Published in final edited form as:

Leuk Res. 2016 May ; 44: 1–7. doi:10.1016/j.leukres.2016.02.009.

Down-regulation of EZH2 expression in myelodysplastic syndromes

Monica Cabrero^{a,*}, Yue Wei^{a,*}, Hui Yang^a, Irene Ganan-Gomez^a, Zach Bohannon^a, Simona Colla^a, Matteo Marchesini^a, Guillermo Montalban Bravo^a, Koichi Takahashi^a, Carlos Bueso-Ramos^b, and Guillermo Garcia-Manero^a

^aDepartments of Leukemia, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, United States

^bDepartments of Hematopathology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, United States

Abstract

EZH2 genetic mutations are common in myelodysplastic syndrome (MDS), which implies that this gene has a pathophysiological role in the disease. To further characterize molecular alterations of EZH2, and their potential prognostic impact in MDS, we assessed EZH2 RNA expression in primary bone marrow CD34+ cells from 78 patients.

We found that 47% of patients have reduced EZH2 expression compared to normal controls. Further analyses revealed that EZH2 is significantly underexpressed in patients bearing chromosome 7 or 7q deletions (7-alt) when compared to controls, diploid patients, and patients with other cytogenetic alterations ($p < 0.05$). In survival analysis, we found a non-significant trend toward overall survival (OS) being better among patients with EZH2 underexpression (median OS 55 vs. 36 months; $p = 0.71$). Importantly, this trend became significant when the analysis was restricted to the subset of cases without alterations in chromosome 7 (62 vs. 36 months; $p = 0.033$).

Furthermore, our previous work has identified a spectrum of innate immune genes in MDS CD34+ cells that are deregulated via abnormal promoter histone methylation. Because *EZH2* is a key regulator of histone methylation, we assessed the relationship between deregulation of these genes and *EZH2* underexpression. We observed that the mRNA levels of 11 immune genes were higher in the *EZH2* under-expression group and that immune gene expression was significantly higher in patients with concomitant *EZH2* underexpression and *KDM6B* (also known as *JMJD3*, an H3K27 demethylase) overexpression.

Taken together, these data indicate that EZH2 underexpression may have unique impact on the molecular pathogenesis and prognosis in MDS and be an important marker for patients without chromosome 7 alteration.

*Corresponding authors. ggarciam@mdanderson.org (M. Cabrero).

Conflict of interest

The authors declare no conflicts of interest.

Keywords

Myelodysplastic syndromes; EZH2; Histone methylation; Epigenetics

1. Introduction

Myelodysplastic syndromes (MDS) are a complex group of malignant myeloid disorders arising from bone marrow (BM) hematopoietic stem and progenitor cells [1]. The molecular patho-physiology of MDS was largely unknown until recent advances in next-generation sequencing (NGS), which have allowed for the identification of a spectrum of recurrent mutations in MDS [2]. Importantly, the biological functions of most of these frequently mutated genes in MDS are involved in DNA/histone modification and RNA splicing, which emphasizes the pathophysiological roles of epigenetic and splicing regulatory machineries in MDS [3].

Among the recurrently mutated genes in MDS, the chromosome 7q gene *Enhancer of Zeste homolog 2* (*EZH2*) encodes a key member of the polycomb repressive complex 2 (PRC2) and is responsible for the regulation of the methylation of lysine 27 on histone 3 (H3K27) as well as the associated transcriptional repression [4]. Importantly, in contrast to the biological gain-of-function properties of *EZH2* mutations that have been identified in epithelial malignancies and lymphoma [5,6], mutations of *EZH2* in MDS and MDS/myeloproliferative neoplasia overlap syndromes are generally loss-of-function mutations and appear to be associated with worse outcomes [7,8]. However, in contrast to the extensively investigated mutational landscape of *EZH2* in MDS, the status of *EZH2* gene expression in MDS has not yet been widely studied.

