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Research paper

# Altered EZH2 splicing and expression is associated with impaired histone H3 lysine 27 tri-Methylation in myelodysplastic syndrome

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ABSTRACT

*Background:* EZH2 (enhancer of zeste homolog 2) is a histone H3K27 methyltransferase involved in the pathogenesis of various hematological malignancies. In myelodysplastic syndromes (MDS), loss of function of EZH2 is known to contribute to pathogenesis, however the pattern of *EZH2* mRNA and protein expression in MDS has not been extensively characterized.

*Material and methods:* A total of 26 patients diagnosed with MDS were analyzed in this study. The relationship between EZH2 expression in patient bone marrow samples, evaluated by RT-PCR and immunoblotting, and patient characteristics were analyzed. The function of truncated EZH2 proteins was examined in vitro.

*Results: EZH2* expression levels and transcript sizes varied considerably between patients, but there was no relationship with the percentage blast component of patient samples. Cloning and sequencing of amplified RT-PCR fragments demonstrated that patients expressed multiple *EZH2* transcripts containing insertions or deletions, with or without frameshift, mainly induced by altered splicing. All identified frameshift mutations were found to be 5′ to the functional SET domain, and resulted in truncated protein translation. Altered patterns of *EZH2* expression was observed in patients with or without alterations in genes involved with RNA splicing, *SRSF2*, *U2AF1* and *SF3B1*. Functional analysis in vitro revealed that C-terminally truncated EZH2, lacking the SET domain, may impair the methyltransferase function of wild-type EZH2 in a dominant negative fashion.

*Conclusion:* Our findings suggest that the loss of function of EZH2 induced by aberrant splicing, and/or *EZH2* mutations resulting in the production of C-terminally truncated proteins, may be involved in MDS pathogenesis.

## 1. Introduction

Myelodysplastic syndromes (MDS) is a heterogeneous myeloid lineage disorder characterized by various degrees of cytopenia and predisposition to acute myeloid leukemia [1]. Recent advances in pathophysiological investigation using next generation sequencing (NGS) technology have indicated that mutations in genes involved in the regulation of histone methylation, acetylation and DNA methylation, such as *TET2* [2], *IDH1/2* [3,4], *DNMT3A* [5,6], *EZH2* [7,8], and *ASXL1*[9], or RNA splicing, such as *U2AF1*, *SRSF2*, *SF3B1* [10], and *ZRSR2,* play a fundamental role in the pathogenesis of the disease [11–13].

The *EZH2* (enhancer of zeste homolog 2) gene, located on chromosome 7q, codes for the functional enzymatic component of Polycomb Repressive Complex 2 (PRC2) [14]. PRC2 is responsible for regulating the transcription of hematopoietic stem cells [15,16], and is comprised of EZH2, suppressor of zeste 12 (SUZ12), embryonic ectoderm development (EED), and RBAP48 proteins. EZH2 is the catalytic subunit of PRC2 and catalyzes mono-, di-, or tri-methylation of lysine (K)-27 of histone H3 (H3K27me1, H3K27me2, and H3K27me3, respectively), resulting in transcriptional repression of the affected target genes [17]. *EZH2* is thus a major epigenetic regulator, and is involved in a pathogenesis of various hematological malignancies. Of relevance to the current study, the deletion of chromosome 7q, and mutations in *EZH2* that lead to loss of function, are involved in the development of MDS [7,8,18–20]. For example, a homozygous *ezh2* gene deletion in mice induces a MDS/myeloproliferative neoplasm overlapping disorder[20]. A recent report has also indicated that mutations in *SRSF2*, a splicing machinery component, result in mis-splicing of *EZH2* [21] and the production of a C-terminally truncated EZH2 protein lacking histone

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methyltransferase activity. Meanwhile, somatic gain-of-function mutations targeting the *EZH2* SET domain have been identified in 7–22% of follicular lymphomas and germinal center B-cell (GCB) type diffuse large B-cell lymphoma (DLBCL), and lead to elevated H3K27 trimethylation [22–26]. Further experiments have revealed that mutated *EZH2* can also cooperate with dysregulated expression of *BCL2* and *TP53* in the accelerated development of germinal center like lymphoma [27].

Such observations suggest that altered splicing and expression of *EZH2* may be relevant for the pathogenesis of MDS, but the expression of *EZH2* in this disease has not yet been characterized. In this study, we therefore analyzed the relationship between *EZH2* expression patterns and the clinical characteristics of MDS patients.

## 2. Materials and methods

### 2.1. Patients

A total of 26 patients diagnosed with MDS at Nagoya University Hospital between August 2009 and February 2013 were analyzed in this study. The study protocol for the experimental use of patient bone marrow (BM) samples was approved by the Institutional Review Board of Nagoya University Hospital, and complied with all provisions of the Declaration of Helsinki and the Ethics Guidelines for Human Genome/Gene Analysis Research issued by the Ministry of Health, Labour and Welfare in Japan. All patients were diagnosed with MDS according to the 4th edition of the World Health Organization (WHO) classification [1], and the risk of the disease was classified by the International Prognostic Scoring System (IPSS) [28]. BM samples to be used as normal controls were obtained from healthy volunteer donors or patients with non-myeloid hematological diseases, after obtaining written informed consent.

