Transferrin internalization in MEFs was measured by adjusting the procedure to a monolayer culture. MEFs were incubated in serum-free medium at 37°C for 2 hours and then in the presence of dye-conjugated transferrin at 37°C for various time periods. After treatment of cells with an acid buffer to remove cell surface transferrin, cells were harvested by trypsin-EDTA treatment and processed for flow cytometry. In the indicated cases, endocytosis and recycling were measured separately using biotinylated transferrin that was prepared by coupling Holo-Transferrin (R&D Systems) and NHS-SS-biotin (Thermo SCIENTIFIC). In the internalization assay, MEFs grown on gelatin-coated dishes were incubated in serum-free medium at 37°C for 2 hours and then in the presence of 20 μg/ml biotinylated transferrin for 30 minutes at 4°C, washed, and further incubated at 37°C for various times. Then, the surface-bound biotinylated transferrin was stripped by treating cells with 50 mM sodium 2-mercaptoethane sulfonate (MESNA) in TNB buffer (50 mM Tris-HCl [pH 8.6], 100 mM NaCl, 0.2% [w/v] BSA) for 30 minutes at 4°C, and MESNA was quenched by 75 mM iodoacetamide in TNB buffer for 30 minutes at 4°C. Lysates were prepared in lysis buffer (10 mM Tris-HCl [pH 7.4], 50 mM NaCl, 1 mM EDTA, 0.2% [w/v] BSA, 0.1% [w/v] SDS, 1% [v/v] Triton X-100) and applied to ELISA plates coated with anti-transferrin antibodies. After washing, antibody-trapped biotinylated transferrin was detected using HRP-conjugated streptavidin (DAKO). In the recycling assay, MEFs were incubated with 20 μg/ml biotinylated transferrin for 40 minutes at 37°C, followed by reduction with MESNA and incubation with 200 μg/ml unlabeled transferrin at 37°C for various times. Preparation of cell lysates and ELISA were the same as that above.

To monitor endocytosis and intracellular transport of c-KIT, MEFs were transfected by signal sequence-tagged EYFP-c-KIT (51) (provided by J. Duyster, Technical University of Munich, Munich, Germany) using a 4D-Nucleofector System (Lonza), incubated with 100 ng/ml SCF for different times, fixed with 4% (w/v) paraformaldehyde in PBS, permeabilized with 0.1 (v/v) Triton X-100 in PBS, and then processed for immunofluorescence detection. To examine the possible involvement of ARF GTPases in the intracellular transport of internalized c-KIT, COS7 cells were transfected with dominant-active forms of ARF1(Q71A), ARF5(Q71A), or ARF6(Q67A) (provided by K. Nakayama, Kyoto University, Kyoto, Japan) together with EYFP-c-KIT. To monitor the internalization of the EGFR, MEFs were incubated with 100 μg/ml Alexa Fluor 488–conjugated EGF (Molecular Probes).

Immunoblotting, immunofluorescence, and RT-PCR. The immunoblotting and immunofluorescence protocols used were as previously described (6). Anti-SMAP1 and anti-SMAP2 antibodies were purchased from Sigma-Aldrich. Anti-SMAP1 antibody was raised against amino acids 210–306 of the SMAP1 protein (440 amino acids), as specified by the manufacturer. The mAbs used were as follows: anti-panARF (Affinity Bioreagents); anti-clathrin heavy chain (Thermo Fisher Scientific); anti–c-KIT and anti-ARF6 (both from Santa Cruz Biotechnology Inc.); anti-ubiquitinated proteins (BIOMOL); anti-Rab11, anti-EEA1, anti-Grb2, and anti–phospho-tyrosine (all from BD Transduction Labs); anti-Rab5, anti-Rab7, anti-phosphorylated ERK1/2, and anti-ERK1/2 (all from Cell Signaling Technology); anti-Hrs (provided by N. Tanaka, Miyagi Cancer Research Center, Natori, Japan); and anti-LBPA (provided by T. Kobayashi, Riken Institute, Wako, Japan). Lysotracker was purchased from Molecular Probes. For immunoprecipitation, BMMCs were lysed with a buffer consisting of 25 mM HEPES-KOH (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100, 2 mM DTT, 5 mM NaF, 5 mM Na_2VO_4, and a mixture of protease inhibitors (Roche Diagnostics). The cellular fluorescence intensities and extent of fluorescence colocalization were measured using a confocal microscope, LSM-510, and the LSM5 Image Examiner tool (Zeiss). Colocalization coefficient was calculated as the ratio of $pixels^{colocalized\ c\text{-}Kit}$/$pixels^{total\ c\text{-}Kit}$. For RT-PCR, RNA was extracted from isolated cells using the TRIzol reagent and reverse transcribed using SSRT II (Invitrogen). The primers used were as follows: for *SMAP1*, 5′-CTGAGGGAGGAGGACAACAAGTAC-3′ (forward on exon 1), 5′-GAAGCCAATCTTCCAGAGAAC-3′ (forward on exon 3), and 5′-GTAACGGTAGACAGGGTAGCAGGT-3′ (reverse on exon 9); for *SCF*, 5′-GAAGAAAACGCACCGAAGAA-3′ (forward) and 5′-TAAGGCTCCAAAAGCAAAGC-3′ (reverse).

Pull-down assay using GST-GGA1. A *DH5α* strain of *E. coli* was transformed by a *GST-GGA1* fusion cDNA (52) (provided by K. Nakayama). Bacteria were grown in LB media and treated with 0.4 mM IPTG for 60 minutes to induce protein expression. Cells were lysed in B-PER buffer (Pierce), and the lysate was centrifuged to obtain a supernatant containing GST-GGA1. Bone marrow cells were lysed with a buffer consisting of 150 mM KCl, 2 mM $MgCl_2$, 10% (v/v) glycerol, 1 mM DTT, 1 mM EGTA, 1 mM EDTA, 1% (v/v) Triton X-100, 50 mM Tris-HCl (pH 8.0), and 1× protease inhibitors and incubated with purified GST-GGA1 that had been coupled with glutathione-Sepharose beads (Pierce). The bound protein was recovered by eluting with SDS-loading buffer and processed for immunoblot analyses.

Statistics. Statistical significance was evaluated using 2-tailed Student's *t* test, and differences of $P < 0.05$ were considered statistically significant.

Study approval. Animal protocols were reviewed and approved by the Animal Studies Committee of the Tohoku University.

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AUTHORSHIP CONTRIBUTION

LLS, AJW, JH, and Y-YY performed the experiments. LLS, AJJ, and JCB analyzed the results and made the figures. XZ performed the statistical analysis. JF, JJ, MRG, and JCB provided clinical samples. AJJ, RB, MRG and JCB designed the research and wrote the paper.

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EED mutants impair polycomb repressive complex 2 in myelodysplastic syndrome and related neoplasms

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Polycomb recessive complex 2 (PRC2) is an epigenetic regulator that marks repressive chromatin domain through trimethylation of histone H3 lysine 27 (H3K27).[1,2] Recently, inactivating mutations of EZH2, the catalytic subunit of PRC2, have been identified in subsets of myeloid disorders including myelodysplastic syndrome (MDS), and are predicted to inactivate PRC2 function.[3–5] PRC2 comprises four core components, EZH2, EED, SUZ12 and RBBP4 (also known as RbAp48). Although EZH2 possesses the methyltransferase activity of PRC2, EZH2 is inactive on its own, and direct interaction of EED to EZH2 is required for EZH2 to fully exert its enzymatic activity.[1,2,6] In addition, EED binds to trimethylated H3K27 (H3K27me3) through the so-called 'aromatic cage' composed of three aromatic amino acids (Phe97, Trp364 and Tyr365) to activate PRC2.[1,7] A previous report demonstrated that PRC2 complexes possessing an aromatic-cage mutation in EED show severely reduced enzymatic activities in the presence of H3K27me3 peptides.[7] EED haploinsufficiency (Leu196Pro) in mice leads spontaneously to a myeloproliferative disorder,[8] and exposure of hypomorphic (Ile193Asn) homozygotes to genotoxic stresses gives rise to tumorigenesis.[8,9] These findings strongly suggest that dysfunction of EED might be involved in the pathogenesis of myeloid disorders. Here we searched for EED mutations in MDS and related diseases.

