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Research paper

Transcription factors implicated in late megakaryopoiesis as markers of outcome after azacitidine and allogeneic stem cell transplantation in myelodysplastic syndrome

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ABSTRACT

The hypomethylating agent azacitidine (AZA) is used to treat higher-risk myelodysplastic syndromes (HR-MDS) and elderly patients with low-blast count acute myeloid leukemia (LBC-AML). Platelet recovery is an early predictor of AZA response. We prospectively studied the expression profile of transcription factors, critical for late megakaryopoiesis and changes in their expression after AZA treatment in patients with HR-MDS and LBC-AML enrolled in the BMT-AZA trial (EudraCT number 2010-019673-15). Twenty-five additional patients with low-risk (LR)-MDS were also studied.

At the time of diagnosis, GATA2 mRNA levels were significantly higher in MDS as compared to controls, with increasing levels from LR- to HR-MDS/AML. RUNX1 expression was also significantly higher in MDS, as compared to controls, but no differences were found between LR- and HR-MDS. Looking at biomarkers of response, we found that patients AZA responsive had higher basal GATA1 and lower FLI1 expression, compared to those with stable or progressive disease after treatment. Univariate analysis showed that increased GATA2 mRNA expression was associated with a worse overall survival. Our findings suggest that high GATA2 expression is a poor prognostic marker for survival in patients with HR-MDS and LBC-AML treated with azacitidine. Moreover, GATA1 and FLI1 mRNA expression may predict response to AZA treatment.

1. Introduction

Myelodysplastic syndromes (MDS) are a group of diseases characterized by ineffective haematopoiesis, with dysplastic features of hematopoietic cells, and peripheral blood (PB) cytopenias [1]. Thrombocytopenia (platelets $< 100 \times 10^9$/L) is a frequent finding in MDS, occuring in 40%–65% of patients, and is the first sign of disease in about 30% of cases [2].

Platelet formation is a complex process including megakaryocyte maturation and platelet assembly and shedding [3,4] driven by several transcription factors (GATA1-2, RUNX1, NFE2, FLI1, FOG1) [3]. The involvement of these transcription factors in late megakaryopoiesis has been previously demonstrated [5–7]. For instance, the Paris-Trousseau syndrome, an inherited disorder associated with thrombocytopenia and increased tendency to bleeding, has been closely related to FLI1 hemizygous loss [5]. In addition, several human hematopoietic diseases such as acute megakaryoblastic leukemia and familial platelet disorders are linked to mutations in GATA1 and RUNX1 genes, respectively

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[6,6,7]. All these transcription factors, interacting with each other, regulate both the activation of megakaryocyte (MK) lineage precursor genes and the repression of genes involved in other hematopoietic lineages commitment.

In MDS, MK differentiation is impaired, with significant morphologic changes in megakaryocytes in the bone marrow (BM), including the presence of micro- and mononuclear megakaryocytes, dumbbell-shaped nuclei, hyper-segmentation and multinuclearity with multiple isolated nuclei. Platelets are often dysmorphic in the PB, with the presence of giant platelets, or anisometry [8].

Outcome in higher-risk MDS (HR-MDS) significantly improved after the introduction of hypomethylating treatment (HMT), in particular azacitidine (AZA) in Europe [9], which has become the standard therapy in these patients. AZA is also currently used in the treatment of elderly patients with low-blast count acute myeloid leukemia (LBC-AML). Following AZA treatment, complete remission (CR) rates of 15–20% have been reported in prospective studies and confirmed in "real-life" patient cohorts, and the overall response rate, including partial response (PR) and haematological improvement (HI), reaches up to 50% [9,10]. In the vast majority of patients, 4–6 cycles of treatment are needed to obtain most of responses. In about 30% of patients, BM evaluation after 4–6 treatment cycles shows disease stability [10,11]. In these patients, the decision of whether to continue or stop treatment with azacitidine is challenging, thus the identification of prognostic factors of response to AZA treatment may be helpful to support this decision.

Clinical markers of response include Eastern Cooperative Oncology Group (ECOG) performance status, presence of blasts in the PB, heavy transfusion dependence, and intermediate/high risk cytogenetics [12]. In addition, rapid platelet recovery after the second cycle of treatment has been shown to predict response to HMT [12–16].

