ARTICLE

Immunohistochemical loss of enhancer of Zeste Homolog 2 (EZH2) protein expression correlates with *EZH2* alterations and portends a worse outcome in myelodysplastic syndromes

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EZH2 coding mutation (*EZH2*^{MUT}), resulting in loss-of-function, is an independent predictor of overall survival in MDS. *EZH2* function can be altered by other mechanisms including copy number changes, and mutations in other genes and non-coding regions of *EZH2*. Assessment of EZH2 protein can identify alterations of EZH2 function missed by mutation assessment alone. Precise evaluation of EZH2 function and gene-protein correlation in clinical MDS cohorts is important in the context of upcoming targeted therapies aimed to restore EZH2 function. In this study, we evaluated the clinicopathologic characteristics of newly diagnosed MDS patients with *EZH2*^{MUT} and correlated the findings with protein expression using immunohistochemistry. There were 40 (~6%) *EZH2*^{MUT} MDS [33 men, seven women; median age 74 years (range, 55–90)]. *EZH2* mutations spanned the entire coding region. Majority had dominant *EZH2* clone [median VAF, 30% (1–92)], frequently co-occurring with co-dominant *TET2* (38%) and sub-clonal *ASXL1* (55%) and *RUNX1* (43%) mutations. *EZH2*^{MUT} MDS showed frequent loss-of-expression compared to *EZH2*^{WT} (69% vs. 27%, $p = 0.001$). Interestingly, NINE (23%) *EZH2*^{WT} MDS also showed loss-of-expression. *EZH2*^{MUT} and loss-of-expression significantly associated with male predominance and chr(7) loss. Further, only EZH2 loss-of-expression patients showed significantly lower platelet counts, a trend for higher BM blast% and R-IPSS scores. Over a 14-month median follow-up, both *EZH2*^{MUT} ($p = 0.027$) and loss-of-expression ($p = 0.0063$) correlated with poor survival, independent of R-IPSS, age and gender. When analyzed together, loss-of-expression showed a stronger correlation than mutation ($p = 0.061$ vs. $p = 0.43$). In conclusion, immunohistochemical assessment of *EZH2* protein, alongside mutation, is important for prognostic workup of MDS.

Modern Pathology (2022) 35:1212–1219; https://doi.org/10.1038/s41379-022-01074-y

INTRODUCTION

Myelodysplastic syndromes (MDS) are a heterogeneous group of acquired clonal hematopoietic stem cell disorders characterized by ineffective hematopoiesis, dysplasia of hematopoietic cells, recurrent genetic abnormalities and an inherent risk for transformation to acute myeloid leukemia[1]. Clinical outcomes of patients with MDS are variable due to the multiple genetic and epigenetic alterations involving different cellular pathways that affect hematopoietic cell survival, growth, proliferation and apoptosis[2–5]. Large-scale high-throughput sequencing studies have shown substantial complexity in the genetic landscape of MDS[4,6]. Nearly 90% of MDS cases have recurrent somatic gene mutations involving the core components of one or more pathways: RNA splicing machinery *(SF3B1, SRSF2, U2AF1)*, DNA epigenetic regulation (*TET2, DNMT3A, IDH1/2*); chromatin modification (*ASXL1, EZH2*), tyrosine kinase signaling (*RUNX1, RAS, RAF)* and DNA repair response (*TP53)*[3,7,8]. Somatic mutations involving genes *RUNX1, SRSF2, EZH2, ETV6, TP53* and *ASXL1* are independent predictors of poorer outcome in MDS patients[3,7,9,10].

Despite these advances, it has been challenging to incorporate gene mutation data formally into any of the current prognostic scoring systems for risk-stratification. This is due to the high degree of complexity and permutations of a multiple variables that affect the outcome, such as combinations of co-mutations, sequence of co-mutations, variant allele frequencies (VAF) as well as the presence of additional alterations at the level of gene copy number, mRNA and protein that lead to similar downstream consequence[2,6,11]. For accurate prognostication and appropriate use of targeted therapies, there is not only a need for mutation detection but also characterization of multitude of alterations leading to similar clinicopathologic features, and to apply this knowledge for novel "individualized" therapeutics.

Enhancer of zeste 2 homologue 2 (*EZH2*) is a gene that encodes a histone methyltransferase, which is an enzymatic component of the polycomb repressive complex 2 (PRC2) complex, that is crucial for epigenetic silencing of genes involved in stem cell renewal[12,13]. EZH2 protein suppresses the transcription of other genes and is an essential regulator of hematopoietic stem cells[12–14]. Unlike germinal center d-

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Received: 6 August 2021 Revised: 15 March 2022 Accepted: 16 March 2022
Published online: 3 May 2022

erived B-cell lymphomas where most cases show an *EZH2* Y641 hotspot gain-of-function mutation, MDS cases show a spectrum of mutations with a loss-of-function phenotype. Multiple studies have shown that *EZH2* mutations are associated with a poorer prognosis in MDS[3,7,15]. The presence of *EZH2* mutations has been shown to induce therapeutic resistance to cytotoxic drugs in AML[16].

EZH2 gene mutation is only one of several mechanisms that can alter its function. *EZH2* function can be disrupted by copy number changes, mutations in the non-coding region, and mis-splicing at the mRNA level caused by other gene mutations, all of which lead to the downregulation of protein. Assessment of EZH2 protein expression can identify loss of downstream EZH2 function resulting from multiple alterations that may be missed by mutation assessment alone. Targeting EZH2 is more challenging in myeloid malignancies due to the loss-of-function phenotype, but multiple therapeutic strategies to restore EZH2 function, such as targeting HOXA9 and other genes derepressed by EZH2, small molecules to inhibit demethylases to modify methylation signatures and addition of bortezomib to cytarabine-based therapies, are currently under exploration[16–19]. Since most of these approaches involve restoration of EZH2 function, their application is not restricted to mutations alone, but extend to all mechanisms that lead to decreased EZH2 protein expression. Hence, there is a need for precise evaluation of *EZH2* mutations as well as protein expression by immunohistochemistry, which is simple and feasible in clinical laboratories, in well-characterized clinical MDS cohorts.

