Impact of splicing factor mutations on clinical features in patients with myelodysplastic syndromes

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Abstract

Splicing factor gene mutations are found in 60–70% of patients with myelodysplastic syndromes (MDS). We investigated the effects of splicing factor gene mutations on the diagnosis, patient characteristics, and prognosis of MDS. A total of 106 patients with MDS were included. The percentage of patients with MDS with ring sideroblasts (14.15%) as per the 2017 WHO classification was significantly higher than that of patients with refractory anemia with ring sideroblasts (2.88%) as per the 2008 WHO classification (P = 0.005). Splicing factor mutations were detected in 32 patients (13 *SF3B1,* 8 *U2AF1,* and 11 *SRSF2*), and the mutations were mutually exclusive. Significant differences were observed in the mean corpuscular volume, platelet count, bone marrow myeloid:erythroid ratio, and megakaryocyte count in patients with different mutations. *SRSF2* mutations were associated with a high cumulative incidence of red blood cell transfusion dependence, while *SF3B1* mutations were associated with a low cumulative incidence of platelet concentrate transfusion dependence. Presence of *SF3B1* mutation was a significant univariate predictor of overall survival, but become nonsignificant in the multivariate model. Although many factors also could affect survival, these results suggest that splicing factor mutations contribute to distinct MDS phenotypes, including patient characteristics and clinical courses.

Keywords: myelodysplastic syndromes, RNA splicing factors, gene mutations, blood transfusion, prognosis

Introduction

Myelodysplastic syndromes (MDS) are heterogeneous diseases within the wider spectrum of myeloid hematological neoplasm, which is characterized by peripheral blood cytopenia and a potential risk of progression to acute myeloid leukemia (AML). Recent studies have exploited the advantages of next-generation sequencing to shed some light on the genetic landscape of MDS [1, 2]. Various genetic mechanisms play a role in disease pathogenesis, including epigenetic regulation, transcription, signal transduction, and splicing machinery. The heterogeneity of MDS phenotypes is thought to be associated with the diversity of genetic events [3-5]. MDS-related mutations have been detected even in older people without prior history of hematologic malignancies [6, 7].

Somatic mutations in the RNA splicing factor genes *SF3B1*, *U2AF1*, and *SRSF2* have been detected in approximately 60%-70% of patients with MDS. Of note, *SF3B1* mutation is strongly associated with MDS with ring sideroblasts (MDS-RS) and favorable prognosis [1, 4, 8], as reflected in the 2017 revision of the World Health Organization (WHO) classification of myeloid neoplasm and acute leukemia [9]. Thus, the proportion of each MDS subtype in the population may be changed through redefinition of disease subtypes and adoption of new criteria. Furthermore, the presence of *U2AF1* or *SRSF2* mutations may have an effect on the clinical phenotypes and disease prognosis [10-12]. However, the association between splicing factor mutations and phenotypes and prognosis remains unclear. Therefore, the present study sought to investigate the effect of splicing factor gene mutations on the diagnosis, patient characteristics, and prognosis of MDS.

Materials and Methods

Study design and patient eligibility

We prospectively collected samples of patients with hematological disorders from our institutions (Tokyo, Japan). All patients provided written informed consents. Study participants were selected from our database according to the following inclusion criteria: (1) diagnosis of MDS, (2) age ≥ 20 years, and (3) patients that visited hospitals between January 2011 and December 2017. Diagnosis was made according to the 2008 and 2017 WHO classifications [9, 13]. Clinical and hematological data were retrospectively collected. This study was approved by the appropriate institutional research ethics boards and carried out in accordance with the Declaration of Helsinki.

