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ER, SOCS1, p15, E-cadherin and RARB are more Likely to be Members of the Methylator Phenotype of Adult and Childhood AML and Their Methylation is Primarly Regulated by an Overexpression of DNMT 3A

Erişkin ve Çocukluk Cağı AML Oluşumunda ER, SOCS1, p15, E-cadherin ve RARB Promoter Metilasyonu Metilatör Fenotip Üyesi Olmaya Aday genlerdir ve Metilasyondan DNMT 3A Başlıca Sorumlu Enzim Olarak Ön Plana Çıkmaktadır

Cumhur G. Ekmekçi[1], Uğur Özbek[2]

[1]Acıbadem Labgen Genetic Diagnosis Center, İstanbul, Turkey
[2]Department of Medical Genetics, Acıbadem University, İstanbul, Turkey

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ABSTRACT

Objectives: The hypermethylator phenotype is used as a term for both acute myeloid leukemia (AML) and other tumors. We analyzed the methylation statuses of ten genes (DAP-kinase, SOCS1, ER, p15, Ecadherin, RARβ, p16, GSTP1, HIC1 and p73) and correlated them with the expression of DNA methyltransferases (DNMTs).

Material and Method: We analyzed the methylation profiles using methylation specific PCR and COBRA and the expression of DNMTs (DNMT 1, 3A and 3B) by quantitative RT-PCR in 25 pediatric and 25 adult AML samples.

Results: The ER, SOCS1, p15, E cadherin, and RARB genes methylated together significantly in the same patients and members of the methylator phenotype.

Conclusion: Our study demonstrated that the gene DNMT3A was dramatically upregulated and significantly correlated with the methylator phenotype.

Keywords: Methylator phenotype, MSP, epigenetics, tumor-suppressor genes, acute myeloid leukemia, DNA methyltransferases

ÖZ

Amaç: Hipermetilatör fenotipi, hem akut miyeloid lösemi (AML) hem de diğer tümörler için bir terim olarak kullanılır. On genin metilasyon durumlarını analiz ettik (DAP-kinaz, SOCS1, ER, p15, Ecadherin, RARβ, p16, GSTP1, HIC1 ve p73) ve bunları DNA metil transferazların (DNMT'ler) ifadesiyle korele ettik.

Gereç ve Yöntem: 25 pediatrik ve 25 yetişkin AML örneğinde metilasyon spesifik PCR ve COBRA ve DNMT'lerin (DNMT 1, 3A ve 3B) ekspresyonunu kullanarak metilasyon profillerini analiz ettik.

Bulgular: ER, SOCS1, p15, E cadherin ve RARB genleri, aynı hastalarda ve metilasyon fenotipinin üyeleri arasında önemli ölçüde birlikte metillendi.

Sonuç: Çalışmamız, DNMT3A geninin dramatik bir şekilde yukarı regüle edildiğini ve metilasyon fenotipi ile anlamlı şekilde korele olduğunu gösterdi.

Anahtar Kelimeler: Metilatör fenotipi, MSP, epigenetik, tümör baskılayıcı genler, akut miyeloid lösemi, DNA metiltransferazlar

INTRODUCTION

The disruption of epigenetic regulation during malignant transformation can profoundly alter a cellular phenotype, resulting in aberrant cellular proliferation and survival (1). This major epigenetic modification targets methylation of cyctosine (C) bases adjacent to guanin (G) within the dinucleotid repeats of the CG's. This process is shared by both prokaryotic and eukaryotic cells (2) and is carried out by DNA methyltransferases (DNMTs) (3, 4). Methylated promoters CpG islands make the gene transcriptionally silenced. A change in the methylation profiles of certain genes is known to cause various diseases or developmental abnormalities (5-7). Global hypomethylation in human DNA

Corresponding Author/Sorumlu Yazar: Cumhur G. Ekmekçi **E-mail:** cgekmekci2@yahoo.com
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appears to begin early and before the development of tumor formations. A changed equilibrium in cancer cell lines gives rise to hypermethylation of CpG islands - especially the loss of function of critical tumor suppressor genes. The main cause of global hypomethylation followed by hypermethylation of specific genes in tumors is not yet clear. Numerous genes have been shown hypermethylated in various subtypes of cancers (8-12). In various studies a correlation between tumor and hypermethylation has been demonstrated, such as p15 (INK4B) (13), SOCS1 (suppressor of cytokine signaling 1) (14), Dap kinase (15), ER (estrogen receptor) (16) and researchers have tried to prove a relation between hypermethylation of multiple genes and cancer (10, 12, 17). Some human cancers exhibit a hypermethylator phenotype, characterized by concurrent DNA methylation-dependent silencing of multiple genes. The hypermethylator phenotype has been shown in various human neoplasms, such as pancreatic (18), colorectal (19), and gastric cancers (8).