In this study, we evaluated the spectrum of *EZH2* mutations and clinicopathologic features and outcomes. In addition, we correlated the mutation findings with EZH2 protein expression using immunohistochemistry. We show that lack of EZH2 expression correlates with the presence of an *EZH2* mutation in 69% of patients, while a subset (23%) of patients, in the absence of *EZH2* mutation or copy number loss, also showed loss of EZH2 expression. Both *EZH2* mutations and loss-of-expression correlated with poor overall survival, independent of R-IPSS, age and gender, with loss-of-expression showing a stronger correlation than mutation. We conclude that EZH2 protein assessment by immunohistochemistry, alongside mutation analysis, is important for prognostic workup of MDS.

MATERIALS AND METHODS

Case selection

We performed an electronic search of our departmental archives for newly diagnosed MDS patients with available next-generation-sequencing (NGS) mutation data. The clinical and laboratory data were collected from patients' electronic medical records with emphasis on variables that are historically known to be more important in myeloid neoplasms. This study was approved by the Institutional Review Board (IRB) and performed in accord with the Declaration of Helsinki.

Histologic evaluation

The diagnosis of MDS was confirmed based on morphologic review of bone marrow (BM) aspirate and peripheral blood smears and routinely prepared histologic sections of biopsy and/or clot specimens in all patients. For controls, 34 consecutive MDS cases with wild-type *EZH2* and confirmed histologic diagnosis were included. Cases were further subcategorized into different subtypes based on criteria established by the current WHO classification[1].

Immunohistochemistry

Immunohistochemical analysis for EZH2 was performed using anti-EZH2 antibody (clone 6A10; Novocastra CAT#NCL-L-EZH2) on 4-µm sections of decalcified formalin-fixed paraffin embedded BM biopsies using a streptavidin-biotin complex technique. Slides were first deparaffinized and rehydrated followed by heat-induced antigen retrieval using 0.01 M citrate buffer at pH 6.0 in a microwave oven. The slides were stained on a Dako Autostainer (Dako, Carpinteria, CA, USA) with EnVision+ (Dako) staining reagents. After blocking the endogenous peroxidase activity (Dual Endogenous Block, Dako for 10 minutes) and buffer wash, the sections were incubated with anti-EZH2 antibody (clone 6A10; Novocastra CAT#NCL-L-EZH2) at a dilution of 1:5000 for 60 min. Following a buffer wash, the sections were incubated with the EnVision+ Dual Link (Dako/Agilent) detection reagent for 30 min and subsequently treated with a solution of diaminobenzidine (DAB) and hydrogen peroxide (10 min) to produce the visible brown pigment. After rinsing, the color was enhanced with DAB enhancer (Dako) and counterstained with haematoxylin, dehydrated and cover slipped with a permanent medium.

Nuclear EZH2 staining was scored independently by three hematopathologists (A.S., C.B.R and R.K-S.) by using a semi-quantitative approach (blinded to data). The mutation data were unknown during scoring. The percentage of positive BM cells was scored as follows: 0 (no staining, 0%), 1 (1–5%), 2 (6–20%), 3 (21–50%), and 4 (51–100%). The intensity of staining was scored qualitatively from 0 to 3. A multiplicative staining score (H-index) was obtained by multiplying the % positive cells and staining intensity, giving a range of 0–12. H-index of 0–1 was considered negative ($EZH2^{NONEXP}$) implying "loss of expression"; ≥2 was considered expressors ($EZH2^{EXP}$).

Cytogenetic studies

Conventional cytogenetic analysis was performed on unstimulated cultured BM aspirate specimens using standard GTG-banding as described previously[20]. At least 20 metaphases were analyzed. Results were reported using the 2016 International System for Human Cytogenetic Nomenclature (ISCN)[21]. Complex karyotype is defined as having at least three structural and/or numerical chromosomal abnormalities.

Next-generation sequencing

NGS-based mutation analysis was performed using 28-gene or 81-gene panels at CLIA-certified molecular laboratory as previously described[22–24]. The complete gene lists provided in Supplemental Table S1. Briefly, sequencing libraries were prepared from 250 ng of gDNA followed by amplicon-based targeted NGS (Illumina Miseq, San Diego CA, USA). Variant calling required at least 250x bidirectional coverage and 2% variant allele frequency (lower limit of detection; reference genome: GRCh37/hg19). Somatic nature was inferred from literature and online databases. SNPs in ExAC, dbSNP 137/138, and 1000 Genomes were excluded.

Statistical analysis

Overall survival (OS) was defined as the time from diagnosis to death/last follow-up. Patients alive at their last follow-up were censored. The median OS was evaluated by the Kaplan-Meier method. Univariate Cox proportional hazards analyses were used to identify associations between risk factors and survival followed by multivariate Cox analyses. Logistic regression (for continuous) and Fisher's exact test (for categorical variables) were used to assess associations. Statistical analysis was performed using R version 3.5.1.

RESULTS

Study group characteristics

Forty ($n = 40$) patients with MDS with a confirmed somatic mutation in *EZH2* gene ($EZH2^{MUT}$) were identified from a total of 454 newly diagnosed MDS patients (40/454, 8.8%) in the archives of the MD Anderson Cancer Center between years January 1, 2014 and December 31, 2017. There were 33 men and seven women with a median age of 74 years (range, 55–90) at diagnosis. The median BM blast percentage was 4% (range, 0–16%); 24 (60%) cases presented with <5% blasts. Within the sub-categories of the WHO 2016 classification, *EZH2* mutations were preferentially observed in MDS with multilineage dysplasia (MDS-MLD, $n = 16$, 40%), followed by MDS with excess blasts-1 (MDS-EB-1, $n = 9$, 23%), MDS with excess blasts-2 (MDS-EB-2, $n = 6$, 15%), therapy-related MDS (t-MDS, $n = 3$, 8%) and MDS with single lineage dysplasia (MDS-SLD, $n = 2$, 5%), MDS with MLD and ring sideroblasts (MDS-MLD-RS, $n = 2$, 5%) and MDS with single lineage dysplasia and ring sideroblasts (MDS-SLD-RS, $n = 2$, 5%). All three cases of t-MDS had multilineage dysplasia. Using IPSS-R criteria for cytopenias, anemia was most common ($n = 31$, 78%) followed by neutropenia ($n = 27$, 68%) and thrombocytopenia

($n = 26$, 78%). Pancytopenia was present in 18 (45%) patients followed by bi-cytopenia in 10 (25%) patients. Twelve (30%) patients had monocytopenia.