Mutational analysis

DNA was extracted from the bone marrow or peripheral blood cells of patients, as previously described [14]. We analyzed the splicing factor gene mutations (*SRSF2*, *U2AF1*, and *SF3B1*) using the Sanger sequencing method. The mutational hotspots of *SF3B1* (exon 14 and exon 15), *U2AF1* (exon 2 and exon 7), and *SRSF2* (exon 1) were analyzed (primers are described in Supplementary Table 1). Genomic DNA was amplified with Pfu-X (Jena Bioscience, Jena, Germany) or KOD-Plus (TOYOBO, Tokyo, Japan) and

purified with ExoSAP-IT system (Affymetrix, California, USA) following the manufacturer's instructions. DNA sequences were evaluated using SEQUENCHER v5.0 (Gene Codes, Inc.) software and the acquired sequences were aligned to the reference genome (GRCh37/hg19).

Statistical methods

Study groups were compared using the Mann-Whitney or the Kruskal-Wallis test for non-parametric variables and Fisher's exact test for categorical variables. If there were significant differences between groups, differences between individual groups were estimated using the Bonferroni correction method. We also confirmed the differences between the presence or absence of each gene mutation. Cumulative incidence of transfusion dependence or progression to AML was estimated and compared using Gray's method, considering death without each event as a competing risk. For the survival analysis, we used the log-rank test for univariate analysis and Cox proportional hazard model for multivariate analysis. Overall survival (OS) was calculated from the day of diagnosis until death or the last follow-up appointment. The prognostic impact of each gene mutation was separately evaluated in patients with MDS for whom follow-up information was available.

For multivariable analysis, MDS group was adjusted with known risk factors as follows: age (≥ 60 or < 60 years) and IPSS-R (very low/low/intermediate or high/very high) [15]. Threshold and classification were adopted from the previous report [16]. A value of $P < 0.05$ was considered significant in all analyses. All statistical analyses were performed on EZR, a graphical user interface for R, version 2.14.0 (http://www.r-project.org/) [17].

Results

Patients characteristics

We prospectively collected samples of patients with hematological disorders, and a total of 116 patients with MDS were enrolled. Ten patients were excluded owing to the following reasons: therapy-related myeloid neoplasm (n = 5), dry tap (n = 4), and transformation from essential thrombocytosis (n = 1). A total of 106 patients, including 29 females and 77 males, were included in the study (Table 1). The median age at diagnosis was 69.5 (range, 22 to 90) years. In 19 patients, diagnosis was made before the study initiation. Iron staining was performed in 53 of 60 (88.3%) patients with MDS without excess blasts. The revised international prognostic scoring system (IPSS-R) risk distribution were as follows: 5.7% very low (n = 8), 28.3% low (n = 30), 24.5% intermediate (n = 26), 17.9% high (n = 19), and 24.5% very high (n = 26).

Splicing factor gene mutations

The median time between diagnosis and mutation analysis in patients was 26 days (ranging from 0 to 3,391 days). Splicing factor gene mutations were detected in 32 patients (13 with *SF3B1* mutations, 8

with *U2AF1* mutations, and 11 with *SRSF2* mutations), and the mutations were mutually exclusive. The most frequent mutation in *SF3B1* was K700E (n = 7). In *U2AF1*, the most frequent mutation was Q157P (n = 5), followed by S34F (n = 2) and S34Y (n = 1). *SRSF2* mutations included P95H (n = 8) and P95R (n = 3).

Patient diagnosis

Patients were initially diagnosed with MDS with single lineage dysplasia (MDS-SLD, n = 12), MDS with multilineage dysplasia (MDS-MLD, n = 30), MDS with ring sideroblasts with single lineage dysplasia (MDS-RS-SLD, n = 4), MDS-RS-MLD (n = 11), MDS with excess blasts 1 (MDS-EB1, n = 24), MDS-EB2 (n = 22), and MDS, unclassifiable (MDS-U, n = 3). There were no patients with 5q- syndrome. As shown in Table 1, these diagnoses were significantly correlated with genetic mutations ($P < 0.001$). According to the 2008 WHO classification, 14 patients were diagnosed with refractory cytopenia with unilineage dysplasia (RCUD), 42 had refractory cytopenia with multilineage dysplasia (RCMD), 3 patients showed refractory anemia with ring sideroblasts (RARS), 23 patients had refractory anemia with excess blasts (RAEB)-1, 21 had RAEB-2, and 3 patients were diagnosed with MDS-U. Two patients were classified with acute erythroid leukemia. The proportion of patients with MDS-RS (14.15%) as per the 2017 WHO classification was significantly increased from that of patients with RARS (2.88%) as per the 2008 WHO classification ($P = 0.005$). Patients with MDS-RS were formerly diagnosed with 12 RCMD and 3 RARS as per the 2008 WHO classification.