The genomic regions encompassing the EED gene (NCBI accession number NM_003797.2) were sequenced in bone marrow samples obtained from 192 patients with MDS or related diseases (Figure 1a). Detailed clinical information of individual samples was given in our previous report.[10] We identified EED mutations in six cases (Figure 1a and Supplementary Table 1), which were confirmed by repeated amplification and sequencing. None of these mutations are reported in the 1000 genomes database (a deep catalog of human genetic variation)[11] and the Ensemble gene and the transcript sequences currently available. We were unable to evaluate whether the mutations arose from germline or somatic tissues or to examine mRNA expression patterns, due to limited sample availability. Of note, three of the six mutations occurred in patients with chronic myelomonocytic leukemia (CMML) or acute myelogenous leukemia possibly preceded by CMML (Figure 1a). This is consistent with the finding that EZH2 abnormalities are most common in CMML and MDS/myelodysplastic-myeloproliferative neoplasms (MDS/MPN).[4] In addition, we observed no compound mutations of the EED and

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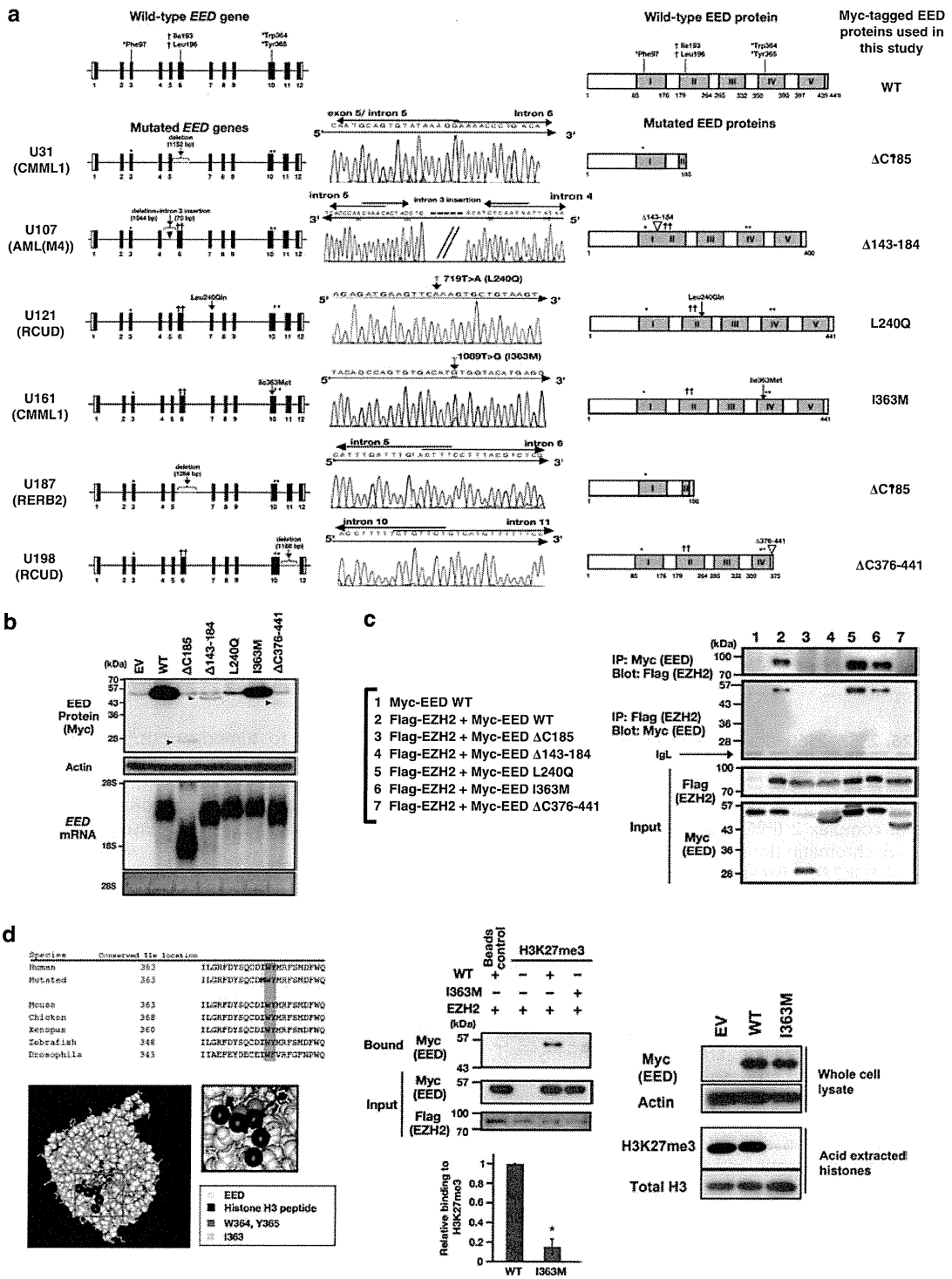
EZH2 genes (Figure 2a), strongly suggesting that these genes may independently affect PRC2 function.

Two distinct mutations lacking a part of intron 5 and the entire exon 6 were found in individuals with CMML1 and refractory anemia with excess blasts 2 (RAEB2) (U31 and U187, respectively) (Figure 1a). In these patients, exon 5 should be either spliced to exon 7 or followed by intron 5, and in each case consequently creates a new stop codon; therefore the mutations are predicted to produce proteins lacking part of the WD40 II motif and all of the WD40 motifs III–V (hereafter referred to as the ΔC185 mutant) (Figure 1a). A genomic region containing exon 5 was absent in an acute myelogenous leukemia (M4) patient with myelodysplasia-related changes arising from CMML (U107) (Figure 1a). In U107 subject, exon 4 should be followed by exon 6 in frame; therefore, the corresponding protein product (hereafter referred to as the Δ143-184 mutant) lacks part of the first and second



WD40 repeats (Figure 1a). One refractory cytopenia with unilineage dysplasia subject (U121) possessed a missense mutation (T719A; CTA > CAA), producing a Leu363Gln amino-acid substitution (hereafter referred to as the L240Q mutant) (Figure 1a). In addition, another CMML1 subject (U161) possessed a missense mutation (T1089G; ATT > ATG), producing an Ile363Met amino-acid substitution (hereafter referred to as the I363M mutant) (Figure 1a). The exon 11 deletion in another refractory cytopenia with unilineage dysplasia subject (U198) is predicted to produce an EED protein that lacks one and a half of the WD40 repeat motifs located at the C terminus (hereafter referred to as the ΔC376-441 mutant), as exon 10 should be followed by exon 12 with premature stop

a

Patient ID[a]	WHO classification	Age	Sex	IPSS[b]	Cytogenetics	SNP array[a]	*EED* mutation	Predicted EED protein	Expression	Binding EZH2	Binding H3K27me3	*EZH2* status
U31	CMML1	64	F	INT-1	46,XX	+Xp	Δexon 6	ΔC185	low	-	ND	WT
U107	AML(M4)	50	M	NA	45,XY,-7	-7	Δexon 5	Δ143-184	low	-	ND	WT
U121	RCUD	32	F	INT-1	46,XX	Normal	T719A	L240Q	low	+	ND	WT
U161	CMML1	35	M	INT-1	46,XY	11qUPD, 18qUPD	T1089G	I363M	normal	+	↓	WT
U187	RAEB2	59	M	HIGH	46,XY,-7,+13	-7,+13	Δexon 6	ΔC185	low	-	ND	WT
U198	RCUD	58	F	INT-1	46,XX	Normal	Δexon 11	ΔC376-441	low	-	ND	WT