Of 39 patients with available karyotype by conventional cytogenetic analysis, 12 (31%) were diploid, 19 (49%) showed a non-complex karyotype, and eight (20%) revealed a complex karyotype (defined here as three or more chromosomal abnormalities). Fourteen (36%) cases showed concurrent abnormality(ies) in chromosome 7 alone or in combination with other changes; a subset of these were confirmed by FISH. These cases included 11 (27.5%) with deletion of whole chromosome 7 (−7; $n = 10$) or long arm of chromosome 7 (−7q; $n = 1$). Three cases showed other abnormalities of chromosome 7 that did not lead to deletion of the *EZH2* locus at band 7q36.1 locus including; r(7)(p12q11.2); r(7)(p11.2q22); and d(1.7)(q10;p10).

A control cohort of 38 consecutive newly diagnosed treatment naïve MDS patients with wild-type *EZH2* (MDS^{EZH2WT}) over a 5-month time period (April 1, 2017 to August 31, 2017) who underwent BM exam was selected. There was no difference in the distribution of BM blast percentage or R-IPSS scores. Compared to *EZH2* wild-type MDS, *EZH2* mutated MDS showed male predominance (54% vs. 83%; $p = 0.014$) and frequent loss in chromosome 7 (8% vs. 36%; $p = 0.001$). There were no appreciable differences with respect to the types of morphologic dysplasia or lineages involved by dysplasia between *EZH2* mutated and *EZH2* wild-type MDS. The clinicopathologic characteristics of MDS patients with and without *EZH2* mutations are summarized in Table 1.

EZH2 gene mutation characteristics

Using NGS myeloid panel that covers the entire coding region (exons 2–20) of *EZH2*, we identified the study cohort of 40 patients with 45 *EZH2* mutations: 35 patients with a single mutation and five patients with double mutations. Of the mutations, 28 (62%) were missense, five (11%) were nonsense and 12 (27%) were frameshift types. *EZH2* mutations spanned the entire coding region: 16 (36%) involved exons 18 and 19 (Fig. 1A). Nearly half ($n = 21$, 47%) mutations were within the catalytic "SET" domain. Based on the support from evidence in the literature and online databases, all of these mutations are considered to be somatic.

Among *EZH2* mutated MDS, 33 (83%) cases harbored at least one additional mutation (Fig. 1B). The most common concurrently mutated genes in the decreasing order of frequency were: *ASXL1* ($n = 22$, 55%), *RUNX1* ($n = 17$, 43%), *TET2* ($n = 15$, 38%), *SF3B1* (25%), *TP53* (14%), and *DNMT3A* (10%) (Table 2). *EZH2* mutated MDS had a significantly higher frequency of concurrent mutations in *ASXL1* (55% vs. 26%; $p = 0.012$) whereas *IDH2* mutations were absent (0 vs. 11%; $p = 0.05$). The mutational frequencies of *RUNX1* and *TET2* were higher but not significantly different from wild-type. Sixteen (40%) patients showed mutations in at least two of three genes: *ASXL1*, *RUNX1* or *TET2*, including seven patients with all three genes mutated in addition to *EZH2*. Of note, many patients showed a second sub-clonal mutation within the same gene (double mutations), including *EZH2* ($n = 6$), *RUNX1* ($n = 4$), *TET2* ($n = 4$), and *ASXL1* ($n = 2$). Figure 1C illustrates the spectrum of concomitant epigenetic modifier gene mutations in *EZH2* mutated and wild-type groups: DNA methylation regulators (*DNMT3A*, *TET2*, *IDH1*, *IDH2*), histone modifiers (*ASXL1*) and genes in PRC2 complex (*EED*, *SUZ12*).

The median VAF of *EZH2* mutation was 30% (range, 1.3–92). Twenty-one (53%) were greater than the median suggesting that *EZH2* was a dominant mutant clone. Eleven (24%) mutations showed a VAF of >60%, suggesting either a homozygous/ biallelic *EZH2* mutation or a concurrent loss of heterozygosity due to chromosome 7 alterations. Twelve (27%) mutations were a minor clone (VAF < 20%), although, in two of these cases the percentage of non-myeloid cells (lymphocytes and plasma cells) was high (48% and 49%, respectively) due to hemodilution. These findings suggest that *EZH2* mutation is a dominant mutant clone in MDS and likely to be a primary driver. By comparing the VAFs of *EZH2* with co-mutated genes, we determined that *TET2* mutations were frequently co-dominant ($n = 11$, 85%) and that *RUNX1* mutations were frequently sub-clonal [$n = 10$, 77%]. *ASXL1* mutations were both sub-clonal [$n = 8$, 47%] and as co-dominant mutations [$n = 9$, 53%] (Fig. 1D).

Table 1. Baseline clinicopathologic characteristics of patients with myelodysplastic syndrome with and without *EZH2* mutations.