Most *EZH2* mutations that have been identified in myeloid neoplasms affect the catalytic SET domain of the protein and disrupt its enzymatic activity [7]. Importantly, another PRC2 complex gene, *ASXL1*, is also recurrently mutated in MDS, and its loss accelerates the latency of MDS/myeloproliferative disease in mice with an *N-RAS* mutation background [9]. Taken together, these results suggest that *EZH2* and associated epigenetic regulators are important for hematopoiesis, and their defects contribute to MDS pathogenesis. This concept prompted us to evaluate the status of *EZH2* gene expression in the bone marrow of patients with MDS and whether its abnormality is part of the epigenetic dysregulation in MDS.

2. Materials and methods

2.1. Isolation of bone marrow mononuclear cells and CD34+ cells

MDS bone marrow specimens were freshly obtained from patients referred to the Department of Leukemia at The University of Texas MD Anderson Cancer Center following Institutional guidelines. Written informed consent was obtained from all donors, and diagnosis was confirmed by a dedicated hematopathologist as soon as the samples were obtained. Bone marrows from healthy individuals were obtained from AllCells (Emeryville, CA). Bone marrow mononuclear cells were isolated using a standard Ficoll gradient

approach. Isolation of CD34+ cells was performed using a MicroBead Kit (Miltenyi, Bergisch Gladbach, Germany) according to the manufacturer's protocol.

2.2. Capture deep sequencing of genomic DNA

Illumina paired-end libraries were generated according to the manufacturer's protocol (Illumina San Diego, CA). Three micrograms of pre-capture library DNA were used for each capture reaction. NimbleGen SeqCap EZ Hybridization and Wash Kits (Roche, Basel, Switzerland) were used following the manufacturer's protocol. We then applied 14–17 cycles of PCR amplification to the samples after hybridization, with the total number being based on yield. After that, captured libraries were quantified by Picogreen and sequenced on the Illumina HiSeq 2000 as 100-bp paired-end reads, following the manufacturer's protocols. Sequencing data were stored in the fastq format, which contained reads and quality information. Fastq files were then mapped and aligned to human reference hg18 using Burroughs-Wheeler alignment (BWA) [10]. To refine the alignment and adjust the quality score provided by sequencer, GATK was used to realign reads and recalibrate the quality score [11]. SNPs were called using AtlasSNP2 (http://www.hgsc.bcm.tmc.edu/cascade-tech-software_atlas_snp-ti.hgsc). The posterior probability was set at 0.9, and the minimum number of variant reads at 3. To identify rare SNPs, candidate SNPs were filtered against dbSNP130, a 1000-genome database, and the Human Genome Sequencing Center (Baylor College of Medicine, Houston, TX) internal database. Indels were called using AtlasIndel (http://www.hgsc.bcm.tmc.edu/cascade-tech-software_atlas2_snp_indel_calling_pipeline-ti.hgsc).

2.3. Real-time RT-PCR

Total cellular RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. For each sample, 200 ng of total RNA were used for reverse transcription (RT) reactions using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) following the manufacturer's protocol. For real-time PCR, primers and probes were purchased from Applied Biosystems and analyzed using an Applied Biosystems Prism 7500 sequence detection system. PCR reactions were performed using 20× Assays-On-Demand gene expression assay mix and TaqMan universal PCR master mix according to the manufacturers' protocols. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Quantitative values were obtained from the cycle number (C_T value) at which an increase in fluorescent signal associated with an exponential accumulation of PCR products was detected. The amount of target gene was normalized to the endogenous reference GAPDH to obtain the relative threshold cycle ($-C_T$) and then related to the C_T of the control to obtain the relative expression level (2^{-C_T}) of the target gene.

2.4. Statistics

All statistical analyses were performed using SPSS v.22 (SPSS, Chicago, IL, USA).

Continuous variables, including gene expression levels, were compared by Mann-Whitney U or ANOVA tests, depending on if they were bivariate or not. When testing gene expression level as a categorical variable defined as over- or underexpressed, we used the chi-square

test. A gene was considered to be underexpressed when we observed expression levels less than 50% of the expression in the controls.

Overall survival (OS) was defined as time from sample collection to death. The Kaplan–Meier method was used to calculate both PFS and OS, and the log-rank test was implemented to analyze the impact of each variable on survival in univariate analysis. Multivariate analysis of variables with significant impact on survival was performed using Cox regression.

