Table 2. MDS Cytogenetic Scoring System

Prognostic subgroups, % of patients	Cytogenetic abnormalities	Median survival,* y	Median AML evolution, 25%,* y	Hazard ratios OS/AML*	Hazard ratios OS/AML†
Very good (4%*/3%†)	−Y, del(11q)	5.4	NR	0.7/0.4	0.5/0.5
Good (72%*/66%†)	Normal, del(5q), del(12p), del(20q), double including del(5q)	4.8	9.4	1/1	1/1
Intermediate (13%*/19%†)	del(7q), +8, +19, i(17q), any other single or double independent clones	2.7	2.5	1.5/1.8	1.6/2.2
Poor (4%*/5%†)	−7, inv(3)/t(3q)/del(3q), double including −7/del(7q), complex: 3 abnormalities	1.5	1.7	2.3/2.3	2.6/3.4
Very poor (7%*/7%†)	Complex: > 3 abnormalities	0.7	0.7	3.8/3.6	4.2/4.9

OS indicates overall survival; and NR, not reached.
*Data from patients in this IWG-PM database, multivariate analysis (n = 7012).
†Data from Schanz et al[8] (n = 2754).

than merely the number of these abnormalities; and modification of the ANC cutpoint to 0.8×10^9/L from 1.8×10^9/L in the IPSS.

Regarding the cytogenetic classification, good correlation was demonstrated regarding the proportional hazard ratios for clinical outcomes (survival and AML evolution) of the subgroups from the recently developed cytogenetic system[8] on which we based our analysis and from our data (Table 2). Because of the higher number of patients analyzed in our database, more cytogenetic subtypes were analyzable for prognosis in our study than had been possible for the IPSS (15 vs 6). A double independent review of the cytogenetic data was performed by the IWG-PM Cytogenetic Committee. Differences between this categorization and that of the IPSS included the finding of complex karyotypes with > 3 abnormalities being distinct from those with 3 abnormalities and with poorer prognosis; chromosome 7 abnormalities were similarly prognostically separable from the Very poor category (when observed in karyotypes with ≤ 3 abnormalities; supplemental Figures 1 and 2, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). The specific cytogenetic abnormalities that now were able to be placed into distinct prognostic subgroups included inv(3)/t3q)/del(3q), del(11q), del(12p), i(17q), +19, double anomalies including del(5q), double abnormalities including del(7q) or monosomy 7, and any other double changes [in addition to the previously IPSS-denoted −Y, del(5q), del(20q), all as single abnormalities] (Table 2).

The distributions of the IPSS cytogenetic categories in the present IWG-PM database was similar to those in the IMRAW database, which generated the IPSS (1): IPSS Good/Intermediate/Poor 73%/15%/12% (IMRAW 70/14/16%). This contrasted with the cytogenetic categorization in the IPSS-R: Very good/Good/Intermediate/Poor/Very poor 4%/72%/13%/4/7%. The IPSS clinical subgroups in our patient cohort were: Low 37%, Intermediate-1 40%, Intermediate-2 16%, and High 7%. These groups were also similar to the IMRAW patients: 33%/38%/22%/7% (1). As both FAB and WHO morphologic classifications were used, refractory anemia with excess blasts in transformation 6%, chronic myelomonocytic leukemia 9%, and isolated del(5q) 4% were represented in our patients.

Model development

Analysis of the marrow blast cutpoints indicated that striking differences were evident for both survival and AML evolution for patients with blasts 0- ≤ 2% (lower risk) versus blasts > 2- < 5%: Cox univariate pairwise comparison hazard ratio 1.4 (95% CI, 1.3-1.5, $P < .001$) for survival and 2.4 (95% CI, 1.9-2.9 $P < .001$) for AML evolution (Figures 1 and 2). Multivariate results confirmed this finding. Thus, we incorporated these distinct categorical values into the scoring model. Further, the statistical analysis of clinical outcomes for blasts > 10-≤ 20% vs > 20-≤ 30% indicated that these values had similar risk: hazard ratio = 1.0 (95% CI, 0.8-1.2, $P = .996$) for survival and 0.8 (95% CI, 0.6-1.1 $P = .174$) for AML evolution (supplemental Figures 3 and 4). Thus, we combined these 2 categories in the scoring model. In addition, this finding of the statistical relevance of the specific blast cutpoints in the combined database was also present in the individual databases, including that of the IMRAW database from which the IPSS[1] had been derived. For the IMRAW patients, the hazard ratios for survival and AML evolution were substantially the same as those for the combined database for both the lower and higher blast group analyses.

Review of the data indicated that baseline depths of cytopenias were statistically and clinically important (Table 3). The relevant cutpoints were: hemoglobin values of < 8, 8-< 10, and ≥ 10 g/dL, platelets of < 50, 50-100 and ≥ 100 × 10^9/L, and ANC of < 0.8 versus ≥ 0.8 × 10^9/L.

The changes from the cutpoints used in the IPSS-R compared with those from the IPSS include (1) separating marrow blasts < 5% into 0%-2% and > 2-< 5%; and (2) providing differing depths of cytopenias; also, as patients with marrow blasts of 10%-20% had similar outcomes as those with 21%-30%; and (3)

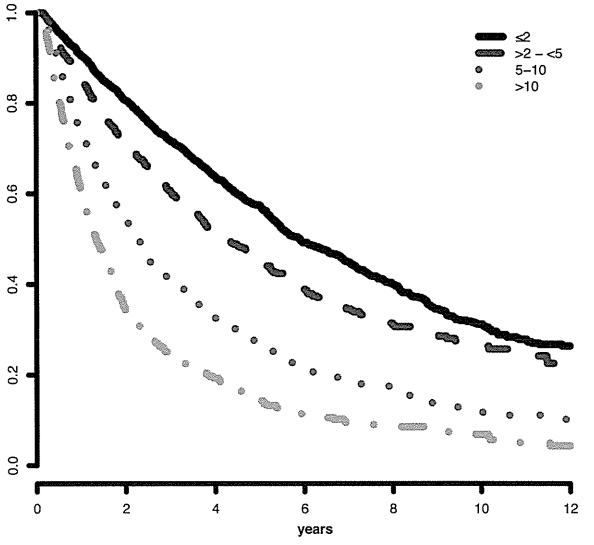

Figure 1. IWG-PM patients marrow blast subgroups. Impact on survival. Survival related to MDS patients' individual marrow blast percent categories (Kaplan-Meier curves, Dxy 0.3, $P < .001$). The number of patients in each category and their proportional representation are shown in Table 1.

