Transcription factors LEF1, PU.1 and IRF8 have decreased expression levels in majority of de novo acute myeloid leukemia patients

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Abstract

Background & Objectives: The lymphoid enhancer-binding factor 1 (LEF1), PU.1 and interferon regulatory factor 8 (IRF8) are three important differentiation genes that are commonly defective and associated with the development of leukaemia. Alternations in the expression of these genes can be resulted in malignancy.

Methods: In this study the expression levels of the genes mentioned were analysed using Real Time PCR with SYBR Green and the ΔΔCT method within 96 patients with acute myeloid leukaemia (AML) and 16 healthy subjects as a normal control.

Results: The results presented in this study revealed that PU.1 and LEF-1 gene expression was significantly lower and IRF8 gene expression levels were significantly higher in patients with AML in comparison with the normal control group (P < 0.0001). Furthermore, Analysis determines that the three genes have moderate positive correlation with each other; correlation between PU.1 and IRF-8 is R: 0.378, P <0.0001, expression of PU.1 and LEF-1: R: 0.399, P <0.0001 and the expression of IRF8 and LEF1: R: 0.320, P: 0.001 in patients with AML. In our study, the relatively strong positive correlation between these genes was observed which is supported by other studies.

Interpretation & conclusion: It can be indicated in this study that when malignancy for unknown reasons that new connections between transcription factors occur which can affect the malignancy process. Our observations suggest that examining the oncogenic role of these genes and discovering new molecular mechanisms formed in the process of malignancy in each of these differentiation genes can play a role in the design of novel diagnostic methods, monitoring and treatment of patients with acute myeloid leukaemia.

Key words: AML, gene expression, IRF8, LEF1, PU.1.

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Introduction

The clonal hematopoietic disorder, acute myeloid leukaemia (AML), is by molecular terms a heterogeneous disease where due to termination of differentiation in the myeloid lineage accumulation of immature progenitors within bone marrow occurs and simultaneously an inability to produce normal blood cells. It is has been widely reported that the formation and behaviour of leukemic cells is perpetrated by impaired regulation of the expression of genes involved in cell growth and differentiation. Three differentiation genes that are commonly defective and associated with the development of leukaemia are transcription factors PU.1, LEF1 and IRF8 which will be investigated in the present study in the context of gene expression in AML.

Of the key transcription factors under investigation is PU.1, an ETS family transcription factor which is encoded by the SPI1 gene and has multiple roles in the normal haematopoiesis process and is necessary for the lymphomyeloid differentiation of stem cells (4,5). Studies report that in certain subtypes of AML, expression of this gene is decreased due to genetic abnormalities and that this reduced level of expression is pivotal for the induction of AML subtype M3, also known as acute promyelocytic leukemia (APL) as evidence shows that the PML-RARA fusion gene, which characterises this AML subtype, has an inverse correlation with PU.1 (6). A number of studies have demonstrated that PU.1 acts as a tumor suppressor gene in AML, for example by disrupting p53 activity (7).

Another transcription gene that will be considered in this study is the lymphoid enhancer-binding factor 1 (LEF1) which belongs to the LEF1/T-cell factor (TCF) transcription factor family (8). LEF1 has a critical role in myeloid differentiation which has recently been reported in human model studies supported by the observation that in normal conditions mRNA levels of LEF1 reaches its maximum levels in the promyelocytic stage of differentiation and experiences reduction in the final granulocytic stage (9). LEF1 mutations have been identified to be associated with high risk events in AML and lymphomas (10). It has been reported that LEF1 can indirectly promote PU.1 expression by repressing the expression of CEBPA and in turn CEBPA can no longer inhibit PU.1 and monocytosis occurs as observed in the case of congenital neutropenia. A direct relationship between LEF1 and PU.1 has also been identified where in normal conditions LEF1 acts as transcriptional inhibitor of PU.1 thusly alterations in LEF1 expression levels affects PU.1. In addition, LEF-1 is an appealing case of research as it has recently been proposed as a prognostic factor as studies have demonstrated that increased expression of LEF-1 is associated with a favourable prognosis in CN-AML (11-13).

