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ORIGINAL RESEARCH

Expression Profiles of DNA Methylation and Demethylation Machinery Components in Pediatric Myelodysplastic Syndrome: Clinical Implications

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Purpose: The aim of this study was to analyse the expression profiles of *DNMT1, DNMT3A, DNMT3B* (components of DNA methylation machinery), *TET2* and *APOBEC3B* (components of DNA demethylation machinery) in pediatric MDS patients and investigate their associations with MDS subtypes, cytogenetics, evolution to acute myeloid leukemia (AML) and $p15^{INK4B}$ methylation level.

Patients and Methods: The expressions of *DNMT1, DNMT3A, DNMT3B, TET2*, and *APOBEC3B* were evaluated in 39 pediatric MDS patients by real-time quantitative PCR (qPCR). The quantification of $p15^{INK4B}$ methylation levels (MtL) was performed in 20 pediatric MDS patients by pyrosequencing. Mann–Whitney test was used to evaluate possible differences between the expression levels of selected in patients and donors, according to MDS subtypes, karyotypes, evolution to AML and $p15^{INK4B}$ MtL. The correlations between the expression levels of the different genes were assessed by Spearman rank correlation coefficient.

Results: We found that *DNMT*s expression levels were higher in pediatric MDS compared to donors [*DNMT1* ($p<0.03$), *DNMT3A* ($p<0.03$), *DNMT3B* ($p<0.02$)]. *TET2* and *APOBEC3B* expression levels did not show a statistically significant difference between pediatric patients and donors. Considering MDS subtypes, patients at initial stage presented *DNMT1* overexpression ($p<0.01$), while *DNMT3A* ($p<0.02$) and *DNMT3B* ($p<0.007$) were overexpressed in advanced subtypes. *TET2* and *APOBEC3B* expression did not differ in MDS subtypes. *DNMT1* ($p<0.03$), *DNMT3B* ($p<0.03$), and *APOBEC3B* ($p<0.04$) expression was higher in patients with normal karyotypes, while patients with abnormal karyotypes showed higher *DNMT3A* expression ($p<0.03$). Karyotypes had no association with *TET2* expression. *DNMTs* overexpression was observed in patients who showed disease evolution. A positive correlation was found between *DNMT*s expression and between *APOBEC3B* and *DNMT3A*/*DNMT3B*. However, *TET2* expression was not correlated with *DNMT*s or *APOBEC3B*. $p15^{INK4B}$ MtL was higher in pediatric MDS patients compared with donors ($p<0.03$) and its hypermethylation was associated with increased *DNMT1* expression ($p<0.009$).

Conclusion: Our results suggest that the overexpression of *DNMTs* and an imbalance between the expressions of the DNA methylation/demethylation machinery components play an important role in MDS development and evolution to AML. These results have clinical implications indicating the importance of *DNMTs* inhibitors for preventing or delaying the progression to leukemia in pediatric MDS patients.

Keywords: pediatric myelodysplastic syndrome, *DNMTs*, *TET2*, *APOBEC3B*

Introduction

Myelodysplastic syndrome (MDS) comprises a heterogeneous group of clonal hematopoietic stem cell diseases. MDS is characterised by ineffective hematopoiesis,

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presence of dysplasias, peripheral blood cytopenia and an increased risk of evolution to acute myeloid leukemia (AML).[1,2] MDS has a higher incidence in adults and elderly individuals. By contrast, MDS is a rare disease in children, occurring in approximately 2–7% of the childhood hematological malignancies.[3–6]

Pediatric patients with MDS have some distinct genetic, epigenetic and clinical characteristics when compared to adult patients.[4–6] Thus, pediatric MDS has a specific classification proposed by Hasle and colleagues in 2003[5] and revised in 2016.[6] These patients can be classified as refractory cytopenia of childhood (RCC), refractory anemia with excess of blasts (RAEB) and refractory anemia with excess of blasts in transformation (RAEB-t).[5,6] Recently, Locatelli and Strahm (2018) used for the advanced pediatric MDS the WHO classification of myeloid neoplasms. So, MDS with ≥2% of blasts in peripheral blood (PB) or 5-19% of blasts in bone marrow (BM) is classified as myelodysplastic syndrome with excess of blasts (MDS-EB).[7] However, in pediatric MDS classification, the subtype MDS-EB in transformation (MDS-EB-t) is maintained. This subtype is characterized by 20-29% of blasts in the BM.[6,7]

Due to the heterogeneity and rarity of pediatric MDS, molecular alterations involved in initial and subsequent stages during its development and progression to AML are not well defined. Several studies in adult patients indicate the important role of epigenetic changes in the pathogenesis of MDS.[6–9] This epigenetic nature can be explained as follows: (1) among the most frequently mutated genes in MDS, approximately 45% are responsible for the regulation of epigenetic mechanisms and (2) MDS is considered the most responsive disease to treatment with DNA methyltransferase inhibitors.[9,10] However, there are few studies focused on the genes that act on the epigenetic mechanisms in pediatric patients with MDS, and these studies have shown that mutations in these genes are rare.[11] Nevertheless, epigenetic mediators could be dysregulated by other mechanisms, such as transcriptional alterations and primarily involve components of the DNA methylation and demethylation machinery to result in aberrant methylation profiles.[12]

Aberrant promoter hypermethylation involving genes encoding cell adhesion molecules, cell cycle regulators and tumor suppressor genes have been associated with hematopoiesis dysregulation. In MDS, a high frequency of hypermethylation of $p15^{INK4B}$, cadherin 1 (*CDH1*), death-associated protein kinase (*DAPK*) and suppressor of cytokine signalling *(SOCS-1*) has been observed.[13] In pediatric MDS, $p15^{INK4B}$ methylation has been associated with disease pathogenesis and poor prognosis.[14]

DNA methylation is considered a guardian of hematopoietic stem cell fate because it acts to maintain the balance of these cells, their self-renewal capacity, and differentiation in specific hematopoietic cell populations.[12] DNA methyltransferases (DNMTs) are enzymes responsible for catalysing the insertion of a methyl group on carbon 5 of a cytosine in the CpG context. DNMT1 is associated with the maintenance of DNA methylation patterns, while DNMT3A and DNMT3B mediate de novo methylation.[15] By contrast, DNA demethylation can occur passively during replication through the inhibition of the methylation maintenance process or actively and independent of DNA replication.[16] Active demethylation is initiated by two independent pathways. The first involves the progressive oxidation of 5-methylcytosine (5mC) and is catalysed by the ten-eleven-translocation (TET) family of enzymes; the second is driven by the apolipoprotein B mRNA editing enzyme (APOBECs) family, which deaminates 5mC and 5-hydroxylmethyl cytosine (5hmC).[17–19] In both cases, mispairing takes place and the base excision repair machinery replaces the modified base by an unmethylated cytosine.[18]

The balance between the enzymes that act on DNA methylation and demethylation is essential for the maintenance of genomic stability and is referred to as the DNA methylation and demethylation machinery.[18] It has been suggested that increased expression of de novo or maintenance *DNMT*s contributes to the development of leukemia by inducing aberrant hypermethylation of important regions in the genome.[20] Up to date, to the best of our knowledge, there are no studies focusing on *DNMTs* expression in pediatric MDS, and only one study evaluated *TET2* expression in pediatric patients.[21] Even in adult patients, few studies have been performed to analyse the expression of DNA methylation and demethylation machinery components.[17,22] Although the APOBEC family is an important component in the demethylation machinery[18,19] and APOBEC3B has been described as a driving mutagenic agent during cancer development and progression,[23] no studies involving the APOBEC family have been performed in MDS. Thus, the aim of this study was to analyse the expression of *DNMT1, DNMT3A, DNMT3B* (components of the DNA methylation machinery), *TET2* and *APOBEC3B* (components of the DNA demethylation machinery) in pediatric patients with MDS and investigate their associations with MDS subtypes, cytogenetics, evolution to AML and the $p15^{INK4B}$ methylation levels of gene to verify the role of epigenetic alterations during pediatric MDS pathogenesis.