### 2.2. Cell lines

To evaluate the expression of *EZH2*, the cell lines Marimo (AML M2) [29], TK6 (T cell lineage blast crisis of CML) [30,31], and MEG-01 (Megakaryocytic blast crisis of CML) [32] were established in house. The following cell lines were obtained from other institutes: Kasumi-1 (AML M2) from Hiroshima University (Hiroshima, Japan), MOLM-13 (AML M5a) and MOLM-14 (AML M5a) from Fujisaki Cell Center, Hayashibara Biochemical Laboratories (Okayama, Japan), and NB4 (AML M3) from Dr M. Lanotte (Hôpital Saint-Louis, Paris, France). All other cell lines were obtained from distributors including the ATCC (Manassas, VA, USA) and the DSMZ (Braunschweig, Germany). 293T cells and NB4 cells were cultured in Dulbecco Modified Eagle Medium (DMEM) and Iscove's Modified Dulbecco Medium (IMDM), respectively, while all other cell lines were cultured in RPMI-1640 medium. All media were supplemented with 10% of fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin.

### 2.3. DNA and RNA preparation, reverse transcriptase PCR, and genomic PCR analysis

To extract genomic DNA and total RNA, BM mononuclear cells (BMMNCs) were purified with Ficoll-Paque solution (GE Healthcare, Little Chalfont, UK). Total RNA was extracted using TRIzol solution (Invitrogen in Thermo Fisher Scientific, Waltham, MA, USA) and then cDNA was prepared with the GeneAmp RNA PCR Core Kit (Applied Biosystems in Thermo Fisher Scientific) according to the manufacturer's protocol. Genomic DNA was extracted with the QIAamp DNA Blood Mini Kit (QIAGEN, Venlo, Netherland). To detect *EZH2*, *ACTB, SRSF2*, *U2AF1*, and *SF3B1* mRNA, the primers listed in **Supplemental Table S1** were used. Total *EZH2* mRNA was amplified using KOD plus neo polymerase (TOYOBO, Tokyo, Japan). Other mRNA fragments of *EZH2* (EZH2-RT1), *SRSF2*, *U2AF1*, and *SF3B1*, were amplified using LA-Taq (Takara Bio, Otsu, Japan), while EZH2-RT2, EZH2-RT3, EZH2-RT4, were amplified with Go Taq Green (Promega, Madison, MI, USA). To analyze real-time quantitative RT-PCR for the *EZH2* gene (EZH2-qRT), the TaqMan Gene Expression Assay whose probe recognized the SET domain sequence was used (Probe Hs 01016789; ABI 7300 Real-Time PCR system, Applied Biosystems), with *GAPDH* evaluated as an internal control.

### 2.4. Sequencing analysis

Fragments of coding DNA sequence (CDS) of *EZH2* amplified from patient BMMNCs were sub-cloned into pCRII-TOPO plasmid (Invitrogen), and then sequenced using the ABI 3500 genetic analyzer (Applied Biosystems). Mutation analysis was performed with Seq Scanner 2 (Applied Biosystems). Samples from patients UPN 11 and 26 were analyzed by targeted sequencing using the TruSight Myeloid Sequencing Panel (Illumina, San Diego, CA, USA), as previously described [33]. The mRNA sequence of the Homo sapiens enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2), transcript variant 1 (https://www.ncbi.nlm.nih.gov/nuccore/NM_004456.4) was used as the reference for mutation analysis.

### 2.5. Cytogenetic analysis and single nuclear polymorphism array analysis

Chromosomal G-banding was performed by the LSI Medience Corporation (Tokyo, Japan). To identify single nuclear polymorphism (SNP), loss of heterozygosity (LOH), and copy-neutral LOH (namely uniparental disomy or gene conversion), BM DNA was analyzed using the 250k Nsp GeneChip-SNP array (Affymetrix in Thermo Fisher Scientific, Santa Clara, CA, USA), as previously described [34].

### 2.6. EZH2 expression vector and transfection/transduction procedures

To develop EZH2 expression vectors, fragments of either full length *EZH2*, C-terminally truncated *EZH2* missing the SET domain (EZH2ΔSET), and C-terminally truncated *EZH2* missing the D2, CXC and SET domains (EZH2ΔD2), were obtained by PCR and sub-cloned into pcDNA4/HisMax TOPO (Invitrogen). Transient transfection of control and expression vectors into 293T cells was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Forty-eight hours later, transfected cells were harvested and the expression of proteins confirmed by immunoblotting.