The final gene that will be considered in the present study is interferon regulatory factor 8 (IRF8) which encodes for a protein also known as interferon consensus sequence-

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CLINICAL RESEARCH AND METHODS
binding protein (ICSBP) (14,15). The fundamental function of IRF8 lies in the regulation of myeloid cell fate moreover in cells of the myeloid lineage IRF8 expression is controlled through means of the myeloid master regulator PU.1. This gene also like PU.1 acts as a tumour suppressor gene in myeloid lineages (16). Recent studies report that IRF-8 is expressed in MDPs, CDPs and in committed granulocyte and monocyte progenitors but absent in GMPs and CMPs which reiterates the crucial function this gene has normal haematopoietic differentiation (14,15). In the case of loss of function IRF8 promotes granulocytic differentiation and represses monocytic differentiation in a disproportionate manner whilst over activation induces the opposite effect. Furthermore, the dysregulation of IRF8 is associated with the development of MDS to AML as seen in murine models and human subjects (17). Moreover, it was observed that in the MDS-AML model there is a positive correlation between IRF8 and PU.1 as lower levels of PU.1 expression were specifically accompanied by lower levels of IRF8 expression in AML patients although no such correlation was observed in normal haematopoietic stem cells (18).

In the present study, we focus on three differentiation related genes, LEF1, PU.1 and IRF8, which have been described as tumour suppressor genes where changes in expression of these genes have been associated to the development of AML. By means of real-time PCR expression levels of the aforementioned genes were measured in AML patients and healthy subjects to analyse relative expression of each gene and further analysed in terms of AML subtype, patient characteristics and to identify potential correlations between the expression levels of these genes.

Methods and materials

Patient samples
Ninety six bone marrow (BM) and peripheral blood (PB) samples with collaborated clinicians were obtained from newly diagnosed patients with de novo AML and 18 samples from healthy subjects (used as a normal control) between the years 2013 to 2015 ( Sample size evaluated with formula).

The patients were referred to Mofid and Emam Khomeini hospital, Tehran, Iran. In addition, Shahid Beheshti University of Medical Science, Tehran, Iran and Department of Medical Research were laboratory involved in our study. Then receiving informed consent according to institutional guidelines the median age of individuals in this study was 47 years with a range of 2 to 87 years and mean age of 45.39 years. Samples were taken from 44 female and 52 male subjects. The number of patients in each subgroup FAB includes: 10 patients with M0, 20 patients with M1, 14 patients with M2, 30 patients with M3, 14 patients with M4, 7 patients with M5 and one patient with M6. Furthermore, patients were divided into three subgroups based on morphological differentiation status: 34 patients without distinction (M0/M1/M2), 30 patients with granulocytic differentiation (M3) and 21 patients with monocytic differentiation (M4/M5).

RNA isolation, cDNA synthesis, real-time PCR
Total cellular RNA was extracted from BM and PB using an RNeasy kit (Qiagen, Germany). Following extraction, the amount and quality of RNA was measured by a NanoDrop (Thermo Scientific, USA). All samples showed high purity (OD 260/280 nm ratio >1.8) and integrity. Subsequently, 2µL (0.5mg) RNA was transcribed into cDNA to a final volume of 20µL using a Thermo kit (USA). An aliquot of 1/10th of the resulting cDNA (1µL) was used as substrate for qRT-PCR amplification.

Primers specific to PU.1, IRF8, LEF1 and ABL ( house keeping gene) were designed via Oligo 7.56 software (Table 1) which subsequently allowed levels of PU.1, IRF8, LEF1 and ABL mRNA expression in patients and healthy subjects to be detected by qRT-PCR (Rotor Gene 6000, Bosch). The components in the qRT-PCR reaction for each gene consisted of 1µL of template cDNA, 1µL forward and reverse primer, 7µL of RealQ Plus 2x Master Mix Green-Low ROX (Ampliqon, Denmark) and 6µL water for a total reaction volume of 15µL. For each qRT-PCR reaction a standard curve was produced, using five consecutive 1:10 dilutions cDNA sample (1, 0.1, 0.01 and 0.001). The thermal cycling conditions for each reaction included an initial hold at 95°C for 10 minutes followed by 40 cycles of primary denaturation at 95°C for 10 seconds and annealing/ extension at 65°C for 15 seconds and a final extension at 72°C for 10 minutes. Additionally, negative controls were included in the assay and the assay was performed in duplicate. The relative quantification of mRNA expression for each sample (fold change=FQ) was calculated using the Livak method (2-ΔΔct ) (19).