Materials and Methods

Patients and Controls

Bone marrow (BM) cells were obtained from 39 pediatric patients with primary MDS between 2007 and 2017. These patients included 23 boys (59%) and 16 girls (41%). The mean age of the patients was 7 years (ranging from 1 to 18 years). Patients were diagnosed at the National Cancer Institute (INCA) and Martagão Gesteira Institute of Pediatrics (IPPMG, UFRJ). The diagnosis and classification were made according to the criteria proposed by WHO,[1] but the subtype MDS-EB-t was retained in pediatric classification of MDS.[6,7] Twenty-seven patients (69%) were classified as RCC, seven (18%) as MDS-EB and five (13%) as MDS-EB-t (Table 1). None of these patients had been previously treated for malignancy. Bone marrow cells were also obtained from 13 healthy pediatric bone marrow transplantation donors as controls, including eight boys (61.5%) and five girls (38.5%). The mean age of the healthy pediatric donors was 10 years (ranging from 4 to 18 years). The bone marrow samples were collected from the Bone Marrow Transplantation Center (CEMO) at the National Cancer Institute (INCA), Rio de Janeiro, Brazil and Pediatric and Puericulture Martagão Gesteira Institute (IPPMG), Federal University of Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil. This study was reviewed and approved by Ethics Committees of the National Cancer Institute and IPPMG-UFRJ, and was conducted in accordance with the Declaration of Helsinki.

Table 1 Patients Characteristics

Patients	Number
MDS	39
Gender	
Male	23 (59%)
Female	16 (41%)
Age (years), mean (range)	7 (1 −18)
MDS Subtypes	
RCC	27 (69.2%)
MDS-EB	7 (18%)
MDS-EB-t	5 (12.8%)
Number of Cytopenias	
0	2 (5%)
1	17 (43.6%)
2	9 (23%)
3	11 (28.2%)
BM Blasts (%)	
<5%	27 (69.2%)
5–19%	7 (18%)
20–29%	5 (12.8%)
Cytogenetics	
Normal	22 (56.4%)
Abnormal	17 (43.6%)
Evolution MDS→AML	
No	28 (71.8%)
Yes	11 (28.2%)

Abbreviations: MDS, myelodysplastic syndrome; RCC, refractory cytopenia in childhood; MDS-EB, MDS with excess of blasts; MDS-EB-t, MDS with excess of blasts in transformation; BM, bone marrow; AML, acute myeloid leukemia.

Analysis of *DNMTs*, *TET2*, and *APOBEC3B* Expression in Pediatric MDS

Total RNA was extracted from bone marrow cells with TRIzol reagent (*Life Technologies*, USA) according to the manufacturer's protocol and stored at −70°C. Two micrograms of total RNA were digested with DNAse amplification grade I (*Life Technologies*, USA) to remove any genomic DNA contaminant. The RNA was reverse transcribed into complementary DNA (cDNA) with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's instructions. Quantitative PCR reactions were performed in 10 µL containing 1x SsoFast EvaGreen Supermix (BIO-RAD, USA), 1.8 µM of each primer and 1 µL of cDNA (diluted 10-fold). Reactions were performed in a Rotor-Gene 6000 thermocycler (Qiagen, Germany) with an initial denaturation at 95°C for 30 s, followed by 40 cycles of 5 s at 95°C and 5 s at 60°C. *B-actin* mRNA levels were used as a reference for normalisation. The following primers sequences were used for quantitative PCR: (1) *DNMT1*, 5′-AAC TCC AAG ACC CAC CCT CC-3′ (forward) and 5′-CAG ACT CGT TGG CAT CAA AGA T-3′ (reverse); (2) *DNMT3A*, 5′-GGC TCT TCT TTG AGT TTG ACT TCT ACC-3′ (forward) and 5′-GAT GTC CCT GTC ACT AAC-3′ (reverse); (3) *DNMT3B*, 5′-ATC AGG ATG GGA AGG AGT TT-3′ (forward) and 5′-TCG GAG AAC TTG CCA TCG CC-3′ (reverse); (4) *TET2*, 5′-GCT GGG ACT ACT GCT GCT GCA CT-3′ (forward) and 5′-ACG CAA GCC AGG CTA AAC A-3′ (reverse); (5) *APOBEC3B*, 5′-ACC CAT CCT CTA TGG TCG GA-3′ (forward) and 5′-GCT TGA AAT ACA CCT GGC CTC-3′ (reverse) and (6) *B-actin*, 5′-TGG AAC GGT GAA GGT GAC A-3′ (forward) and 5′-ATG TGC AAT CAA AGT CCT CGG C-3′ (reverse). The relative expression of all genes was calculated using the $\Delta\Delta C_T$ method with the median levels of healthy controls as a normaliser.[24]

Analysis of *p15*^{INK4B} Promoter Methylation Levels in Pediatric MDS

The quantification of *p15*^{INK4B} promoter methylation levels (MtL) was performed in BM cells DNA samples from 20 pediatric patients with MDS and four healthy pediatric donors according to Kim and colleagues in 2013.[14] Bisulfite-converted DNA was sequenced using a pyrosequencing system (PSQTM 96MA, Qiagen, Germany). PCR reactions were performed with 0.2 mM/µL of each primer (forward and reverse), 1X PCR Buffer, 0.2 mM/µL of dNTPs, 1.5 mM/µL of $MgCl_2$, 1 u/µL Platinum Taq^{TM} (Invitrogen, CA, USA), and 100 ng of bisulfite-modified DNA in a final volume of 50 µL. PCR cycling conditions consisted of an initial denaturation at 95ºC for 10 mins, followed by 50 cycles of denaturation at 95ºC for 20 s, annealing at 57ºC for 20 s and elongation at 72ºC for 20 s, and a final elongation for 5 mins. PCR quality was confirmed on 2% agarose gels with ethidium bromide staining. Hot-start PCR was performed with HotStarTaq Master Mix kit (Qiagen 203445) and pyrosequencing was performed in accordance with the manufacturer's protocol (Qiagen). The target CpGs were evaluated by converting the resulting pyrograms into numerical values for peak heights and calculating the average of all CpG sites analyzed at *p15*^{INK4B} promoter. We used the mean and standard deviation (SD) [mean (0.84) + 2 SD (1.17) = cut-off (3.18)] of the donors for determining hypermethylated samples.[14]

Statistical Analyses

Comparisons of the *DNMTs, TET2,* and *APOBEC3B* expression between patients and donors, MDS subtypes, karyotypes and evolution from MDS to AML were performed using the Mann–Whitney *U*-test. Correlations between the expression levels of each pair of genes considered in this study were evaluated by the Spearman rank correlation coefficient. The analyses of *p15*^{INK4B} MtL and its association with the expression of DNA methylation and demethylation machinery components were performed using the Mann–Whitney *U*-test. Statistical analyses were performed using GraphPad Prism (Graphpad Software, USA). A p-value < 0.05 was considered statistically significant in all analyses.