Table 3. IPSS-R prognostic score values

Prognostic variable	0	0.5	1	1.5	2	3	4
Cytogenetics	Very good	—	Good	—	Intermediate	Poor	Very poor
BM blast, %	≤ 2	—	> 2%- < 5%	—	5%-10%	> 10%	—
Hemoglobin	≥ 10	—	8- < 10	< 8	—	—	—
Platelets	≥ 100	50-< 100	< 50	—	—	—	—
ANC	≥ 0.8	< 0.8	—	—	—	—	—

— indicates not applicable.

the category of marrow blasts > 10%-30% usefully described the statistical impact of this parameter compared with having separated these groups in the IPSS.

The IPSS-R prognostic risk categories were determined by combining the scores of these main 5 features (Table 4). The model permitted the definition of 5 well-separated prognostic categories for both survival and AML evolution in the IPSS-R (Very low, Low, Intermediate, High, Very high) rather than the 4 categories that are present in the IPSS (Tables 4 and 5; Figures 3 and 4). These risk categories describe scores for 70-year-old patients.

Survival duration and time to AML evolution for patients within these 5 prognostic categories are shown in Table 5 and Figures 3 and 4. As indicated in Table 5, in which hazard ratios are shown, ~ 56% of the patients were in the lower risk (Very low and Low) and ~ 23% were in the higher (High and Very high) risk prognostic subgroups for both of these clinical outcomes. For both survival and time to AML evolution, the individual centers' Dxy's were in good agreement with that for the total patient cohort.

Ready extrapolation is available to adjust the score for patients of any age by use of the following formula: (years − 70) × [0.05 − (IPSS-R risk score × 0.005)], add the result to the sum of the 5 major variables. Patient age clearly had major impact on survival (ie, decreased survival with aging), but not for AML evolution (Figure 5; supplemental Figure 5). Table 6 provides specific survival data within each risk category for patients of differing ages. Figure 6 provides a nomogram, based on the just noted formula, which visually describes the method to determine predicted survival based on patient's age and risk status, generating age-adjusted IPSS-R categorization (IPSS-RA).

Additional significant differentiating features for predicting survival were found, although their impact on prognostic score was relatively low compared with the 5 major features and age. These were: performance status, serum ferritin, LDH, and possibly β_2-microglobulin (supplemental Table 1). For determining the contribution of each of these features to the patient's risk category, the numerical values (with the categorized values for each variable) are indicated in the table and should be added to the raw scores of the major variables. This table provides multivariate *P* values as well as indicating the incremental contribution of a feature to the already defined score. Of note, none of these variables was a statistically significant additive feature for predicting AML evolution.

As shown in Table 7, differences were noted in the proportion of our patient cohort who died with or without developing AML in relation to their initial prognostic risk category. Of the patients who died, if observed until death, the proportions dying with leukemia in the groups were 13%-33%, positively related to their higher-risk categories.

Distinction between IPSS-R and IPSS

A summary of the refinements of the IPSS-R beyond the IPSS is shown in Table 8. The IPSS-R model showed effective separation of the IPSS patient risk categories and more effectively discriminated prognostic risk for these patients than the IPSS, as indicated by the higher Dxy values (.43 vs .37 for survival, .52 vs .48 for AML evolution; Table 1). Data indicated that 99% of the patients in the IPSS-R Very low and Low risk subgroups encompassed those who had been classified as IPSS Low and Intermediate-1; 81% of those in the IPSS-R High and Very high risk subgroups had been classified as IPSS Intermediate-2 and High (Figure 7, Kendall tau = 0.73). The IPSS-R Intermediate category (20% of the patients) was composed of 73% IPSS Intermediate-1, 19% Intermediate-2, 7% Low, 1% High (Table 9). In the IPSS lower risk group (Low/Intermediate-1), 27% of these patients were shifted into higher risk IPSS-R categories (mainly Intermediate). At the other prognostic extreme, 18% of the IPSS higher-risk (Intermediate-2/High) were downstaged into lower-risk IPSS-R categories (predominantly IPSS-R Intermediate).

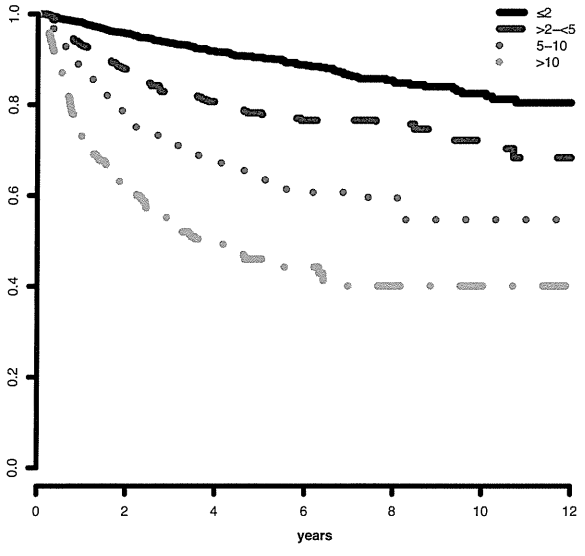

Figure 2. IWG-PM patients marrow blast subgroups: Impact on AML evolution. Progression to AML related to MDS patients' individual marrow blast percent categories (Kaplan-Meier curves, Dxy 0.47, $P < .001$). The number of patients in each category and their proportional representation are shown in Table 1.

Table 4. IPSS-R prognostic risk categories/scores

Risk category	Risk score
Very low	≤ 1.5
Low	> 1.5-3
Intermediate	> 3-4.5
High	> 4.5-6
Very high	> 6

Table 5. IPSS-R prognostic risk category clinical outcomes

	No. of patients	Very low	Low	Intermediate	High	Very high
Patients, %	7012	19	38	20	13	10
Survival, all*		8.8	5.3	3.0	1.6	0.8
		(7.8-9.9)	(5.1-5.7)	(2.7-3.3)	(1.5-1.7)	(0.7-0.8)
Hazard ratio		0.5	1.0	2.0	3.2	8.0
(95% CI)		(0.46-0.59)	(0.93-1.1)	(1.8-2.1)	(2.9-3.5)	(7.2-8.8)
Patients, %	6485	19	37	20	13	11
AML/25%*†		NR	10.8	3.2	1.4	0.73
		(14.5-NR)	(9.2-NR)	(2.8-4.4)	(1.1-1.7)	(0.7-0.9)
Hazard ratio		0.5	1.0	3.0	6.2	12.7
(95% CI)		(0.4-0.6)	(0.9-1.2)	(2.7-3.5)	(5.4-7.2)	(10.6-15.2)

NR indicates not reached.
*Medians, years (95% CI), $P < .001$.
†Median time to 25% AML evolution (95% CIs), $P < .001$.