Since platelet recovery appears to be an early predictor of AZA response, we studied the expression profile of transcription factors, critical for the late MK development, in a well-characterized cohort of patients enrolled in BMT-AZA multicenter trial [17]. The aims of our work were to characterize expression of genes involved in megakaryocyte development, and identify early biomarkers predictive of treatment response, disease evolution and patient survival.

2. Materials and methods

2.1. Sample and patient cohort

A total of 51 patients, 45 with *de novo* HR-MDS and 6 with LBC-AML, with a median age of 60 years (range 36–65 years) enrolled in BMT-AZA multicenter trial (EudraCT number 2010-019673-15), were studied [17]. An additional cohort of 25 patients with *de novo* low-risk MDS (LR-MDS), with a median age of 69 years (range 46–86 years) were included in the study. Diagnosis was established according to the World Health Organization (WHO) classification, whereas International Prognostic Scoring System (IPSS) and revised-IPSS (IPSS-R) were used for prognostic evaluation [1,18,19]. All HR-MDS and LBC-AML patients were homogeneously treated with the standard azacitidine regimen (75 mg/sqm/day subcutaneously for 7 days every 28 days) for at least 4 cycles, followed by allogeneic stem cell transplantation (HSCT) in 41 patients. The median number of cycles of AZA treatment was 4 (range 1–11), and evaluation of AZA response was performed according to the International Working Group (IWG) criteria for MDS [20] and AML [21]. Patients were considered responders if they achieved complete remission (CR), partial remission (PR), marrow CR (mCR), or haematological improvement (HI). On the contrary, patients that exhibited stable (SD) or progressive disease (PD) were considered resistant.

Detailed patient characteristics are reported in Tables 1 and S1. BM samples from 14 healthy individuals (6 females, 8 males; median age: 37 years, range: 16–64 years), were used as controls.

According to the declaration of Helsinki, all patients and controls gave informed consent to the study, which was approved by the Ethical Committees of University of Tor Vergata and Policlinico Agostino Gemelli.

Table 1
Patient characteristics.

Patient Characteristics (n = 51 patients)		
Age (median, range)		60 (36-65)
BM-blasts % median (range)		13 (0-30)
Blood counts median (range)	Hb (g/dl)	9.4 (7.1-13.3)
	Neutrophils (10^9/L)	0.6 (0.1-37)
	Platelets (10^9/L)	84 (6-662)
WHO 2016 Classification (n)	HR-MDS	45 (88.2%)
	LBC AML	6 (11.8%)
Karyotype (n = 46)	Normal	19 (41.3%)
	Trisomy 8	2 (4.3%)
	−7	10 (21.7%)
	Del 5q	1 (2.2%)
	Complex (≥3 abn)	11 (24%)
	Other	3 (6.5%)
IPSS (n = 49)	Low/Int-1	2 (4.1%)
	Int-2	29 (59.2%)
	High	18 (36.7%)
R-IPSS (n = 38)	Very low/low	3 (7.9%)
	Intermediate	7 (18.4%)
	High	12 (31.6%)
	Very high	16 (42.1%)
HMT response (n = 51)	CR	11 (21.6%)
	PR	9 (17.6%)
	HI	2 (3.9%)
	SD	23 (45.1%)
	PD	6 (11.8%)
HSCT (n = 51)	Yes	41 (80.4%)
	No	10 (19.6%)

BM: bone marrow, HR: higher risk, LBC: low blasts count, IPSS: International Prognostic Scoring System, R-IPSS: Revised International Prognostic Scoring System, HMT: hypomethylating therapy, CR: complete response, PR: partial response, HI: hematological improvement, SD: stable disease, PD: progression disease, HSCT: Hematopoietic Stem Cell Transplantation.