### 2.7. Immunoblotting

BMMNCs and cultured cells were lysed as described previously [35,36]. NaF (50 mM) and $Na_3VO_4$ (1.68 mM) were routinely added to prevent post-lysis changes in phosphorylation. After centrifugation (11,700*g* for 10 min), concentrations of lysate were calculated using the Bradford method, and then sample buffer containing 5% 2-mercaptoethanol was added. After boiling for 5 min, samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride membranes, and blocked with 5% skimmed milk in TBS-Tween buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, and 0.05% Tween 20). Immunoblotting was carried out using primary antibodies appropriately diluted in TBS-Tween buffer, with 5% bovine serum albumin and 0.05% sodium azide. For immunoblotting of EZH2, FLAG, H3K27me3, and GAPDH, the following primary antibodies were used: anti-EZH2, recognizing the N-terminus of EZH2 protein (ab3748; Abcam, Cambridge, UK), anti-FLAG (Sigma-Aldrich, St. Louis, MO, USA), anti-H3K27me3 (EMD Millipore, Billerica, MA, USA), and anti-GAPDH (Santa Cruz Biotechnology, Dallas, TX, USA). Horseradish peroxidase-conjugated secondary monoclonal antibodies (GE Healthcare) were added and activated with Amersham ECL-prime (GE Healthcare). Images were obtained using a LAS 4000 mini bio-imager (Fujifilm, Tokyo, Japan) and analyzed with

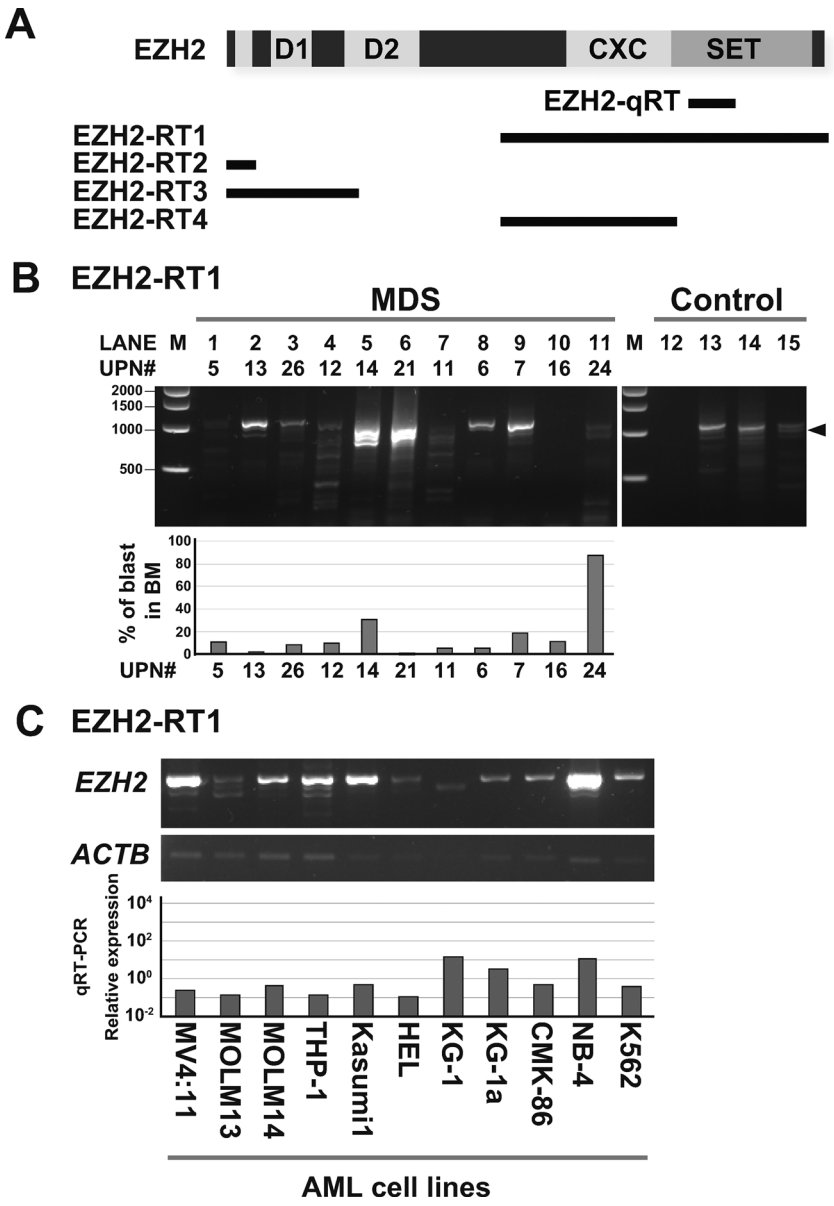


**Fig. 1. Analysis of *EZH2* mRNA expression in MDS patient samples and AML cell lines. (A)** Schematic of the EZH2 protein and expected products from RT-PCR and quantitative RT-PCR (qRT-PCR) analyses, including EZH2-RT1, EZH2-RT2, EZH2-RT3, EZH2-RT4 and EZH2-qRT. **(B)** Semi-quantitative RT-PCR products in agarose gel electrophoresis (upper panel) are shown, with the blast% in BM cells indicated in the lower panel. Note the multiple band sizes amplified using EZH2-RT1 primers in MDS patient samples compared to the control samples from patients with non-myeloid hematological diseases. The bands amplified in normal controls are indicated by the black arrowheads. **(C)** Semi-quantitative RT-PCR using AML-derived cell lines, showing the uniform bands amplified using EZH2-RT1 primers; ACTB (beta actin) was analyzed as an amplification control. The relative expression of *EZH2* relative to *GAPDH* was analyzed by quantitative RT-PCR (lower graph).