Results

Analysis of *DNMTs*, *TET2*, and *APOBEC3B* Expression in Pediatric Patients with MDS

The analysis of *DNMTs* expression levels in pediatric patients with MDS showed a higher expression compared with the donors [*DNMT1* (p<0.03), *DNMT3A* (p<0.03), and *DNMT3B* (p<0.02)] (Figure 1A–C). Among all *DNMTs, DNMT3B* showed the highest expression level compared with *DNMT1* and *DNMT3A. TET2* and *APOBEC3B* expression levels did not show a statistically significant difference between pediatric patients and the donors (Figure 1D and E).

We observed a linear correlation between the expression levels of *DNMT1* and *DNMT3A* (r= 0.52; p<0.001); *DNMT1* and *DNMT3B* (r= 0.42; p<0.01); *DNMT3A* and *DNMT3B* (r= 0.68; p<0.0001); *DNMT3A* and *APOBEC3B* (r=0.52; p<0.001); and *DNMT3B* and *APOBEC3B* (r=0.71; p<0.0001) (Figure 2A, B, E, G and I, respectively). The expression levels of *DNMT1* and *APOBEC3B* were not significantly correlated (r=0.22; p=0.18). The same profile was observed for *TET2* expression and *DNMTs* or *APOBEC3B* expression (Figure 2C, D, F, H and J, respectively).

Associations of *DNMTs*, *TET2*, and *APOBEC3B* Genes Expression with the MDS Subtypes, Cytogenetics and Disease Evolution

When considering MDS subtypes, we observed a higher *DNMT1* expression (p<0.01) at initial stage (RCC) (Figure 3A) and a higher expression of *DNMT3A* (p< 0.02) (Figure 3B) and *DNMT3B* (p<0.007) (Figure 3C) in advanced stages (MDS-EB/MDS-EB-t) compared to controls. *TET2* and *APOBEC3B* expression did not significantly differ between MDS subtypes and controls (Figure 3D and E).

In relation of cytogenetics (Table S1), we found that *DNMT1* (p<0.03), *DNMT3B* (p<0.03) and *APOBEC3B* (p<0.04) expression levels were higher in patients with normal karyotypes (Figure 4A, C and E), while patients with abnormal karyotypes showed a higher *DNMT3A* expression (p < 0.03) relative to donors (Figure 4B). Karyotypes had no association with *TET2* expression (Figure 4D).

We also analysed the association between *DNMTs*, *TET2* and *APOBEC3B* expression and the evolution from MDS to AML. The evolution of disease was observed in 28% of the patients (11/39). Patients who progressed to AML had higher expression levels of *DNMT1, DNMT3A*, and *DNMT3B* when compared to donors (p<0.04, p<0.02 and p<0.005, respectively) (Figure 5A–C). *TET2* and *APOBEC3B* expression did not differ between patients who showed evolution from MDS to AML and controls (Figure 5D and E). Table 2 contains a summary of all analyses performed comparing *DNMTs*, *TET2* and *APOBEC3B* expression between patients and donors, and

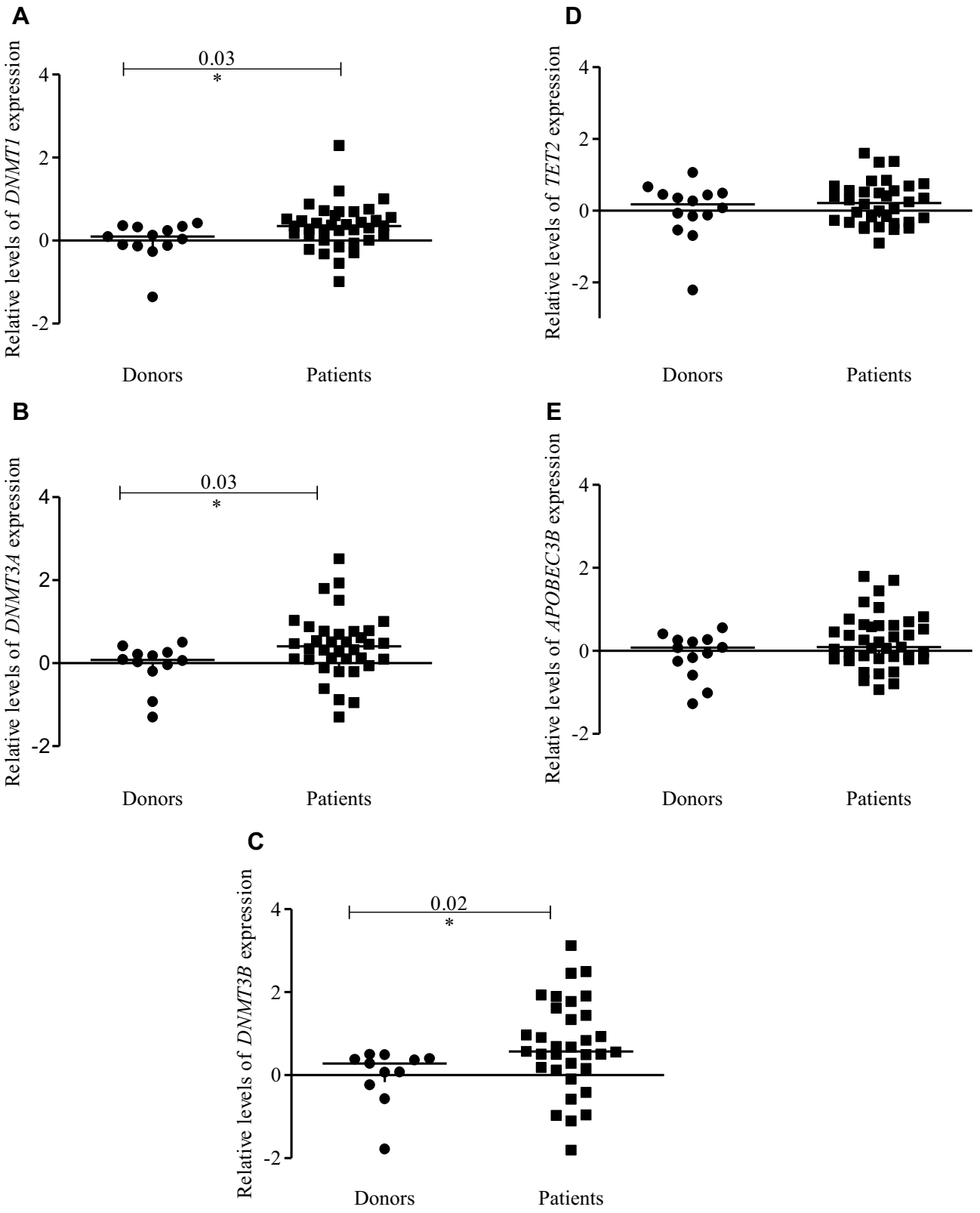

Figure 1 Expression profiles of *DNMT*s, *TET2*, and *APOBEC3B* in pediatric patients with MDS and healthy pediatric donors. Dot-plots (log10 scale on the Y-axis) show the expressions for (**A**) *DNMT1*, (**B**) *DNMT3A*, (**C**) *DNMT3B*, (**D**) *TET2* and (**E**) *APOBEC3B*.