DNMTs have been categorized in three different families DNMT1, DNMT2, and DNMT3. These enzymes are encoded by different genes in mammals (6, 24-26). DNMT3a and DNMT3b are classified as de novo methyltransferases and have been shown to have overlapping functions with DNMT1 (24, 27, 28). While It has been demonstrated that an increased DNA methylation capacity accompanies an increase in DNMT transcripts observed during progressive stages of colon cancer (29), another study reported an opposite result for the same diseases (30). In hemotologic malignancies, an overexpression of DNMT1 was shown in 12 leukemia samples including AML, ALL and MDS (31).

In this study, we expanded our previous study population to get improved results to help understand the epigenetic rearrangement in AML tumorogenesis. We analyzed the methylation of tumor suppressor genes previously confirmed to be methylated in AML, including DAP-Kinase, SOCS1, ER, p15 (10, 32-34) and E-Cadherin (10) RARβ (Retinoic aside receptor beta) (32), p73 (35). Our AML patients have a distinct geographical and ethnic background. We also wanted to revisit the pattern of methylation of 3 genes, including p16 (INK4A), GSTP1 (glutathion S transferase P1) and HIC1 (hypermethylated in cancer1), that are rarely methylated in AML (10, 36). After obtaining the methylation profiling of multiple genes then we address the expression level of DNA methyltransferases' importance in the aberrant methylation of multiple tumor suppressor genes.

MATERIAL AND METHOD

Patients

Fifty DNA samples were obtained from the 25 pediatric and 25 adult AML patients participating in our study. The median age of the pediatric patients was 9 years (ranging from 1.2 to 17) and the median age of the adult patients was 34 years (ranging from 20 to 74). Thirty one patients were males and 19 were females. The diagnosis of AML was made based upon standard clinical and laboratory criteria, including histocytomorphologic appearance and immunophenotypes. Morphologic subtyping was determined according to FAB classification. The study was approved by the appropriate IRB.

Sodium Bisulfite Treatment of DNA

Genomic DNA was extracted from mononuclear cells using the Puregene DNA isolation kit (Gentra, Minneapolis, MN) and treated with sodium bisulfite as previously described (16). In brief, 5µg of DNA were denatured at 42°C for 30 min with 0.4M NaOH, incubated in 10mM hydroquinone, 3M sodium bisulfite (Sigma, St Louis, MO) at 55°C for 16 hours and purified with the GeneClean III kit (Bio 101, Vista, CA). Prior to ethanol precipitation, the DNA was desulfonated in 0.4M NaOH for 15 min at 37°C. The DNA was resuspended in water and stored at -80°C.

Methylation-Specific PCR (MSP) and Restriction Digestion

The bisulfite-treated DNA was used in methylation-specific PCR (MSP) reactions for 9 genes using primers - specific for the methylated and unmethylated forms (31). The amplifications reactions contained 25 pmoles of each primer, 200µM dNTP, 1U Hotstart Taq polymerase and 1x Q buffer (Qiagen, Valencia, CA) with variable amounts of $MgCl_2$ and cycling conditions. The methylation of ER was assessed by COBRA as previously described (33). The bisulfite-treated DNA was first amplified with specific primers and the products were subsequently digested with HinfI and run in a 4% agarose gel. PCR products were also cloned and sequenced to correlate methylation at the HinfI site of ER with 9 other neighboring CpG sites. The MSP primers and PCR conditions were the same as in the previous report (31).

RNA Isolation

Total RNA isolation was performed using the RNeasy total RNA purification kit (Qiagen, Germany) according to the manufacturer's recommendations. For this purpose, WBC was separated from the bone marrow or a peripheral blood sample of patients. For isolation, the RNA of lysed cells was absorbed in a silica-gel based membrane, DNase treated, and washed and eluted with 30µL RNase-free water by centrifugation.

cDNA Synthesis

cDNA synthesis was performed from isolated total RNA by M-MuLV reverse transcriptase (RevertAid M-MuLV reverse transcriptase, Fermantas). The reverse transcription was done as follows: 5µg of template total RNA from each sample was annealed with 0.2 µg random hexamer at 70°C for 5 min and kept in ice. Extension reactions contained 5mM MgCL2, 50mM KCL, 10mMTris-HCL, 1.0mM final concentration of dNTP mix, 20 unit of RNase inhibitors, 200 units of reverse transcriptase and template mixture. The extension reaction was performed as follows: 60 min at 42°C and the reaction was then stopped by heating at 99°C for 5 minutes.