Parameters	*EZH2* mutated $n = 40$	*EZH2* wild-type $n = 38$	*p* value
Gender M: F	33: 7	21: 17	0.014
Age: median (range)	74 (55–90)	71 (41–88)	
CBC (median, range)			
WBC (k/μL)	2.7 (0.5–24.7)	2.8 (0.3–25.8)	
ANC (k/μL)	1.2 (0.2–16.6)	1.3 (0.1–18.7)	
Platelet (k//μL)	72 (9–794)	94 (10–331)	
Hgb (g/dL)	8.7 (6–13.1)	9.1 (7–11.1)	
MCV (fl)	93.5 (80–118)	99 (66–116)	
PB blasts	0 (0–14)	0 (0–13)	
BM morphology			
BM blast% (median, range)	4 (0–15)	4 (0–17)	
BM cellularity	60 (10–95)	70 (20–95)	
% ring sideroblasts	0 (0–67)	5 (0–77)	
AML transformation rate	13 (33%)	7 (20%)	0.1981
WHO subtype of MDS			
SLD	2 (5%)	2 (5%)	1
RS-SLD/ RS-MLD	4 (10%)	7 (18%)	0.3419
MLD	16 (40%)	7 (18%)	0.0480
EB-1	9 (23%)	9 (24%)	1
EB-2	6 (15%)	8 (21%)	0.5628
MDS-U	0	1 (3%)	0.4872
Therapy-related	3 (8%)	4 (11%)	0.7082
R-IPSS			
0	2 (5%)	0 (0%)	0.65
1	8 (21%)	8 (23%)	
2	11 (29%)	9 (26%)	
3	8 (21%)	6 (17%)	
4	10 (26%)	12 (34%)	
Karyotype			
Normal	12 (31%)	18 (47%)	0.1646
Not-complex	19 (49%)	9 (24%)	0.0327
Complex	8 (20%)	11 (29%)	0.4372
Chr 7 alteration	14 (36%)	3 (8%)	0.0050

ANC Absolute neutrophil count, *BM* Bone marrow, *EB* Excess blast, *MCV* Mean corpuscular volume, *MLD* Multilineage dysplasia, *SLD* Single lineage dysplasia.

Immunohistochemical EZH2 protein expression in *EZH2* mutated and wild-type MDS

For immunohistochemical evaluation, we evaluated EZH2 protein expression using decalcified BM sections from MDS with mutated and wild-type *EZH2* using a semi-quantitative staining score described under methods. In order to assess the baseline expression of EZH2, we first evaluated EZH2 expression in 11

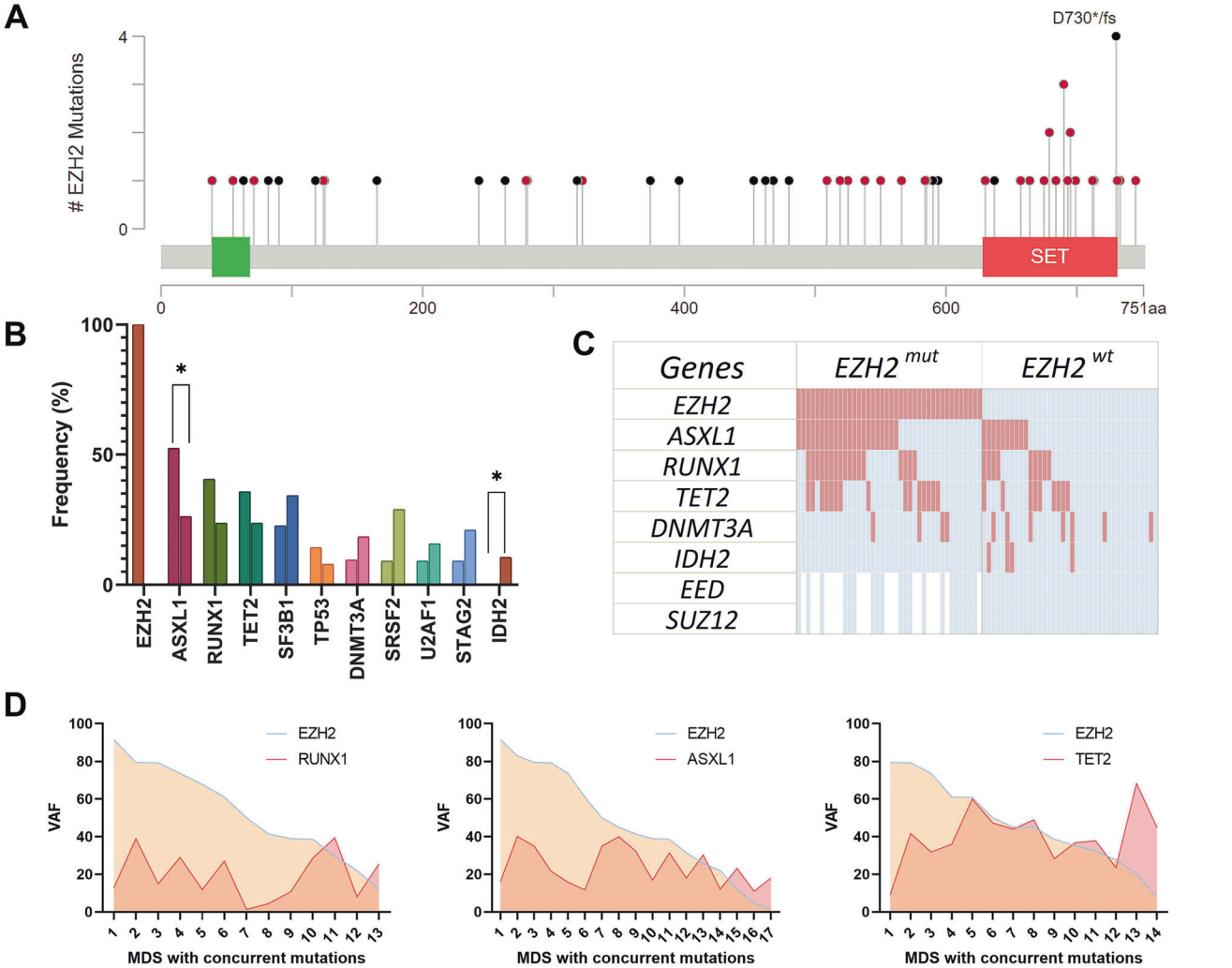


Fig. 1 *EZH2* mutation characteristics of this cohort. A Distribution of mutations along different the coding region of *EZH2*. **B** Highlights the distribution of *EZH2* mutations in different functionally important sections of the gene. Mostly mutations occur in the catalytic "SET" domain. **C** Mutational heatmap in the *EZH2* mutated (study) vs. *EZH2* wild-type (control) cohorts showing epigenetic modifier and related genes: DNA methylation regulators: DNMT3A, TET2, and IDH2, histone modifiers: ASXL1 and EZH2 (including the core components of PRC2 pathway: EED and SUZ12) and *RUNX1*. **D** Clonal relationship between *EZH2* and additional mutations in *RUNX1* (sub-clonal), *ASXL1* (sub-clonal and co-dominant) and *TET2* (co-dominant) based on variant allele frequencies gene exons.