Notes: *Indicates a significant difference ($p<0.05$) calculated by Mann–Whitney *U*-test.

Abbreviations: *DNMT*s, DNA methyltransferases; *TET*, ten-eleven-translocation; *APOBEC*, apolipoprotein B mRNA editing enzyme; MDS, myelodysplastic syndrome.

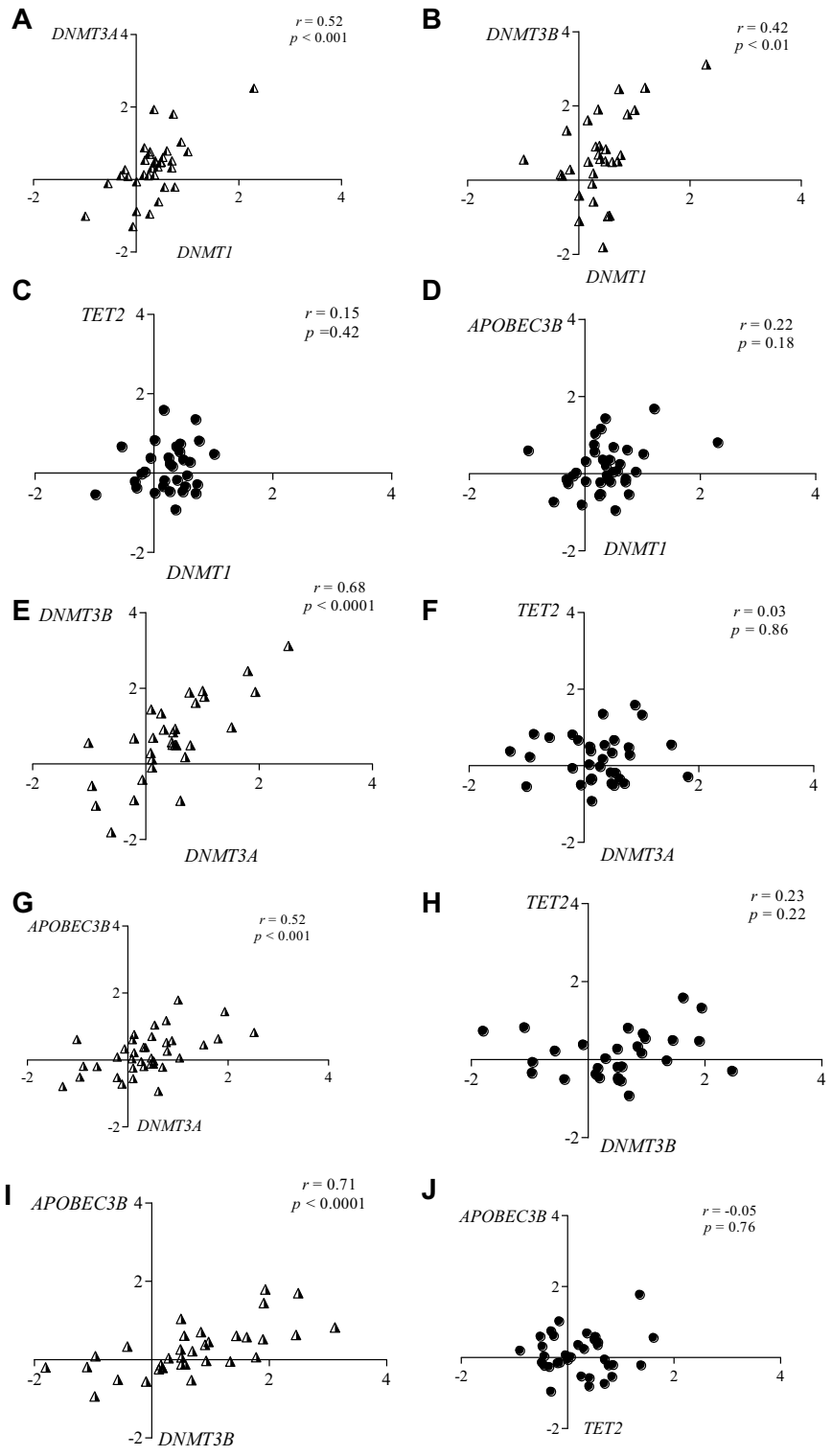

Figure 2 Linear correlation between the expression levels of *DNMTs*, *TET2* and *APOBEC3B* in pediatric patients with MDS. Correlation plots (log10 scale on the X and Y-axis) between the expressions of (**A**) *DNMT1* and *DNMT3A*; (**B**) *DNMT1* and *DNMT3B*; (**C**) *DNMT1* and *TET2*; (**D**) *DNMT1* and *APOBEC3B*; (**E**) *DNMT3A* and *DNMT3B*; (**F**) *DNMT3A* and *TET2*; (**G**) *DNMT3A* and *APOBEC3B*; (**H**) *DNMT3B* and *TET2*; (**I**) *DNMT3B* and *APOBEC3B* and (**J**) *TET2* and *APOBEC3B*.

Notes: *p*-Value calculated by Spearman rank correlation coefficient (*r*).

Abbreviations: *DNMTs*, DNA methyltransferases; *TET*, ten-eleven-translocation; *APOBEC*, apolipoprotein B mRNA editing enzyme; MDS, myelodysplastic syndrome.