MultiGauge software (Fujifilm).

## 3. Results

### 3.1. EZH2 expression in MDS patient samples

To evaluate *EZH2* mRNA expression in BMMNCs from MDS patients, we attempted to amplify the C-terminal domains of *EZH2*, including the CXC and SET domains, using a primer pair indicated as EZH2-RT1 (Fig. 1**A**). In the 11 patients whose samples were evaluable, *EZH2* mRNA expression levels varied considerably, however this did not correlate to the percentage blast component of the BM. Meanwhile, *EZH2* expression in the four normal control samples was relatively low. Interestingly, multiple *EZH2* transcript sizes were detected in MDS samples, with amplicons smaller than the predicted wild-type transcript detected in several patients (Fig. 1**B**). In contrast, in acute leukemia cell lines, *EZH2* amplicon sizes were mostly uniform and the expression level was also consistent (Fig. 1**C**). These data indicate that the expression of *EZH2* varies both in terms of intensity and transcript size between individual MDS patients, but is not dependent on BM blast percentage (Table 1).

### 3.2. Multiple transcripts and protein sizes for EZH2 in MDS patients

Amplification of *EZH2* mRNA from MDS patient samples revealed complex patterns of transcript expression that were difficult to resolve by agarose gel. Considering that previous work has indicated that the loss of EZH2 function contributes to the pathogenesis of MDS, we hypothesized that aberrant *EZH2* transcripts may accumulate in patients with higher expression of *EZH2* mRNA. To investigate our hypothesis, the *EZH2* coding region was amplified from total RNA extracted from BMMNCs of 10 patients that demonstrated relatively strong mRNA expression, and the subsequent fragments were sub-cloned into plasmid vectors and sequenced. In addition, we sub-cloned and sequenced the *EZH2* coding region from the BM of normal controls. The structures of randomly picked clones of these *EZH2* fragments are shown in Fig. 2**A**. All 48 types of clones from 10 MDS patients demonstrated a variety of patterns of alterations in coding sequence, representing insertions or deletions, with or without frameshift; no clone with an *EZH2* wild-type sequence (Genbank: NM_004456.4) was identified. All frameshift alterations (indicated as FS in Fig. 2**A**) were observed 5′ to the SET domain, resulting in premature termination of the coding sequence and the predicted translation of an impaired EZH2, lacking either the

**Table 1**
Patient characteristics.

| | Total |
|---|---|
| Patient number (%) | 26 (100%) |
| Age | 70.5 [17–83] |
| Median[range] | 20 (77) |
| > 60 years old | |
| Sex | 19 (73) |
| male | |
| Subtype | |
| MDS(RCUD/RCMD) | 9 (35) |
| RAEB-1/2 | 7 (27) |
| AML with myelodysplasia-related changes | 7 (27) |
| MDS/MPN (CMMoL) | 4 (15) |
| IPSS* | |
| Lower (Low/Int-1) | 16 (62) |
| Higher (Int-2/High) | 10 (38) |
| Karyotype | |
| G banding | |
| Normal karyotype | 11 (42) |
| Ch. 7 abnormalities | 8 (31) |
| Other abnormalities | 7 (27) |
| SNP array | |
| Normal karyotype | 5 (19) |
| Ch. 7 abnormalities | 5 (19) |
| Other abnormalities | 8 (31) |
| Not tested | 8 (31) |
| Mutation | |
| *EZH2* | 3 (12) |
| *SRSF2* | 3 (12) |
| *U2AF1* | 3 (12) |
| *SF3B1* | 1 (4) |

Abbreviations: MDS, myelodysplastic syndromes; RCUD, refractory cytopenia of unlineage dysplasia; RCMD, refractory cytopenia with multilineage dysplasia; RAEB, refractory anemia with excess blasts; AML, acute myeloid leukemia; MPN, myeloproliferative neoplasms; CMMoL, chronic myelomonocytic leukemia; IPSS, international prognostic scoring system; Int, intermediate; Ch, chromosome; SNP, single nucleotide polymorphism.

* IPSS was evaluated when bone marrow samples were obtained.

catalytic SET domain or both the SET domain and the protein interaction D2 domain (Fig. 2**B**). Multiple clones were also detected in normal controls but the expression level was generally lower than that observed in MDS patients (data not shown). Moreover, the summary of altered *EZH2* transcripts indicated in Fig. 3**A** demonstrates that recurrent alternative splicing events (exon inclusion, skipping, alternative splicing, and intron retention) occurred between MDS patients. Since the percentage of transcripts encoding C-terminally truncated EZH2 protein lacking the SET domain (SET domain-dead transcripts) varied, it was difficult to identify a correlation between the expression of these transcripts and mutations in splicing machinery genes (*SRSF2, U2AF1* and *SF3B1*) or chromosomal 7 deletion (Fig. 3**B**). These data suggest that the function of EZH2 in the BM of MDS patients may be altered by two mechanisms, namely altered overall expression, and the generation of aberrant C-terminally truncated proteins resulting from altered splicing.