b

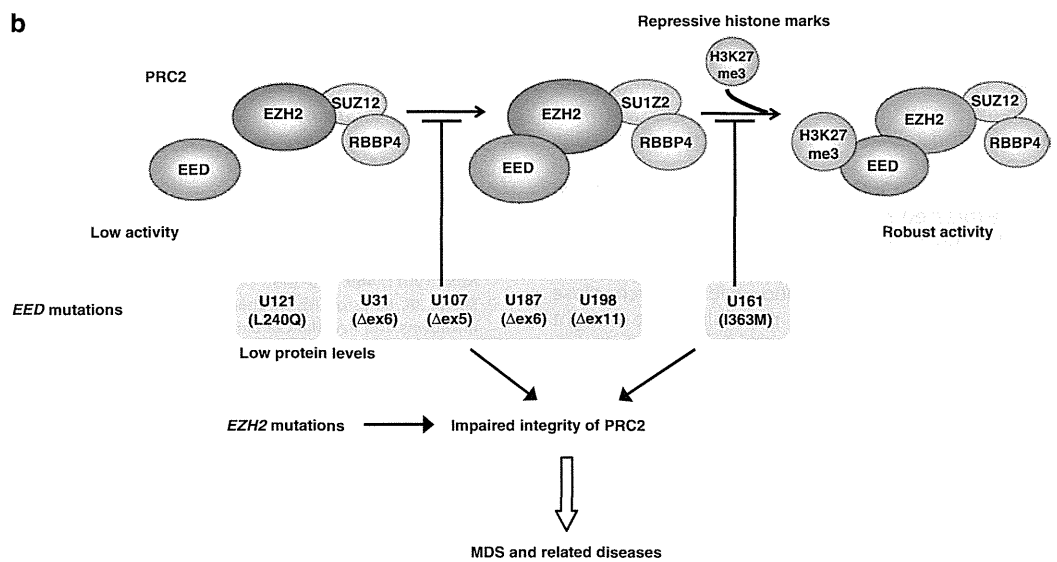


Figure 2. *EED* mutations in MDS and related diseases. (**a**) Characterization of samples with *EED* mutation. [a]Patient ID and SNP array are as previously described.[10] [b]Classification is according to the International Prognostic Scoring System. ND, not determined. (**b**) Impaired integrity of PRC2 promotes the pathogenesis of MDS and related diseases. *EED* exon deletions and L240Q mutation inhibit the PRC2 function through lower expression of EED proteins with or without impaired binding to EZH2. The I363M mutation interrupts the active interaction between H3K27me3 and EED.

Figure 1. Functionally defective mutations of *EED* in MDS and related diseases. (**a**) *EED* mutations detected in individuals with MDS and related diseases. The exon/intron structure, nucleotide sequence chromatogram, predicted protein structure and the Myc-tagged EED proteins used in this study are shown. I-V, WD40 repeat motifs. Dagger symbols indicate amino acids whose mutations were linked to myeloproliferative disorder in mice. Asterisks indicate amino acids necessary for binding to H3K27me3. In exon-deleted subjects (U31, U107, U187 and U198), PCR spanning the deletion point resulted in two distinct bands. Sequence chromatograms show the results from direct sequencing of the purified smaller bands, whereas the larger bands represented the corresponding wild-type *EED* sequences (data not shown). (**b**) EED protein and mRNA levels following forced expression in 293T cells. Cells were harvested with 2% SDS-sample buffer for western blot analysis (top) or Trizol reagent for northern blot analysis (bottom). Arrowheads indicate poorly expressed proteins of exon deletion mutants. EV, empty vector. (**c**) Interaction of EZH2 with EEDs. 293T cells were transfected with plasmids expressing the proteins indicated. The amounts of plasmids were adjusted to achieve similar protein expression levels at the stage of transfection. Cell lysates were immunoprecipitated and analyzed by western blot using anti-Flag (EZH2) and anti-Myc (EED) antibodies. Input represents 0.5% or 2% of cell lysate used for IP (Myc or Flag, respectively). IgL, immunoglobulin light chain. (**d**) I363M mutant decreases global H3K27me3 levels through impaired interaction to H3K27me3. Left: The alignment of amino-acid sequences surrounding Ile363. Blue-shaded boxes indicate well-conserved aromatic cage residues. The yellow-shaded box indicates the Ile363Met mutation found in subject U161 CMML. (Protein DataBank ID code: 3IIW). The boxed area is magnified on the right side. Middle: Myc-tagged wild-type EED (WT) or I363M mutant was co-expressed with Flag-tagged EZH2 in 293T cells. Cell lysates were analyzed in a pull-down assay using H3K27me3 peptide by western blot using an anti-Myc antibody. Input represents 0.5% of cell lysate used for the pull-down assay. Relative binding efficiencies (WT = 1) were estimated by normalizing the densitometry values representing the bound EED against those from the 'Input EED'. Bar graph: mean binding ± s.e. from three independent experiments. $*P = 0.0028$ (Student's *t*-test). Right: Myc-tagged wild-type EED (WT) or I363M mutant was retrovirally transduced in NIH3T3 cells. Acid-extracted histones were analyzed by western blot using an anti-H3K27me3 or an anti-total H3.

codon (Figure 1a). In subjects with a deletion (U31, U107, U187 and U198), the deletion points demonstrated the joining of the 5′ part and 3′ part with 1- to 8-base-pair microhomology (Figure 1a), which suggests the possibility that these deletions resulted from recombination with unequal crossover.[12]

We first evaluated the cellular expression levels of the mutant proteins following forced expression in 293T cells. The result showed that exon deletions (ΔC185, Δ143-184, L240Q and ΔC376-441) and L240Q mutant expressed significantly less protein than did either the wild-type or I363M (Figure 1b, top, indicated by arrowheads), despite a similar level of expression of the corresponding mRNAs (Figure 1b, bottom). These results indicate that the exon deletions and L240Q impaired translation and/or stability of the EED proteins, and suggest that these mutated forms are hypomorphic, and thus are functionally defective. Interaction of EED with EZH2 was demonstrated to be necessary for the full activity of PRC2;[2] hence we next examined the binding ability of the mutant proteins to EZH2. As expected from the results of the previous studies showing that WD repeats are important for EED–EZH2 interaction,[6,13] the ΔC185, Δ143-184 and ΔC376-441 failed to bind to EZH2 (Figure 1c). L240Q, in contrast, showed comparable binding ability to EZH2 as compared with wild-type EED. Thus, these results suggest that exon deletions and an L240Q mutation disrupt the functional integrity of PRC2, owing to poor protein expression coupled with or without their inability to bind EZH2.

On the other hand, the I363M mutant retained the ability to bind EZH2 and was expressed at a level similar to that of the wild-type EED (Figures 1b and c), suggesting that this mutant could incorporate into the PRC2 complex comparably with the wild type. However, substitution of an amino acid in such close proximity to the cage residues raised the possibility that it might affect the EED-H3K27me3 interaction, and therefore PCR2 function (Figure 1d, left).[7] We compared the binding of wild-type and I363M mutant EED in a pull-down assay that employed a synthetic H3K27me3 peptide ligand. Intriguingly, the I363M substitution significantly inhibited the EED-H3K27me3 interaction when co-expressed in the presence of EZH2 (Figure 1d, middle). In addition, global H3K27me3 levels were severely decreased in cells stably overexpressing the I363M mutant (Figure 1d, right), suggesting that the PRC2 complex incorporating the I363M mutant is functionally compromised, possibly through impaired structural integrity of the aromatic cage.