Quantification of mRNA

For quantification, we used real-time quantitative PCR (LightCycler, Roche Diagnostics GmbH, Germany). PCR reactions were performed using Fast Start DNA Syber Green I mix (Roche, Mannheim, Germany), 10 pmol of each forward and reverse primer and 2µL cDNA of each sample. For each sample the

Table 1. Primers used for expression analyses

Gene	Name	Primer Sequence
DNMT1	DNMT1-F	5'GCG GAG CCA ACG GAT GAA GC-3'
	DNMT1-R	5'CAC CAG CAT CAG GCC CAG GC-3'
DNMT3A	DNMT3A-F	5'CAG CTT CCA CGT TGC CTT CT-3' 5'-
	DNMT3A-R	CAT CTG CAA GCT GTC TCC CTT T-3'
DNMT3B	DNMT3B-F	5'CCA ACA ACA CGC AAC CAG AG-3'
	DNMT3B-R	5'GCC ACA AGA CAA ACA GCC ATC-3'
Beta-2-microglobulin	B2M-F	5'-TGCCGTGTGAACCATGTGAC-3'
	B2M-R	5'ACCTCCATGATGCTGCTTACA-3'

Table 2. The methylation frequency of genes in pediatric, adult and total AML patients

	P15	SOCS-1	E-CAD	RARB	P16	5-HIC	GSTP-1	DAP-KIN	ER	P73
Pediatric	0.32	0.36	0.08	0.04	0	0	0	0.56	0.42	0.17
Adult	0.36	0.32	0.2	0.2	0	0	0	0.56	0.28	0.18
Total	0.34	0.34	0.14	0.12	0	0	0	0.56	0.35	0.18

amount and the quality of RNA was normalized with the Beta-2 microglobin gene. The PCR conditions were 60 seconds at 95°C (1 cycle) for denaturation, followed by 45 cycles of 10 seconds at 95°C, 5 seconds at 55°C and 20 seconds at 72°C'. After amplification for melting curve analyses 1 cycle of 0 second at 95°C, 10 seconds at 65°C and 0 seconds at 95°C'. For preparation of standards we used a serial dilution of DNA and each dilution was used twice. Light Cycler PCR products were also analyzed on agarose gel in order to determine PCR quality. Primers used for expression analyses are shown in Table 1. Beta -2-Microglobulin (B2M) was used as a reference housekeeping gene.

We used peripheral CD34+ hematopoietic stem cell from 5 healthy donor as a control for expression analysis.

Statistical Analysis

All tests were done using SPSS Version 17.0 (SPSS Inc.; Chicago, IL, USA) and $p<0.05$ was considered statistically significant with only 2-sided results of the tests. To test if the methylation of one loci was cooperating with the other loci, methylation statuses were analyzed using the Fisher Ki-square test or Pearson test results. We analyzed for a correlation between the methylation of those ten genes, comparing the methylation status of each gene (M or U) with the Wilcoxon rank sum test. The results of the expression analyses were tested by the nonparametric Mann-Whitney U Test. Correlation bivariate analysis was used to compare the expression level of DNMTs and the methylation index.

RESULTS

We analyzed the methylation status of 10 genes in 25 adult AMLs (20-74 years old, median age 34) and in 25 pediatric AMLs (1.2-17 years old, median age 9). DNA was sufficient to complete analyses of 9 genes and 40/50 samples were analyzed for p73.

Methylation specific PCR (MSP) was used to determine methylation of p15, p16, E-cadherin, RARβ, SOCS1, GSTP1, p73, DAP-Kinase and HIC1. MSP reactions for each gene were first standardized using in-vitro methylated DNA (IVM), which yielded a positive product with primers for the methylated form (32). Under our experimental MSP conditions a consistent absence of an amplification signal for the methylated form was observed in 20 different DNA samples obtained from peripheral blood mononuclear cells from healthy individuals. This indicated that methylation was tumor specific.

Due to the heterogeneity described in the methylation pattern of the estrogen receptor gene (ER), we used a COBRA approach to determine the status of the ER gene (34). This data was further confirmed by bisulfite-sequencing. Several PCR products that were cloned and sequenced confirmed that not only the CpG site at the HinfI site was methylated but also 9 other CpG sites were included in the amplification fragment.

The frequency of methylation of each gene notably varied. The frequency of methylation for the pediatric and adult group is shown in Table 2.

The percentage of methylation in the pediatric group of patients was 32% for p15, 36% for SOCS1, 8% for E-cadherin, 4% for RARβ, 56% for DAP-Kinase, 42% for ER, 17% for p73 and in the adult group, 36% for p15, 32% for SOCS1, 20% for E-cadherin, 56% for DAP-Kinase, 28% for ER, and RARβ and 18% for p73.

Three other genes, p16, GSTP1 and HIC1 were unmethylated in all samples (Table 2).

We analyzed the correlation between the pattern and extent of methylation in AML and clinicopathological features, including age and the FAB subclass. We compared the frequency of methylation in each gene between the pediatric group (n=25) and the adult group (n=25), we observed that some genes were varied. While RARβ was more common in adults than in children (20% and 4%, respectively), ER was more common in children than in adults (42% and 28%, respectively). Similarly, the methylation of E-cadherin was more frequent in adults (20%) than in children (8%). Our data clearly shows that AML in pediatric and in young adults tends to have a greater number of methylated genes than in children or older patients.