### *3.3. Association of aberrant EZH2 expression and mutations in splicing machinery*

Recent progress in our understanding of the pathogenesis of MDS has revealed that mutations in splicing machinery genes are also involved in the development of the disease [11–13]. We thus explored whether the expression of aberrant *EZH2* transcripts was associated with mutations in *EZH2* and/or genes involved in mRNA splicing (*SF3B1*, *SRSF2* and *U2AF1*). We found that the expression level and sizes of amplicons produced using the EZH2-RT2 primer set, which amplifies a preserved 5′ region of the *EZH2* sequence, were consistent between samples regardless of mutation status. In contrast, multiple amplicons were generated using the EZH2-RT3 and EZH2-RT4 primer sets (which amplify 3′ regions of the gene), particularly in patients with *SRSF2* and *U2AF1* mutations (Fig. 4).

### *3.4. Association of EZH2 protein expression with H3K27 tri-methylation status in MDS BM cells*

A previous report has revealed that a loss of function of EZH2, leading to decreased activity of H3K27me3, is involved in MDS pathogenesis. We therefore explored the association between EZH2 expression and H3K27 tri-methylation status in patient BMMNCs. Proteins of the expected size were detected by immunoblotting in normal BMMNC samples from healthy donors, or patients with non-myeloid or no malignancies (C1 to C4 in Fig. 5), while multiple proteins sizes were detected in samples from MDS patients, in keeping with the existence of truncated EZH2 transcripts. However, in the majority of MDS samples, we failed to detect H3K27 tri-methylation by immunoblotting. Together, these data suggest that the aberrant EZH2 proteins may lose methyltransferase activity, resulting in lower levels of endogenous H3K27me3 (Fig. 5).

Finally, we explored whether C-terminally truncated EZH2 proteins might act as dominant negative mutants, competing with wild-type EZH2 for the methylation of H3K27me3. Three different types of FLAG-tagged EZH2, namely a shorter form of the wild-type protein lacking five amino acids (WT2), a C-terminally truncated form lacking the SET domain (EZH2ΔSET), and a C-terminally truncated form lacking the D2, CXC and SET domains (EZH2ΔD2), were transiently overexpressed in 293T cells (Fig. 6**A**). We found that when WT2 was co-expressed with either EZH2ΔSET or EZH2ΔD2, endogenous H3K27me3 was indeed decreased in a dose-dependent manner (Fig. 6**B** and **6C**). These data indicate that truncated EZH2 mutants in MDS might impair wild-type EZH2 function, further contributing to MDS pathogenesis.

## 4. Discussion

In this study, we explored *EZH2* expression in primary MDS patient samples. The data indicate that the expression of *EZH2* varies considerably in patients' BM cells, and is associated with the generation of aberrant *EZH2* transcripts and/or loss of wild-type EZH2 function. Moreover, truncated EZH2 proteins appear to inhibit the histone H3 trimethylation function of normal EZH2 in a dominant negative fashion. These observations suggest that production of aberrant *EZH2* transcripts, induced by alterations in splicing machinery genes, might results in a defective EZH2 phenotype, irrespective of the overall expression level for this gene. Considering that alternative transcripts were observed in almost all of our MDS patients, impaired functionality of EZH2 by this route may be an important mechanism in MDS pathogenesis, over and above the known importance of chromosome 7q deletion or mutations in the *EZH2* gene.

Mutations in splicing machinery genes such as *SF3B1*, *SRSF2,* and *U2AF1*, leading to altered *EZH2* expression, are thought to be involved in the development of MDS [12,13], and those mutations alter pre-mRNA splicing patterns[21,37,38]. Makishima et al. confirmed aberrant splicing of the *TET2* gene in *U2AF1* mutated cells, and aberrant splicing of the *RUNX1* gene has been reported for *U2AF1*-, *U2AF26*-, and *SRSF2*-mutated cells[37]. In our experiments, common deletions or insertions in the *EZH2* sequence were observed between MDS patients, with almost all abnormalities conforming with the GT-AG rule, implying that they were induced by alternative splicing. The aberrant mRNA transcripts result in truncated EZH2 proteins, mostly without the functional SET domain. In addition, the 82 bp insertion that we identified between exons 9 and 10 of *EZH2* is similar to the alternative exon usage reported in the *SRSF2*-mutated mouse (Kim et al. [21]), in which a premature termination codon results in a C-terminally truncated EZH2 protein. Overall, 48 different transcript variants were identified among the 10 MDS patients, and hence it was not possible to identify a correlation with chromosomal abnormalities or splicing machinery