Model validation

After construction and acceptance of the IPSS-R within the IWG-PM, an external validation cohort of 200 MDS patients from the Medical University of Vienna was evaluated and demonstrated good comparability of demographic features with the global IWG-PM database (supplemental Table 2). Their median age was 71 years, 83% were > 60 years, and the male/female ratio was 1.2:1 with a median follow-up time of 4.6 years. A similar proportion of these patients composed the cytogenetic, clinical, and IPSS-R subgroups, as did the global IWG-PM cohort (supplemental Table 2). Our IPSS-R multivariate model fit these data well, exhibiting high prognostic power, as indicated by the high Dxy's and the clearly differing temporal medians and hazard ratios between prognostic risk categories for both survival and AML evolution (Table 10, supplemental Table 2), including age-related survival (supplemental Table 3). Cox model analyses also supported the improved prognostic ability compared with the IPSS.

Discussion

We herein describe the IPSS-R, which provides useful advances and more discriminatory prognostic risk assessment beyond the IPSS for assessing clinical outcomes in MDS (Table 8). Although the IPSS has been an important standard for assessing prognosis of primary untreated adult MDS patients over the past decade, additional refinements[27] and prognostic variables have been suggested as providing meaningful differences for patient clinical outcomes.[2-5,9-20] In addition, cytogenetic subgroups have recently been defined as providing improved prognostic evaluation of clinical outcomes of primary MDS patients.[8] A number of prior prognostic systems in addition to the IPSS have demonstrated merit, although the relative value of each variable was unclear. Thus, in this collaborative IWG-PM project, with the large number of patients evaluated from multiple coalesced databases (n = 7012), we have integrated the various recently independently defined

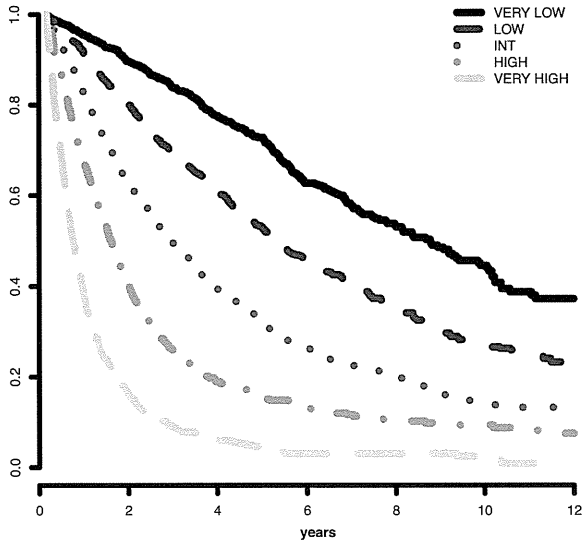

Figure 3. Survival based on IPSS-R prognostic risk-based categories. Survival related to MDS patients' prognostic risk categories (Kaplan-Meier curves, n = 7012; Dxy 0.43, $P < .001$). The number of patients in each category and their proportional representation are shown in Table 1.

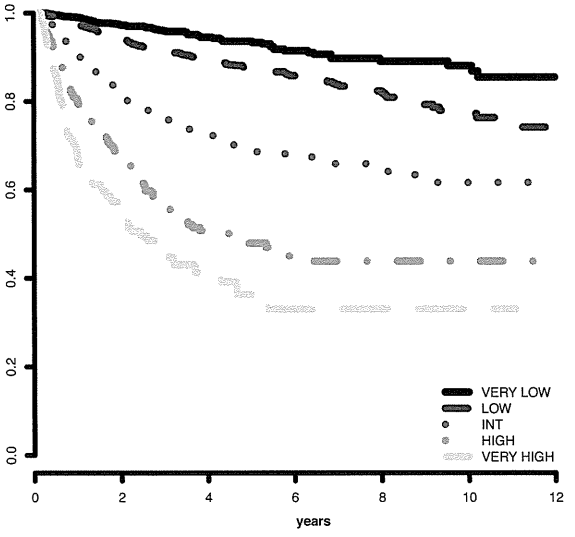

Figure 4. AML evolution based on IPSS-R prognostic risk-based categories. Progression to AML related to MDS patients' prognostic risk categories (Kaplan-Meier curves, n = 6485; Dxy 0.52, $P < .001$). The number of patients in each category and their proportional representation are shown in Table 1.

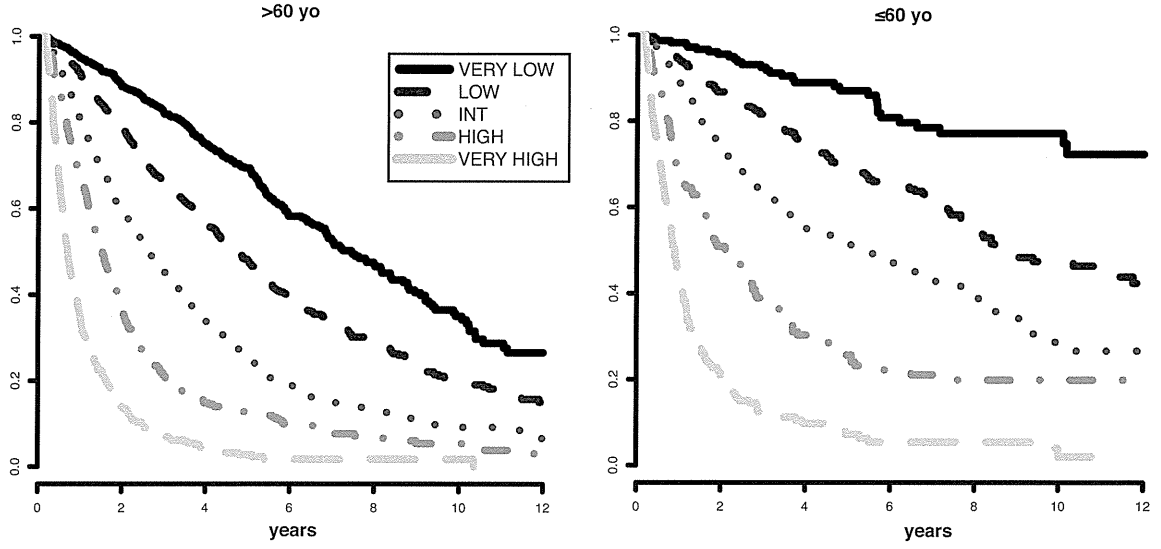

Figure 5. Survival based on patient ages > 60 years vs ≤ 60 years related to their IPSS-R prognostic risk-based categories (Kaplan-Meier curves). Age-related differential survivals are shown for patients in all groups, particularly for those in lower risk categories.

clinical factors for MDS in a comprehensive method and assessed their relative prognostic impact. Multivariate analyses demonstrated that the same major features present in the IPSS (cytogenetic subgroups, marrow blast percentage, and cytopenias) retained major prognostic impact in IPSS-R (in descending order of statistical weight). However, more precise prognostication of these clinical outcomes (survival and AML evolution) in the IPSS-R was demonstrated by effective refinement of these features within the IPSS-R (depth of cytopenias, splitting of marrow blasts < 5%, and more precise cytogenetic subgroups). The IPSS-R also demonstrated improved predictive prognostic power with more precise prognostic categories[5] versus 4 groups in the IPSS. In particular, a substantial proportion of those patients previously placed within the IPSS Intermediate-1 and -2 categories were more precisely separated into all 5 IPSS-R categories.