apparently healthy individuals with no prior/ concurrent diagnosis of malignancy or clonal hematopoiesis (negative for mutations using the same comprehensive NGS panel). EZH2 showed nuclear positivity in most erythroid and myeloid precursors and a subset of megakaryocytes; within myeloid precursors, maturation was associated with loss of EZH2 expression, with segmented neutrophils showing virtually absent protein. This was established as the normal staining pattern. The median H-score was 9 (range, 6–12).

Next, we scored the nuclear EZH2 expression in 29 MDS with $EZH2^{MUT}$ (those who had adequate quality decalcified biopsy cores) and 34 MDS with wild-type *EZH2*. Twenty of 29 (69%) $EZH2^{MUT}$ MDS showed loss of EZH2 protein ($EZH2^{NONEXP}$) whereas 9 (31%) retained protein expression ($EZH2^{EXP}$). Among *EZH2*-wild-type MDS, 9 (27%) cases showed loss and 25 (74%) cases retained protein expression. Based on these data, EZH2 expression was lost more frequently in $EZH2^{MUT}$ MDS compared to $EZH2^{WT}$ ($p = 0.001$), and hence correlated with absence of *EZH2* mutation. Representative images of different patterns of staining in all three groups are shown in Fig. 2.

We were particularly interested in the nine patients with $EZH2^{WT}$ MDS that showed loss of EZH2 protein expression ($EZH2^{NONEXP}$). These included two patients with deletions of chromosome 7 [monosomy 7, and del(7q31)] and five patients who had concurrent mutations in other related genes: four with coexisting *SRSF2* mutations and one patient with mutations in *ASXL1*, *U2AF1*, and *KMT2A*. In the latter five patients, mutations in *SRSF2* and *U2AF1* could have possibly led to mis-splicing of wild-type EZH2 and/or EZH2 protein disruption. The remaining two patients did not show any other concurrent genomic alterations leading to protein loss. On the contrary, nine patients with $EZH2^{MUT}$ MDS had preserved EZH2 expression ($EZH2^{EXP}$), six had mutations located in the SET domain and two had loss of entire chromosome 7. The negative predictive value of a preserved EZH2 expression for lack of *EZH2* mutation was 76% (Fisher's exact test).

Due to differences in *EZH2* mutation vs. EZH2 expression, we also compared the differences in clinicopathologic features and outcomes between $EZH2^{EXP}$ and $EZH2^{NONEXP}$ MDS, in addition to $EZH2^{MUT}$/$EZH2^{WT}$ subgroups. $EZH2^{NONEXP}$ MDS showed frequent loss of chromosome 7 (35.7% vs. 5.9%, $p = 0.004$) compared to $EZH2^{EXP}$ MDS, similar to $EZH2^{MUT}$ MDS. In addition, patients with $EZH2^{NONEXP}$ MDS showed significantly lower median platelet count (68 vs. 112, $p = 0.041$) and a trend for higher R-IPSS scores and BM blast% compared with $EZH2^{EXP}$ MDS. There were no

Table 2. Multivariate analysis showing that the presence of *EZH2* mutation and *EZH2* loss of protein correlated with poor survival, independent of R-IPSS.

Multivariable models		
Variable	**HR, 95% CI**	***p* value**
Model 1		
EZH2 mutated	3.12 (1.14–8.57)	0.027
Age (per year)	1.02 (0.97–1.08)	0.420
Gender = M	0.90 (0.33–2.40)	0.830
R-IPSS (per point)	1.86 (1.23–2.82)	0.003
Model 2		
EZH2 expressor (IHC)	0.24 (0.083–0.66)	0.006
Age (per year)	1.03 (0.97–1.09)	0.300
Gender = M	0.61 (0.21–1.75)	0.360
R-IPSS (per point)	1.67 (1.02–2.76)	0.043
Model 3		
EZH2 mutated	1.66 (0.47–5.82)	0.430
EZH2 expressor (IHC)	0.30 (0.085–1.057)	0.061

When mutation and protein expression were analyzed together, loss of protein expression is more strongly correlated with poor survival than the mutation.

significant differences in the types of morphologic dysplasia or lineages involved by dysplasia between EZH2 non-expressors and expressors. There were no significant differences in mutations of other genes between EZH2 non-expressors and expressors.

Correlation of *EZH2* mutation and EZH2 protein loss with survival

Over the course of the study, 34 (85%) patients received therapy with hypomethylating agent(s). Over a median follow-up of 14 months (range, 1–45), 17 (42.5%) patients died and 12 (30%) patients transformed to acute myeloid leukemia (AML).

$EZH2^{MUT}$ MDS patients had a significantly worse overall survival (OS) compared to $EZH2^{WT}$ [20 months vs. not reached (NR), HR 2.953 (1.352–6.449), $p = 0.0066$] (Fig. 3A). $EZH2^{NONEXP}$ MDS patients (with loss or deficient EZH2 expression), irrespective of the *EZH2* mutation status, had a significantly worse overall survival compared to $EZH2^{EXP}$ MDS patients (NR vs. 20 months, HR 5.231 (2.205–12.41), $p = 0.0002$) (Fig. 3B). There was no significant survival difference between MDS patients with *EZH2* mutations located in the catalytic "SET" domain compared to those with *EZH2* mutations located in other domains. (Supplemental Fig. S1A). There was no significant survival difference in *EZH2* mutated MDS patients between those with or without deletion of chromosome 7q36 locus (Supplemental Fig. S1B).