2.2. In vitro megakaryocytic differentiation

To study normal megakaryocytic development, CD34+ progenitor cells were purified from healthy cord blood (CB) by positive selection using the midi-MACS immune-magnetic separation system (Miltenyi Biotec, Bergisch Gladabach, Germany), according to the manufacturer's instructions. Median CD34+ cell purity, assessed by flow cytometry using a monoclonal PE-conjugated anti-CD34 antibody, was 95% (range 92–98%). Purified human hematopoietic progenitor cells were grown in serum-free medium containing BSA (10 mg/ml), transferrin (1 mg/ml), low-density lipoproteins (40 µg/ml), insulin (10 µg/ml), sodium pyruvate (10^{-4} M), L-glutamine (2×10^{-3} M), rare inorganic elements (Sn, Ni, Va, Mo and Mn (+/-)) supplemented with iron sulphate (4×10^{-8} M) and nucleosides (10 µg/ml each) containing recombinant Human Thrombopoietin Protein (TPO) (100 ng/ml). Megakaryocytic differentiation of CD34+ cells was induced and evaluated by May Grunwald-Giemsa staining (Sigma-Aldrich, St. Louis, Mo, USA), cytologic and immunophenotypic analyses, as previously reported [22].

2.3. RNA expression profiles

Following separation of BM-mononuclear cells (MNCs) by Lympholyte-H density gradient separation (Cedarlane, Euroclone, Italy), total RNA was isolated from patient and control samples. Complementary cDNAs used for reverse transcription quantitative (Q)-PCR were synthesized using the QuantiTect Reverse Transcription Kit (Qiagen), according to the manufacturer's instructions.

Messenger RNA (mRNA) expression level of specific transcription factors involved in late megakaryopoiesis (GATA1-2, FLI1, FOG1,

NFE2, RUNX1) was evaluated using a semiquantitative PCR assay (iQ SYBR Green Supermix, Bio-Rad). GAPDH was used as reference gene. Gene expression levels of MNCs from 51 patients with HR-MDS or LBC-AML and 25 patients with LR-MDS were compared to those of 14 normal BM-MNCs. Primers used for each amplification reaction are listed in Table S2. A melting curve (62 °C – 95 °C) was generated at the end of each run to verify the specificity of the reactions. Specific gene expression values were expressed as $2^{-\Delta Ct}$, where ΔCt was Ct (test gene) - Ct (reference gene).

2.4. RUNX1 sequencing

Following separation of BM-mononuclear cells (MNCs) by Lympholyte-H density gradient separation (Cedarlane, Euroclone, Italy), DNA was isolated from all HR-MDS and LBC-AML patients, using a QIAamp DNA Mini Kit (Qiagen srl, Milan, Italy), following the manufacturer's instructions. Detection of RUNX1 mutations was performed by next generation sequencing (NGS) using the Myeloid Solution (MYS) panel by SOPHiA GENETICS. Sequencing was performed on an Illumina MiSeq with 300 bp paired-end reads, yielding an average read depth > of 1000 reads per amplicon. All mutations identified were confirmed in independent experiments.

2.5. Statistical analysis

Patients' and disease characteristics were summarized by means of cross-tabulations for categorical variables or by quintiles for continuous variables. Non parametric tests were used to evaluate differences among groups (Fisher exact test and Wilcoxon test for categorical and continuous variables respectively). Pearson test was used to estimate the correlation between the expression levels of platelet differentiation markers and characteristics of patients. Cox regression models were used to assess the effect of clinical and biologic factors on Overall Survival (OS) and Progression Free Survival (PFS) in univariate and multivariate analysis, after assessment of proportionality of hazards. Confidence intervals were calculated at 95% level and all tests were two-sided, accepting $p \leq 0{\cdot}05$ as indicating a statistically significant difference. All analyses were performed using R (R Foundation for Statistical Computing, Vienna, Austria) system software.

3. Results

3.1. Expression of platelet differentiation markers during normal megakaryocytic differentiation and in MDS/AML samples

Megakaryocyte development was studied using an *in vitro* differentiation model starting from hematopoietic CD34+ progenitor cells isolated from healthy CB. As previously reported by Bianchi et al., [23] and Pang et al., [24] we found that GATA1, NFE2, FLI1 and FOG1 mRNA expression increased at late stages (day 14) of megakaryocytic differentiation. On the other hand, GATA2 expression levels were stable at all stages of differentiation, whereas RUNX1 expression slightly decreased from day zero to day 10 and then increased at day 14, at levels similar to that of the starting point (Fig. 1).