3. Results

3.1. Patient cohort

Bone marrow specimens were collected from 78 patients with MDS. The diagnoses of these patients were: refractory anemia with or without ringed sideroblasts (RA/RARS, N = 12), refractory cytopenia with multilineage dysplasia (RCMD/RCMD-RS, N = 14), refractory anemia with excess blasts (RAEB, N = 32), refractory anemia with excess blasts in transformation/acute myeloid leukemia (RAEB-T/AML, N = 3), chronic myelomonocytic leukemia (CMML, N = 12), MDS-unclassified (MDS-U, N = 4), and 5q syndrome (N = 1).

Fifty-seven of these patients were newly diagnosed with MDS, and 61 of them had not received hypomethylating agents (HMA) before the sample collection. The remaining 17 patients had already been diagnosed and treated with HMA before the sample was collected. Other major clinical characteristics of patients are summarized in Table 1.

Three patients carried *EZH2* mutations, including missense (nt148511133), nonsense (nt148524257), and splicing mutations (nt148524257). All 3 of these patients with genetic alterations of *EZH2* were diagnosed as having CMML.

3.2. RNA expression of *EZH2* in the whole patient cohort and relation with clinical variables

We separated the CD3-/CD34+ cell population from patient bone marrow specimens and studied *EZH2* mRNA expression in this hematopoietic stem/progenitor cell compartment. Quantitative RT-PCR assays were performed in CD34+ cells from both MDS patients (n = 78) and healthy individuals (N = 9). When we compared MDS patients to normal controls, low *EZH2* expression, which was considered anything less than 50% of that of normal individuals, was present in 37 patients (47%). The mean relative *EZH2* mRNA levels were 1.0008 (standard deviation [SD]: 0.594) in controls and 0.9227 (SD: 1.196) in patients, and there was no significant difference between patients and controls ($p = 0.159$).

We also analyzed the influence of previous treatment on *EZH2* expression. The 17 patients who had already received HMA were treated for a median of 6 months (range: 2–25) before sample collection. There were no significant differences in *EZH2* mRNA expression between newly diagnosed patients and patients who had been previously treated ($p = 0.35$) (Fig. 1a).

Furthermore, there were no significant differences between the various diagnoses ($p = 0.49$) (Fig. 1b).

Regarding to the relation between *EZH2* expression and International Prognostic Scoring System (IPSS) [12], there were no significant differences between low (mean: 1.13, SD: 1.648), intermediate (mean: 0.97, SD: 1.07), and high (mean: 0.307, SD: 0.294) IPSS risk groups ($p = 0.115$) (Fig. 1c). Although there is a trend toward lower *EZH2* expression among higher-risk patients, this could be due to the close relation between chromosome 7 alterations and high-risk IPSS status.

3.3. *EZH2* RNA expression and patient karyotype

We subsequently analyzed *EZH2* expression in patients with various karyotypes. Among all karyotypes, *EZH2* was significantly underexpressed in patients with chromosome 7 alterations (7-alt), including 7q- or 7 monosomy. The median level of *EZH2* mRNA expression in the subset of patients with 7q- or 7 monosomy was 40% compared to the controls (0.398 vs. 1.0008; $p = 0.006$) (Fig. 1c). Comparing 7-alt with other cytogenetic abnormalities (including 5q del [n = 1], +9 [n = 1], +16 [n = 1], +8 [n = 1], 11q del [n = 1], -Y [n = 1], t(7; 20) [n = 1], inv 3 [n = 2], and 20q del [n = 2]) and diploid samples, we also found that 7-alt was the only cytogenetic group associated with lower *EZH2* expression ($p = 0.041$) (Fig. 1d). When we assessed the correlation between 7-alt and *EZH2* expression, 32.4% of the patients with *EZH2* underexpression had 7-alt, whereas only 9.8% of patients with normal *EZH2* expression or overexpression had 7-alt ($p = 0.013$).