To quantify the extent of methylation in our series of AML, we calculated a methylation index (MI=ratio between the number of genes methylated and the number of genes analyzed) for the 7 genes that demonstrated some degree of methylation (Figure 1). The MI for the pediatric group ranged from 0-0.714, with an average of 0.284 corresponding to 1.99 genes/sample and for the adult group this ranged from 0-0.856, with an average of 0.305 corresponding to 2.13 genes/sample. Among all the samples 50% of AMLs (25/50) showed methylation of at least two genes and four or more tumor suppressor genes methylated in 20% of AMLs (4 or more gene methylation frequency was in 6 samples corresponding to 24% in the pediatric group and 4 samples corresponding to 16% in the adult samples). The distiribution of methylation index in patients of different ages was shown in Figure 2. There was no statistical difference for the distribution of MI between the pediatric and the adult group (p=0.38).

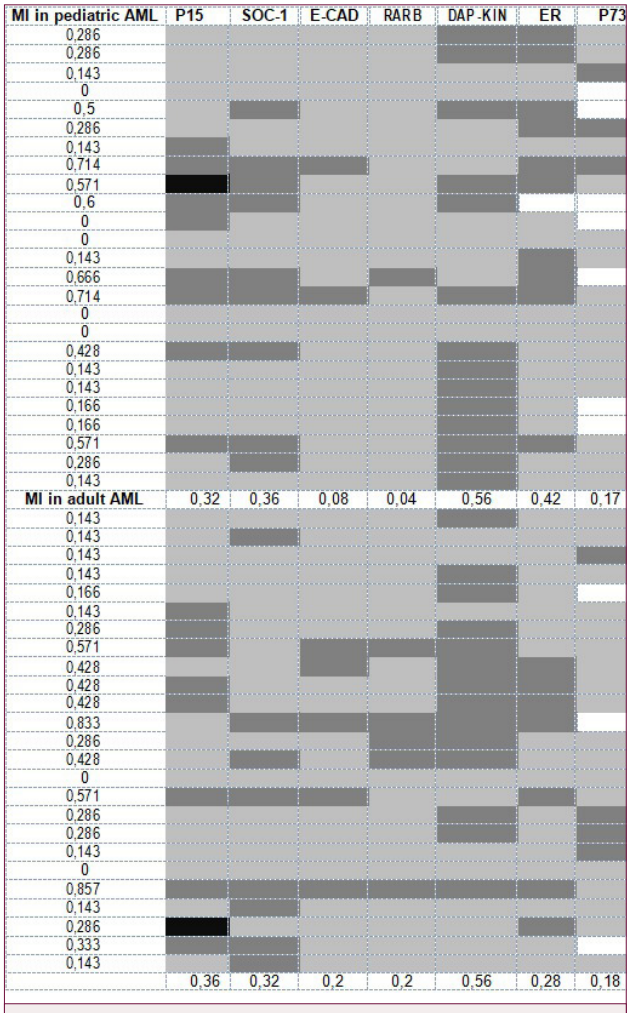

Figure 1. Methylation index (MI) and methylation pattern of genes in pediatric and adult AML patients. Filled grey boxes indicate methylation; hatched boxes indicate unmethylated gene; filled black boxes indicate full methylation and white boxes indicate no data avalible

We analyzed the coexistence of interrelationship between two different loci methylation status to understand whether methylation of one locus is affected by the methylation status of another locus. We performed this for the pediatric and adult groups and also for the total number of patients by the two-sided Pearson X^2 or Fisher's exact test. We identified a statistical association between ER and E cad (p=0.005), SOCS1 and p15 (p=0.015), SOCS1 and E cad (p=0.037), ER and p15 (p=0.009), E

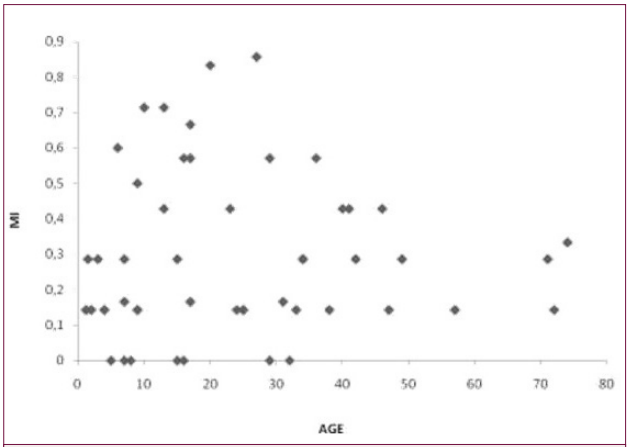

Figure 2. Distiribution of methylation index (MI) according to age (years)