By multivariate analysis that also included age, gender and R-IPSS, both *EZH2* mutation ($p = 0.027$) and loss of protein expression (0.0063) correlated with poor survival, independent of R-IPSS [Table 2. When mutation and protein expression were analyzed together, protein expression [HR 0.30 95% CI: 0.085–1.057; $p = 0.061$) showed a stronger correlation with survival than mutation (HR: 1.66 95% CI: 0.47–5.82; $p = 0.43$).

EZH2 immunohistochemistry in MDS at the time of AML transformation

To explore the value of immunohistochemical assessment of EZH2 protein expression in MDS cases transformed to AML (AML-MRC), we performed EZH2 immunohistochemical staining on six cases of $EZH2^{MUT}$ MDS and four cases of $EZH2^{WT}$ MDS, at the time of MDS diagnosis and AML transformation. All cases had the same *EZH2* mutations detected at both time points. The median blast count was 35% (range, 21–89%). Two $EZH2^{MUT}$ patients had concurrent del(7q23). We also included three cases of $EZH2^{MUT}$ *de novo* AML without a history of MDS (but meeting the criteria for AML-MRC based on dysplastic morphology in >50% of cells in at least two lineages).

Among six $EZH2^{MUT}$ MDS cases that transformed to AML, four (67%) showed loss-of-expression ($EZH2^{NONEXP}$) whereas two (33%) retained protein expression ($EZH2^{EXP}$). The immunohistochemical EZH2 protein expression results were similar at the time of MDS and AML in all cases. The two cases with discordant $EZH2^{EXP}$ included the following: one patient with 89% blasts and mutations in *EZH2* p.N693K (VAF 30%) and *NRAS* (VAF 79%); second patient with 21% blasts with multiple mutations involving *EZH2* p.G628A (VAF 33%), *ASXL1* p.G644fs (32%), *RUNX1* p.R320* (28%), *TET2* p.Q916* (35%) and *TET2* p.S1671fs (31%). All four $EZH2^{WT}$ cases retained protein expression ($EZH2^{EXP}$) at the time of MDS and AML. All three cases of $EZH2^{MUT}$ de novo AML showed loss-of-expression. The details are provided in Supplemental Table S2. These preliminary findings suggest that data could be extrapolated to AML cases. The findings need to be confirmed in a larger number of AML cases and the clinical significance needs to be evaluated within the context of prognostic attributes pertinent to AML, which is beyond the scope of the current study.

DISCUSSION

EZH2 encodes a histone methyltransferase with an important role in chromatin modification and epigenetic changes[12,13]. The frequency of *EZH2* mutation in newly diagnosed MDS in this study was ~5%, within the reported ranges from other studies (range, 2–13%)[3,4,13]. *EZH2* mutations in MDS patients included missense, nonsense and frameshift subtypes distributed throughout the entire coding region without a hot-spot location. A third of the mutations clustered in exons 18 and 19 within the SET domain, the main catalytic site of the methyl transferase enzyme, in accordance with other studies[12,25]. This is in contrast to *EZH2* mutations in low-grade B-cell lymphomas and diffuse large B-cell lymphomas, which are often a gain-of-function mutation in tyrosine 641 (Y641), also located in SET domain[26–28]. Others have shown that *EZH2* mutation is a poor prognostic biomarker, which we confirm in this study. Therefore, knowledge of the EZH2 mutation spectrum is important to design clinical NGS panels that include the whole coding region for workup of myeloid neoplasms.

The observed distribution of *EZH2* mutations, with a substantial proportion of nonsense and frameshift mutations, together with a strong association with concurrent disruption of the other *EZH2* allele, frequently by chromosomal changes (monosomy or segmental deletions), supports the loss-of-function phenotype for *EZH2* alterations in MDS and other myeloid malignancies[29,30]. This statement is further corroborated by this study showing an association of *EZH2* mutations with loss of protein expression in MDS. Frequent loss of chromosome 7 or del(7q) also points to *EZH2* as a tumor suppressor gene[13,31], underscoring the importance of additional testing, beyond NGS-based mutational analysis, to look for these concurrent alterations in other *EZH2* allele using copy number analysis such as single nucleotide polymorphism (SNP) arrays to identify cytogenetically cryptic aberrations and/or copy-neutral loss of heterozygosity.

About 90% of cases of *EZH2* mutated MDS had concurrent mutations, most frequently of another chromatin modifier ASXL1 (~45%). Based on the comparison of mutant allele burdens of *EZH2* and *ASXL1* genes, *ASXL1* appears to be a secondary subclone following an *EZH2* initiating event[4,32,33]. In contrast, *TET2* mutation consistently showed a parallel clonal size with a similar or higher allelic frequency than *EZH2*. Six (15%) patients showed an additional mutation in *TP53*, half of which had abnormalities of chromosome 5 as previously recognized[34]. These interesting