SF3B1 gene mutations were observed in 11 of 15 (73.3%) patients with MDS-RS. As two patients had RS < 15% (5% and 7%), these were diagnosed with MDS-RS by the presence of *SF3B1* mutations. Two patients with *SF3B1* were diagnosed as MDS-EB. *U2AF1* mutations were detected in eight patients; of these, 4 had MDS-EB1 and one each was diagnosed with MDS-EB2, MDS-MLD, MDS-RS-MLD, and MDS-U. *SRSF2* mutations were detected in 11 patients, of which 4 had MDS-EB1, 2 were diagnosed with MDS-EB2, 4 with MDS-MLD, and 1 had MLD-SLD. Accordingly, *U2AF1* and *SRSF2* mutations were more common in MDS-EB.

Clinical characteristics associated with splicing factor gene mutations

We analyzed differences in specific baseline characteristics between patients carrying different splicing factor mutations. In terms for peripheral blood findings (Fig. 1), no significant differences were observed in white blood cell count, neutrocyte count, and hemoglobin levels between the patient groups. However, mean corpuscular volume (MCV) showed a significant difference ($P = 0.015$). MCV values were higher in patients carrying *SF3B1* mutations (median108 fL, range 99-118 fL, $P = 0.007$) than in those without mutation (median101 fL, range 78-125 fL). In contrast, the MCV value in patients carrying *U2AF1* mutations tended to be low (median 95.2 fL, range 85.3-104 fL, $P = 0.075$). Furthermore, a significant difference was reported between the MCV value in patients carrying *SF3B1* mutations and those with

U2AF1 mutations ($P = 0.008$). In contrast, *SRSF2* had no impact on MCV values ($P = 0.252$). The platelet count of 11 of 13 patients with *SF3B1* mutations was over 100×10^9/L. Median platelet count in patients carrying *SF3B1* mutations was 171×10^9/L (range, 60-441) and significantly higher than that reported in other groups ($P = 0.045$). The presence of *SF3B1* mutations was associated with higher platelet count ($P = 0.007$), whereas no association was observed between high platelet count and *U2AF1* and *SRSF2* mutations ($P = 0.409$ and $P = 0.721$, respectively). No significant difference was found in ferritin, iron, and WT-1 expression levels among the mutation status.

We analyzed bone marrow (BM) findings (Fig. 2). No significant differences were observed in all nucleated cell count (ANC) and blast fraction between patients with different mutations. However, the myeloid to erythroid (M/E) ratio was significantly different between all patient groups ($P = 0.007$). *SRSF2* mutations were associated with a high M/E ratio (median 3.78, ranging from 0.63-33.0, $P = 0.001$). In contrast, *U2AF1* mutations were associated with low M/E ratios (median of 0.69, ranging from 0.51-1.43, $P = 0.016$). In addition, a significant difference was observed in megakaryocyte count between groups ($P = 0.004$). Compared to patients without *SF3B1* mutation, megakaryocyte count in patients with *SF3B1* mutations was significantly higher ($P = 0.023$) and it was almost normal level, which was not observed in patients with *U2AF1* mutations ($P = 0.146$) or *SRSF2* mutations ($P = 0.583$).