3.4. *EZH2* RNA expression and clinical outcome: survival and response to hypomethylating agent treatment

To investigate the potential prognostic impact of *EZH2* expression in MDS, we analyzed the association between *EZH2* expression and survival in MDS. In a univariate OS model, we found that patients with a lower *EZH2* expression had better OS, although this was not statistically significant (median OS: 55 vs. 36 months; $p = 0.71$), which is discordant with previous published data on *EZH2* mutations [2]. To better characterize this effect, and given the strong correlation between *EZH2* underexpression and 7-alt, which could be a confounding factor in the prognostic impact of *EZH2*, we further analyzed OS in those patients without alterations in chromosome 7, and we found that in this set of patients, *EZH2* underexpression is significantly associated with a better OS (median OS: 62 vs. 36 months; $p = 0.033$). When we compare both *EZH2* underexpressing and non-underexpressing patients and those with 7-alt, we found that these 3 subgroups of patients had significantly different outcomes: median OS was 13 months for 7-alt (n = 14) versus 36 for *EZH2* normal expression or overexpression (n = 27) and 62 months for *EZH2* underexpression (n = 19); $p < 0.001$ (Fig. 2).

We also performed a multivariate OS analysis by Cox regression both in the whole cohort and in non-7-alt patients. For the whole group, chromosome 7 alterations (hazard ratio [HR]: 4.83 [95% confidence interval (CI): 2.1–10.8]; $p < 0.001$) and white blood cell count (WBC) (HR: 1.026 [95% CI: 1.008–1.044]; $p = 0.004$) showed significant impact on OS. Then we performed the multivariate analysis on non-7-alt patients and found that *EZH2*

underexpression (HR: 0.3 [95% CI: 0.1–0.89]; $p = 0.03$) was an independent prognostic factor among these patients. Also WBC (HR 1.04 [95% CI: 1.019–1.063]; $p < 0.001$) and high-risk IPSS status (HR: 15.4 [95% CI: 2.3–100.6]; $p = 0.004$) (Table 2).

Although we did not observe significant differences in *EZH2* expression between treatment groups (Fig. 1a), to address the possible bias of including patients that had received HMA treatment before sample collection, we repeated the survival analysis for only untreated patients ($n = 60$). We analyzed the impact of both *EZH2* underexpressing or non-underexpressing patients and those with 7-alt and did not observe a poor prognosis associated with lower *EZH2* expression in this subset of patients (median OS: 14 months for 7-alt vs. 78 months for *EZH2* normal or overexpression vs. a median that has not yet been reached for *EZH2* underexpression; $p = 0.001$).

To better understand the interaction between *EZH2* and treatment status, we selected those patients untreated at the time of sample collection and reviewed treatment response records and correlated them with *EZH2* expression. After sample collection, 33 patients received later treatment with HMA, and the overall response rate (ORR) was 63% ($n = 21/33$) according to IWG criteria [13]. We observed that the ORR was 33% among 7-alt patients, 75% in the *EZH2* underexpression group, and 71% in the *EZH2* normal or overexpression group ($p = 0.178$), which indicates that *EZH2* expression is not a predictor of a better or worse response to treatment.

3.5. *EZH2* RNA expression and innate immune gene deregulation

Our group previously found that the overexpression of a set of innate immune genes contributes to MDS pathogenesis and is related to deregulation of histone methylation. [14, 15] Because *EZH2* is a key regulator of histone methylation, we assessed the relationship between *EZH2* underexpression and the deregulation of key downstream genes. To exclude the effects of cytogenetic defects and *EZH2* mutations, we studied a subset of 29 patients with normal karyotypes and wildtype *EZH2*. Among them, 14 (48%) had *EZH2* underexpression. mRNA expression of all 11 genes examined was significantly higher in this subset of patients when compared to controls. We then compared mRNA expression of 11 innate immune genes known to be overexpressed in MDS between the patients with *EZH2* underexpression and others. We observed that mRNA levels of all 11 immune genes were higher in the *EZH2* under-expression group and statistically significant for the genes *KDM6B* (also known as JMJD3), *IL-8*, *IL-1B*, *TLR-2*, and *S100-A9* (Table 3).

Because *EZH2* functions as a histone H3K27 methyltransferase and *KDM6B* is a H3K27 demethylase that is known to also regulate key *EZH2* targets, we hypothesized that *EZH2* downregulation and *KDM6B* overexpression could have a potential combinatorial effect on target gene expression. We divided patients into subgroups based on the patterns of expression of *EZH2* and *KDM6B* and observed that patients with concomitant *EZH2* underexpression and *KDM6B* overexpression had significantly higher expression of innate immune genes compared to patients who only had dysregulation in one of these genes and those in whom both genes had normal expression (Fig. 3b).