Analogous to the IPSS, based on our clinical outcome data, lower-risk patients are composed within the IPSS-R Very low and Low categories; higher-risk patients are composed within the High and Very high categories. However, as shown in Table 9, the IPSS-R has permitted improved refinement of risk categories for the IPSS Intermediate-1 and Intermediate-2 patients because a substantial portion of the patients who would have been categorized as IPSS Intermediate-1 are now in the IPSS-R Low category; a substantial portion of the patients who would have been categorized as IPSS Intermediate-2 are now in the IPSS-R High category. In other words, the "better risk" IPSS Intermediate-1 patients have been categorized into the lower-risk IPSS-R category; the "poorer risk" IPSS Intermediate-2 patients are now in the higher-risk IPSS-R category. Remaining within the IPSS-R Intermediate category are those who, indeed, have "intermediate" risk (Tables 5 and 7). On review, the clinical outcome data indicate that the IPSS-R Intermediate category appears closer to the initial IMRAW IPSS Intermediate-1 group (survival 3.0 years for IPSS-R Intermediate vs 3.5 years for IPSS Intermediate-1) than it is to IPSS Intermediate-2[1]; the proximity of AML evolution for the IPSS-R Intermediate category is also closer to Intermediate-1 than to Intermediate-2 (ie, the "lower risk" patient group). However, per Table 7, the proportion of patients dying with leukemia for the

Table 6. IPSS-R survival related to age

	IPSS-R prognostic risk categories				
Ages, y	**Very low**	**Low**	**Intermediate**	**High**	**Very high**
All	8.8	5.3	3.0	1.6	0.8
≤ 60	NR	8.8	5.2	2.1	0.9
	(13.0-NR)	(8.1-12.1)	(4.0-7.7)	(1.7-2.8)	(0.8-1.0)
> 60-70	10.2	6.1	3.3	1.6	0.8
	(9.1-NR)	(5.3-7.4)	(2.5-4.0)	(1.5-2.0)	(0.7-1.0)
> 70-80	7.0	4.7	2.7	1.5	0.7
	(5.9-9.0)	(4.3-5.3)	(2.4-3.1)	(1.3-1.7)	(0.6-0.8)
> 80	5.2	3.2	1.8	1.5	0.7
	(4.2-5.9)	(2.8-3.8)	(1.6-2.6)	(1.2-1.7)	(0.5-0.8)
≤ 60 (median, 52)	NR	8.8	5.2	2.1	0.9
> 60 (median, 74)	7.5	4.7	2.6	1.5	0.7
≤ 70 (median, 62)	13.3	7.7	3.9	1.7	0.9
> 70 (median, 77)	5.9	4.2	2.5	1.4	0.7

Data are median (95% CI).
NR indicates not reached.

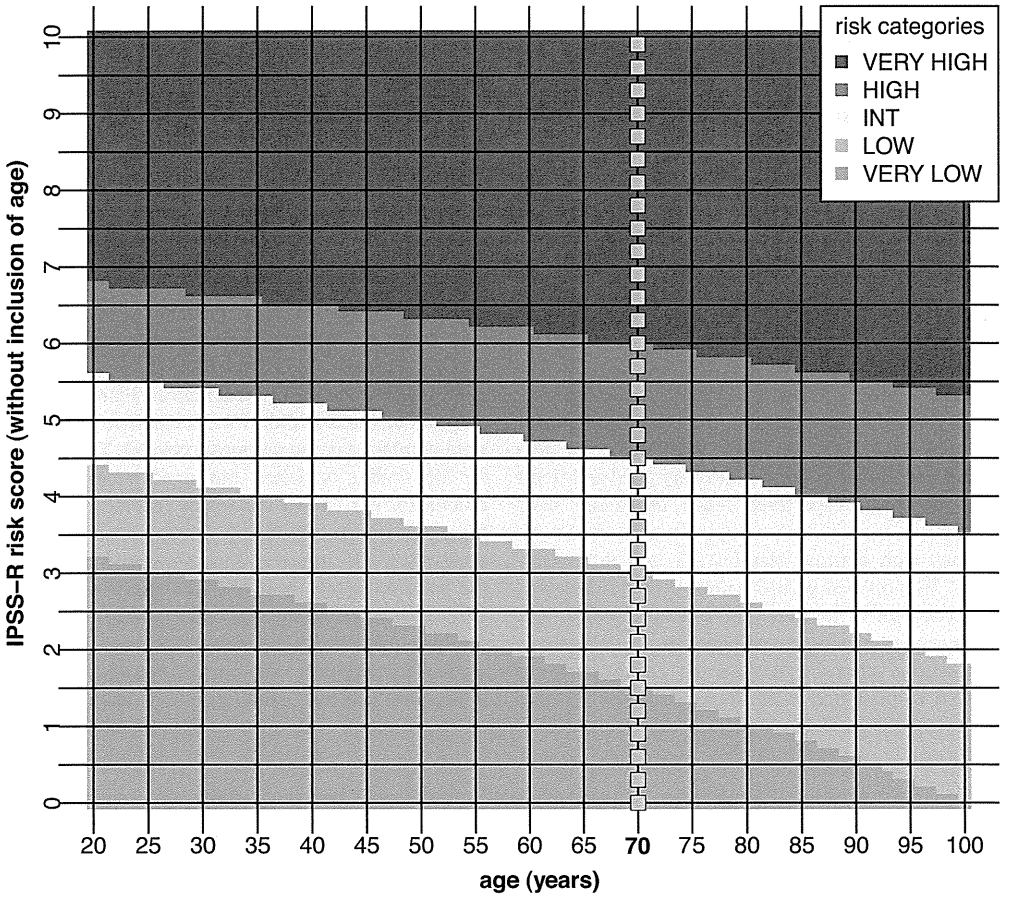

Figure 6. Age-adjusted IPSS-R risk categories. The nomogram describes predicted survival based on patient age and IPSS-R risk status (IPSS-RA). To determine an age-adjusted risk categorization, for example, follow the horizontal line, starting at the IPSS-R risk score 3.5 on the vertical axis (Int [Intermediate] risk category per Table 4) to the age of the patient and record the color at that point. If the patient is 45 years, the 3.5′-line and the vertical 45-year line cross in the gray field, placing the patient in the Low risk category, whereas if the patient is 95 years the 3.5′-line and the 95-year line cross in the yellow field, placing the patient in the Intermediate risk category. As indicated, for most patients in the Very high risk category there is no change of risk group, whereas for most patients in the lower risk categories there is greater possibility of category change. Note the "dotted" vertical line at 70 years, which is at the median age of the IWG-PM patient cohort from which the basic risk category scores were calculated (ie, without need for age correction for these patients). The formula to generate the age-adjusted risk score in the figure: (years − 70) × [0.05 − (IPSS-R risk score × 0.005)]. Example: For the 45-year-old patient with an IPSS-R risk score of 3.5 (Intermediate risk): (45-70) × [0.05 − (3.5 × 0.005)] = −0.81. Thus, 3.5-0.8 = 2.7 [age-adjusted IPSS-R score, IPSS-RA: Low risk].