Prognostic effects of splicing factor gene mutations

We investigated the association between each gene mutation and prognosis in patients for whom follow-up information was available. The mean observational period was 366 days (ranging from 4 to 3,917 days). Patients were classed as transfusion-dependent if they had at least two transfusions within 8 weeks. Patients on temporary transfusions were excluded from being transfusion-dependent. Among the 101 patients with available transfusion information, 69 had become red blood cell (RBC) transfusion-dependent and 41 showed platelet concentrate (PC) transfusion dependence. During the study period, progression to AML was documented in 24 patients. Erythropoiesis-stimulating agent (ESA) was administered to 12 patients, while hypomethylating agent was administered to 41 patients; allogeneic stem cell transplants were performed in nine patients. Overall, 37 patients died.

The cumulative incidence of transfusion dependence is shown in Fig. 3. The 1-year incidence was 53.8% (95% confidence interval [CI]: 42.8%-63.6%) for RBC transfusion and 29.7% (95% CI: 20.5%-39.5%) for PC transfusion. The cumulative incidence of RBC transfusion dependency was significantly higher in patients with *SRSF2* mutations than in patients without *SRSF2* mutations ($P = 0.012$). All patients with *SRSF2* mutations became RBC transfusion-dependent within 2 years. In contrast, *SF3B1* mutations were significantly associated with PC transfusion independence ($P = 0.025$). During the follow-up period, only one patient that progressed to AML needed constant PC transfusion. No association was observed between the presence of *U2AF1* mutations and future risk of RBC or PC transfusion dependency.

Univariate analysis demonstrated that patients with *SF3B1* mutations had significantly better OS than

those without *SF3B1* mutations (Fig. 4A, $P = 0.038$). In contrast, the presence of *U2AF1* or *SRSF2* mutations had no significant impact on OS (Fig. 4B and 4C). In addition, high or very high IPSS-R and age (≥ 60 years) were significant risk factors for OS ($P < 0.001$ and $P = 0.044$, respectively). Multivariate analysis for OS showed that only IPSS-R (high and very high) was an independent risk factor (hazard ratio [HR] 3.483, 95% CI: 1.625-7.469, $P = 0.001$, Table 2). Although not statistically significant, patients with *SF3B1* mutations tended to have better OS ([HR] 0.245, 95% CI: 0.032-1.851, $P = 0.173$). Splicing factor gene mutations had no significant effects on the cumulative incidence of AML progression (Supplementary Fig. 1).

Discussion

In this study, we first focused on the diagnoses according to the 2008 and 2017 WHO classifications. The frequency of RARS diagnosis (2.88%) as per the 2008 WHO classification was comparable to that reported in previous Japanese studies [16, 18], whereas a total of 15 patients (14.15%) were diagnosed with MDS-RS as per the 2017 WHO classification. The proportion of MDS-RS as per the 2017 WHO classification was higher than that of RARS as per the 2008 WHO classification. This difference was mainly associated with the reclassification of RCMD to MDS-RS-MLD. Previous reports have described the same shift by reclassification [19, 20]. In addition, we routinely performed both iron staining and *SF3B1* mutation analysis, which may have contributed to the increased number of MDS-RS. The proportion of MDS-RS in our study seemed to be lower than that reported in a German study [20], probably due to ethnic differences. This might influence the frequency of splicing factor gene mutation because *SF3B1* gene mutation is one of the most frequent gene mutations in patients with MDS [21]. In fact, splicing factor gene mutation was detected in 30.19 % in this study: this rate was relatively small compared to previous nation-wide studies [1, 21]. Also, it seemed that the lack of *ZRSR2* gene mutation status may have influence this result. *ZRSR2* is the 4th frequent splicing factor gene mutated in patients with MDS [1, 21]. We did not examine the mutation; however, this should be ascertained by further studies.