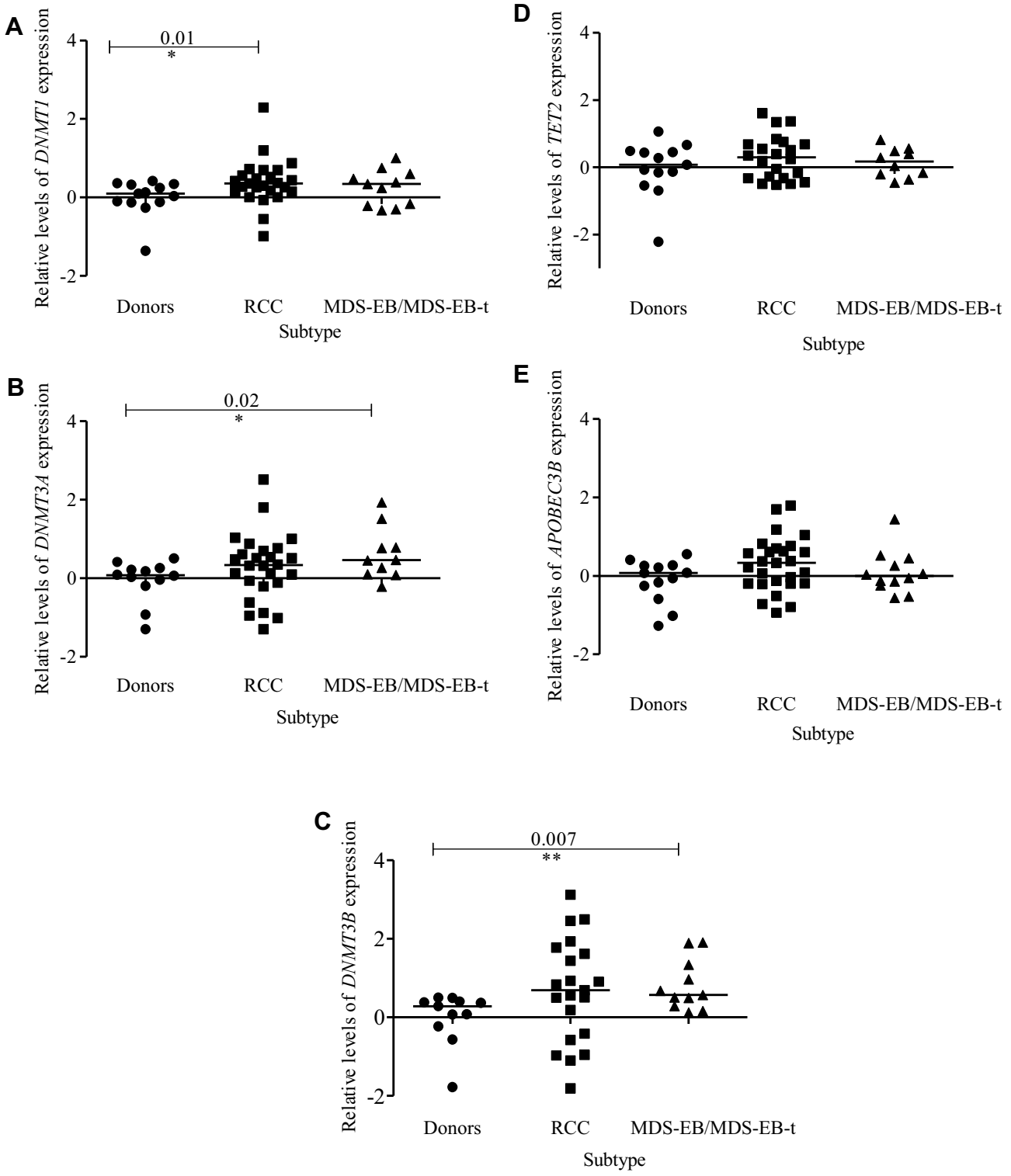

Figure 3 Expression profiles of *DNMTs*, *TET2*, and *APOBEC3B* according to the pediatric MDS subtypes and healthy pediatric donors. Dot-plots (log10 scale on the Y-axis) show the expression of (**A**) *DNMT1*, (**B**) *DNMT3A*, (**C**) *DNMT3B*, (**D**) *TET2* and (**E**) *APOBEC3B* in donors, RCC (initial MDS stage), MDS-EB/MDS-EB-t (advanced MDS stages).

Notes: *Indicates a significant difference ($p<0.05$) and **Indicates a significant difference ($p<0.01$) calculated by Mann–Whitney *U*-test.

Abbreviations: *DNMTs*, DNA methyltransferases; *TET*, ten-eleven-translocation; *APOBEC*, apolipoprotein B mRNA editing enzyme; MDS, myelodysplastic syndrome; RCC, refractory cytopenia of childhood; MDS-EB, MDS with excess of blasts; MDS-EB-t, MDS with excess of blasts in transformation.

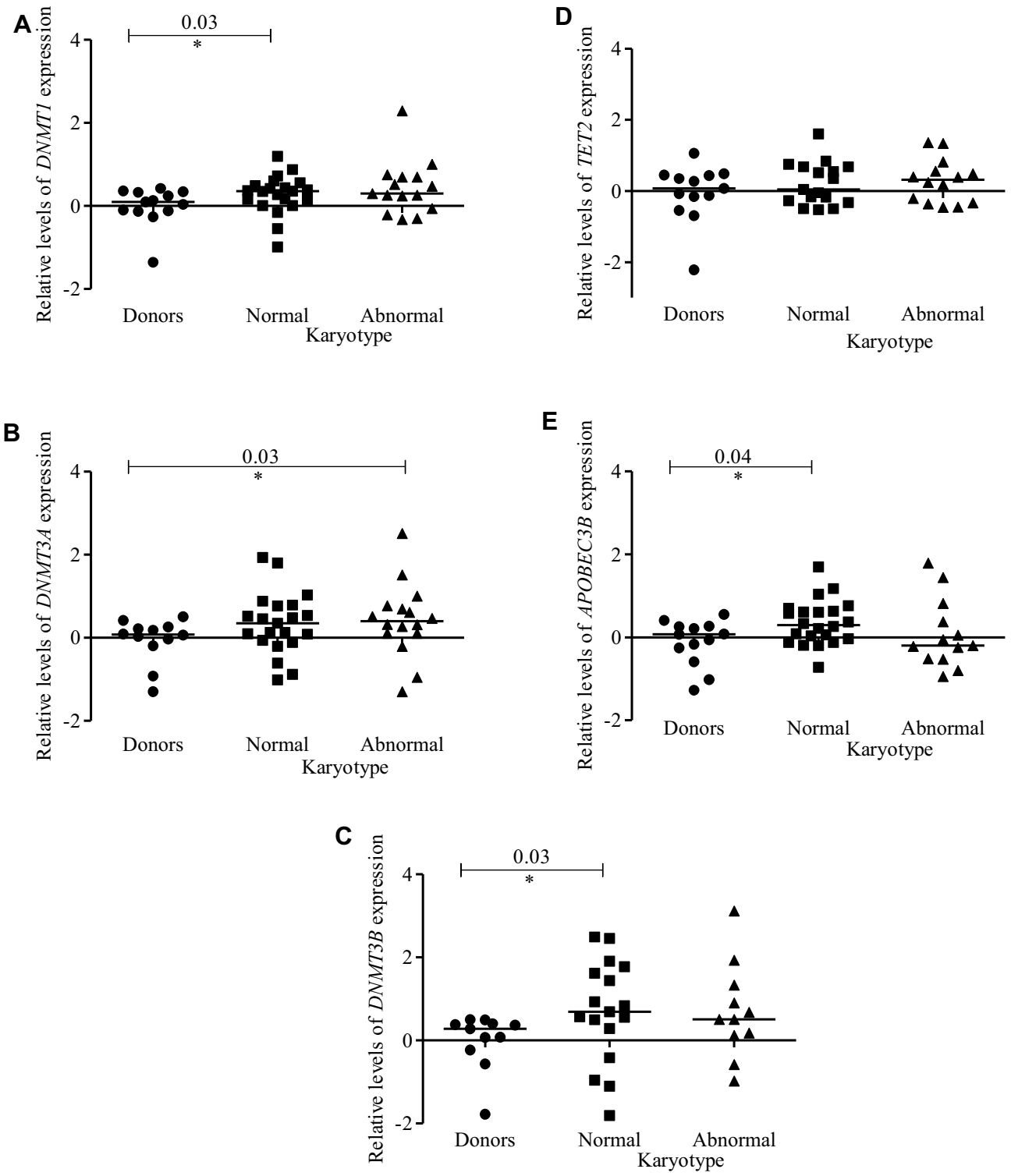

Figure 4 Expression profiles of *DNMTs*, *TET2*, and *APOBEC3B* according to cytogenetics (normal and abnormal karyotypes). Dot-plots (log10 scale on the Y-axis) show the expressions of (**A**) *DNMT1*, (**B**) *DNMT3A*, (**C**) *DNMT3B*, (**D**) *TET2* and (**E**) *APOBEC3B* in donors and patients.