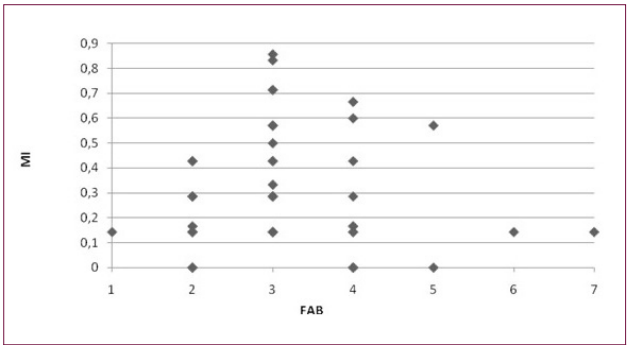

Figure 3. Distribution of methylation index (MI) according to FAB groups

cad and RARB (p=0.029), E cad and p15 (p=0.037) but did not identify any association between ER and SOCS1 (P=0.053). In the pediatric group there was a statistical association between SOCS1 and p15 (p=0.001), ER and SOCS1 (p=0.032), ER and E cad (p=0.032), SOCS1 and E cad (p=0.049), Dap-Kin and p73 (p=0.034). For only the adult group, we identified an association between p15 and ER (p=0.021), E cad and RARB (p=0.038), ER and E cad (p=0.012), RARB and Dap-kin (p=0.027).

We also analyzed the correlation between the methylation of those ten genes, comparing each gene's methylation status (M or U) with the Wilcoxon rank sum test. We calculated the MI for the rest of the loci and we obtained significant results for P15 (p=0.002), E cadherin (p=0.0001), RAR Beta (p=0.011), ER (p=0.0001) but no significance was found for DAP kinase (p=0.106) and p73 (p=0.240).

It is clear that concurrent methylation is also prevalent in pediatric AML and that the subset of genes affected is similar in both age groups, although the frequencies may differ and the number of samples unmethylated in the 10 loci is higher in the pediatric group (n=5 and n=2 for adult). The extent of methylation also appears to be slightly higher in adults (median MI=0.305) than in children (median MI=0.284).

Table 3. a, b. The methylation index in pediatric, adult and total AML FAB groups. The median value of methylation index (a); the average methylation index (b)

a	**FAB M2**	**FAB M3**	**FAB M4**
Pediatric	0	0.29	0.23
Adult	0.29	0.43	0.14
Total	0.17	0.33	0.17
b	**FAB M2**	**FAB M3**	**FAB M4**
Pediatric	0.06	0.38	0.29
Adult	0.29	0.43	0.1
Total	0.2	0.41	0.24

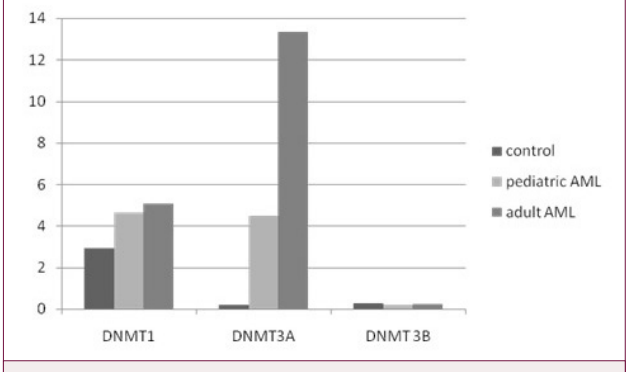

Figure 4. Distribution of methylation index (MI) according to FAB groups

The methylation of RARβ in 18% of cases was almost always accompanied by a significant methylation at other loci. Indeed, the average MI of AML samples with RARβ methytion was 0.67. A similar correlation was found for E-cadherin (the average MI for samples with E-cadherin methylation was 0.65), but not for p73. However, p73 was methylated in only 2/20 samples.

Among AMLs that carried a methylation of DAP-Kinase, methylation of p15 or SOCS1 were equally distributed. However, the methylation of ER occurred more frequently in association with DAP-Kinase. Only a very small proportion of AMLs (1/28, 3%) were unmethylated for these 4 loci (DAP-Kinase, p15, SOCS1 and ER). Since p15 and ER methylation have been recently described to be of prognostic significance, we assessed the fraction of AMLs that carry methylation in these clinically relevant markers. A large fraction of our series (18/28, 64%) had methylation of ER and/or p15.

We also analyzed any possible relation within FAB and MI. The distribution of the methylation index of each sample in the different FAB groups are shown in Figure 3. With respect to FAB classification, there was a general tendency of higher methylation in M3 (n=21, median MI=0.33, average MI=0.405). Table 3. shows the number of affected samples in M2, M3, and M4 with their median and average MI. Unlike the M3 samples, in M2 patients there was a tendency to have less methylation, especially in the pediatric group-unmethylated in 10 loci were 5 samples and 3 of them were M2, 2 of them were M4 patients.

Expression analyses of DNA methyltransferases were carried out on the samples of available cDNA of 12 pediatric (1.2-17 years old, median age 8) and 13 adult (20-72 years old, median age 41) AML samples. Most of the patients were in the FAB3 subclass (pediatric 8, adult 9). Only in one adult patient could the DNMT3A not be analyzed.