Splicing factor gene mutations cause aberrant pre-mRNA splicing, which could affect the function of several downstream target genes; however, the precise mechanism is questionable [22-27]. We, therefore, analyzed the association between splicing factor gene mutations and clinical phenotypes. *SF3B1* mutations were frequently detected in patients with MDS-RS [1, 28]. A recent multicenter study reported that patients with macrocytic anemia may be categorized according to MCV values [29]. In the present study, patients carrying *SF3B1* mutations showed high MCV values, whereas those with *U2AF1* mutations had relatively low values. MCV value may be influenced by splicing factor mutations. In addition, patients carrying *SF3B1* mutations showed high platelet and megakaryocyte counts at baseline and were subsequently unlikely to become PC transfusion-dependent. These findings suggest that the effect of *SF3B1* is mainly based on erythropoiesis rather than platelet production. *SF3B1* gene has been

associated with iron homeostasis and mitochondrial metabolism [30]. Therefore, it is possible that the difference in the splicing target genes reflected the distinctive phenotypes. This hypothesis is supported by clustering analysis results reported by Malcovati et al., wherein patients with *SF3B1* mutations clustered as a distinct group [11].

More than half of the patients carrying *U2AF1* or *SRSF2* gene mutations were classified as MDS-EB1 or MDS-EB2. A previous study showed decreased platelet count in patients carrying the *U2AF1* gene mutation [12]; however, we failed to observe this phenomenon in the present study. One possible explanation was that only three patients in our cohort had the $U2AF1^{S34}$ mutation, which was associated with low platelet count. Patients carrying *U2AF1* gene mutations showed significantly lower BM M/E ratio. On the other hand, patients carrying *SRSF2* gene mutations showed high BM M/E ratios and high cumulative incidence of RBC transfusion dependency. Zhu et al. suggested that high BM erythroblasts as well as serum ferritin value reflected ineffective erythropoiesis [31]. In this study, patients carrying *U2AF1* gene mutations showed low M/E ratio but failed to show high ferritin value. In addition, the cumulative incidence of RBC transfusion dependence in patients carrying *SRSF2* gene mutations was much higher than that in patients carrying *U2AF1* gene mutations. To the best of our knowledge, no study has investigated the BM M/E ratios and future cumulative incidence of transfusion dependence by splicing factor gene mutations. *U2AF1* and *SRSF2* gene mutations have been reported to induce erythroid and granulocytic proliferation and differentiation [32, 33], which may be influenced by a downstream effect on target genes or co-existing mutations [4, 22, 34-36]. Unfortunately, we lacked genotype information for any genes other than splicing factor genes in our study cohort. Further studies are warranted to shed light on the pathophysiology of these mutations.

Patient survival studies have shown the association between *SF3B1* gene mutations and good prognosis [2, 11, 37]. Mutation in both *U2AF1* and *SRSF2* genes are known to be risk factors for survival and AML progression [10, 12, 34, 38]. In this study, the presence of *SF3B1* mutations was associated with better OS; however, no statistical significance was observed after adjusting for IPSS-R and age. We speculate that this result was partly because 11 of 13 patients with MDS carrying *SF3B1* mutations were classified under very low or low risk in IPSS-R. Tennant et al. reported that a high MCV value was associated with favorable OS [39]. In the present study, patients with *SF3B1* mutations showed high MCV values; therefore, the clinical impact of high MCV may reflect the presence of *SF3B1* genetic variations. In contrast, Wang et al. reported that MCV value was not a prognostic factor in MDS patients with normal karyotype [40]. Further investigation of *SF3B1* mutations is needed, given their association with the cytogenic abnormalities -Y and del(11q) [41, 42], which was newly defined as a very good cytogenetic abnormality in 2017 WHO classification. Furthermore, neither *U2AF1* nor *SRSF2* gene mutations showed any prognostic impact in our study. Prognosis analysis should be limited to patients without excess blasts [43]; however, we were unable to follow practice owing to the small patient number. An alternative effective strategy is the addition of mutational data to the IPSS-R [37, 44].