Table 1. Nucleotide sequences of primers used for ABL, PU.1, IRF8 and LEF1 qRT-PCR reactions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>ABL</td>
<td>AGTCTCAGGATGCAAGGTGCT</td>
<td>TAGGCTGGGCGCTTTTGTAA</td>
</tr>
<tr>
<td>PU.1</td>
<td>AGAGAGGAGCTTGCCCGCTATG</td>
<td>GTAAATGGTCGCTATGGCCT</td>
</tr>
<tr>
<td>IRF8</td>
<td>CAGCCACTTGGAAGAGCAG</td>
<td>TGTTATGGTCGAGAATTGCT</td>
</tr>
<tr>
<td>LEF1</td>
<td>GAGAAGGGCGATTTAGCTG</td>
<td>AGGTTTGTGCTTGTGCTGCC</td>
</tr>
</tbody>
</table>
**Results**

**Variable expression levels of PU.1, IRF8 and LEF1 in AML**

By means of real-time PCR, Ct values were obtained for LEF1, PU.1, IRF8 and ABL (housekeeping gene) and subsequently the mean Ct values were measured for all genes for both AML patients and the normal control group (Table 2). For each gene, an expression level in the range of 95% confidence interval was defined as a normal or intermediate expression level for a healthy population. Based on these levels parameters for a range of low expression and high expression for each was also defined (Table 2).

Table 2: Average of Ct subjects were obtained for 4 genes ABL, PU.1, LEF1 and IRF8 in patients and healthy according to the above table. An expression level in the range of 95% confidence interval (0.76 – 1.90), (1.04 – 3.36) and (0.79 – 2.23) defined for the average PU.1, LEF1 and IRF8 expression level in the healthy population, was considered 'intermediate', respectively. The majority of patients (64/96),(91/96) and (71/96) had a LOW expression level of PU.1, LEF1 and IRF8, respectively.

PU.1 and LEF1 expression is significantly lower in AML patient samples in comparison to healthy subjects.

The resulting Ct values obtained for each gene were normalised against the internal housekeeping gene, ABL, for both AML patients and the normal control group and subsequently compared. An initial comparison by application of the Livak formula (ΔΔCT) demonstrated that expression levels of the genes PU.1 and LEF1 were lower in AML patients in comparison to the normal control group with a 1.28 and 9.17 fold decrease, respectively (Figure 1, A and B). A statistical analysis by means of a T-test determines that there is a significant difference in expression levels between the two groups for both PU.1 (P < 0.0001) and LEF1 (P < 0.0001) (Figure 1, C and D).
Figure 1: Relative expression of PU.1 and LEF1 in 96 AML patients and 18 healthy patients was measured from Ct values and normalized against a reference gene (ABL). A,B) There is a 1.28 fold decrease in PU.1 expression and a 9.17 fold decrease in LEF-1 expression in AML patients in comparison to the normal control group. C) A mean LEF-1 expression level of 0.24 ± 0.04 (SEM) was measured in AML patients in comparison to a mean LEF-1 expression level of 2.20 ± 0.54 in the normal control group. A significant difference (P< 0.0001) between LEF-1 expression in AML patients and healthy subjects was identified. D) A mean PU.1 expression level of 1.04 ± 0.17 (SEM) was measured in AML patients in comparison to a mean PU.1 expression level of 1.33 ± 0.26 in the normal control group. A significant difference (P< 0.0001) between LEF-1 expression in AML patients and healthy subjects was identified.

IRF8 expression is significantly higher in AML patient samples in comparison to healthy subjects.

As with the PU.1 and LEF1 genes, expression of IRF8 was analysed by normalising the mean Ct values against the housekeeping gene and a comparison of relative expression between AML patients and normal control group was performed by means of the same statistical analyses. In contrast to PU.1 and LEF1, IRF8 gene expression was higher in AML patients than the normal control group with fold increase of 1.87 (Figure 2, A). The increase of IRF8 expression level in AML patients was deemed statistically significant by application of the T-test (P < 0.0001) (Figure 2, B). In our study, this gene significantly increased compared to control group but similar to this study but as in this study when the overexpression is studied in detail in patients we found that only 15% (15 of 96 patients) had increased expression and the rest reduced expression (Figure 3).