We used CD34+ peripheral hematopoietic stem cells from 5 healthy donors and performed the same analyses in order to get a comparison of expression level of DNMT's. In the pediatric vs. control groups, the only significance was in the expression level of DNMT3 (p=0.006 for DNMT3A, and p=0.833 for DNMT 1; p=0.916 for DNMT 3B) but in adult group in addition to DNMT3A (p=0.003), there was an increase in the expression of DNMT1 (p=0.027), but not in the expression of DNMT 3B (p=0.171) (Figure 4). When we compared the total number of patients with the control group, we only observed a difference for DNMT3A (p=0.002) but not for DNMT1 (p=0.254); DNMT 3B (p=0.419). When we compared adult and pediatric groups, the only difference was observed in DNMT1 expression (p=0.044, and for DNMT 3A, p=0.253, DNMT 3B, p=0.419).

When we performed correlation analyses between MI and expression level of DNMT's we observed the relation of DNMT3A and MI (p=0.045). But we did not find any correlation between MI and DNMT1 or DNMT3B expression (DNMT1 p=0.981, DNMT 3B p=0.152). Figure 5. shows the relation DNMT 1, DNMT3A and DNMT3B with MI.

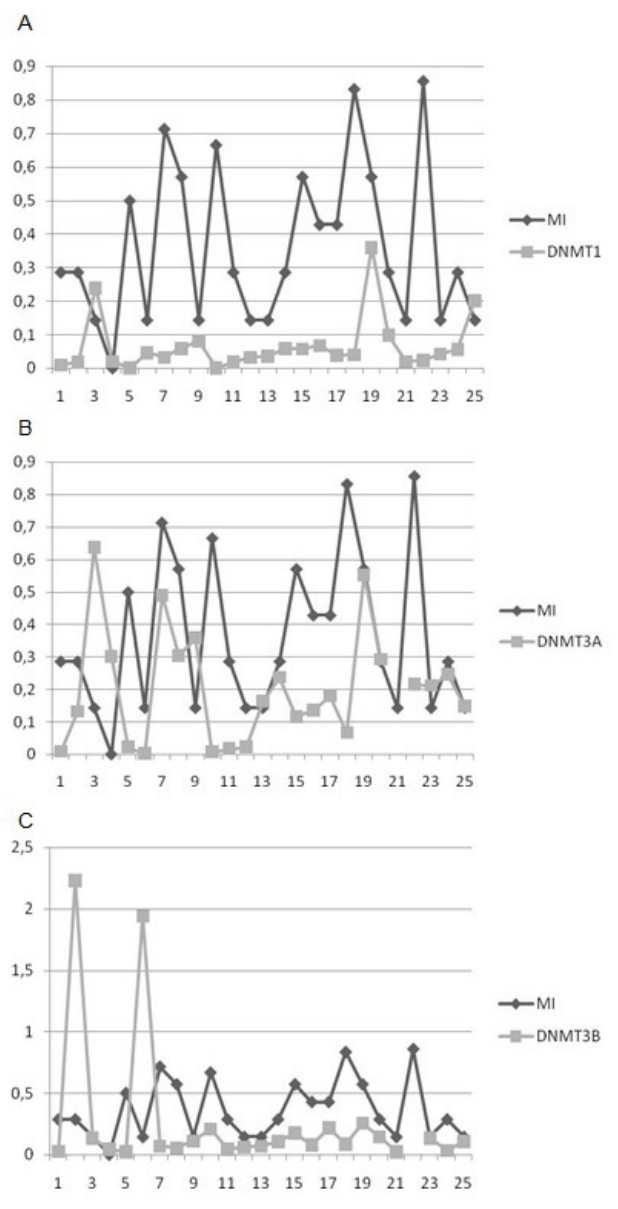

Figure 5. Correlation between methylation index (MI) and DNMT expression; (a) DNMT1; (b) DNMT3A; (c) DNMT3B expression

DISCUSSION

We performed methylation analysis at ten different loci and conducted expression analysis of DNMTs (DNMT1, 3A, 3B) in AML patients (25 pediatric and 25 adult). Ten candidate genes that had had epigenetic modification mostly in lymphoid and myeloid malignancies were chosen for methylation analysis. These included p15, p16, p73, ER, SOCS1, E-cadherin, RAR beta, 5-HIC, DAP kinase, GSTP1. Except for the ER gene, all hypermethylation of the 5' regions had been assayed by methylation-specific PCR while we performed COBRA for only ER. In this group of patients, 25 of the samples had cDNA available to analyze the expression level of DNMTs (DNMT1, 3A, and 3B). As a result, we analyzed methylation and expression of DNMTs in the same sample to research the correlation and affect of DNMTs on methylation patterns in the AML sample group.

Indeed, some AML samples exhibited no aberrant methylation (5 were pediatric, corresponding to 20% and 2 were from the adult group corresponding to 8%-14% in total) we observed that 86% of the samples showed methylation in at least one locus and 23% of the pediatric and 16% of adult samples were methylated in 4 or more loci. Among these ten genes, there was no clear difference between the adult and pediatric form of AML.