In summary, all the six mutated forms of EED displayed functional defects involving changes: (i) protein stability, (ii) interaction with EZH2 and/or (iii) binding to H3K27me3, thereby impairing PRC2 function (Figure 2b). We suggest that, in addition to inactivating mutations of catalytic EZH2,[3–5] non-catalytic EED mutations exclusively perturb PRC2-mediated epigenetic regulation and substantially contribute to the pathogenesis of MDS and related diseases (Figure 2b). Recently, Score *et al.*[14] reported a set of defective gene mutations of PRC2 constituents, including an EED point mutation, Gly255Asp, in 148 MDS/MPN cases. Our data suggest that various types of defective EED mutations contribute to the MDS pathogenesis. Analysis of more samples would clarify the clinical features of patients with EED mutation(s) in MDS and related diseases. Our findings highlight that recurrent mutations in PRC2 may constitute a new molecular-based disease category of myeloid malignancies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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LETTERS TO THE EDITOR

Novel splicing-factor mutations in juvenile myelomonocytic leukemia

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Myelodysplastic syndromes (MDS) and myelodysplastic/myeloproliferative neoplasms (MDS/MPN) are heterogeneous groups of chronic myeloid neoplasms characterized by clonal hematopoiesis, varying degrees of cytopenia or myeloproliferative features with evidence of myelodysplasia and a propensity to acute myeloid leukemia (AML).[1] In recent years, a number of novel gene mutations, involving *TET2, ASXL1, DNMT3A, EZH2, IDH1/2,* and *c-CBL,* have been identified in adult cases of chronic myeloid neoplasms, which have contributed to our understanding of disease pathogenesis.[2–7] However, these mutations are rare in pediatric cases, with the exception of germline or somatic *c-CBL* mutations found in 10–15% of chronic myelomonocytic leukemia (CMML) and juvenile myelomonocytic leukemia (JMML),[8] highlighting the distinct pathogenesis of adult and pediatric neoplasms.[9]

Recently, we reported high frequencies of mutations, involving the RNA splicing machinery, that are largely specific to myeloid neoplasms, showing evidence of myeloid dysplasia in adult.[10] Affecting a total of eight components of the RNA splicing machinery (U2AF35, U2AF65, SF3A1, SF3B1, SRSF2, ZRSR2, SF1 and PRPF40B) commonly involved in the 3′ splice-site (3′SS) recognition, these pathway mutations are now implicated in the pathogenesis of myelodysplasia.[10] To investigate the role of the splicing-pathway mutations in the pathogenesis of pediatric myeloid malignancies, we have examined 165 pediatric cases with AML, MDS, chronic myeloid leukemia (CML) and JMML for mutations in the four major splicing factors, *U2AF35, ZRSR2, SRSF2,* and *SF3B1,* commonly mutated in adult cases.

Bone marrow or peripheral blood tumor specimens were obtained from 165 pediatric patients with various myeloid malignancies, including *de novo* AML ($n=93$), MDS ($n=28$), CML ($n=17$) and JMML ($n=27$), and the genomic DNA (gDNA) was subjected to mutation analysis (Supplementary Table 1). The status of the RAS pathway mutations for the current JMML series has been reported previously (Supplementary Table 2).[11,12] Nineteen leukemia cell lines derived from AML (YNH-1, ML-1, KASUMI-3, KG-1, HL60, inv-3, SN-1, NB4 and HEL), acute monocytic leukemia (THP-1, SCC-3, J-111, CTS, P31/FUJ, MOLM-13, IMS/MI and KOCL-48) and acute megakaryoblastic leukemia (CMS and CMY) were also analyzed for mutations. Peripheral blood gDNA from 60 healthy adult volunteers was used as controls. Informed consent was obtained from the patients and/or their parents and from the healthy volunteers. We previously showed that for *U2AF35, SRSF2* and *SF3B1,* most of the mutations in adult cases were observed in exons 2 and 7, exon 1, and exons 14 and 15, respectively.[10] Therefore, we confirmed mutation screening to these 'hot-spot' exons. In contrast, all the coding exons were examined for *ZRSR2,* because no mutational hot spots have been detected. Briefly, the relevant exons were amplified using PCR and mutations were examined by Sanger sequencing, as previously described.[10] The Fisher's exact test was used to evaluate the statistical significance of frequencies of mutations for *U2AF35, SF3B1, ZRSR2* or *SRSF2* in adult cases and pediatric cases. This study was approved by the Ethics Committee of the University of Tokyo (Approval number 948-7).

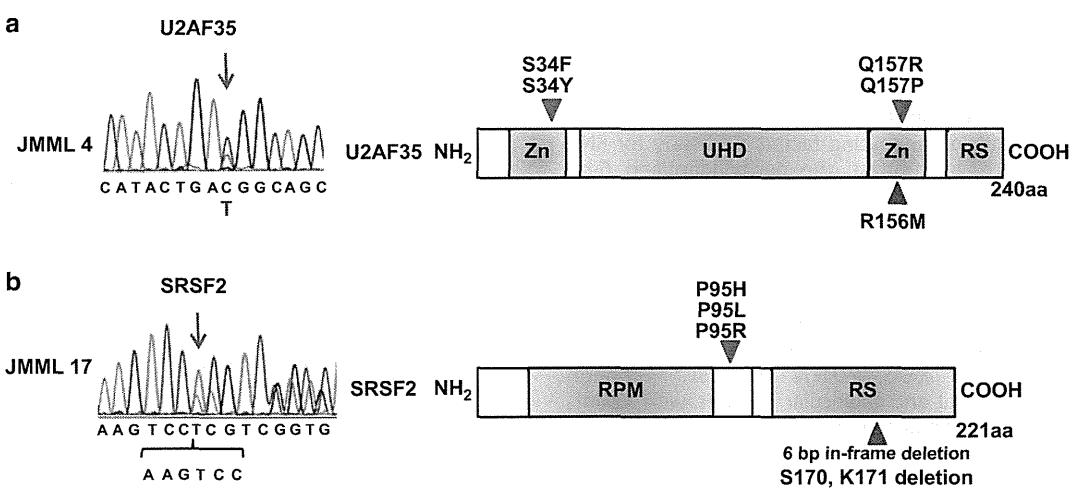

Figure 1. Novel *U2AF35*and *SRSF2* mutations detected in JMML cases. (**a**) Left panel: sequence chromatogram of a heterozygous mutation at R156 in N-terminal zinc-finger motifs of *U2AF35* detected in a JMML case (JMML 4) is shown. Mutated nucleotides are indicated by arrows. Right panel: illustration of functional domains and mutations of U2AF35. Red arrow heads indicate hot-spot mutations at S34 and Q157 detected in the adult cases.[10] Blue arrow head indicates the missense mutation at R156. (**b**) Left panel: sequence chromatogram of a 6-bp in-frame deletion (c.518-523delAAGTCC) in *SRSF2* detected in JMML 17 is shown. Mutated nucleotides are indicated by arrows. Right panel: illustration of functional domains and mutations of SRSF2. Red arrow head indicates hot-spot mutation at P95 frequently detected in the adult cases.[10] Blue arrow head indicates a 6-bp in-frame deletion leading to deletion of S170 and K171.

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No mutations were identified in the 28 cases with pediatric MDS, which included 13 cases with refractory anemia with excess blasts, 5 with refractory cytopenia of childhood, 2 with Down syndrome-related MDS, 2 with Fanconi anemia-related MDS, 2 with secondary MDS and 4 with unclassified MDS. Similarly, no mutations were detected in 93 cases with *de novo* AML or in 17 with CML, as well as 19 leukemia-derived cell lines. Our previous study in adult patients showed the frequency of mutations in *U2AF35*, *SF3B1*, *ZRSR2* or *SRSF2* to be 60/155 cases with MDS without increased ring sideroblasts and 8/151 *de novo* AML patients, emphasizing the rarity of these mutations in pediatric MDS ($P<5.0\times10^{-6}$) and AML ($P<0.02$) compared with adult cases. We found mutations in two JMML cases, JMML 4 and JMML 17. JMML 4 carried a heterozygous *U2AF35* mutation (R156M), whereas JMML 17 had a 6-bp in-frame deletion (c.518-523delAAGTCC) in *SRSF2* that resulted in deletion of amino acids S170 and K171 (Figure 1). Both nucleotide changes found in *U2AF35* and *SRSF2* were neither identified in the 60 healthy volunteers nor registered in the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) or in the 1000 genomes project, indicating that they represent novel spliceosome mutations in pediatric cases.

U2AF35 is the small subunit of the U2 auxiliary factor (U2AF), which binds an AG dinucleotide at the 3′SS, and has an essential role in RNA splicing.[13] With the exception of a single A26V mutation found in a case of refractory cytopenia with multilinage dysplasia, all the U2AF35 mutations reported in adult myeloid malignancies involved one of the two hot spots within the two zinc-finger domains, S34 and Q157, which are highly conserved across species, suggesting the gain-of-function mutations.[10] In JMML 4, the R156M U2AF35 mutation affects a conserved amino acid adjacent to Q157, suggesting it may also be a gain-of-function mutation, leading to aberrant pre-mRNA splicing possibly in a dominant fashion.