IRF8 expression is significantly higher in AML patient samples in comparison to healthy subjects.
Figure 2: Relative expression of IRF8 in 96 AML patients and 18 healthy patients was measured from Ct values and normalized against a reference gene (ABL). A) There is a 1.87 fold increase in IRF8 expression in AML patients in comparison to the normal control group. B) A mean IRF8 expression level of 2.83 ± 1.09 (SEM) was measured in AML patients in comparison to a mean IRF8 expression level of 1.51 ± 0.34 in the normal control group. A significant difference (P< 0.0001) between IRF8 expression in AML patients and healthy subjects was identified.

Figure 3: IRF8 gene expression in patients and healthy subjects

PU.1 expression is significantly lower in APL patient samples in comparison to other AML subtypes and healthy subjects.

Expression levels of PU.1 in each FAB subtype (M0-M5) were compared to the normal control group and a statistically significant difference was observed in the APL-M3 subgroup when compared to all other subtypes as a single subgroup (non-M3) (P = 0.027), that there is a 2.27 fold decrease in expression (Figure 4, A). Subsequently, the patients in this study were classified by broader disease subgroups instead of the FAB classification they were initially assigned by. Patients were assigned to a subgroup based on three morphological differentiation statuses; without differentiation (M0/ M1/M2 subgroup, 34 patients), granulocytic differentiation (APL-M3 subgroup, 30 patients) and monocytic differentiation (M4/M5 subgroup, 21 patients). When PU.1 expression levels in these subgroups were compared to the normal control group a significant difference was observed between M3 group with normal group (P=0.001), but not the other subgroups. (Figure 4, B) The Tukey test was applied to analyse the relationship of PU.1 expression between AML subgroups and it was observed that PU.1 expression in patients with the APL subtype was significantly lower than the other monocytic differentiation(M4/M5) and undifferentiated subgroups(M0/M1/M2)(P = 0.022 and P = 0.027) (Figure 4, B).
Figure 4: Normalized relative expression of PU.1 in 96 AML patients and 18 healthy patients based on varying disease subtypes. A) PU.1 expression is analysed in patients based on their FAB classification. Patients classified as M3 and non-M3: a 2.27 fold decrease in gene expression levels in the M3 subgroup. B) Patients are classified into three disease subgroups based on differentiation status without differentiation (M0/M1/M2 subgroup), granulocytic differentiation (M3 subgroup) and monocytic differentiation (M4/M5 subgroup). PU.1 expression in comparison to the normal control group was only significantly lower in the M3 subgroup.

PU.1 expression is significantly higher in AML patients with ‘some’ MPO activity. Patient samples were assessed for MPO activity by peroxidase staining and subsequently graded and sub-grouped based on the percentage of MPO positive cells: rare (<3%), few (10%), some (50%), most (80%) and strong (100%). PU.1 expression levels were evaluated based on the aforementioned MPO grades and compared. The ANOVA test was applied for normal distribution and as a result a significant correlation (P=0.007) between PU.1 expression and MPO subgroups was observed. The Tukey test was applied to reveal the relationship between PU.1 expression and specific MPO grade and it was observed that PU.1 expression levels in patients with ‘some’ peroxidase activity was significantly higher than ‘rare’ (P-value=0.016) and ‘strong’ (P-value= 0.021) grades (Figure 5).

Figure 5: Normalized relative expression of PU.1 in 96 AML patients graded by MPO activity. Patients are graded ‘rare’ to ‘strong’ based on the percentage of MPO positive cells. Patients graded with ‘some’ MPO activity, those with the intermediate number of MPO positive cells, had PU.1 expression levels which were significantly higher in comparison ‘rare’ and ‘strong’ group. A significant difference is observed between PU.1 expression levels in the ‘some’ grade in comparison to the ‘rare’ grade (lowest number of MPO positive cells) (P=0.016, also a significant difference is observed between PU.1 expression levels in the ‘some’ grade in comparison to the ‘strong’ grade (highest number of MPO positive cells) (P=0.021).
Correlation between PU.1, LEF1 and IRF8 in AML patients:
An analysis by means of statistical test was conducted to identify any correlation between the expression of PU.1, LEF1 and IRF8 genes in AML patients. Analysis determines that the three genes have moderate positive correlation with each other; correlation between PU.1 and IRF-8 is R: 0.378, P < 0.0001(Figure 6 A), expression of PU.1 and LEF-1: R: 0.399, P < 0.0001 (Figure 6 B) and the expression of IRF8 and LEF1: R: 0.320, P: 0.001(Figure 5C) in patients with AML. In comparison, an analysis of the correlation between these genes in healthy subjects determines that there no correlation exists in the normal status; PU.1 and IRF8: R: 0.469, P: 0.049, PU.1 expression and LEF1: R: 0.281, P: 0.259 and between the expression of IRF8 and LEF1: R: 0.418, P: 0.084.