IPSS-R Intermediate category is distinctively worse than for the lower-risk categories. As survival is the major endpoint for most MDS clinical trials, and of predominant concern to patients and caregivers, it seems reasonable to suggest placement of IPSS-R Intermediate patients into the lower-risk group regarding their potential therapeutic management. However, given the distinctiveness of this patient category, assessment of these patients within both lower- and higher-risk treatment protocols appears warranted. Clinical trial evaluation and recommendations by practice guidelines committees will be needed to substantiate this point. Use of the additional differentiating features (eg, age, performance status, ferritin, LDH; see below) could be of particular value for categorization of these patients.

A major component of this schema was the provision of 5 cytogenetic subgroups (vs 3 in the IPSS) based on an increased number of specific prognostic chromosomal categories[15] versus 6 in the IPSS. This increase in defined cytogenetic categories, with their increased prognostic weight, was the result of a larger number

Table 7. Mortality of MDS patients with or without AML evolution

		Patients who		
Risk category	No. (%) of patients	Died, no. (%)	Died with AML, no. (%)	Died without AML, no. (%)
Very low	1313	350 (27)	46 (13)	304 (87)
Low	2646	1053 (40)	174 (17)	861 (83)
Intermediate	1433	782 (55)	205 (26)	568 (74)
High	898	633 (71)	207 (33)	421 (67)
Very high	722	619 (86)	193 (31)	422 (69)
Total	7012	3437 (49)	825 (24)	2576 (76)

Table 8. Refinements of the IPSS-R beyond the IPSS

1. New marrow blast categories
≤ 2%, > 2%-< 5%, 5%-10%, > 10%-30%
2. Refined cytogenetic abnormalities and risk groups
16 (vs 6) specific abnormalities, 5 (vs 3) subgroups
3. Evaluation of depth of cytopenias
Clinically and statistically relevant cutpoints used
4. Inclusion of differentiating features*
Age, Performance Status, serum ferritin, LDH; β_2-microglobulin†
5. Prognostic model with 5 (vs 4) risk categories
Improved predictive power

*For survival.
†Provisional.

Table 9. Distribution (%) of IWG-PM patients who would previously have been categorized by IPSS now categorized by IPSS-R

IPSS	Very low	Low	Intermediate	High	Very High
Low (37)	44	52	4	0	0
Intermediate-1 (40)	6	45	38	10	1
Intermediate-2 (16)	0	1	24	45	30
High (7)	0	0	3	19	78
Total	19	38	20	13	10

% indicated within rows. Kendall tau = 0.73.

of patients available for analysis of some of the relatively rare cytogenetic categories. This increased number of cases permitted specific characterization of many of the cytogenetic subgroups that had previously been labeled in the IPSS as "other" and also separated the prior Good and Poor groups into Very good and Good and Poor and Very poor, respectively, thus improving their prognostic accuracy.

Splitting patients with marrow blasts < 5% into those with 0%-2% and > 2-< 5% provided groups with very low risk versus low risk features. The issue of splitting this "low blast group" into 2 separate subgroups may present a challenge for reporting these values in some routine clinical laboratories. However, these differences in blast enumeration were reproducible within the various databases from the different institutions in our study. The discriminatory lower blast percentages should be of particular importance in helping to ensure balanced representation of patients in clinical trials.

The presence of 10%-20% marrow blasts had similar impact on clinical outcomes as did 21%-30% blasts. Thus, these 2 categories were combined in the prognostic model. The underlying reason(s) for this finding is unclear but could relate to the stringent entry criteria for our patient cohort (eg, excluding treated patients and those with high circulating blasts or patients' innate biologic similarity). Of interest, this similarity of clinical outcomes for patients within both the low marrow blast group and the 10%-30% blast group was also demonstrated in the IMRAW database from which the initial IPSS was generated.

Scoring the depth of cytopenias by subdivision at clinically and statistically relevant cutpoints rather than solely counting their