SRSF2, better known as SC35, is a member of the serine/arginine-rich (SR) family of proteins.[14] SRSF2 binds to a splicing-enhancer element in pre-mRNA and has a crucial role not only in constitutive and alternative pre-mRNA splicing but also in transcription elongation and genomic stability.[14] All mutations thus far identified in adult cases exclusively involved P95 within the intervening sequence between the N-terminal RNA-binding domain and the C-terminal RS domain.[10] This region interacts with other SR proteins, again suggesting that the P95 mutation may result in gain-of-function.[10] This proline residue is thought to determine the relative orientation of the two flanking domains of SRSF2, and a substitution at this position could compromise critical interactions with other splicing factors necessary for RNA splicing to take place. In contrast, the newly identified 6-bp in-frame deletion in JMLL 17 results in two conserved amino acids, S170 and K171, within the RS domain. Although it may affect protein–protein interactions, the functional significance of this deletion remains elusive.

JMML is a unique form of pediatric MDS/MPN characterized by activation of the RAS/mitogen-activated protein kinase signaling pathway; in 90% of cases, there are germ line and/or somatic mutations of *NF1*, *NRAS*, *KRAS*, *PTPN11* and *CBL*.[8] Although JMML shares some clinical and molecular features with CMML, its spectrum of gene mutations suggests that it is a neoplasm distinct from CMML.[15] This was also confirmed by the current results that the splicing-pathway mutations are rare in JMML, whereas they are extremely frequent (~60%) in CMML.[10] Although the two JMML cases carrying the splicing-pathway mutations had no known RAS-pathway mutations, both the pathway mutations frequently coexisted in CMML.[8]

To summarize, no mutations of *SF3B1*, *U2AF35*, *ZRSR2* or *SRSF2* are found in pediatric MDS and AML. In our study, except for *ZRSR2*, mutations were examined focusing on the reported hot spots in adult studies, raising a possibility that we may have missed some mutations occurring in other regions. However, these hot spots represent evolutionally conserved amino acids and have functional relevance, it is unlikely that the distribution of hot spots in children significantly differs from adult cases and as such, we could safely conclude that mutations of *SF3B1*, *U2AF35*, *ZRSR2* and *SRSF2* are rare in myeloid neoplasms in children. Finally, mutations of *U2AF35* and *SRSF2* may have some role in the pathogenesis of JMML, although further evaluations are required.

CONFLICT OF INTEREST

The authors declare no conflict interest.

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Sequencing histone-modifying enzymes identifies UTX mutations in acute lymphoblastic leukemia

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Mutations affecting epigenetic regulators have long been known to have a crucial role in cancer and, in particular, hematological malignancies.[1,2] One of the earliest epigenetic factors described altered in leukemia was the mixed lineage leukemia (*MLL*) protein which is found translocated in 10% of adult acute myeloid leukemia (AML), 30% of secondary AML and >75% of infants with both AML and acute lymphocytic leukemia (ALL). *MLL* is a SET domain-containing protein, which is recruited to many promoters and mediates histone 3 lysine 4 (H3K4) methyltransferase activity, thought to promote gene expression.[3]

In addition to *MLL* fusions, recently, somatic mutations of *UTX* (also known as *KDM6A*), encoding an H3K27 demethylase, were described in multiple hematological malignancies, including multiple myeloma and many types of leukemia cell lines.[4,5] H3K27 methylation is generally thought to cause gene repression. Complimentary to *UTX*, mutations of *EZH2*, a H3K27 methyltransferase, have been reported in both lymphoid and myeloid tumors (Figure 1).[6,7] These mutations lead to altered EZH2 activity and influence H3K27 in tumor cells. Mutations in EZH2, EED and SUZ12, which all cooperate in Polycomb repressive complex 2 have been recently described in early T-cell precursor ALL.[8] Similarly, point mutations affecting the functional jumonji C (jmjC) domain of UTX inactivates its H3K27 demethylase activity. In addition, UTX associates with MLL2 in a multiprotein complex, which promotes H3K4 methylation, and recently *MLL2* has also been found mutated in cancer, further pointing to a common and complex epigenetic deregulation in cancer.[9] In line with the growing evidence for epigenetic regulators as important in tumorigenesis, additional mutations affecting epigenetic regulators such as SETD2, a H3K36 methyltransferase, KDM3B, a H3K9 demethylase, and KDM5C, a H3K4 demethylase, have been reported and are associated with distinct gene expression patterns (Figure 1).[4]

Though the clinical significance of these findings remains to be explored, it is evident that epigenetic deregulation is having an important role in both lymphoid and myeloid leukemogenesis. Furthermore, with novel drugs at hand, such as histone deacetylase inhibitors or demethylating agents that can target and reverse epigenetic alterations, understanding the underlying molecular aberrations is of growing interest.[10] We therefore undertook an effort to examine the prevalence of somatic mutations in genes encoding histone-modifying proteins, in particular, *KDM3B*, *KDM5C*, *UTX*, *MLL2*, *EZH2* and *SETD2*, which previously were reported mutated in cancer.[4,5]

For an initial screen, we analyzed banked diagnostic primary leukemia samples from 44 childhood B-cell ALL and 50 adult AML patients, and, where available, used bone marrow samples obtained in complete remission to validate the somatic nature of the mutations. Samples had been collected with patient/parental informed consent from patients enrolled on Dana–Farber Cancer Institute protocols for childhood ALL (DFCI 00-001 (NCT00165178), DFCI 05-001 (NCT00400946)) or AML treatment protocols of the German-Austrian AML Study Group (AMLSG) for younger adults (AMLSG-HD98A (NCT00146120), AMLSG 07-04 (NCT00151242)), and the study was approved by the IRB of the participating centers.

Using conventional Sanger sequencing of primary leukemia sample-derived genomic DNA, we first screened all coding exons in which mutations have been reported previously.[4,5] Initially, we analyzed a total of 36 of 174 exons (*KDM3B* (2/24), *KDM5C* (9/26), *UTX* (7/29), *MLL2* (8/54), *EZH2* (1/20) and *SETD2* (9/21)) and found 7 non-synonymous tumor-specific aberrations. In AML, we found one *EZH2* mutation (p.G648E) in a t(8;21)-positive, and two *MLL2* missense mutations (p.R5153Q and p.Y5216S; Table 1) and one

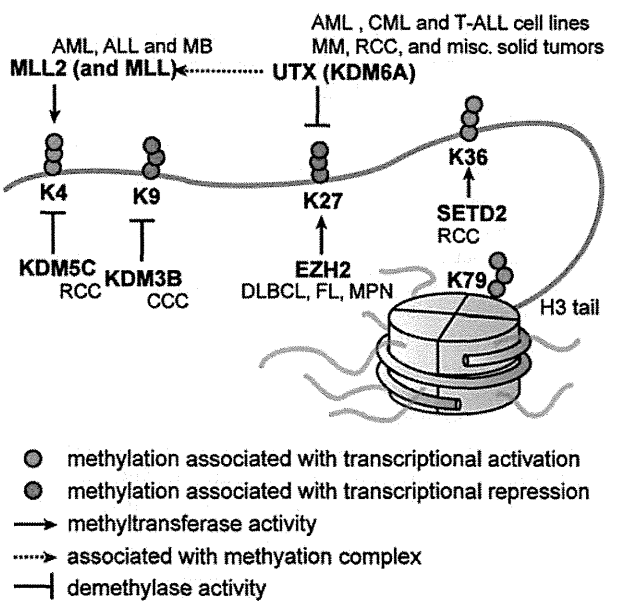

Figure 1. Histone 3 methylation and selected histone demethylases and methyltransferases. Cancers are shown in italics next to the mutated protein they are associated with. MM, multiple myeloma; FL, follicular lymphoma; DLBCL, diffuse large B-cell lymphoma; RCC, renal cell carcinoma; CCC clear cell carcinoma; MPN, myeloproliferative neoplasm; MB, medulloblastoma.