In summary, we defined the incidence of MDS subtypes in a cohort according to the 2017 WHO classification and showed the clinical impact of splicing factor gene mutations, including specific patient characteristics and transfusion dependency. This research sheds some light on the mechanisms underlying the phenotypic heterogeneity of MDS.

Conflict of interest

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Table 1. Patient characteristics

Variables	Splicing factor gene mutation				
	No mutation	*SF3B1*	*U2AF1*	*SRSF2*	*P* value
	n = 74	n = 13	n = 8	n = 11	
Age, median (range), years	68 (29-89)	77 (57-83)	72.5 (53-85)	72 (22-90)	0.217
Sex					
Female	23	4	1	1	0.405
Male	51	9	7	10	
Diagnosis					
MDS-SLD/MLD	36	0	1	5	< 0.001
MDS-RS-SLD/MLD	3	11	1	0	
MDS-EB	33	2	5	6	
MDS-U	2	0	1	0	
IPSS-R					
Very low/Low/Intermediate	39	11	5	7	0.218
High/Very high	33	2	3	4	
Transfusion before diagnosis					
No	62	11	7	9	0.214
Yes	3	1	0	2	
Hepatomegaly					
No	52	9	5	8	0.105
Yes	2	0	2	0	
Splenomegaly					
No	41	6	3	5	0.343
Yes	14	3	4	3	

MDS, myelodysplastic syndrome; SLD, single lineage dysplasia; MLD, multilineage dysplasia; RS, ring sideroblast; EB, excess blast; U, unclassifiable; IPSS-R, revised international prognostic scoring system

Table 2. Multivariate analysis of risk factors for OS

Variables	Hazard ratio (95% CI)	*P* value
Age, years		
< 60	1	Ref.
≥ 60	1.96 (0.68-5.654)	0.156
IPSS-R		
Very low/Low/Intermediate	1	Ref.
Very high/High	3.483 (1.625-7.469)	0.001
SF3B1		
Wild-type	1	Ref.
Mutation	0.245 (0.032-1.851)	0.173

OS, overall survival; CI, confidence interval; Ref., reference; IPSS-R, revised international prognostic scoring system

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Figure legends

Fig. 1 Peripheral blood findings associated with splicing factor gene mutations. **(A)** White blood cell count (WBC), **(B)** neutrocyte count (Neu), **(C)** hemoglobin level **(**Hb), **(D)** mean corpuscular volume (MCV), **(E)** platelet count (Plt), **(F)** serum iron, **(G)** serum ferritin, and **(H)** *WT-1* gene expression level (WT-1). The results of the statistical analysis (*P* value) are shown on each panel.

Fig. 2 Bone marrow findings associated with splicing factor gene mutations. **(A)** All nucleated cell count (ANC), **(B)** blast fraction (Blast), **(C)** myeloid to erythroid (M/E) ratio, and **(D)** megakaryocyte count (Mgk). The results of the statistical analysis (*P* value) are shown on each panel.

Fig. 3 The association of splicing factor gene mutations with cumulative incidences (CI) of transfusion dependence. CI of red blood cell (RBC) transfusion dependence were compared in patients with or without mutations of *SF3B1* **(A)**, *U2AF1* **(B)**, or *SRSF2* **(C)**. CI of platelet concentrates (PC) transfusion dependence were compared in patients with or without mutations of *SF3B1* **(D)**, *U2AF1* **(E)**, or *SRSF2* **(F)**. The results of the statistical analysis (*P* value) are shown on each panel.

Fig. 4 Kaplan-Meier curves for the probabilities of overall survival were compared in patients with or without mutations of *SF3B1* **(A)**, *U2AF1* **(B)**, or *SRSF2* **(C)**. The results of the statistical analysis (*P* value) are shown on each panel.

Supplementary Fig. 1 Cumulative incidences of AML progression were compared in patients with or without mutations of *SF3B1* **(A)**, *U2AF1* **(B)**, or *SRSF2* **(C)**. The results of the statistical analysis (*P* value) are shown on each panel.