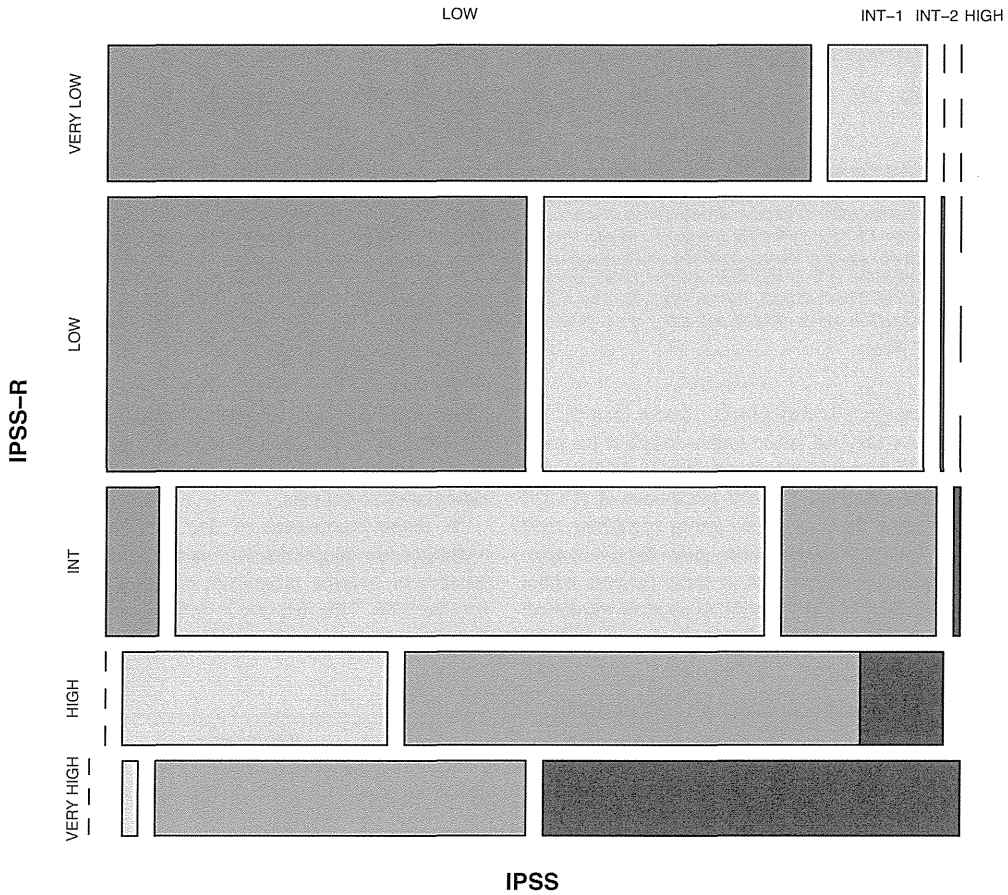

Figure 7. Comparison of IPSS-R and IPSS subgroups within the IWG-PM database patient cohort. Vertical axis represents IPSS-R categories' and horizontal axis, IPSS categories. The proportion of patients in each category is shown in Table 9. Kendall $\tau = 0.73$.

Table 10. IPSS-R prognostic risk category: clinical outcomes of Medical University of Vienna patients (n = 200)

	Very low	Low	Intermediate	High	Very high
Patients, %	21	38	18	14	8
Survival					
All*	9.3	6.3	3.4	1.2	0.6
Hazard ratio	0.8	1	2.1	4.3	9.4
95% CI	0.4-1.5	0.7-1.5	1.3-3.5	2.4-7.7	4.3-20.8
AML transformation					
AML/25%*†	NR	NR	2.4	0.8	0.6
Hazard ratio	0	1	8.0	18.7	52.2
95% CI	0-∞	0.3-3.9	3.1-20.5	7.0-49.7	13.8-198.2

NR indicates not reached.
*Medians, years (95% CI; $P < .001$).
†Median time to 25% AML evolution (95% CI; $P < .001$).

presence was demonstrated as being useful. The degree of anemia is an important correlate of poor clinical outcomes in MDS[27] and appears to be a good surrogate for RBC transfusion dependence.[28] In this regard, low hemoglobin levels have recently replaced RBC transfusion dependence as a prognostic parameter of the WPSS.[28] Underlying this finding, chronic anemia may contribute to the high nonleukemic mortality related to cardiac disease in MDS patients.[2,3,28]

The other cytopenia cutpoints also have clinical relevance. The ANC of 0.8×10^9/L (in IPSS-R) is associated with higher potential infectious risk rather than that of 1.8×10^9/L (in the IPSS).[29,30] Severe thrombocytopenia has been associated with increased morbidity and poor survival in MDS patients.[29,31-33]

The impact of age was a major prognostic parameter for overall survival, although not for AML evolution. This effect has previously been shown with the IPSS and in other studies.[1,29,34-36] In the IPSS-R prognostic model, the data are shown for age 70 years (the near median age of our patient cohort). However, to incorporate the model for different patient ages (IPSS-RA), we provide a formula (in "Results"), which permits statistical adjustment of survival prognosis for patients of all ages. This formula and the Figure 6 nomogram for calculating the impact of age for modifying the risk score/category provide resources for clinicians and for trial design and analysis. These age-related survival data are also shown in Table 6, Figures 5 and 6, and supplemental Figure 5. Our approach herein for providing age as an important, although optional, feature to assess predicted survival permits this variable to be used if total mortality risk is the aim but to not be used if solely disease-related risk is the objective. Age as a variable has some prognostic influence in all risk groups, but with more impact in lower than in higher-risk patients.

Additional differentiating features in the IPSS-R were additive to the 5 major parameters for predicting survival, albeit not for AML evolution: performance status, serum ferritin, and LDH levels. Serum LDH,[9-11] ferritin,[12] and β_2-microglobulin[13,14] have previously been shown to have prognostic importance for survival in MDS. Thus, our analyses have helped determine that these clinical features, of the many previously reported, were also reproducible in our large patient cohort after multivariate analysis. Relevant is that, although these features had some additive impact on survival (often moving patients either into a higher or lower risk category based on dichotomized values), this effect was relatively minor for determining prognostic risk categories compared with that of the 5 major features (see gains in Dxy's and score points shown in supplemental Table 1, which would be added to the basic IPSS-R prognostic score values seen in Table 3). None of these features had additive prognostic impact on the potential for AML transformation.

Our data indicated the importance of performance status as contributing to prognosis for survival in MDS. Other studies have demonstrated the impact of comorbidities on survival in MDS,[5,18-20,37-39] which may in part be reflected by performance status.

The negative impact of elevated serum ferritin levels for survival in our patients may relate to prior RBC transfusions contributing to iron overload and its complications or may reflect the severity of the anemia and degree of ineffective erythropoiesis because of the patients' poorer innate marrow function.[2,12,40] In addition, as serum ferritin is an inflammatory marker and this value was obtained early in our patients' disease courses (ie, before high RBC transfusion burden), this abnormality may reflect the effects of inflammatory cytokines in MDS.[41,42]

Although high serum β_2-microglobulin levels had significant negative impact on survival in our patients, as this feature was essentially reported from only one institution in our cohort, we have included this as a provisional predictive parameter. In addition, renal dysfunction alters these levels and could confound these results.

Marrow fibrosis did not show incremental prognostic value for clinical outcomes in our study despite previous reports demonstrating poor prognosis of this morphologic feature.[15-17] The absence of this variable as an additive factor could relate to the low number of patients assessed for this feature (19%) as well as the variable ways the degree of fibrosis was reported from the different institutions in our study.

Comparison of the IPSS-R with the IPSS categorizations of our IWG-PM patients indicated that a substantial proportion of patients within the IPSS lower-risk group (27%) would be "upstaged" with the IPSS-R categorization, and 18% in the higher risk group would be "downstaged" by IPSS-R. Such findings have implications for more precisely evaluating patient prognosis and their potential management.

Our data showed that the risk of dying related to leukemic evolution, for our patients observed until death, was increased in those with more advanced prognostic risk categories, Table 7). Thus, mortality from complications of bone marrow failure (without leukemic evolution) and patient comorbidities plays a major role in the clinical outcome of the lower-risk patients in contrast to the more prominent role of leukemic evolution in the higher-risk patients. The proportion of patients dying is lower in our patent cohort compared with the patient group within the original IMRAW patient sample,[1] predominantly because of a lower proportion of our patients dying after AML transformation. This was probably related to the more stringent entry criteria used in our current patient cohort.