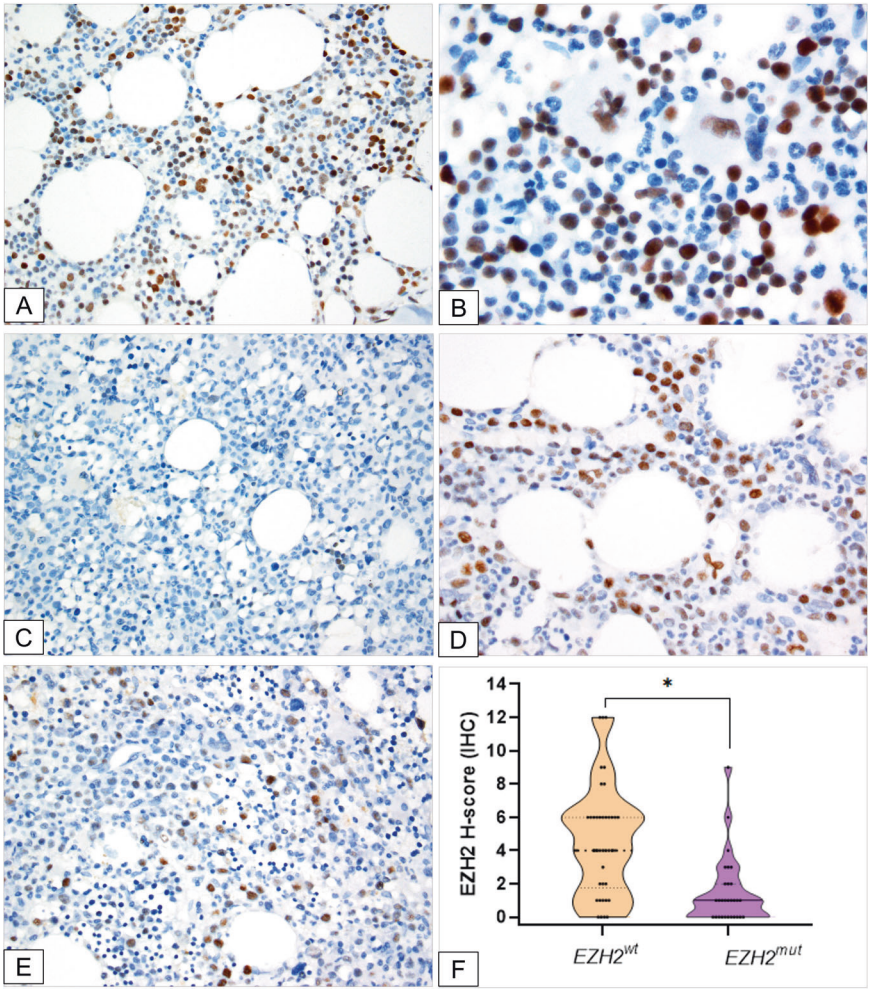
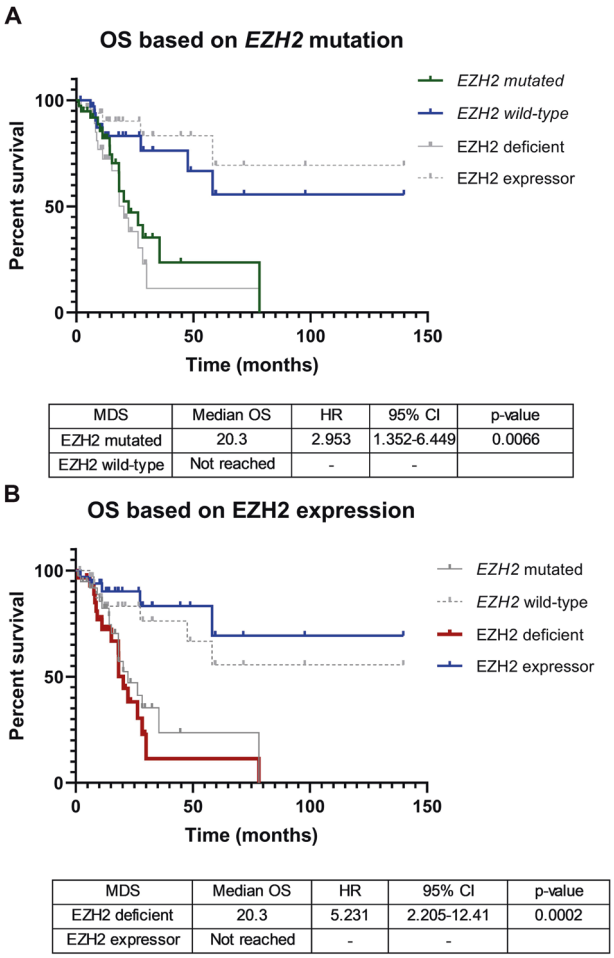


Fig. 2 Representative examples of EZH2 protein expression using immunohistochemistry. A EZH2 stain on bone marrow biopsy of apparently healthy individual showing nuclear positivity in erythroid and myeloid precursors, and megakaryocytes. **B** At higher magnification, EZH2 protein is lost with maturation of myeloid precursors: segmented neutrophils show virtually absent protein expression. **C** *EZH2* mutated MDS with loss of EZH2 protein (EZH2 deficient). **D** EZH2 wild-type MDS with retained EZH2 protein (EZH2 expressor MDS). **E** *EZH2* wild-type MDS with loss of EZH2 protein (EZH2 deficient). **F** Violin plot showing significantly decreased EZH2 protein expression (measured by H-score) in *EZH2* mutated MDS compared to wild-type.

clonal relationships based on bulk NGS need to be confirmed using single-cell mutation studies. Nevertheless, MDS patients with both *EZH2* and *TET2* mutations had a worse survival compared to MDS patients without *TET2* mutations, supporting the synergistic effects of this combinatorial mutational change as suggested by others[35–37]. We found a similar prognostic effect of additional *RUNX1* mutations, but not with *ASXL1* or *TP53* mutations (data not shown). This result may reflect *EZH2* and *ASXL1* being part of the same chromatin modification system of polycomb repressive complex 2 (PRC2) and PRC1, respectively, both of which directly or indirectly result in methylation of lysine 27 on histone H3 of DNA[30,38–40]. Loss of EZH2 promotes MDS by expansion of MDS-initiation cells in *RUNX1* S291fs mutated mice[15].