4. Discussion

In this study, we found that although there is no significant difference in *EZH2* mRNA expression between MDS patient and healthy control bone marrow progenitor cells, there is a very significant downregulation of *EZH2* expression in patients with 7-alt. The genomic localization of the *EZH2* gene at chr7q36.1 strongly suggests that haploinsufficiency is the underlying mechanism of this phenomenon. Indeed, haploinsufficiency of multiple genes have been recently identified as contributors to the pathogenesis of MDS. For instance, downregulation of chromosome 5q genes *RPS14* [16], *EGR1*, and *APC* [17] are involved in the pathological progression of MDS with 5q deletion. Haploinsufficiency of the important hematopoietic transcription factor *GATA2* also plays an important role in MDS and AML [18]. *MYBL2* [19], a cell cycle regulatory tumor suppressor gene located in a commonly deleted region of chromosome 20q, has also been shown to be downregulated via the same mechanism in MDS and myeloproliferative neoplasms. In this scenario, our findings together with previous studies of *EZH2* in MDS by Nikoloski et al. [7] provide evidence that *EZH2* haploinsufficiency is associated with MDS.

Another striking finding of this study is the potential impact of *EZH2* underexpression on OS among those patients who do not have a poor prognostic marker, such as chromosome 7 alteration. *EZH2* mutations are thought to be related to poor prognosis. For instance, Bejar et al. demonstrated that *EZH2* mutations are predictors of poor OS [2]. Compared to these studies, our work has two major differences. Previous studies [2,20] focused mainly on genetic alterations of *EZH2* in MDS, whereas we investigate RNA expression of the gene in patients. Similarly, those previous conclusions related to the adverse effect of *EZH2* mutation on patient survival were mostly based on observations of patients with various cytogenetic features (such as chromosome 7 alteration). Therefore, although the differences in the patient cohorts could explain our differing results, this could also mean that the clinical impact of *EZH2* genetic alteration could depend on concomitant events, such as alteration in chromosome 7. Furthermore, these reports taken together reflect the complex biopathological role of *EZH2* and its alterations in the pathogenesis and progression of MDS. Indeed, recent mouse studies have related *EZH2* deletion with resistance to the progression to leukemia [21], which was consistent with the observations that few de novo AML patients have *EZH2* mutations [22,23].

More recent work has begun to address *EZH2* expression and its alteration in addition to the more well-known role of *EZH2* mutation in MDS. Karopongse et al. showed that *EZH2* expression is lower in CD34+ MDS cells and that this downregulation was related to inflammatory and immune responses [24]. Mechanistically, *SRSF2* mutation was recently shown to lead to the mis-splicing of *EZH2* RNA and consequent nonsense-mediated decay (NMD) of the transcript [25]. However, *SRSF2* mutation is consistently associated with adverse outcomes among MDS patients, which seems to be contradictory to our observation that *EZH2* downregulation is associated with better survival in a subset of patients. One explanation is that the spliceosome gene mutations, including *SRSF2* mutation, are associated with global alterations of gene expression and splicing, which potentially include other disease-relevant genes in MDS. Therefore, further investigation is necessary.

Our results in the diploid and wildtype-*EZH2* subsets have also demonstrated that, without abnormal karyotype or *EZH2* mutation, downregulation of *EZH2* alone is associated with the overexpression of a series of innate immune and inflammatory genes. These genes are known to be deregulated in MDS [14], and one gene of particular interest is *KDM6B*, which encodes a demethylase of H3K27. Furthermore, mRNA expression of a number of innate immune/inflammatory genes is also significantly higher in *EZH2*-underexpressing patients, and promoter H3K27 methylation of some of these genes, such as *IL-8*, has been previously shown to be regulated by *KDM6B* [14]. Although *EZH2* encodes a component of an H3K27 methyltransferase complex, these results suggest that *EZH2* downregulation and *KDM6B* upregulation may simultaneously contribute to dysregulation of H3K27 methylation at gene promoters and thus promote the abnormal expression of disease-relevant genes. We demonstrated this by analyzing immune gene expression in patients with both *EZH2* and *KDM6B* dysregulation and found that this dual dysregulation results in a higher expression of innate immune genes than the alteration of *EZH2* or *KDM6B* alone. Our analyses of the association between *EZH2* downregulation and the overexpression of innate immune genes in non-diploid patients in our cohort found that, among all the candidate genes analyzed, only *IL-8*, *IL1B*, *TLR2* and *S100A9* were significantly higher in *EZH2*-low patients. These observations suggest more complicated molecular mechanisms in non-diploid MDS. Detailed molecular and functional characterizations of *EZH2*, *KDM6B*, and related epigenetic regulators of innate immune genes in MDS pathogenesis are definitely required. We have not studied associative effect between *EZH2* downregulation and other mutations in the outcome of these patients.