Notes: *Indicates a significant difference ($p < 0.05$) calculated by Mann–Whitney *U*-test.

Abbreviations: *DNMTs*, DNA methyltransferases; *TET*, ten-eleven-translocation; *APOBEC*, apolipoprotein B mRNA editing enzyme; MDS, myelodysplastic syndrome.

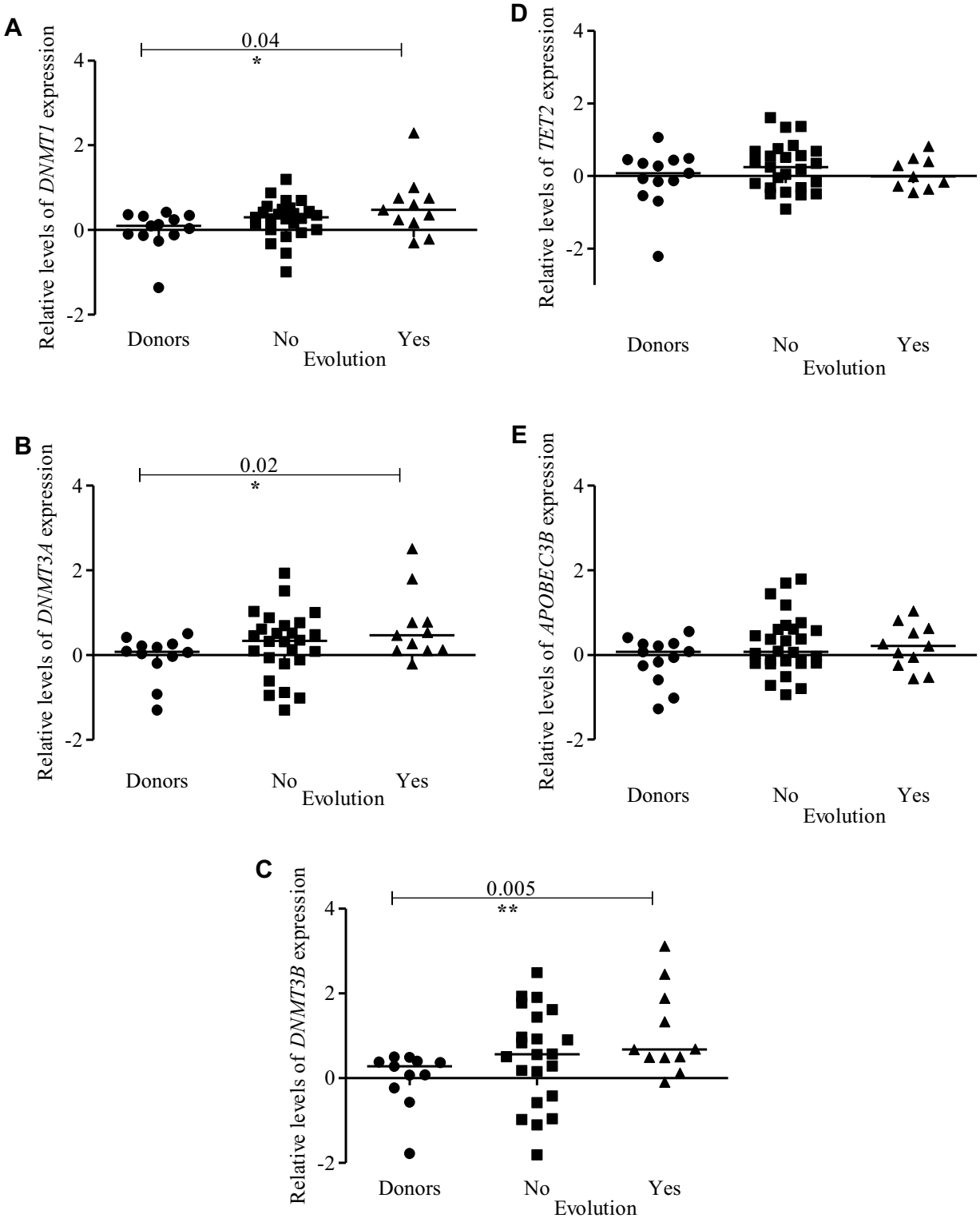

Figure 5 Expression profiles of *DNMTs*, *TET2*, and *APOBEC3B* according to evolution from MDS to AML. Dot-plots (log10 scale on the Y-axis) show the expressions of (**A**) *DNMT1*, (**B**) *DNMT3A*, (**C**) *DNMT3B*, (**D**) *TET2*, and (**E**) *APOBEC3B* in healthy donors, patients who did not show disease evolution and patients who showed disease evolution.
Notes: *Indicates a significant difference ($p<0.05$); **Indicates a significant difference ($p<0.01$) calculated by Mann–Whitney *U*-test.
Abbreviations: *DNMTs*, DNA methyltransferases; *TET*, ten-eleven-translocation; *APOBEC*, apolipoprotein B mRNA editing enzyme; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia.

Table 2 Summary of All Analyses Performed Comparing *DNMTs*, *TET2* and *APOBEC3B* Expressions Between Patients and Donors, and According to MDS Subtypes, Karyotypes, Evolution from MDS to AML

	DNMT1	***DNMT3A***	***DNMT3B***	***TET2***	***APOBEC3B***
Donors vs Patients	*p*<0.03*	*p*<0.03*	*p*<0.02*	*p<0.66*	*p<0.16*
MDS Subtypes	**p Value**				
Donors vs RCC	*p*<0.01*	*p*<0.1	*p*<0.06	*p*<0.3	*p*<0.08
Donors vs MDS-EB/MDS-EB-t	*p*<0.3	*p*<0.02*	*p*<0.007*	*p*<0.9	*p*<0.7
RCC vs MDS-EB/MDS-EB-t	*p*<0.6	p<0.4	*p*<0.9	*p*<0.7	*p*<0.2
Cytogenetics	**p Value**				
Donors vs normal karyotypes	*p*<0.03*	*p*<0.1	*p*<0.03*	*p*<0.5	*p*<0.04*
Donors vs abnormal karyotypes	*p*<0.08	*p*<0.03*	*p*<0.06	*p*<0.5	*p*<1.0
Normal vs abnormal karyotypes	p<0.7	*p*<0.7	*p*<0.7	*p*<0.6	*p*<0.08
Evolution from MDS→AML	**p Value**				
Donors vs no disease evolution	*p*<0.06	*p*<0.1	*p*<0.09	*p*<0.5	*p*<0.2
Donors vs disease evolution	*p*<0.04*	*p*<0.02*	*p*<0.005*	*p*<0.9	*p*<0.2
No disease evolution vs disease evolution	*p*<0.2	*p*<0.3	*p*<0.4	*p*<0.5	*p*<0.9

Note: *$p<0.05$.

Abbreviations: *DNMTs*, DNA methyltransferases; *TET*, ten-eleven-translocation; *APOBEC*, apolipoprotein B mRNA editing enzyme; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; RCC, refractory cytopenia of childhood; MDS-EB, MDS with excess of blasts; MDS-EB-t, MDS with excess of blasts in transformation.

according to MDS subtypes, karyotypes and evolution from MDS to AML.