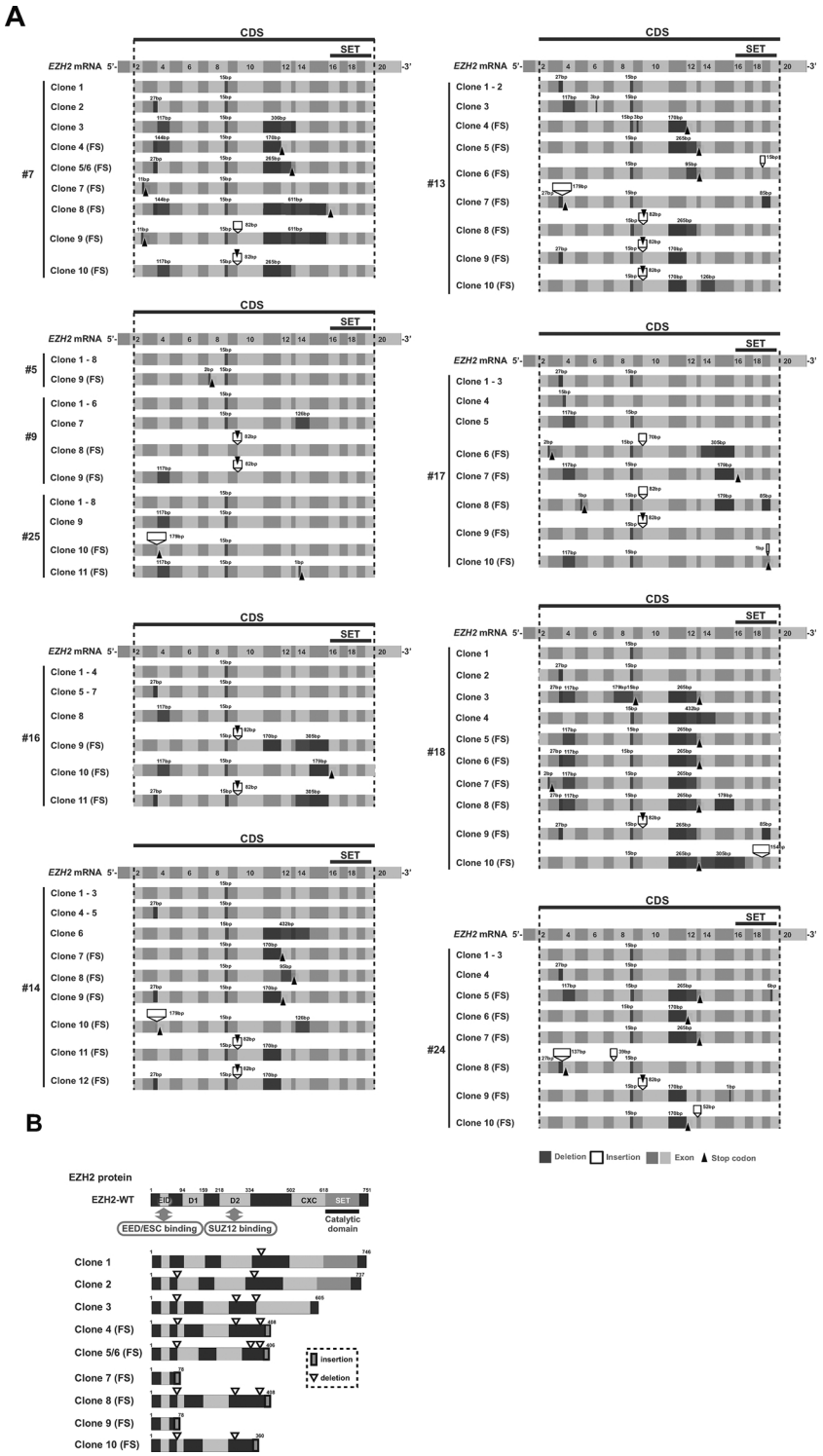


**Fig. 2. EZH2 transcription variants and putative proteins in MDS patients. (A)** The structure of various *EZH2* transcripts amplified from 10 MDS patients are shown. Deletions, insertions, and stop codons are indicated by black squares, white squares, and black triangles, respectively; CDS, coding sequence. **(B)** The structure of putative EZH2 proteins resulting from translation of the disrupted mRNA transcripts from patient UPN7 are shown. The insertions and deletions in these putative proteins are indicated by gray squares and open inverted triangles, respectively.

mutations. Taken together however, our findings suggest that loss of EZH2 function may occur more frequently in MDS patients than previously expected, not only via genetic loss and/or mutation of the *EZH2* gene, but also aberrant mRNA expression resulting from altered splicing, potentially promoted by mutated spliceosomal proteins. Further investigation using comprehensive genetic analyses is required, to detect abnormalities in other spliceosomal genes and splice site mutations in *EZH2*.

A previous study has indicated that patients with *SRSF2* mutation have faster transition to AML, leading to a shorter overall survival [13]. In the present study, we were unable to evaluate clinical outcomes with azacitidine treatment in relation to mutations in *SRSF2* or other splicing machinery genes, due to an insufficient number of patients. Since a recent report indicates that decitabine efficacy is better in patients with *TP53* mutation [39], the efficacy of DNA methyltransferase inhibitors can clearly be influenced by mutation status and further investigations are thus warranted.