Figure 6: Statistical analysis by means of Pearson’s chi-squared test reveals dependence and relation between the expression of PU.1, LEF1 and IRF8. A) Correlation between PU.1 and LEF1 in 96 AML patients was determined to be positive and significant (P < 0.0001, r= 0.378). B) Correlation between PU.1 and IRF8 in 96 AML patients was determined to be positive and significant (P < 0.0001, r=0.399). C) Correlation between IRF8 and LEF1 in 96 AML patients was determined to be positive and significant (P = 0.001, r= 0.320).
Discussion

Several studies together, approve our results associated with decreased expression of PU.1 in AML patients. In studies pointed out that there is a relationship between PU.1 low expression and AML, the absence of this gene leads to stimulation of AML and partial inhibition of this gene is common in AML patients. In other types of leukemia such as CML also showed reduced expression of the transcription factor (20-22). Thus, this gene in our study has maintained its tumour suppressor role and found decrease and with its decrease in expression also leads to increased tendency towards leukemia development. In contradictory studies to ours it was shown that IRF8 has significant decrease in expression. In CML it was shown that IRF8 acts as a tumour suppressor as STAT5 is a target gene of the BCR-ABL fusion gene its expression is suppressed (23). Also, reported that decreased expression of this gene in addition to CML and is associated with AML. Since this gene can regulate the survival of progenitors, therefore it can act as a tumour suppressor gene with its decrease cause myelogenous leukemia formation (14). The absence of this gene leads to granulocytic differentiation and blocking of monocytic differentiation and vice versa. But in a study showed that increased expression of WT-IRF8 transcripts (expressed in hematopoietic stem cell senescent and early leukemic blasts) and SV-IRF8 (identified in malignant cell lines and leukemic blasts in AML patients due to aberrant promotor hypermethylation IRF8 becomes normal) is significantly associated worse with RFS than AML. Since the decreased expression of the gene associated with haematopoiesis dysfunction and promotion of leukaemia it was unexpected that increased expression of the WT transcript is associated with adverse prognosis. Increased expression of this gene likewise to its decrease can have detrimental effects on normal haematopoiesis. Increased expression of this gene blocks the neutrophil differentiation and promotes differentiation towards monocytic, macrophage and DC types. If the overexpression of this gene leads to leukemic cells dendritic cells improperly transport, this change in performance may partly explain the adverse prognosis. In our study, this gene significantly increased compared to control group but similar to this study but as in this study when the overexpression is studied in detail in patients we found that only 15% (15 of 96 patients) had increased expression and the rest reduced expression (Figure 3). LEF1 in our study showed significant decreased expression, in various studies the role of this gene is described as factor in granulocyte differentiation due to decreased expression observed in patients with congenital neutropenia. Moreover, it seems that LIFE-1 gene in leukemic cells acts as a tumour suppressor gene. So its reduction of expression allows leukemic cells to proliferate and increase in growth. (8, 10). Studies have shown that overexpression of this gene is associated with a more favourable prognosis in patients with APL. The findings showed that increased expression of this gene in CN-AML is associated with favourable outcome even if the molecular and clinical risk factors are known. Studies have showed that LEF1 is significantly overexpressed compared to controls but the contradictory results were obtained in our study and in one patient increased expression compared to the normal control was identified. As low expression of LFE1 in AML patients with increased blasts in the bone marrow and is associated with progression of AML. Since this gene is a transcription factor important in the granulocytic differentiation thereby reduction of LEF1 expression may participate in the differentiation block in AML and MDS blasts which an increase in blast percentage and white blood cells in patients with CN-AML is a reflection of reduction of LEF1 expression.