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Favorable outcome of patients who have 13q deletion: a suggestion for revision of the WHO 'MDS-U' designation

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ABSTRACT

To characterize bone marrow failure with del(13q), we reviewed clinical records of 22 bone marrow failure patients possessing del(13q) alone or del(13q) plus other abnormalities. All del(13q) patients were diagnosed with myelodysplastic syndrome-unclassified due to the absence of apparent dysplasia. Elevated glycosylphosphatidylinositol-anchored protein-deficient blood cell percentages were detected in all 16 with del(13q) alone and 3 of 6 (50%) patients with del(13q) plus other abnormalities. All 14 patients with del(13q) alone and 2 of 5 (40%) patients with del(13q) plus other abnormalities responded to immunosuppressive therapy with 10-year overall survival rates of 83% and 67%, respectively. Only 2 patients who had abnormalities in addition to the del(13q) abnormality developed acute myeloid leukemia. Given that myelodysplastic syndrome-unclassified with del(13q) is a benign bone marrow failure subset characterized by good response to immunosuppressive therapy and a high prevalence of increased glycosylphosphatidylinositol-anchored protein-deficient cells, del(13q) should not be considered an intermediate-risk chromosomal abnormality.

Key words: glycosylphosphatidylinositol-anchored protein-deficient, cells, bone marrow failure, 13q deletion, immunosuppressive therapy.

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Introduction

Numerical karyotypic abnormalities such as -7/del(7q) and del(13q) are occasionally seen in patients with bone marrow (BM) failure who do not exhibit typical signs of myelodysplasia. The 2008 World Health Organization (WHO) criteria defined this subset of BM failure as myelodysplastic syndrome-unclassified (MDS-U) because patient progression to leukemia was still possible. However, no large patient study has been conducted to explore an association between del(13q) and pre-leukemia.[1] Several anecdotal reports have shown that BM failure patients with del(13q) responded to immunosuppressive therapy (IST) and had a favorable prognosis.[2,3] However, the incidence of BM failure with del(13q) and its relationship with immune pathophysiology of BM failure remain unclear.

Several studies have identified the presence of small populations of glycosylphosphatidylinositol-anchored protein-deficient (GPI-AP$^-$) blood cells as a significant factor predicting a good response to IST in patients with aplastic anemia (AA) and low-risk myelodysplastic syndromes (MDS).[4,5] Immune mechanisms are, therefore, thought to be involved in the increase in the GPI-AP$^-$ cells in this type of BM failure, though the exact mechanisms responsible for the increase in the GPI-AP$^-$cells remain unknown. Given that BM failure with del(13q) is likely to respond to IST, this type of BM failure may be associated with the presence of small populations of GPI-AP$^-$ cells. It is essential to precisely characterize BM failure with del(13q) because the present WHO definition of an intermediate-risk abnormality may lead to inappropriate treatment of potentially benign BM failure with hypomethylating agents or allogeneic stem cell transplants from unrelated donors. To address this issue, the present study analyzed clinical and genetic features of 22 BM failure patients possessing del(13q) by comparing them to BM failure patients with a normal karyotype.

Design and Methods

Study subjects

Clinical records were analyzed for 1,228 BM failure patients: 733 with aplastic anemia (AA), 495 with low-risk MDS, including 286 with refractory cytopenia with unilineage dysplasia (RCUD), 149 with

The online version of this article has a Supplementary Appendix.

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refractory cytopenia with multilineage dysplasia (RCMD) and 60 with MDS-U, whose blood samples were sent to our laboratory between May 1999 and July 2010 for screening of GPI-AP$^-$ granulocytes and erythrocytes. BM smear slides were reviewed by 2 independent hematologists. BM cellularity was defined as the percentage of BM volume occupied by hematopoietic cells in the trephine biopsy specimens. Hypocellular marrow was defined as less than 30% cellularity in patients under the age of 70 years, or less than 20% cellularity in patients 70 years and over.[6] Chromosomal analysis was performed and described according to the International System for Human Cytogenetic Nomenclature (ISCN).[7] Responses to IST were defined according to the established criteria.[8] The ethics committee of Kanazawa University Graduate School of Medical Science approved the study protocol, and all patients provided their informed consent prior to sampling.

Monoclonal antibodies

Monoclonal antibodies (mAbs) used for flow cytometry are shown on the *Online Supplementary Table S1*.

Flow cytometry for detecting GPI-AP$^-$ cells

All blood samples were analyzed within 24 h of collection to avoid false positive results due to cell damage. Staining with each mAb was performed according to the lyse-stain protocol as previously described.[5,9] The presence of CD55 CD59 glycophorin A$^+$ erythrocytes at the level of 0.005% and over and/or CD55 CD59 CD11b$^+$ granulocytes at the level of 0.003% or over was defined as an abnormal increase ('positive') based on the results obtained from 183 healthy individuals.[10] With careful handling of samples and elaborate gating strategies, cut-off values can be lowered to these levels without producing false positive results.[10-12]

Cell sorting and FISH analysis

GPI-AP$^+$ and GPI-AP$^-$ granulocytes from 2 patients with del(13q) (unique patient numbers (UPNs) 3 and 7) were sorted using a FACSAria III cell sorter (BD Bioscience, Franklin Lakes, NJ, USA) and subjected to fluorescence *in situ* hybridization (FISH) analysis using a D13S319-specific probe (Vysis, Voisins-le-Bretonneux, France) as previously described.[13]

Genome analysis of deleted region in patients with del(13q)

Genomic DNA was isolated from peripheral blood cells of 7 patients with del(13q) (UPNs 1, 3, 4, 5, 7, 8 and 22) and subjected to SNP array-based genome-wide analysis of genetic alterations using GeneChip® 250K arrays (Affymetrix, Santa Clara, California, USA) according to the manufacturer's protocol. Genomic and allele-specific copy numbers were calculated using Copy Analyser for GeneChip® (CNAG) software as previously described.[14,15]

Statistical analysis

Prevalence of increased GPI-AP$^-$ cells among different patient populations was compared using the χ^2 test. Time-to-event variables were analyzed using the Kaplan-Meier method, and groups were compared with the log rank test. Two-sided *P* values were calculated and $P<0.05$ was considered statistically significant. All statistical analyses were performed using the JMP software program version 8.0 (SAS Institute, Cary, NC, USA).

Results and Discussion

Of the 1,228 patients with BM failure, 22 possessed del(13q) (1.8%) that were demonstrated by G-banding; their clinical features are summarized in Table 1. Sixteen patients had only the del(13q) abnormality (which we define as 13q^{-alone}) while the remaining 6 patients had other abnormalities, which we define as 13q$^{-+other}$. Of these 6, 2 had -Y, one had -20, one had del(7q), one had +8, and one had +mar in addition to the del(13q) abnormality. The presence of the del(13q) clone was confirmed by FISH when the number of del(13q) revealed by the G-banding method was less than or equal to two. Median age was 64.5 years old, and BM was hypocellular in 16 patients (12 with 13q^{-alone} and 4 with 13q$^{-+other}$), normocellular in 4 (2 with 13q^{-alone} and 2 with 13q$^{-+other}$), not evaluable in 2 with 13q^{-alone}. All patients with del(13q) were diagnosed with MDS-U due to the absence of significant dysplasia that would fulfill the criteria for MDS as defined by the 2008 WHO classification. All patients were classified as Int-1 according to the International Prognostic Scoring System (IPSS), except for UPN17 who had an IPSS score of 1.5 (Int-2).

As shown in Table 1, GPI-AP$^-$ cells that accounted for from 0.006% to 12.342% (median 0.137%) of granulocytes were detected in all 16 13q^{-alone} patients. FISH analysis of sorted GPI-AP$^-$ and GPI-AP$^+$ granulocytes revealed that del(13q) cells were derived from non-*PIGA* mutant hematopoietic stem cells (HSCs) (Figure 1A). On the other hand, the prevalence of elevated GPI-AP$^-$ cell percentages in 13q$^{-+other}$ patients and those with a normal karyotype (637 patients with AA and 300 with MDS) was 50% (3 of 6) and 43% (405 of 937), respectively ($P<0.001$).