P16 is one of the cell's cycle regulatory genes and is considered as a tumor suppressor gene. Similar to p15, rarely has it been found methylated in leukemia (37), but the methylation of p16 has been associated with disease progression in adult T cell leukemia (38). Merki *et al*. (10) indicated that hypermethylation was found to be limited to p15 and did not extend to p16 in AML patients (p15 methylation 68%, p16 methylation 30%). Our results also confirmed that p15 methylation occurred more frequently than p16 methylation in AML. Nevertheless in our study, the methylation ratio was lower than previously reported and we found that 31% of the samples had been methylated in the p15 gene and no methylation in the p16 promoter. Even when we increased the number of samples that we analyzed we could not identify methylation in the p16 genetic region. These results were unlike previous reports (10, 12, 39, 40).

Hypermethylation of the HIC1 has been associated with poor prognosis and disease progression in AML (36, 41, 42). It has been reported that 10% of AML patients were methylated in HIC1 (10). Hypermethylation was observed in 83% of AML patients but these studies included methylation analysis of the intron 2 and exon 3 regions of the gene and intron 2 seem to be more likely related with leukemia in previous reports. In contrast we analyzed the methylation of the 5 prime (5 HIC) gene and we observed no methylation in our patient group.

Methylation of GSTP1 rarely presented itself in hematopoietic cancer but Merki *et al*. (10) found no methylation in 12 samples among adult forms of AML. In our larger series there was no methylation in GSTP1 (neither the adult nor pediatric sample groups).

E cadherin methylation has been found in hematopoietic malignancies, as well as in some solid tumors like prostate and breast cancer (10, 17, 18, 29, 30). Gutierrez *et al*. published that E cadherin methylation was very common in pediatric ALL (17). Merki *et al*. (10) showed extensive methylation of E cadherin in 9 out of 13 sample AML cases. The E cadherin gene methylation pattern was heterogeneous and the degree of methylation was high in the 3' region of the gene. We found only 14% of E cadherin genes were methylated in the total number of patients and this percentage is very low compared with Merki's AML data and Gutierrez's ALL data.

Dap-Kinase had been shown necessary for programmed cell death in the presence of γ interferon (11, 12) and its concor-

dant hypermethylation protects cell from γ interferon induced apoptosis. It has been shown that a loss of expression of Dap-Kinase in lung cancer cells increases the metastatic potential of a tumor. The epigenetic modification of Dap-Kinase has been offered as a marker for B cell malignancies. Dap-kinase methylation was also found related to myelodysplastic syndrome and therapy related AML (15, 45). In our study, we observed the most frequent methylation in DAP Kinase without any difference in the pediatric and adult forms of diseases (56%, 56% respectively). Although we observed DAP Kinase most frequently methylated loci among 10 loci in AML we could not demonstrate any relation with the clinical outcome and any correlation with methylation statuses of other loci.

One of the seven suppressors of the cytokine signaling (SOCS1-7) gene is SOCS1, which has a tumor-suppressor activity. Methylation of this gene has been found very frequent in HCC (hepatocellular carcinoma) (65%). For newly diagnosed AML patients, methylation analysis of the SOCS1 gene was done in a large series (n=89) by Chen *et al* (14). They had observed methylation of this gene in 60% of the patients. SOCS1 methylation seems to be more frequent in the presence of t(15;17) translocation comparing with the t(8;21) (76% vs 11%). In our study we observed the methylation of SOCS1 in 34% of all the samples.

ER methylation (ERM) had been found significantly heterogeneous among the FAB categories by Li et al. (44). A decerease in ER methylation with an increase in ageing and a lower ERM had been observed in M4, M5 in a large series of AMLs (44). Similarly Toyato *et al*. (12) studied 14 different loci methylations for 35 adult AMLs and it was found that ERM positively correlated with methylation of all the remaining 13 loci and methylation of ER indicated a positive association with the age of the patient. In contrast to these results ER hypermethylation has been associated with better prognosis in AML (44). Southern blot analysis has shown ER gene methylation in acute and chronic leukemia at around 50-90% (13-21). Interestingly Merki *et al*. (10) found that hypermethylation for each CpG in the ER was smaller than the 50% which was most probably the methylation of only one allele in a small series of patient samples. They also pointed out that a higher ERM had been observed with males and an inverse correlation of a decreasing ERM was present within the increase count of WBC and platelet.

Epigenetic studies revealed that methylation of CpG islands are increased with age. An acceleration in the methylation profiles with ageing is one of the proposals for epigenetic studies. Similar results have been found previously that, in normal appearing colonic epithelium (19, 29, 30) and other tissue ageing, was associated with an increase in ER methylation. We found the methylation of ER was more frequent in the pediatric group (42%) than the adult AML (28%). Our study indicated that there was a tendency to be more frequently methylated in a pediatric group than in an adult group and this was just the opposite of the previous proposal. Also in overall epigenetic modification we could not observe any age dependency. In our patient group there was no statistical significance between age and MI. There was no significant difference when compared with the adult and pediatric MI results. In our sample group, among the 10 loci for only RARB methylation there was a tendency towards a higher methylation in the adult AML than in pediatric AML. However this was not statistically significant (p=0.189).