Analysis of $p15^{INK4B}$ Promoter Methylation and Its Association with the Expression of DNA Methylation/ Demethylation Machinery Components

The analysis of $p15^{INK4B}$ promoter MtL in 20 patients showed that three had no methylation (15%), 11 had methylation (55%) and six were hypermethylated (30%). When comparing the patients who presented methylation with the donors, we observed a statistically significant increase in the first group ($p<0.03$) (Figure 6A) and when subdividing MDS cases in methylated and hypermethylated, increased MtL were observed in the latter group (Figure 6B). Next, we compared *DNMTs* expression in the different groups defined by $p15^{INK4B}$ methylation status and showed increased *DNMT1* expression in patients with methylation (p<0.02) and hypermethylation (p<0.009) when compared to donors (Figure 6C). For the other genes (*DNMT3A, DNMT3B, TET2*, and *APOBEC3B*) no statistically significant differences were observed (data not shown).

Discussion

In the present study, we described the expression levels of the DNA methylation and demethylation machinery components in pediatric patients with MDS. *DNMT1, DNMT3A*, and *DNMT3B* were overexpressed in pediatric MDS when compared with healthy pediatric donors. The initial stage of MDS showed a higher expression of *DNMT1*, while in advanced stages an increased expression was observed for *DNMT3A* and *DNMT3B. DNMTs* overexpression in MDS was previously demonstrated in adult patients.[25,26] Langer et al observed *DNMT1, DNMT3A* and *DNMT3B* overexpression in initial stage and in advanced stage when compared to control group.[25] Hopfer et al showed a higher expression of *DNMT3A* and *DNMT3B* in advanced stages relative to initial stage.[26]

DNMTs activity is essential for normal hematopoiesis as well as in the process of blood cell differentiation.[20,26] DNMT1 has been shown to be important for HSC self-renewal, niche retention, and multilineage hematopoietic differentiation.[27] DNMT1 overexpression has already been associated with aberrant methylation in tumour suppressor genes.[12] DNMT3A controls the balance between self-renewal and differentiation in normal hematopoiesis[28] and alterations in this enzyme result in HSC expansion, impaired differentiation and seems to be associated with

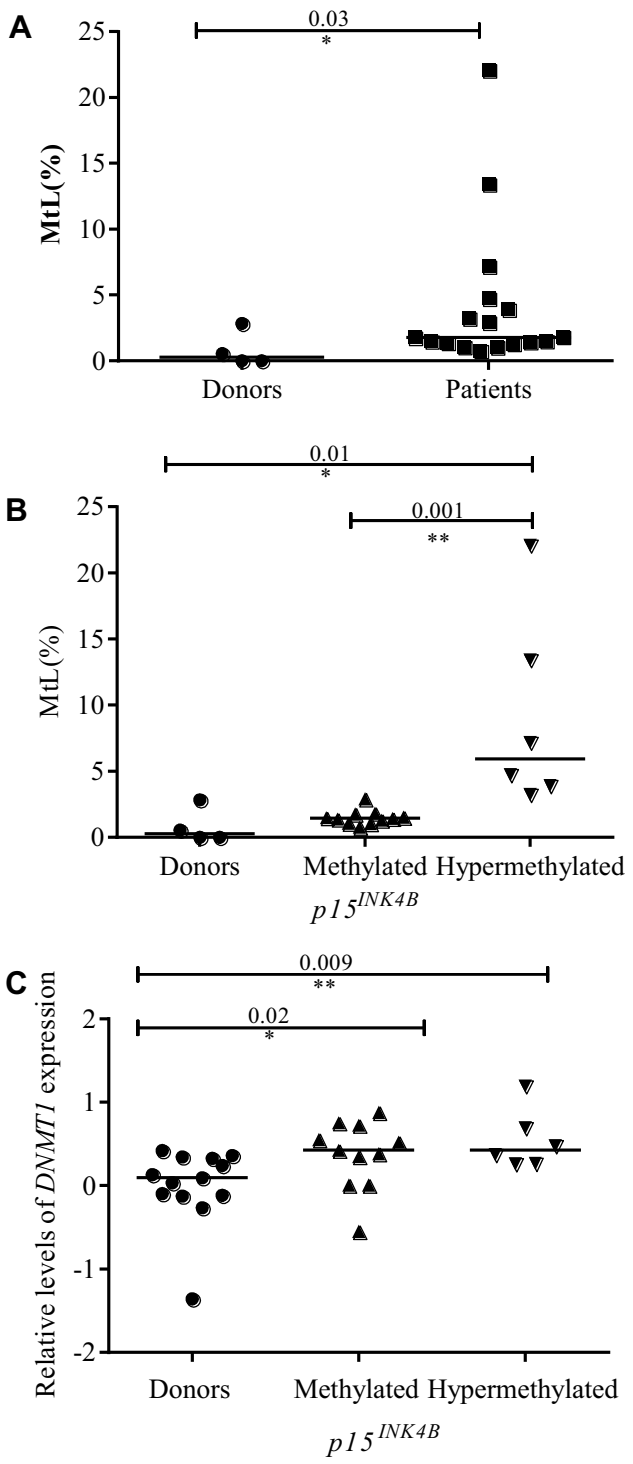

Figure 6 *p15*^{INK4B} promoter methylation profile in pediatric MDS and its association with *DNMT1* expression. (**A**) The comparison of *p15*^{INK4B} methylation profile between patients (who showed any detectable methylation) and donors, (**B**) Methylation level of *p15*^{INK4B} in pediatric MDS according to the methylation cut-off established by the standard deviation (SD) [mean (0.84) + 2 SD (1.17) = cut-off (3.18)] of the donors and (**C**) Dot-plots (log10 scale on the Y-axis) show the association between *p15*^{INK4B} gene methylation profile and *DNMT1* expression.

Notes: *Indicates a significant difference ($p<0.05$); **Indicates a significant difference ($p<0.01$) calculated by Mann–Whitney *U*-test.

Abbreviations: *DNMT1*, DNA methyltransferase 1; *p15*^{INK4B}, Cyclin-dependent kinase 4 inhibitor B; MtL, quantification of methylation level.

more advanced MDS subtypes.[26,28] DNMT3A and DNMT3B together play a role in the epigenetic repression of stem cell gene networks during HSC differentiation.[29] Recently, it has been demonstrated that *DNMT3B* acts as a master regulator of transcription in the pediatric AML genome.[30]

As DNMT1 acts on maintenance methylation while DNMT3A and DNMT3B mediate de novo methylation,[15] our results suggest that the three enzymes act in the development of the disease and mainly in its evolution to AML. However, it is noteworthy that the increased expression of *DNMT3A* and *DNMT3B* suggesting that new patterns of methylation occur, especially in patients with disease evolution. In fact, some studies have identified aberrant methylomes in adult patients with MDS, as well as hypermethylation of specific genes, particularly during the course of AML.[10,12,17,26,31] In our results, we observed an association between *p15*^{INK4B} promoter methylation and higher *DNMT1* expression levels. In this way, it is possible that *DNMT3*A and *DNMT3*B produce new methylation patterns during the development of the MDS and that *DNMT1* acts by maintaining these patterns.