Fourteen 13q^{-alone} patients were treated with cyclosporine (CsA) alone,[6] CsA and antithymocyte globulin (ATG)[6] or CsA and anabolic steroids;[2] all achieved either a hematologic improvement in two or three lineages or complete remission (CR), while the response rate to IST in 13q$^{-+other}$ patients was 40%. No case was IST-dependent, and response was durable after the cessation of the treatment after patients achieved CR. The clinical course of one patient (UPN 4) who responded to CsA alone and entered CR, despite the fact that G-banding of BM cells showed all 20 dividing cells to be del(13q), has been previously reported.[16] Ninety-six AA patients with the normal karyotype were treated with CsA and ATG (n=47) or CsA±anabolic steroids (n=49). Seventy-eight percent of AA patients responded to IST. Among 19 MDS patients (RCUD, n=14; RCMD, n=5) with a normal karyotype who have been treated with ATG plus CsA (n=3) or CsA with or without anabolic steroids (n=16), 63% responded to IST.

None of the 17 13q^{-alone} patients progressed to advanced MDS or acute myeloid leukemia (AML) during the follow-up period of 3-108 months (median 52 months), while 2 of 6 13q$^{-+other}$ patients (one with -20, one with del(7q)) developed AML. The 10-year overall survival rates of patients with 13q^{-alone}, patients with 13q$^{-+other}$, AA patients with a normal karyotype and MDS (RCUD, n=38; RCMD, n=20; MDS-U, n=8) patients with a normal karyotype were 83%, 67%, 85% and 57%, respectively ($P=0.0003$, log rank test on 3 degrees of freedom) (Figure 1B). The 10-year overall survival rates of AA patients with a normal karyotype with and without increased GPI-AP$^-$ cells and MDS (38 with RCUD, 20 with RCMD and 8 with MDS-U) patients with a normal karyotype with and without increased GPI-AP$^-$ cells were 85%, 84%, 66% and 55%, respectively ($P=0.0011$, log rank test on 4 degrees of freedom) (Figure 1C). The percentage of del(13q) clones revealed by G-banding increased in 5 patients and decreased in 3 after successful IST. (*Online Supplementary*

Figure S1) No patient developed clinical features of paroxysmal nocturnal hemoglobinuria (PNH).

SNP array analysis of peripheral blood cells from 7 $13q^{-alone}$ and $13q^{+other}$ patients indicated the region from 13q13.3 to 13q14.3 to be commonly deleted (Figure 1D).

The current retrospective study with a large number of BM failure patients revealed distinctive clinical features of BM failure with del(13q) abnormalities. The 1.8% incidence of del(13q) patients was comparable to that of a recent study (1.9%) based on 2,072 patients with MDS,[17] for which detailed diagnoses of patients with del(13q) were not provided. All del(13q) patients in our study were classified as MDS-U due to the absence of significant dysplasia. We have previously reported that response to IST was remarkably high in 9 patients with del(13q). The present study, which used a different patient cohort, confirmed our previous finding.[2] Between these 22 patients and the 9 patients that we reported in 2002, only 2 developed AML and 22 responded to IST. The overall and leukemia-free survival spans of del(13q) patients treated with IST were as long as AA patients with normal karyotypes treated with IST. These findings suggest that the del(13q) clone in BM failure patients represents the presence of immune pathophysiology rather than clonal disorder associated with AML risk.

Table 1. Clinical features of bone marrow failure patients with del(13q) alone (patients 1-16) or del(13q) plus other abnormalities (patients 17-22).

UPN	Age (years)	Sex	Months from diagnosis to sampling	Dysplasia	Cellularity	Cytogenetics	% of del(13q) cells	Break point	% GPI(-) granulocytes	% GPI(-) RBCs	Previous therapy	Treatment	Response	Outcome	AML transformation	LFS (months)
1	64	F	54	None	hypo	46,XX,del 4/20 (13)(q?)	20	13q(?)	0.042	0.015	No	CsA+AS	HI-2	alive	No	67+
2	42	M	0	None	hypo	46,XY,del(13)(q12q14) 1/20	5	13(q12q14)	3.511	0.562	No	CsA	CR	alive	No	79+
3	47	F	0	None	hypo	46,XX, del(13)(q?) 2/20	10	13q(?)	2.101	0.601	No	ATG+CsA	HI-3	alive	No	24+
4	50	F	4	Erythroid	hypo	46,XX,del(13)(q12q22) 20/20	100	13(q12q22)	0.111	0.013	No	CsA	CR	alive	No	44+
5	65	F	5	None	hypo	46,XX,del(13)(q12q14) 3/20	15	13(q12q14)	0.009	0.008	No	ATG+CsA	CR	alive	No	43+
6	21	M	1	None	hypo	46,XY, del(13)(q?) 6/20	30	13q(?)	0.038	0.003	No	ATG+CsA	HI-3	alive	No	15+
7	52	M	1	Erythroid	NE	46,XY, del(13)(q?) 19/20	95	13q(?)	12.342	0.524	PSL	CsA	HI-3	alive	No	3+
8	87	F	1	None	normo	46,XX,del(13)(q12q22) 9/20	45	13(q12q22)	0.37	0.095	No	CsA	HI-3	alive	No	15+
9	63	F	16	None	hypo	46,XX,del(13)(q12q14) del(13)(q21q31) 5/20	25	13(q12q14), 13(q21q31)	0.006	0.665	PSL	ATG+CsA	HI-3	alive	No	29+
10	74	F	3	None	hypo	46,XX, del(13)(q12q14) 7/13	54	13(q12q14)	0.504	N/A	No	ATG+CsA	HI-3	death (cancer)	No	38
11	54	F	0	None	hypo	46,XX,del(13)(q14q22) 40/40	100	13(q14q22)	0.125	0.008	No	Allo-BMT	NE	alive	No	74+
12	53	M	43	None	hypo	46,XY,del(13)(q14.3)	14	13q14.3	0.281	0.539	No	ATG+CsA	HI-3	alive	No	108+
13	85	M	1	None	hypo	46,XY,del(13)(q?) 2/20	10	13q(?)	0.031	0.01	No	No treatment	NE	death	No	10
14	77	F	3	Erythroid	NE	46,XX,del(13)(q?) 8/20	40	13q(?)	3.125	1.65	No	CsA	CR	alive	No	45+
15	56	M	1	Erythroid	normo	46,XX,del(13)(q12q14) 6/20	30	13(q12q14)	0.069	0.036	No	CsA	HI-2	alive	No	24+
16	74	M	37	None	hypo	46,XY,del(13)(q?) 7/20, 47,X,+Y 7/20	35	13q(?)	0.171	0.441	No	CsA+AS	HI-2	alive	No	52+
17	69	M	1	None	hypo	46,XY,del(7)(q22), del(13)(q12q14) 3/20	15	13(q12q14)	0	0	No	CsA+AS	NR	death	Yes	8
18	68	F	1	None	normo	45,XX,del(13)(q12q22),-20 2/20	10	13(q12q22)	0	0	No	VitK	NE	death	Yes	7
19	75	M	2	None	hypo	45,X,-Y.del(13)(q?) 2/20	10	13q(?)	0	0.003	PSL	CsA	NR	alive	No	71+
20	81	M	17	None	hypo	47,XY,+8,del(13)(q?) 19/20	95	13q(?)	6.851	0.272	No	CsA	NE	alive	No	67+
21	57	F	122	Erythroid	normo	46,XX,del(13),+mar 10/20	50	del(13)	0.522	1.075	AS	CsA+AS	HI-3	alive	No	146+
22	66	M	1	Erythroid	hypo	45,X.-Y,del(13)(q12q14) 15/20	75	13(q12q14)	0.149	0.209	No	CsA	HI-2	alive	No	11+
Median	**65**						**30**		**0.137**	**0.095**						

UPN: unique patient number; M: male; F: female; normo: normocellular marrow; hypo: hypocellular marrow; GPI-AP-granulocytes, glycosylphosphatidyl-inositol anchored protein-deficient granulocytes; GPI-AP-erythrocytes, glycosylphosphatidyl-inositol anchored protein-deficient erythrocytes; CsA: cyclosporine; ATG: antithymocyte globulin; AS: anabolicsteroid; Allo-BMT: allogeneic bone marrow transplant; VitK: vitamin K; CR: complete remission; HI-2: hematologic improvement in two lineages; HI-3: hematologic improvement in three lineages; NR: no response; NE: not evaluable; AML: acute myeloid leukemia; LFS: leukemia-free survival.