At the time of diagnosis, GATA2 mRNA levels were significantly higher in MDS compared to control samples (fold change = 5.3, $p < 0.0001$), with increasing levels from LR- to HR-MDS/AML (fold change = 2.1, $p = 0.0271$ and fold change = 7, $p < 0.0001$, respectively, Fig. 2). RUNX1 expression was also significantly higher in MDS, as compared to controls (fold change = 2.1, $p = 0.0024$), with no differences between LR- and HR-MDS subtypes. There were no differences in NFE2, FLI1, GATA1 and FOG1 gene expression between controls and HR-MDS/AML samples (data not shown).

Since the RUNX1 gene is a frequently mutated gene in patients with HR-MDS or LBC-AML [25], we screened the RUNX1 gene using the NGS technology. We found pathogenic mutations of RUNX1 in 18 out of 48 HR-MDS/LBC-AML, accounting for 37.5% of patients, but there were no correlations between the presence of RUNX1 mutations and its RNA expression level (Fig. S1).

3.2. Baseline gene expression levels according to patient characteristics

We then looked for associations between mRNA expression patterns (GATA1, GATA2, RUNX1, NFE2, FLI1 and FOG1) and clinical features of patients with HR-MDS/LBC-AML, including age, haemoglobin, white blood cell, BM-blast, platelet and neutrophil counts. As shown in Table S3, we only found weak correlations between expression of megakaryocytic differentiation markers and patient characteristics. Also, there was no correlation between IPSS, IPSS-R, karyotype, mutational status of epigenetic modifiers (DNMT3A, IDH1, IDH2 and TET2), and mRNA expression levels of megakaryocytic differentiation factors, except for a trend towards higher GATA2 mRNA expression levels in high-risk IPSS patients as compared to intermediate-2 risk ($p = 0.0506$).

Looking for biomarkers of response to AZA, we found that GATA1 and FLI1 mRNA baseline expression were predictors of AZA treatment response. In detail, patients responsive to AZA had higher basal GATA1 mRNA expression compared to those with stable or progressive disease after treatment ($p = 0.0111$, Fig. 3A). On the contrary, lower basal FLI1 mRNA levels were predictive of AZA response ($p = 0.0382$, Fig. 3B). This was reflected by the significantly different ratio between GATA1 and FLI1 mRNA baseline expression in responsive, unresponsive patients ($p = 0.0002$) and controls ($p = 0.0091$ and $p = 0.0853$, respectively, Fig. 3C). Bone-marrow samples collected after 4 AZA cycles were available from 20 patients (15 responsive and 5 unresponsive patients). At this time-point, FLI1 mRNA expression increased to normal levels in responders, but not in patients with stable or progressive disease (Fig. S2A). On the other hand, other gene expression levels did not change after 4 AZA cycles (Fig. S2), with no correlations with patients' clinical characteristics (Table S4).

3.3. Influence of expression of differentiation markers on patients' survival

We studied the role of the expression of platelet differentiation markers on the survival outcomes. The univariate analysis showed that GATA2 was the only gene associated with OS (Table 2A) and PFS (Table 2B), with higher GATA2 expression being associated with significantly worse OS (HR: 1.072; $p = 0.0220$) and PFS (HR: 1.054; $p = 0.0546$). The multiple Cox regression analysis adjusted for IPSS, response status, age, hemoglobin, platelet count and hematopoietic cell transplantation (HCT)-specific comorbidity index, confirmed that expression of GATA2 mRNA, considered as continuous variable, was the only independent prognostic factor for OS (HR: 1.095; $p = 0.0049$) and PFS (HR: 1.076; $p = 0.0136$) (Table 3).

4. Discussion

In the present study, we investigated the relevance and prognostic value of the expression of several transcription factors involved in late megakaryocyte differentiation in a cohort of HR-MDS or LBC-AML patients enrolled in the multicenter trial BMT-AZA [17]. The results show that RUNX1 and GATA2 mRNA expression is higher in MDS patients than in healthy donors, probably reflecting the impairment of megakaryocytic differentiation in MDS. RUNX1 and GATA2 transcription factors play essential roles in the maintenance of the hematopoietic stem and progenitor cell compartment during normal hematopoiesis [23,24,26]. Abnormal expression of RUNX1 has been frequently reported in solid tumors and in hematological diseases, in particular AML with normal karyotype, and its overexpression has been associated with poor prognosis [27–29]. RUNX1 expression was up-regulated in our patients, regardless of RUNX1 mutational profile and IPSS.