In addition to mutations in the coding region, loss of EZH2 protein function could be disrupted by multiple upstream alterations that are beyond the detection capacity of targeted NGS± copy number analysis, such as mutations in non-coding intronic or splice-site locations, mis-splicing of mRNA due to mutations in other splicing factor gene mutations among others. Bulk RNA sequencing of CD34+ cells showed EZH2 mRNA downregulation in MDS patients even without monosomy 7/del (7q), suggesting alternate mechanisms of downregulation of these genes[41,42]. Hence, quantification of EZH2 protein expression can be a surrogate measure of EZH2 function. In this study, we evaluated the functional disruption of EZH2 by immunohistochemistry. Due to loss-of-function phenotype of *EZH2* mutations in MDS, the expression levels correlated with mutation status in 73% of MDS patients, unlike diffuse large B cell lymphoma cases, where EZH2 expression was independent of mutation status[43,44]. The possible reasons for the incomplete correlation in the remaining (27%) MDS patients are elaborated below.

Among the 9 $EZH2^{WT}$ MDS cases with loss of EZH2 protein expression, two patients had deletion of 7q36.1 locus which might have led a dose dependent decrease in EZH2 expression[45]. *EZH2* overlaps one of the three commonly deleted regions (CDRs) in MDS with del(7q)[41,42]. Four patients had concurrent mutations in *SRSF2* which causes mis-splicing of EZH2 mRNA leading to nonsense mediated decay and consequently lower protein levels[46]. One additional patient had mutation in *U2AF1*, another splicing factor gene that might lead to similar effect. Four patients were co-mutated with *ASXL1*, also a component of PRC1/2 complex, leading to reduction of EZH2 expression. In the remaining two $EZH2^{WT}$ MDS patients, the reasons for EZH2 protein loss are unclear. There was no *EZH2* deletion by karyotyping or copy number evaluation by targeted NGS. Copy-neutral loss-of-heterozygosity of 7q was not assessed but is rare in MDS. Cabrero



MDS	Median OS	HR	95% CI	p-value
EZH2 mutated	20.3	2.953	1.352-6.449	0.0066
EZH2 wild-type	Not reached	-	-	

MDS	Median OS	HR	95% CI	p-value
EZH2 deficient	20.3	5.231	2.205-12.41	0.0002
EZH2 expressor	Not reached	-	-	

Fig. 3 Survival analysis. A comparison of overall survival (OS) between MDS patients segregated by (**A**) *EZH2* mutation status and (**B**) EZH2 protein expression status. EZH2 protein expression status was able to segregate these patients better than mutation status.

et al. have shown that ~50% of $EZH2^{WT}$ MDS with diploid karyotype had downregulation of EZH2 mRNA in CD34+ cells[29]. There is a possibility of mutations in other major proteins of the PRC2 complex that are closely related with EZH2[47]. Only two genes [embryonic ectoderm development (EED)1 and suppressor of zeste (SUZ)12] were included in the panel and tested wild-type. The status of the other components of PCR2, such as *JARID2, AEBP2, EZH1* etc are unknown[48].

The reasons for preserved EZH2 expression in nine $EZH2^{MUT}$ MDS cases include: first, the possibility of residual normal/non-neoplastic clones in the bone marrow. Second, we cannot completely exclude the possibility of these *EZH2* variants being rare germline polymorphisms, although these specific variants have been shown to be somatic alterations in the literature and COSMIC databases. These variants were not identified in gNOMAD or EXAC databases. Eight of these patients had disruption of SET domain with median VAF of 68% (range, 1–91). Nevertheless, this finding brings to light the challenges and importance of standardization of NGS interpretation using functional studies that is partly addressed by protein correlation. It is noteworthy that evaluating the mutational status of *EZH2* in myeloid malignancies is not as straightforward as lymphoid neoplasms[26,49].

Finally, we correlated both *EZH2* mutations and immunohistochemistry expression levels with clinicopathologic findings and outcomes. Although not formally incorporated into risk-stratification models, studies have shown that *EZH2* is an independent predictor of worse survival in MDS patients[3,7,15,50]. While both EZH2 mutation and protein loss correlated with poor survival in MDS, independent of R-IPSS, age and gender, protein expression showed a stronger correlation with survival than mutation ($p = 0.061$ vs. $p = 0.43$). Remarkably, even within this small cohort of $EZH2^{MUT}$ MDS, the OS of patients with preserved and loss of EZH2 expression was significantly different (Supplemental Fig. S1C). A similar observation was noted among $EZH2^{WT}$ MDS (Supplemental Fig. S1D). This is important for therapy. *EZH2* alterations are dominant clones which occur early in the disease course, making this an important and attractive targetable epigenetic regulator. Currently, multiple therapeutic approaches to restore the function of EZH2 are underway and show encouraging results[19], which are not restricted to MDS cases with *EZH2* mutations alone. Hence, there is a need for development and correlation of assays such as immunohistochemistry that are feasible for routine clinical workup for baseline and response assessment. The findings from this study highlight the prognostic value of EZH2 protein expression in MDS, regardless of the presence of mutation. Validation of the current findings in larger clinical cohorts of MDS patients, in the context of EZH2 targeted therapeutic strategies is needed. Overall, this study highlights the limitation of using NGS data "in silo" for prognostication of MDS and confirms that the epigenetic and genetic complexity of MDS extends beyond mutations.

DATA AVAILABILITY

The datasets generated for the current study are not publicly available due to patient privacy concerns but are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS

AS: Data collection, analysis, manuscript writing and final approval. CC: Data analysis, scientific input, manuscript writing and final approval. GM-B, KS, CB-R, KP, SL, CO, AQ, JDK, MJR, SK, HK, GG-M, LJM: Data collection; scientific input, manuscript writing and final approval. RK-S: Concept and Design of the study, data collection, analysis, interpretation, manuscript writing and final approval.

FUNDING

This work was supported in part by The University of Texas MD Anderson Cancer Center Institutional Start-up Funds (RK-S), the Institutional Research Grant (RK-S) and by the NIH/NCI award number P30 CA016672 (Research Histology Core Laboratory at MD Anderson Cancer, Houston, Texas).

COMPETING INTERESTS

The authors declare no competing interests.

ETHICAL APPROVAL

This study was approved by the Institutional Review Boards of all the participating institutions in accordance with the Declaration of Helsinki.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41379-022-01074-y.

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