Transformation of patient 17 (UPN17) to AML could be attributed to the coexistence of del(7q), which is associated with high risk of AML evolution.[18]

The percentage change of del(13q) clone following IST varied from one patient to another (*Online Supplementary Figure S1*) in a similar way in which the percentage of GPI-AP$^-$ cells changed in the present study (*data not shown*), which is consistent with our previous findings regarding *PIGA* mutant HSCs.[10] Given that effective removal of immune mechanisms by IST does not consistently lower the percentage of del(13q) clone, it is speculated that preferential expansion of del(13q) clones by the immune mechanisms at the onset of BM failure[10] may lead to the escape from immunological pressure, as in the case of *PIGA*

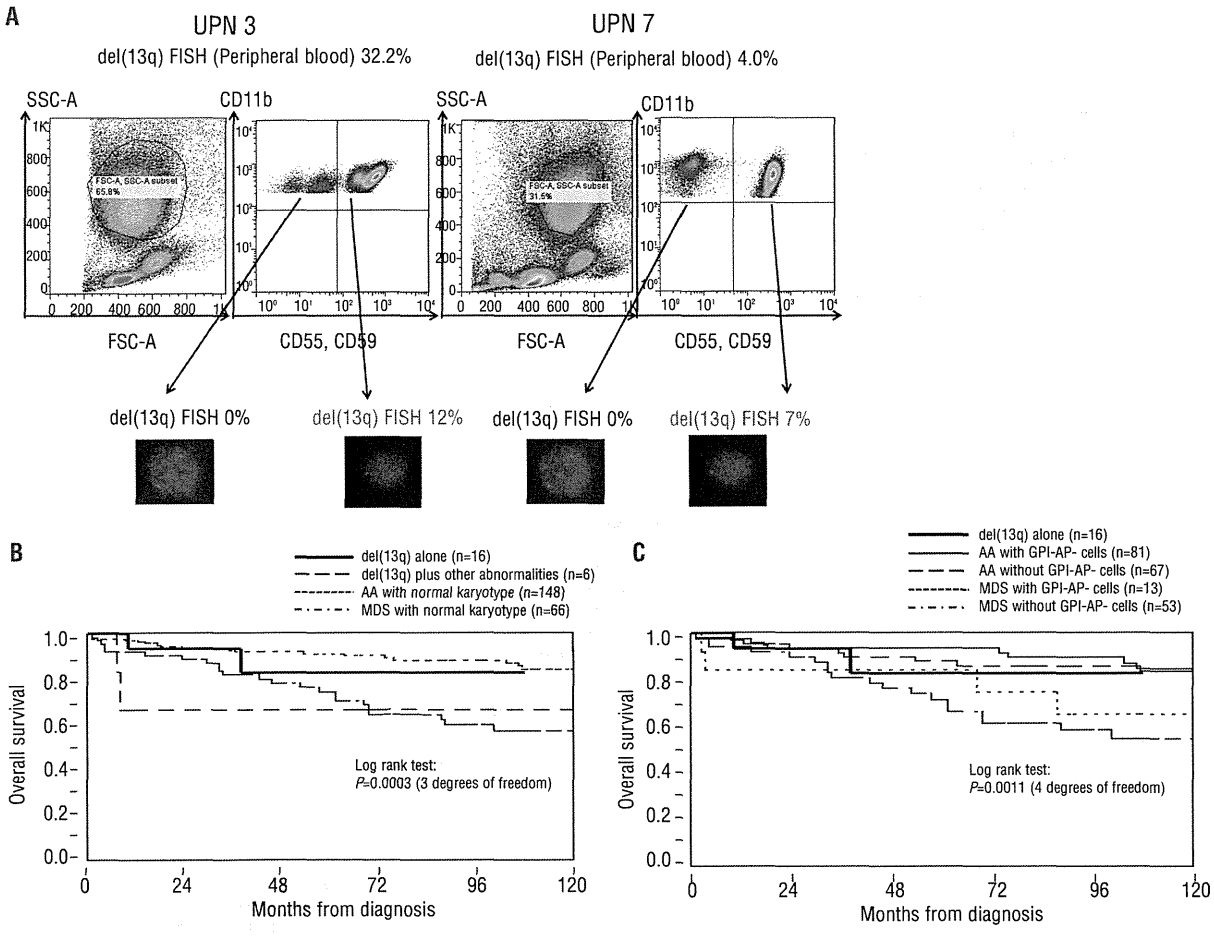

Figure 1. (A) Glycosylphosphatidylinositol-anchored protein (GPI-AP) expression in granulocytes with del(13q). GPI-AP$^-$ and GPI-AP$^+$ granulocytes were separated from peripheral blood cells of 2 patients with del(13q), one (Unique Patient Number (UPN) 3) comprised of 2.1% GPI-AP$^-$/ 32.2% del(13q) granulocytes and one (UPN 7) comprised of 12.3% GPI-AP$^-$/ 4.0% del(13q) granulocytes. Both GPI-AP$^-$ and GPI-AP$^+$ fractions were subjected to FISH analysis using a 13q probe (13q14.3); del(13q) clones were detected only in the GPI-AP$^+$ fraction of each patient (red), suggesting that del(13q) cells are derived from non-PIGA mutant hematopoietic stem cells. (B) Ten-year overall survival rates of patients with *del(13q), patients with del(13q) plus other abnormalities*, aplastic anemia (AA) patients with a normal karyotype and myelodysplastic syndrome (MDS) patients with a normal karyotype. All 66 MDS patients with a normal karyotype had Int-1-risk IPSS scores. (C) Ten-year overall survival rates of patients with del(13q), aplastic anemia (AA) patients with a normal karyotype with and without increased GPI-AP$^-$ cells and myelodysplastic syndrome (MDS) patients with a normal karyotype with and without increased GPI-AP$^-$ cells. (D) Deleted gene loci regions of 7 patients with del(13q), shown as bold horizontal lines for each UPN under the gene. The shaded box represents the deleted region common to patients, 13q13.3 to 13q14.3, which encodes proteins involved in cytokine signal transduction.

mutant HSCs. It is necessary to identify common mechanisms leading to preferential activation of both PIGA mutant HSCs and HSCs with del(13q) in immune-mediated BM failure to verify these hypotheses.

A possible immune pathophysiology in 13q^{-alone} patients is supported by the markedly high prevalence (100%) of elevated GPI-AP$^-$ cell levels which is linked to the escape of *PIGA* mutant HSCs from an immune system attack.[19] Because the del(13q) abnormality occurs in the GPI-AP$^+$ population, it may play a similar role to the GPI-AP$^-$ cells. SNP array analysis revealed the common deletion of a 15 Mb (13.3 to 14.3) region of 13q in 7 13q^{-alone} and 13q$^{-+other}$ patients. This segment encodes several proteins that regulate cell proliferation and the cell cycle, such as SMAD9 and RB1; both are involved in the signal transduction pathway of transforming growth factor-beta (TGF-β, an important cytokine in regulating HSC dormancy. Cytokine-mediated selection of *PIGA* mutant HSCs has been proposed as a mechanism for preferential proliferation of GPI-AP$^-$ cells,[20] but no supporting evidence has been presented. A previous study demonstrated that GPI-AP$^-$ T cells show decreased sensitivity to herpes virus entry mediator (HVEM) ligands that transmit inhibitory signals through receptors for CD160(21) and TGF-β.[22,23] The presence of del(13q) represents a unique subgroup of immune-mediated BM failure associated with an increase in the percentage of GPI-AP$^-$ cells, where del(13q) and *PIGA* mutant HSCs undergo preferential expansion, possibly due to their decreased sensitivity to cell-cycle inhibitory molecules, such as TGF-β compared to normal HSCs.

In conclusion, MDS-U with del(13q) alone is a benign BM failure syndrome characterized by a good response to IST and a markedly high prevalence of elevated GPI-AP$^-$ cell percentages. Therefore, del(13q) should be eliminated from the list of karyotypic abnormalities representing the intermediate group defined by IPSS,[24] and BM failure with del(13q) should be managed as AA.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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