The small sample size in our study makes it yet difficult to draw relevant conclusions, and it is clearly indicating that, besides genetic alterations, the regulation of *EZH2* expression in MDS pathogenesis are exceedingly complex, and future studies are needed to fully understand the signaling network linking immune signaling, gene expression, and histone methylation status with MDS.

Acknowledgements

MC is funded by the Fundacion Alfonso Martin-Escudero. GGM is supported by The University of Texas MD Anderson Cancer Center Support Grant P30CA016672. GGM is also supported by the Edward P. Evans Foundation, the Fundacion Ramon Areces, grant RP100202 from the Cancer Prevention & Research Institute of Texas (CPRIT), and by generous philanthropic contributions to MD Anderson's MDS/AML Moon Shot Program. GGM partially supported by the Dr. Kenneth B. McCredie Chair in Clinical Leukemia Research endowment.

References

- [1]. Tefferi A, Vardiman JW, Myelodysplastic syndromes, N. Engl. J. Med 361 (2009) 1872–1885. [PubMed: 19890130]
- [2]. Bejar R, Stevenson K, Abdel-Wahab O, Galili N, Nilsson B, Garcia-Manero G, et al., Clinical effect of point mutations in myelodysplastic syndromes, N. Engl. J. Med 364 (2011) 2496–2506. [PubMed: 21714648]
- [3]. Yoshida K, Toki T, Okuno Y, Kanezaki R, Shiraishi Y, Sato-Otsubo A, et al., The landscape of somatic mutations in Down syndrome-related myeloid disorders, Nat. Genet 45 (2013) 1293–1299. [PubMed: 24056718]
- [4]. Vire E, Brenner C, Deplus R, Blanchon L, Fraga M, Didelot C, et al., The Polycomb group protein *EZH2* directly controls DNA methylation, Nature 439 (2006) 871–874. [PubMed: 16357870]