According to our observation ER, SOCS1, p15, E cadherin, RARB promoter and methylation are most likely correlated with each other for individual patient samples. These 5 genes were susceptible to methylation in the same patient samples and did not show methylation in others These genes are more likely to be members of the methylator phenotype for AML. We could not find any correlation between methylation, age and sex in our AML samples.

As multiple genes are frequently methylated in cancer cell lines, this suggests that there is a mechanism which protects these genes from methylation and this mechanism is defective or somehow broken in cancer cells. We also analyzed the expression level of DNA methyltransferases (DNMT1, DNMT3A and DNMT3B) which could be primarily responsible for aberrant methylation in the same AML samples. Our results showed that DNMT3A over-expressed in AML patients and there was a relation between MI and DNMT3A.

Roberson et al. (45) showed that in the tumors of different tissues, the expression of DNMT1, 3A, 3B genes and DNMT3B expression in all tumors is markedly increased. However DNMT1 and 3A have increased moderately. Colon, bladder, kidney and pancreas samples were studied and it was observed that while DNMT3B was significantly over-expressed, the expression level of DNMT1 and 3A was modest (4). Langer *et al*. (46) found that DNMT1 and DNMT3A over-expressed in RA and RAEB subtypes in a large series (n=80) of MDS samples. Additionally in RARS subtypes the only differences in DNMT3A were observed with the control group. They used the cyclin D2 gene for normalization and they also observed similar results when the normalization was performed with PCNA (46). Aoki *et al*. (47) observed an over-expression of DNMT1 when normalized with GAPDH but no up-regulation when normalized with histone H4. Mizuno *et al*. (48) observed an increase in the expression of all 3 types of DNMTs when normalized with GAPDH in their 33 AML samples by competitive PCR (5.3-4.4-11.7 fold mean increase in DNMT1, 3A, 3B respectively). Xie *et al*. (49) also reported an increased expression of all 3 DNMTs in several tumor cell lines. Mizuno *et al*. (48) investigated whether the expression levels of DNMTs correlated with aberrant hypermethylation of the p15 INAK4B tumor suppressor gene in AML cases. They showed that the AML patients with methylated p15 INAK4B expressed a higher level of DNMT1, 3B and potentially 3A using competitive PCR assay (48). They suggested that the over-expressed DNMTs played a role in the aberrant regional hypermethylation observed in AML. In our study we observed that the expression level of DNMT3A correlated with aberrant hypermethylation. An over-expression of DNMT3A was also correlated with MI. We could not observe any correlation between the expression level

of DNMT3B and aberrant hypermethylation using quantitative RT-PCR. It was shown that an increased expression of DNMT3B was associated with worse clinical outcomes and a greater genome-wide methylation burden in pediatric AML (49).

A microarray based study to identify subclasses of adult AML by Bullinger *et al*. (51) identified 2 subclasses and in the first subclass a high level of DNMT3A and 3B expression was indicated with GATA2 transcription factor. With this result they emphasized the potential role of aberrant methylation via high expression of DNMTs (51).

Methylation in 5 of the 10 genes examined correlated with each other (ie, five patients had methylation in one patient while another patient did not methylate in these five genes). As such, these genes are candidate genes for the AML methylator phemotype. The expression of DNMT3A was significantly higher where methylation was observed in these 5 than the rest of the samples in Figure 5.

In several expression studies of DNMTs, it was pointed out that over-expression of DNMTs was observed when the level of mRNA normalization was performed with the housekeeping gene. If the procedure repeated with the proliferation marker PCNA gene then these differences disappeared. Mizuno *et al.* (48) showed that the over-expression was cancelled in 3 leukemia cell lines, while in AML samples DNMTs expression level was found increased in AML and leukemia. However none of these studies demonstrated any clear correlation between DNMTs expression and the number of genes methylated. In addition, a two to ten fold increase of expression in this gene was not found related to age or sex of patients (13).

Correlations between the methylation statuses of ER and E cad, SOCS1 and p15, SOCS1 and E cad, ER and p15, E cad and RARB, E cad and p15 were statistically significant. These may indicate that if there is a methylator phenotype for AML, these 5 genes are the most powerful candidates. However, it is not sufficient to emphasize that there is only one methylator phenotype for one cancer type. There could be multiple methylator phenotypes which build up different types of genes in one tumor type.

This data clearly demonstrated that DNMT3A over-expressed in AML patients when compared with the control group. Our results suggest that DNMT3A over-expression is the most important factor for aberrant methylation according to the correlation with MI and DNMT3A.

Ethics Committee Approval: The authors declared that the research was conducted according to the principles of the World Medical Association Declaration of Helsinki "Ethical Principles for Medical Research Involving Human Subjects", (amended in October 2013).

Informed Consent: Written informed consent was obtained from the patients who participated in this study.

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