Regarding the stringent inclusion criteria in our study, to be more precise with the diagnostic entity of MDS, and as recommended by National Comprehensive Cancer Network practice guidelines for MDS, relative stability of peripheral blood counts for 1-2 months was required to exclude other possible etiologies for their cytopenias, such as drugs, other diseases, or incipient evolution to AML.[43] Exclusion of these patients had minimal influence on the estimates of survival and time to AML evolution (data not shown). In addition, we excluded patients with secondary MDS as their clinical and biologic features (higher degree of AML progression, decreased survival and differing distribution, incidence and types of aberrant and poor risk cytogenetics) distinctively differ from those of primary MDS patients.[44]

An external validation cohort of untreated primary MDS patients from the Medical University of Vienna was evaluated and demonstrated that the IPSS-R model also fit these data well. In particular, the validity of the model for this cohort was indicated by the high prognostic power (ie, Dxy's) and clearly differing temporal medians and hazard ratios between prognostic risk categories for both survival and AML evolution, including age-related survival. Further validation of the IPSS-R in other patient cohorts is warranted.

Some of the patients in the IWG-PM project were also assessed by the WPSS parameters.[2,3] However, because of the relatively low proportion of our patients having several of this system's parameters reported (cellular dysplasia, RBC transfusions), these clinical variables were not included in our analysis. Modification and refinement of the WPSS (WPSS-R) based on the additional features present in the IWG-PM database will be the subject of a separate publication.

In conclusion, the IPSS-R retained continuity with the IPSS and was shown to possess improved prognostic ability for survival and AML evolution compared with the IPSS along with determining additional predictive features, particularly age, having significant impact on survival in primary untreated MDS patients. As such, the IPSS-R should prove beneficial for determination of prognostic status of untreated patients with this disease and aid design and analysis of clinical trials for this disease. Given recent molecular[45-47] and flow cytometric studies[48,49] showing prognostic value in MDS, further investigations to determine the impact of these technologies on the IPSS-R are warranted and ongoing.

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The URLs for a Web-based calculator tool to the IPSS-R are located at http://www.ipss-r.com and at http://www.mds-foundation.org/calculator/index.php.

Authorship

Contribution: P.L.G. designed, performed, and coordinated the research, collected, contributed, analyzed and interpreted the data, and wrote the manuscript; H.T. designed and performed the research, performed the statistical analyses, produced the figures, and edited the manuscript; J.S. collected and contributed data, performed the research, and analyzed and interpreted the data; G.S., G.G.-M., F.S., D.B., P.F., A.L., J.C., O.K., M.L., J.M., S.M.M.M., Y.M., M.P., M.S., W.R.S., R.S., S.T., P.V., T.V., A.A.v.d.L., and U.G. collected and contributed data, analyzed the results and critically revised the paper; J.M.B. collected, contributed, analyzed, and interpreted the data and critically revised the paper; C.F., M.M.L.B., and M.L.S. analyzed and interpreted the data and critically revised the paper; F.D., H.K., A.K., L.M., and M.C. collected and contributed data and reviewed the manuscript; the Cytogenetics Committee members reviewed the cytogenetics data and formulations; and D.H. collected, contributed, analyzed, and interpreted data, designed and performed the research, and edited the manuscript.

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TLL performed the plasma cytokine profiling. CAH provided hematopathology review. All authors approved the final version of the paper for submission.

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Direct binding of Grb2 has an important role in the development of myeloproliferative disease induced by ETV6/FLT3

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FMS-like tyrosine kinase 3 (*FLT3*) is one of the most frequently mutated genes in hematological malignancies.[1] The most common mutations of *FLT3* are internal tandem duplications (ITDs) within the juxtamembrane domain, which occur in 20% to 30% of patients with acute myeloid leukemia (AML).[2,3] Although FLT3 is a potential therapeutic target in AML, recent studies involving FLT3 inhibitors as single agents in patients with AML showed limited clinical responses.[4] FLT3 has been reported to fuse to ETV6 (TEL) in a few cases of myeloid/lymphoid neoplasms with eosinophilia (MLN-eo) carrying a translocation t(12;13)(p13;q12).[5,6] Although it has been shown that ETV6/FLT3 acts as a constitutively active tyrosine kinase, the molecular mechanisms underlying ETV6/FLT3-mediated leukemogenesis remain incompletely understood.[7–9]

We identified a novel *ETV6/FLT3* variant fusion transcript (E/F-1) in a MLN-eo patient (Supplementary Figures S1A and B) and investigated the transforming properties of ETV6/FLT3 *in vivo* using a murine bone marrow transplant (BMT) model.[10,11] E/F-1-transduced recipients developed an aggressive polyclonal myeloproliferative disease (MPD) in 100% of recipient mice with a latency of 3-4 weeks, as evidenced by marked leukocytosis, splenomegaly and massive expansion of myeloid cells in peripheral blood, spleen and bone marrow (Figures 1A–C). In this mice model, eosinophilia was not observed. Flow cytometric analysis of the peripheral blood from E/F-1 mice showed a large population of EGFP$^+$/Mac-1$^+$/Gr-1$^+$ cells. In primary E/F-1 mice, serial passage was performed by transferring a 1:1 mixture of spleen and bone marrow cells to sublethally irradiated recipient mice. This resulted in hematopoietic malignancies in most of the recipient mice receiving five different primary tumors. For three of the primary tumors, it was possible to transmit the MPD for at least one round. In all cases of serial passage, the MPD transformed into aggressive T-lymphoblastic lymphoma (T-LBL) with a latency of 4–17 weeks (Figure 1E, Supplementary Figure S2B). Most lymphoma occurred in the thymus or abdominal lymph nodes, and some of the secondary recipient mice displayed leukocytosis, generalized lymphadenopathy or hepatosplenomegaly. Histopathological examination revealed that the architecture of the lymph nodes and the thymus was completely effaced and that they contained a uniform population of lymphoblasts. The liver showed prominent periportal, lobular and sinusoidal