- [5]. Yu J, Rhodes DR, Tomlins SA, Cao X, Chen G, Mehra R, et al., A polycomb repression signature in metastatic prostate cancer predicts cancer outcome, *Cancer Res.* 67 (2007) 10657–10663. [PubMed: 18006806]
- [6]. Morin RD, Johnson NA, Severson TM, Mungall AJ, An J, Goya R, et al., Somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin, *Nat. Genet* 42 (2010) 181–185. [PubMed: 20081860]
- [7]. Nikoloski G, Langemeijer SM, Kuiper RP, Knops R, Massop M, Tonnissen ER, et al., Somatic mutations of the histone methyltransferase gene EZH2 in myelodysplastic syndromes, *Nat. Genet* 42 (2010) 665–667. [PubMed: 20601954]
- [8]. Abdel-Wahab O, Pardanani A, Patel J, Wadleigh M, Lasho T, Heguy A, et al., Concomitant analysis of EZH2 and ASXL1 mutations in myelofibrosis, chronic myelomonocytic leukemia and blast-phase myeloproliferative neoplasms, *Leukemia* 25 (2011) 1200–1202. [PubMed: 21455215]
- [9]. Inoue D, Kitauro J, Togami K, Nishimura K, Enomoto Y, Uchida T, et al., Myelodysplastic syndromes are induced by histone methylation-altering ASXL1 mutations, *J. Clin. Invest* 123 (2013) 4627–4640. [PubMed: 24216483]
- [10]. Li H, Durbin R, Fast and accurate short read alignment with Burrows-Wheeler transform, *Bioinformatics* 25 (2009) 1754–1760. [PubMed: 19451168]
- [11]. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernysky A, et al., The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data, *Genome Res.* 20 (2010) 1297–1303. [PubMed: 20644199]
- [12]. Greenberg P, Cox C, LeBeau MM, Fenaux P, Morel P, Sanz G, et al., International scoring system for evaluating prognosis in myelodysplastic syndromes, *Blood* 89 (1997) 2079. [PubMed: 9058730]
- [13]. Cheson BD, Greenberg PL, Bennett JM, Lowenberg B, Wijermans PW, Nimer SD, et al., Clinical application and proposal for modification of the International Working Group (IWG) response criteria in myelodysplasia, *Blood* 108 (2006) 419–425. [PubMed: 16609072]
- [14]. Wei Y, Chen R, Dimicoli S, Bueso-Ramos C, Neuberg D, Pierce S, et al., Global H3K4me3 genome mapping reveals alterations of innate immunity signaling and overexpression of JMJD3 in human myelodysplastic syndrome CD34+ cells, *Leukemia* 27 (11) (2013) 2177–2186. [PubMed: 23538751]
- [15]. Chen X, Eksioglu EA, Zhou J, Zhang L, Djeu J, Fortenbery N, et al., Induction of myelodysplasia by myeloid-derived suppressor cells, *J. Clin. Invest* 123 (2013) 4595–4611. [PubMed: 24216507]
- [16]. Dutt S, Narla A, Lin K, Mullally A, Abayasekara N, Megerdichian C, et al., Haploinsufficiency for ribosomal protein genes causes selective activation of p53 in human erythroid progenitor cells, *Blood* 117 (2011) 2567–2576. [PubMed: 21068437]
- [17]. Stoddart A, Fernald AA, Wang J, Davis EM, Karrison T, Anastasi J, et al., Haploinsufficiency of del(5q) genes Egr1 and Apc, cooperate with Tp53 loss to induce acute myeloid leukemia in mice, *Blood* 123 (2014) 1069–1078. [PubMed: 24381225]
- [18]. Spinner MA, Sanchez LA, Hsu AP, Shaw PA, Zerbe CS, Calvo KR, et al., GATA2 deficiency: a protean disorder of hematopoiesis, lymphatics, and immunity, *Blood* 123 (2014) 809–821. [PubMed: 24227816]
- [19]. Heinrichs S, Conover LF, Bueso-Ramos CE, Kilpivaara O, Stevenson K, Neuberg D, et al., MYBL2 is a sub-haploinsufficient tumor suppressor gene in myeloid malignancy, *eLife* 2 (2013) E00825. [PubMed: 23878725]
- [20]. Ernst T, Chase AJ, Score J, Hidalgo-Curtis CE, Bryant C, Jones AV, et al., Inactivating mutations of the histone methyltransferase gene EZH2 in myeloid disorders, *Nat. Genet* 42 (2010) 72. [PubMed: 20023659]
- [21]. Sashida G, Harada H, Matsui H, Oshima M, Yui M, Harada Y, et al., EZH2 loss promotes development of myelodysplastic syndrome but attenuates its predisposition to leukaemic transformation, *Nat. Commun* 5 (2014) 4177. [PubMed: 24953053]
- [22]. Xu F, Li X, Wu LY, Zhang QX, Yang R, Yang YJ, et al., Overexpression of the EZH2, RING1 and BMI1 genes is common in myelodysplastic syndromes: relation to adverse epigenetic alteration and poor prognostic scoring, *Ann. Hematol* 90 (2011) 643–653. [PubMed: 21125401]

- [23]. Papaemmanuil E, Gerstung M, Malcovati L, Tauro S, Gundem G, Van Loo P, et al., Clinical and biological implications of driver mutations in myelodysplastic syndromes, *Blood* 122 (2013) 3616–3627 (QUIZ 99). [PubMed: 24030381]
- [24]. Karopongse E, Yeung C, Byon J, Ramakrishnan A, Holman ZJ, Jiang PY, et al., The KDM2B-let-7b –EZH2 axis in myelodysplastic syndromes as a target for combined epigenetic therapy, *PLoS One* 9 (2014) E107817. [PubMed: 25225797]
- [25]. Kim E, Ilagan JO, Liang Y, Daubner GM, Lee SC, Ramakrishnan A, et al., SRSF2 mutations contribute to myelodysplasia by mutant-Specific effects on exon recognition, *Cancer Cell* 27 (2015) 617–630. [PubMed: 25965569]