IPSS high-risk patients displayed significantly higher GATA2 mRNA levels than patients with lower-risk, suggesting that higher GATA2

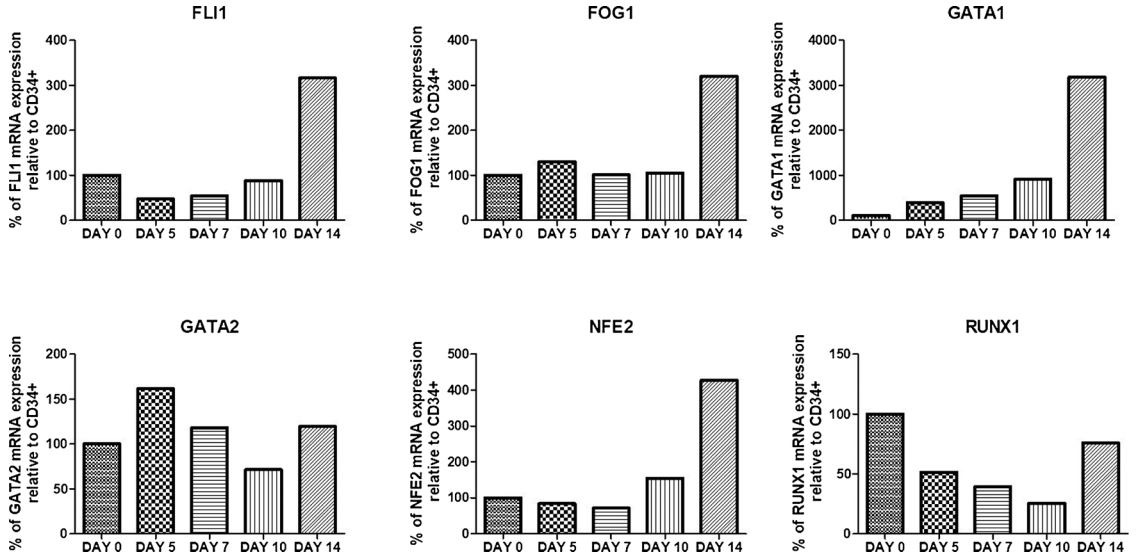

Fig. 1. [37] Y. Wang , P.S. Fan , B. Kahaleh . Association between enhanced type I collagen expression and epigenetic repression of the FLI1 gene in scleroderma fibroblasts Arthritis Rheum. 2006; 54: 2271-2279 mRNA expression of platelet differentiation markers during normal megakaryocytic differentiation. Expression platelet differentiation markers was studied in cord blood CD34+ cells, at time 0 and after differentiation along the megakaryocytic lineage. Specific time points during megakaryocytic differentiation are indicated in the graph.

