account both levels of DNA methylation as well as numbers of concurrently hypermethylated genes, and revealed better OS when more HM genes were present and when levels of methylation were higher. The same was observed for cumulative rates of relapse – they were higher when less DNA methylation was present. These findings are somewhat surprising when considering the general concept that inactivation of TSG might be rather accompanied by a negative prognostic impact due to their role as genome keepers. However, such observations have already been done also by other groups. Most supportive observations to ours were made by Deneberg et al. [26]. They found that CDKN2B methylation, along with increased genome-wide promoter-associated methylation, was connected with better OS and disease-free survival (DFS). Very similar revelations were described by Kroeger et al. who found increased DNA methylation of multiple studied genes connected with favorable prognosis [27]. Furthermore, methylation of CEBPA was shown to be associated with better treatment response, OS and DFS [28]. On the other hand, there are a number of studies reporting adverse outcome due to promoter-associated methylation [29–31].

All these reports, including our data, show the growing evidence of DNA methylation impact on assessing prognosis. This importance is strengthened by increasing numbers of recently revealed mutations in genes playing role in epigenetic mechanisms. More studies are needed to clarify the relation between DNA methylation and patients’ outcome. The present study provides preliminary data regarding the influence of DNA methylation on prognosis of AML patients within intermediate and high risk groups. Obviously, there might be differences between particular genes being hypermethylated and their prognostic impact. We can hypothesize that hypermethylation of genes involved in specific cellular pathways may make these cells more prone and sensitive to chemotherapy treatment. Better understanding of biological differences between AML with less and more DNA methylation may bring clinical benefits for patients.

Conflict of interest

The authors declare no competing financial interests.

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Contributions: HH, JS, JM, CH - designed experiments, wrote and revised the manuscript; JM-mutational and statistical analysis; HH - methylation and statistical analysis; IS, KM-cytogenetic data; OF, AK – mutational analysis; JS, PC – patients’ data.
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2012.05.012.

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