Some studies demonstrated that there is a coordinate action of the *DNMTs*.[32,33] In accordance, a significant correlation between the expression of these enzymes was observed in the present study, suggesting that there is a cooperative action of the *DNMTs* expression in pediatric MDS pathogenesis.

TET2 and APOBEC3B act as components of the DNA demethylation machinery and they have relevant roles in cells homeostasis. *TET2* has been shown to regulate myeloid differentiation and clonal cell expansion during hematopoiesis. Its decreased expression has been previously reported in patients with MDS/AML, and it has also been associated with a poor prognosis.[34,35] APOBEC3B, in addition to its potential role in DNA demethylation, has also been shown to be an important source of mutations in human cancer.[23] Through its deamination activity, APOBEC3B can induce C to T and C to G transitions, leaving specific footprints in tumour DNA. Although this APOBEC-mediated mutational signature has been reported in several solid tumours, it is still poorly explored in the context of hematological malignancies.[19,36] In adult MDS patients, C to T transitions are the most frequent point mutations.[11,31,37] Furthermore, a more recent study with MDS pediatric patients showed that its mutational load is also characterised by C to T substitutions.[19]

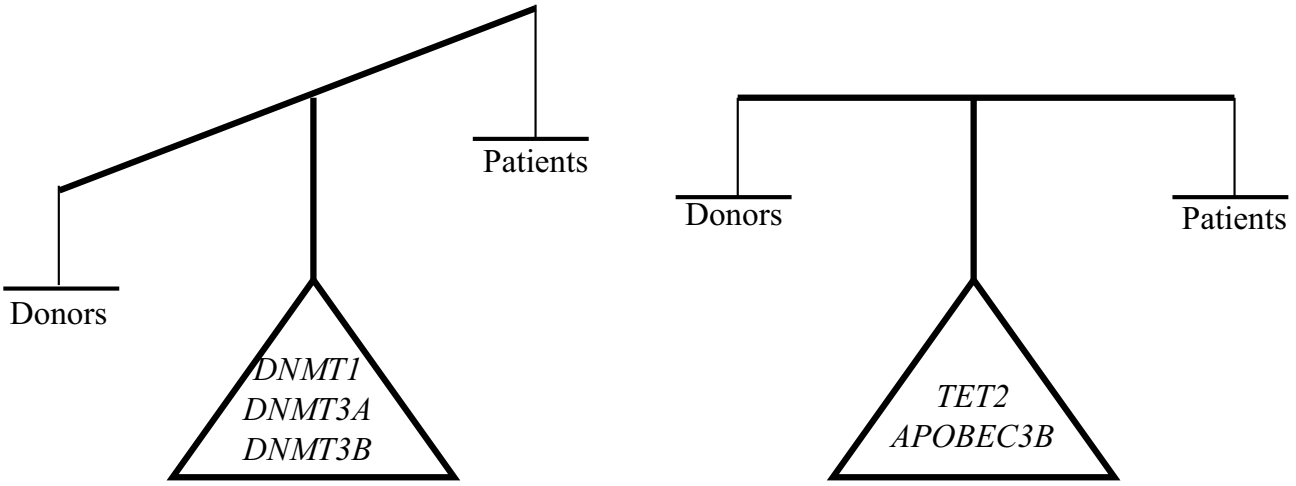

Figure 7 Imbalance of the DNA methylation and demethylation machinery components expression in pediatric MDS.
Abbreviations: *DNMTs*, DNA methyltransferases; *TET*, ten-eleven-translocation; *APOBEC*, apolipoprotein B mRNA editing enzyme; MDS, myelodysplastic syndrome.

In our study, *TET2* and *APOBEC3B* expression levels did not differ between pediatric patients and donors nor according to MDS subtypes. On the other hand, *APOBEC3B* expression levels were higher in patients with normal karyotypes when compared with patients with abnormal karyotypes and with healthy donors. In pediatric MDS, most patients with RCC show normal karyotypes.[38] However, some of these patients will manifest rapid disease evolution, suggesting the contribution of molecular alterations.[9] Our results suggest a possible role of *APOBEC3B* at initial stage of disease.

We also analysed the correlation between the expression of *DNMTs, TET2*, and *APOBEC3B* in pediatric patients with MDS, showing that *DNMTs* and *TET2* expression are not significantly correlated. However, it was possible to observe a correlation between *DNMTs* and *APOBEC3B* expression. Alterations in *DNMTs* also cause DNA imbalances and/or histone modifications, which may lead to chromatin remodelling, genomic instability, and gene expression pattern changes.[39] So, our results reinforce the relevant role of *DNMTs* and also suggests the participation of *APOBEC3B*, possibly contributing to genomic instability in the pathogenesis of pediatric MDS.

Taken together, our results suggest the presence of an imbalance between the DNA methylation and demethylation machinery components during pediatric MDS pathogenesis, with an overexpression of *DNMT1, DNMT3A*, and *DNMT3B* (Figure 7). Our results have clinical implications and indicate the importance of hypomethylating agents for preventing or delaying the progression to leukemia, especially for children undergoing hematopoietic stem cell transplantation (HSCT). Waespe et al demonstrated that azacytidine treatment prior to hematopoietic stem cell transplantation was well tolerated in pediatric patients with advanced MDS and was associated with superior event-free survival.[40] Therefore, laboratory tests for detecting the level of *DNMTs* expression may aid the prediction of disease evolution and may select the patients with MDS for the use of DNA methyltransferases inhibitors.

Since this study was the first to address the alterations in DNA methylation and demethylation machinery components in pediatric patients with MDS, it is necessary to confirm our results in other cohorts to provide a better understanding of the aberrant expression of these genes in the pathogenesis of pediatric MDS.

Conclusion

Our results suggest that the overexpression of *DNMTs* and an imbalance between the expressions of the DNA methylation/demethylation machinery components play an important role in MDS development and evolution to AML. These results have clinical implications indicating the importance of *DNMTs* inhibitors for preventing or delaying the progression to leukemia in pediatric MDS patients.

Abbreviations

APOBEC, apolipoprotein B mRNA editing enzyme; AML, acute myeloid leukemia; BM, bone marrow; cDNA, complementary DNA; *DNMTs*, DNA methyltransferases; HSCs, hematopoietic stem cells; HSCT, hematopoietic stem cell transplantation; MDS, myelodysplastic syndrome; *TET*, ten-

eleven-translocation; MDS-EB, MDS with excess of blasts; MDS-EB-t, MDS with excess of blasts in transformation; MtL, quantification of methylation level; RCC, refractory cytopenia of childhood; 5mC, 5-methylcytosine; 5hmC, 5-hydroxylmethyl cytosine; $p15^{INK4B}$, Cyclin-dependent kinase 4 inhibitor B.

Data Sharing Statement

The data used to support the findings of this study are included within the article and the original data used to support the findings of this study are available from the corresponding author upon request.

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Author Contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was approved by local Ethic Committees, Ethic Committee of the Brazilian National Cancer Institute (CEP #62/10) and Pediatric and Puericulture Martagão Gesteira Institute (CEP 08926213.9.0000.5264). Informed consent was obtained from children's parents.

Disclosure

The authors declare no conflicts of interest in this work.

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