Fig. 1. Analysis of EZH2 mRNA expression in bone marrow CD34+ cells of MDS patients: (a) by treatment status at the time of sample collection; (b) by diagnosis; (c) by IPSS score and (d) by karyotype.

Fig. 2.
Analysis of EZH2 mRNA expression and overall patient survival.

Fig. 3.
Analysis of innate immune genes *IL8*, *IL1b*, *S100A9* and *TLR2* according to different patterns of expression of EZH2 and KDM6B.

Table 1

Clinical characteristics of patients.

Characteristics (N = 78)	No. of patients	%
Age (years)		
Median	67	
Range	33–91	
Sex		
Male	42	54
Female	36	46
BM blast (%)		
Median	5	
Range	0–26	
WBC ($\times 10^9/L$)		
Median	3.4	
Range	1–83	
Hemoglobin (g/dL)		
Median	9.7	
Range	7–15	
Platelet ($\times 10^9/L$)		
Median	65	
Range	4–469	
Neutrophil ($\times 10^9/L$)		
Median	50	
Range	5–86	
Diagnosis		
RA/RARS/RCMD/RCMD-RS	26	33
RAEB/RAEBT	35	45
CMML	12	15
MDS-U	4	5
5q-	1	1
IPSS		
High	10	13
Intermediate-2	21	27
Intermediate-1	25	32
Low	20	26
N/A	2	3
Cytogenetics		
Diploid	50	64
7 monosomy or 7q-	16	21
others	12	15

BM: bone marrow, WBC: white blood cell count, RA: refractory anemia, RARS: refractory anemia with ring sideroblasts, RCMD: refractory cytopenia with multilineage dysplasia, RCMD-RS: refractory cytopenia with multilineage dysplasia and ring sideroblasts, RAEB: refractory

anemia with excess blasts, RAEBT: refractory anemia with excess blasts in transformation, CMML: chronic myelomonocytic leukemia, MDS-U: MDS-unclassified, IPSS: International Prognostic Scoring System.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 2

Multivariate analysis.

	HR	95% CI	<i>p</i> value
Whole cohort			
WBC	1.026	1.008 – 1.044	0.004
Chromosome 7 alterations (n = 16)	4.83	2.1 – 10.8	<0.001
Non 7-alt patients			
<50% EZH2 expression (n = 43)	0.3	0.1 – 0.89	0.003
WBC	1.04	1.019 – 1.63	<0.001
High-risk IPSS (vs. interm/low; n = 10)	15.4	2.3–100.6	0.004

Table 3

Innate immune gene expression in EZH under-expression and non-underexpression diploid patients.

Gene	EZH2 under expressed patients Mean mRNA expression (fold to controls)	EZH2 non underexpressed patients Mean mRNA expression (fold to controls)	<i>p</i> -value
EZH2	0.2063 ± 0.05488	1.838 ± 0.4420	0.002
KDM6B	6.421 ± 2.251	2.119 ± 0.3976	0.01
FPR2	27.50 ± 9.318	15.71 ± 4.288	0.22
IL-8	17.06 ± 5.656	5.212 ± 1.127	0.04
IL1B	16.16 ± 4.372	4.987 ± 0.9131	0.01
TNFA	19.08 ± 11.99	5.364 ± 2.305	0.22
TLR1	16.00 ± 7.163	4.837 ± 0.8559	0.08
TLR2	70.66 ± 20.46	24.42 ± 5.636	0.03
TLR4	8.230 ± 3.772	3.427 ± 1.040	0.19
TLR6	61.08 ± 39.52	3.860 ± 1.430	0.15
S100A8	19.34 ± 4.357	9.817 ± 3.545	0.09
S100A9	15.78 ± 3.053	6.595 ± 2.180	0.02