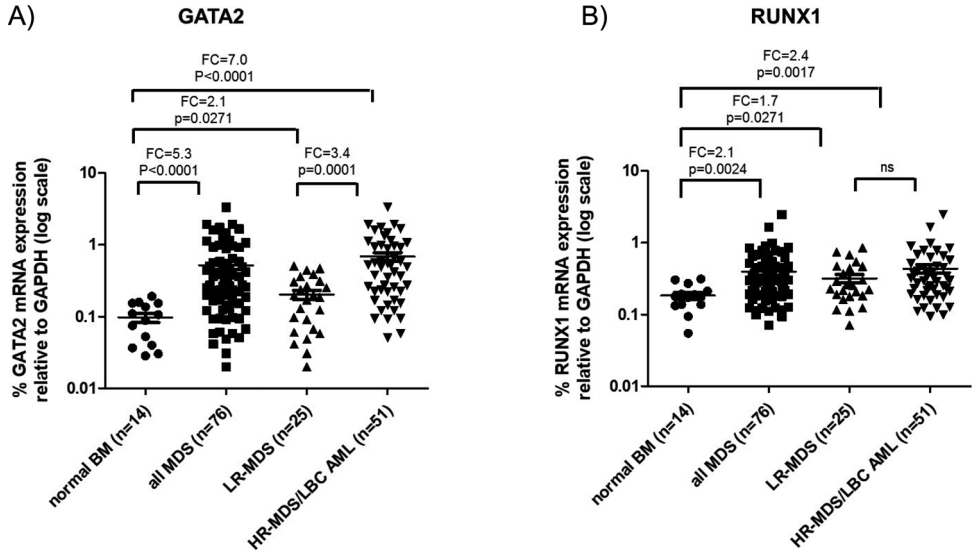

Fig. 2. GATA2 and RUNX1 expression in controls and MDS/AML patient subgroups. A) Expression of GATA2 and B) RUNX1 mRNA in the BM-MNC of patients with LR-MDS and HR-MDS/LBC AML samples collected at the time of initial diagnosis, and in normal BM-MNC. The number of samples studied is indicated in the figure.

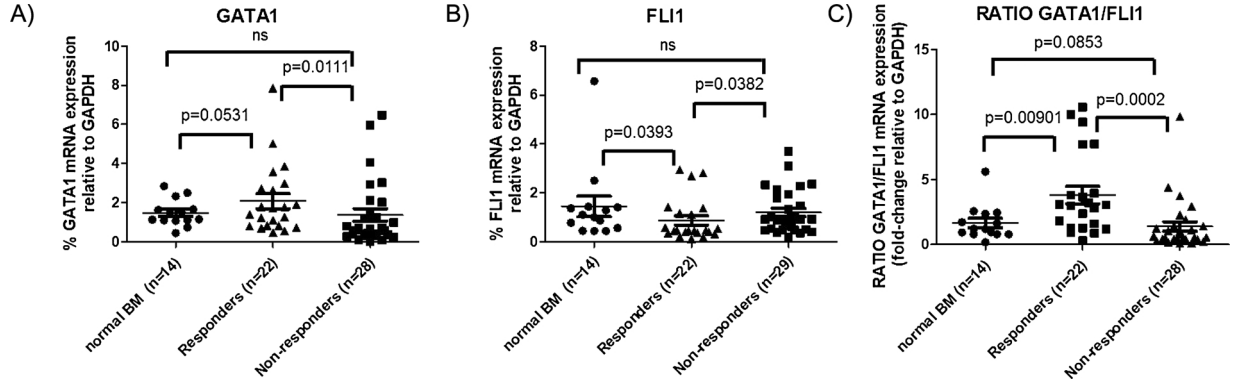

Fig. 3. mRNA expression of platelet differentiation markers in AZA responders and unresponsive patients at baseline and controls. A) Expression of GATA1 and B) FLI1 mRNA was studied in the BM-MNC of responding and unresponsive patients, collected at the time of initial diagnosis and in normal BM-MNC. C) Ratio between GATA1 and FLI1 mRNA expression. The number of samples studied is indicated in the figure.

Table 2A
OS univariate analysis. Significant variations are reported in bold.

	Pr > ChiSq	Hazard Ratio	95% Lower Confidence Limit for Hazard Ratio	95% Upper Confidence Limit for Hazard Ratio
FOG1/GAPDH T0	0.8613	0.998	0.975	1.021
FLI/GAPDH T0	0.5379	0.985	0.940	1.033
GATA1/GAPDH T0	0.3722	0.988	0.962	1.015
GATA2/GAPDH T0	0.0220	1.072	1.010	1.138
RUNX1/GAPDH T0	0.6314	1.021	0.939	1.109
NFE2/GAPDH T0	0.7438	0.997	0.980	1.014

Table 2B
PFS univariate analysis. Significant variations are reported in bold.

	Pr > ChiSq	Hazard Ratio	95% Lower Confidence Limit for Hazard Ratio	95% Upper Confidence Limit for Hazard Ratio
FOG1/GAPDH T0	0.7633	0.997	0.975	1.019
FLI/GAPDH T0	0.3346	0.978	0.934	1.023
GATA1/GAPDH T0	0.3326	0.988	0.963	1.013
GATA2/GAPDH T0	**0.0546**	**1.054**	**0.999**	**1.113**
RUNX1/GAPDH T0	0.8690	0.992	0.903	1.090
NFE2/GAPDH T0	0.5475	0.995	0.977	1.012

Table 3
Multiple Cox regression analysis for survival and PFS. Significant variations are reported in bold.