In the present study, we confirmed that alternative *EZH2* transcripts, with varying levels of expression, are found in primary MDS patient samples. Moreover, this variation contributes to a loss of function for this important protein. However, it also important to consider normal transcript variants for *EZH2*. Using in-silico PCR in the genome

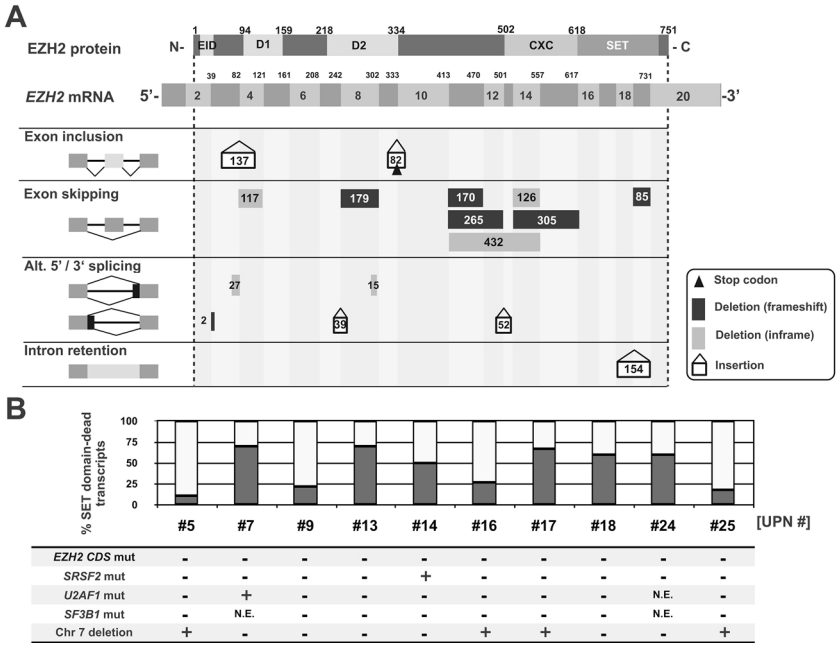


**Fig. 3. Summary of alternative EZH2 transcripts in MDS. (A)** The structure of *EZH2* transcript variants amplified from 10 MDS patients is shown. Recurrent altered splicing events, including exon inclusion, skipping, alternative splicing, and intron retention, are indicated. Frameshift or in-frame deletions, insertions, and stop codons are indicated by black or gray squares, white squares, and black triangles, respectively. The numbers in squares indicate base pairs deleted or inserted. **(B)** The percentage of putative SET domain-deleted transcripts resulting from the translation of disrupted mRNA in each patient. The presence or absence of mutations in *EZH2 CDS*, *SRSF2*, *U2AF1*, *SF3B1*, and chromosome 7 deletion in each patient is shown; N.E., not evaluable.

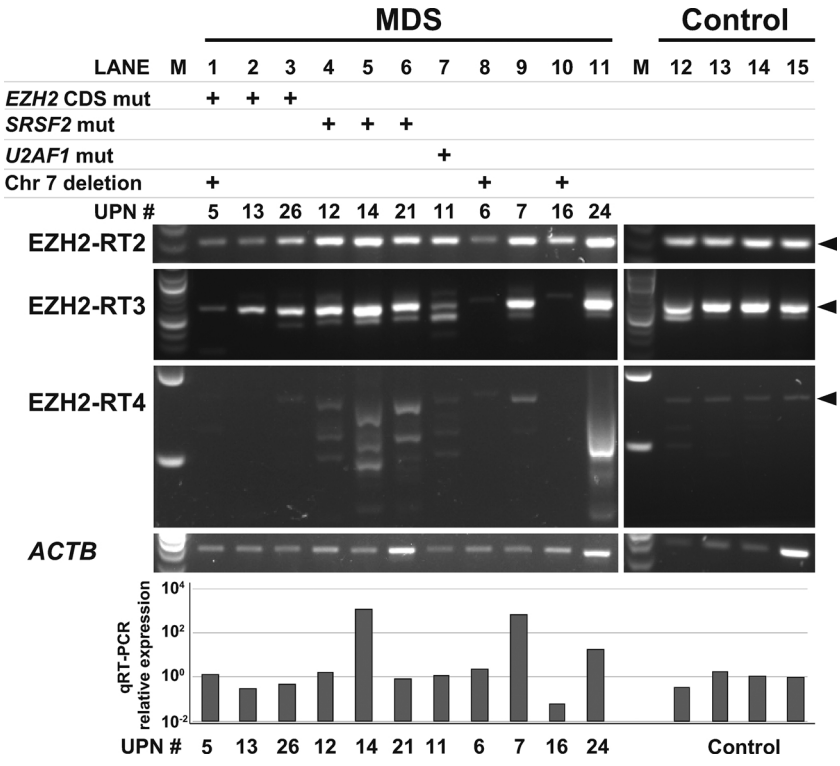


**Fig. 4. Correlation between aberrant mRNA expression and genetic mutations.** RT-PCR products, amplified using the different primer pairs indicated in Fig. 1, were separated by agarose gel electrophoresis. Samples carrying mutations in *EZH2*, *SRSF2*, *U2AF1*, and/or chromosome 7 deletion (+), as detected by chromosomal analysis and SNP array analysis, are indicated above each patient number. Samples without any mutations (mut) in the hot spot of *SF3B1*, *SRSF2* and *U2AF1* are also indicated. Bands that were detected in control samples are indicated by black triangles. The relative expression of *EZH2* relative to *GAPDH* was analyzed by quantitative RT-PCR (lower graph).