The decreased expression of these genes leads to poor patient prognosis and development of malignancy on the order of PU.1 expression based on subgroups: m3/m0/m1/m2/m4/m5. This gene is expressed at the highest levels in monocytes and plays an important role in myeloid differentiation. APL in 98% of cases is accompanied with the PML-RARA fusion gene and the expression of this gene in M3 due to PML-RARA is inhibited as it has been observed that after ATRA increased expression of this gene occurs and differentiation is induced (24). In this study only the M3 subtype was related to the normal control group compared to the other two subtypes. As in other studies our study also showed that PU.1 had significantly decreased expression in APL patients in comparison to the normal control group due to the PML-RARA fusion gene in these patients. This gene in HSCs is expressed lower than CMPs and it this increase in expression which is the factor which promotes differentiation towards monocytes and granulocytes. Expression of this gene was increased from M0 to M5, except in M3. The lowest expression of this gene was found in M0 in blasts with minimal differentiation the lowest expression was seen and average expression in M2 / M1 was shown and expressed higher in M4 / M5. In M2 the fusion gene AML1-ETO is the factor that represses PU.1 inhibition. However the reason M2 was higher compared to M3 is the lack of this fusion gene in 80% of patients. And it was observed that suppression of expression of this gene is essential for the initiation and pathogenesis of APL. Our results on the expression levels of AML subtypes was exactly the same (6). The order of LEF-1 expression in subtypes: M3=M0/M1/M2>M4/M5 which showed this gene is not involved in cell maturation because with increased differentiation this gene showed reduced expression which contradicts this genes role as a differentiation factor. The more mature the cell this gene is more impaired. This gene in its normal state has a role in the differentiation, proliferation and survival of granulocytic progenitors. In our study in detail expression of this gene in subgroup with granulocytic differentiation was higher than subgroup of monocytic differentiation although this difference was no significant (8). The order of IRF8 expression in subtypes: M0/M1/M2<M3<M4/M5. The more mature the cell, the more this gene was expressed which is why the expression of IRF8 was much higher in the M4/M5 subgroup than the undifferentiated subgroup. It is M3 that confirms the fact that IRF8 has the highest expression in in monocytic differentiation which is the case in normal circumstances. Likewise with consideration to cell maturation it has increased expression which can...
be a sign of its proliferation role in AML. In studies it has also been shown that this gene in patients with M5 have overexpression(25, 26).

In our study, the relatively strong positive correlation between these genes was observed which is supported by other studies where it has also been shown that overexpression of PU.1 leads to activation of IRF8 and inhibition of CEBPA, GATA1, GATA2 and KLF4 which are necessary for granulocytic differentiation(6). Since IRF8 acts as a cofactor for PU.1 and based on IRF8 defective mice studies, dysfunction in determining granulocytic and monocytic differentiation and develop towards MDS, slight decreases in PU.1 and defective IRF8 plays a role in the pathogenesis of human leukemia. This means that either IRF8 with lower expression in AML patients with PU.1 lower expression is observed or a positive correlation between the expression of IRF8 and PU.1 exists in LSCs whilst this correlation does not exist in healthy HSCs (17). Furthermore, in other studies it has been reported that LEF1/B-catenin leads to increased regulation of the gene IRF8 (27).

In our study we also showed that between the expression of these three genes in AML patients there is correlation but in the normal control group no correlation was observed. It can be indicated in this study that when malignancy for unknown reasons that new connections between transcription factors occur which can affect the malignancy process. Our observations suggest that examining the oncogenic role of these genes and discovering new molecular mechanisms formed in the process of malignancy in each of these differentiation genes can play a role in the design of novel diagnostic methods, monitoring and treatment of patients with acute myeloid leukaemia(28-32).

Abbreviations:
LEF1, lymphoid enhancer-binding factor 1; IRF8, interferon regulatory factor 8; AML, Acute Myeloid Leukaemia; HSCs, Hematopoietic stem cells; bZIP, basic-leucine zipper; WT, wild-type; BM, Bone Marrow; PB, peripheral blood; S.E.M, standard error of the mean; MDS, Myelodisplastic syndrome; CN-AML, cytogenetically normal acute myeloid leukemia.

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