Parameter		OS				PFS			
		Pr > ChiSq	Hazard Ratio	95% Hazard Ratio Confidence Limits		Pr > ChiSq	Hazard Ratio	95% Hazard Ratio Confidence Limits	
GATA2		**0.0049**	**1.095**	**1.028**	**1.167**	**0.0136**	**1.076**	**1.015**	**1.140**
IPSS	Intermediate (1-2), High (> = 3) *vs* Low(0)	**0.0437**	**2.691**	**1.028**	**7.044**	0.0559	2.312	0.979	5.461
Age as continuous		0.7455	0.990	0.933	1.051	0.6293	1.013	0.960	1.070
Response	CR/PR/HI/mCR *vs* PD/SD	**0.0052**	**0.225**	**0.079**	**0.641**	**0.0024**	**0.234**	**0.091**	**0.598**

expression may reflect the severity of the disease. This finding is in line with data previously reported by Fadilah et al., who showed that GATA2 expression is associated with the severity of dysplasia in MDS patients [30]. Accordingly, in our patient cohort higher GATA2 expression was the only independent factor predicting for significantly worse OS and PFS in univariate and multivariate analysis, although it did not influence treatment response to AZA. Similarly, in patients with AML, treated with conventional chemotherapy, Vicente et al., showed that patients overexpressing GATA2 mRNA had a significantly lower OS and EFS as compared to patients with normal GATA2 mRNA expression [31]. Similar data were reported in 237 pediatric AML patients, of whom 65% had overexpression of GATA2 compared to healthy donors, and this was an independent poor prognostic factor for OS, event-free survival (EFS) and disease-free survival (DFS) [32].

As to response to AZA and expression of transcription factors, we found that GATA1 and FLI1 mRNA levels correlated with AZA response. GATA1 is an essential transcription factor driving the development of multiple hematopoietic lineages, including erythroid and megakaryocytic [33], known to interact with several transcription factors such as FOG1 and FLI1 [24]. FLI1 is an E26 transformation-specific (ETS) transcription factor that is important for GATA1/FOG1 synergy [34], which was shown to bind to the proximal promoter of these genes *in vivo*. FLI1 gene–disrupted mice either have abnormal megakaryocytes with thrombocytopenia [35], or fail to develop recognizable megakaryocytes, depending on the size of the FLI1 gene deletion. In our *in vitro* model mimicking the process of normal megakaryocytic differentiation, GATA1 and FLI1 mRNA expression increased during normal megakaryocytic differentiation. On the contrary, we observed high GATA1 and low FLI1 mRNA expression in our patient cohort, indicating an imbalance between these genes. This deregulation was associated to improved AZA treatment response in our patient cohort. On the contrary, in AML patients treated with intensive chemotherapy, GATA-1 expression had a negative impact on the probability of achieving complete remission [36]. The reversal of FLI1 levels to normal values after 4 cycles of AZA in responding patients may reflect the restoration of the normal megakaryocytic differentiation process. To our knowledge, the tight association of GATA1 and FLI1 mRNA deregulation has not been previously reported in HR-MDS patients. However, FLI1 upregulation following hypomethylating treatment has been previously shown in fibroblast cultures from skin biopsies of patients with systemic sclerosis (SSc) [37]. In this line, it would be interesting to evaluate the contribution of epigenetic regulation of FLI1 in patients with HR-MDS treated with azacitidine.

In conclusion, we report that high GATA2 expression is a poor prognostic marker for OS and PFS in patients with HR-MDS and LBC-AML treated with AZA. Our data also indicate that GATA1 and FLI1 mRNA expression may predict response to AZA. Further studies are warranted to better assess the role of these factors as markers of response to hypomethylating treatment.

Authorship contributions

GF: designed the research study, performed the research, analysed the data, wrote the paper; EF: performed the research, analysed the data, wrote the paper; MC, LF, CF, EC, CG, LL, MP enrolled patients; EV, MS, IZ: performed the research; AP: analysed the data; FLC: designed the research study, enrolled patients; MTV: designed the research study, enrolled patients and wrote the paper. All authors critically reviewed and approved the final version of the manuscript.

Declaration of Competing Interest

Authors declared no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.leukres.2019.106191.

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