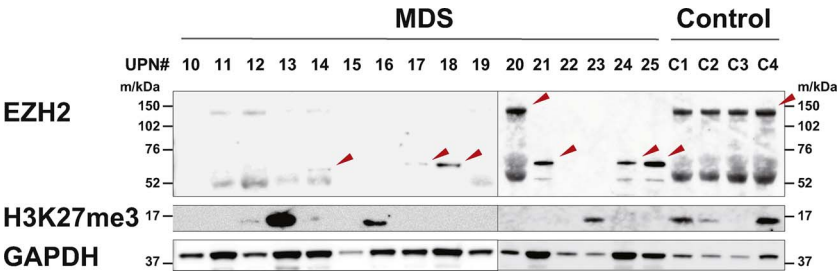


**Fig. 5. EZH2 protein expression in MDS patients.** Immunoblotting for EZH2, H3K27me3, and GAPDH as a loading control, using BM whole cell lysates from MDS patients and normal control donors was performed. Bands of various sizes are indicated by the red arrowheads. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

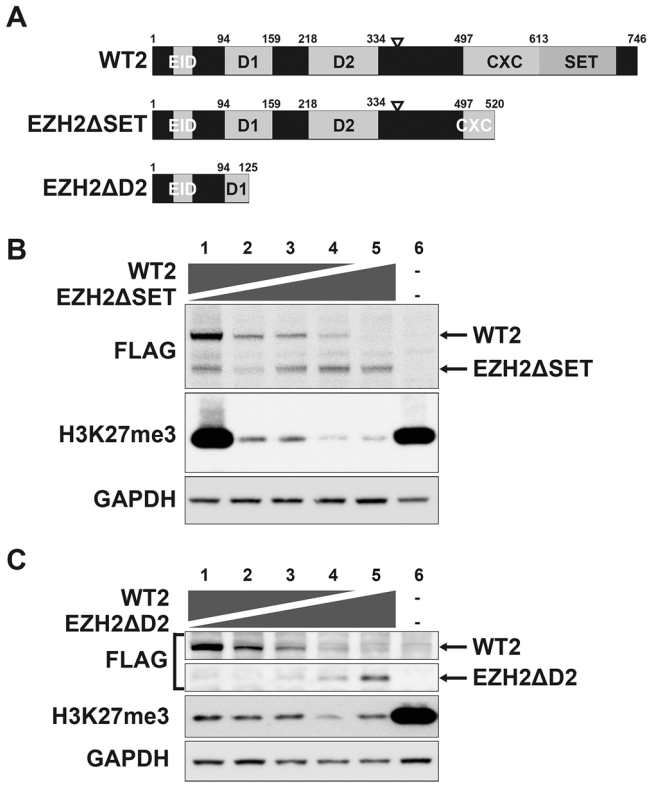


**Fig. 6. Overexpression of C-terminally truncated EZH2 in 293T cells. (A)** Schematic of the structure of the C-terminally truncated EZH2 proteins WT2, EZH2ΔSET, and EZH2ΔD2. WT2 had loss of five amino acids compared to wild-type protein, while EZH2ΔSET and EZH2ΔD2 had truncations of the C-terminal catalytic SET domain and the functional D2 domain, respectively. **(B)** Expression vectors containing WT2 and EZH2ΔSET were co-transfected into 293T cells in inverse ratios, as indicated. Immunoblotting for FLAG, H3K27me3, and GAPDH as a loading control was subsequently performed. **(C)** Co-transfection of WT2 and EZH2ΔD2 into 293T cells, and subsequent immunoblotting, was performed in the same way as in panel (B).

browser, the *EZH2* primer sets used in our study are predicted to amplify a number of products as the result of splicing events. For example, three different sizes of transcript (from 975 to 2029 bp), and six different sizes of transcript (from 2088 to 3251 bp) are obtained as known transcript variants using the EZH2-RT1 and EZH2-Full primer sets, respectively, and we confirmed a number of alternative *EZH2* mRNA transcripts using control samples. Considering the complex pattern of transcripts carrying insertions or deletions that we have identified in this study, in addition to the number of normal variants that may be expressed from *EZH2*, it is reasonable to suggest that aberrant spliceosomal machinery can contribute to MDS pathogenesis.

In this study, we analyzed cells from whole BM, rather than single cells; future studies using sorted blast cells may be useful to demonstrate the biological significance of changes in *EZH2* expression in MDS. Nonetheless, studies of the pattern of *EZH2* expression in primary MDS samples are limited, and our data thus provide important information regarding the significance of *EZH2* splicing and expression in this disease.

## 5. Conclusions

The loss of function of EZH2 induced by abnormal splicing, results in the generation of truncated proteins that are implicated in pathogenesis of MDS.

## Author contributions

C.I., A.T., and H.K. designed research; M.S.-A., C.I., Y.S., and K.S. performed experiments; M.S.-A., C.I., A.T., K.S., and H.K. analyzed and interpreted data; A.T. and H.K. supervised research; M.S.-A., C.I., A.T., K.S., and H.K. wrote the manuscript; and all authors reviewed and revised the manuscript.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2017.10.015.

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