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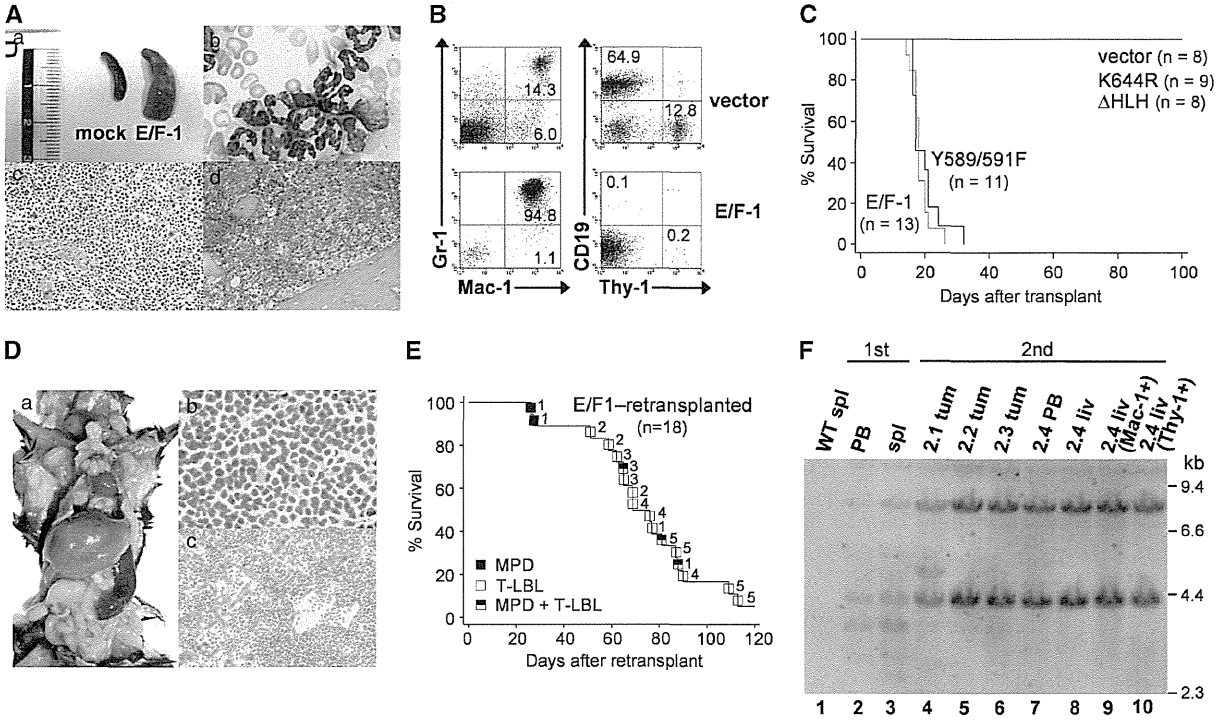

Figure 1. ETV6/FLT3 induces a rapidly fatal MPD in primary recipient mice, which transformed into T-LBL and MPD during serial passage. (**A**) (a) Splenomegaly associated with MPD. (b) Representative May–Giemsa-stained peripheral blood smear of the diseased mouse (× 100). (c, d) Representative hematoxylin and eosin-stained bone marrow (c) and spleen (d) of the diseased mouse (× 20). (**B**) Flow cytometric analysis of cells from the peripheral blood of vector control and E/F-1 mice. The percentages of cells in quadrants of interest are shown. (**C**) Survival curve for recipients of bone marrow transduced with a vector control ($n = 8$), E/F-1 ($n = 13$), Y589/591F mutant ($n = 11$), kinase-inactive K644R mutant ($n = 9$) or deletion mutant of the HLH oligomerization domain of ETV6 (ΔHLH) ($n = 8$). Mice transplanted with a vector control, K644R, or ΔHLH remain free of disease 180 days after transplant. Survival data are cumulative from two or three separate experiments for all retroviral constructs. (**D**) (a) Macroscopic examination of a secondary recipient with T-LBL (b and c) Hematoxylin and eosin-stained lymph node (b) and liver (c) of the diseased mouse (× 100 and × 20, respectively). (**E**) Kaplan–Meier survival analysis of the secondary recipients. Most secondary recipients succumbed to T-LBL or/and MPD. Pairs of recipients transplanted with cells from the same primary donor are indicated by numbers. (**F**) Proviral integrations in cells isolated from primary and secondary recipients. Genomic DNA isolated from the indicated tissues of a wild-type (WT) mouse (lane 1), a primary MPD mouse (lanes 2 and 3) and four secondary T-LBL mice receiving bone marrow and spleen from the same primary MPD mouse (lanes 4–10) was digested with *Eco*RI and analyzed for proviral integrations by hybridization with a DIG-labeled EGFP probe. Lanes 4–6 are DNAs from the tumors of mice with T-LBL. Lanes 7–10 represent lineage analysis from a single secondary mouse, which developed both MPD and T-LBL (2.4). The peripheral blood of the 2.4 mouse contained 83.5% EGFP$^+$Mac-1$^+$ and 10.6% EGFP$^+$Thy-1$^+$ cells, and the liver of the 2.4 mouse contained 27.2% EGFP$^+$Mac-1$^+$ and 58.8% EGFP$^+$Thy-1$^+$ cells at the time of euthanization. The MACS-sorted liver myeloid (Mac-1$^+$) and T cells (Thy-1$^+$) from this mouse were 98.1% and 98.4% pure, respectively. DNA size markers (in kb) are shown on the right. liv, liver; PB, peripheral blood; spl, spleen; tum, tumor.

infiltration by lymphoma cells (Figure 1D). Flow cytometric analysis of spleen cells revealed that the lymphomas typically showed an immature T-cell immunophenotype characterized by expression of both CD4 and CD8 (Supplementary Figure S2A). Affected tissues from secondary diseased mice contained proviral integrations identical to those in the primary MPD mouse (Figure 1F). ETV6/FLT3-induced T-LBL was transplantable, with all tertiary transplant recipients rapidly succumbing to T-LBL at 4–7 weeks after transfer arising from common clones identified in the secondary mice (Supplementary Figure S2C).

Previous studies have shown that FLT3-ITDs induce a lethal MPD in mice and that tyrosine residues 589 and 591 in the juxtamembrane domain of FLT3 are critical for STAT5 phosphorylation and generation of the MPD phenotype.[10] We also demonstrated the corresponding results for FLT3-ITD in our murine BMT experiment (Supplementary Figures S3A and B). On the other hand, mice that received the double tyrosine-to-phenylalanine mutant of E/F-1 at sites 589 and 591 (Y589/591F) in the juxtamembrane domain of FLT3 developed a similar MPD (Supplementary Table S1). There was no significant difference in survival between recipients of E/F-1 vs Y589/591F, with both mice groups succumbing to a fatal MPD within a median survival time of 18 and 19.5 days, respectively ($P = 0.284$; Figure 1C). The Y589/591F mutation did not abrogate STAT5, Erk1/2 and Akt activation in Ba/F3 cells transformed by E/F-1 (Supplementary Figure S4), which is consistent with the previous studies using a deletion mutant of the FLT3 juxtamembrane domain in ETV6/FLT3.[9]

Growth factor receptor-binding protein 2 (Grb2) is an adaptor protein known to bind several receptor tyrosine kinases. Grb2 binds the scaffolding protein Grb2-associated binder 2 (Gab2) and contributes to survival signaling in ligand-activated wild-type FLT3.[12] A recent study has shown that tyrosines 768, 955 and 969 of FLT3 are the direct Grb2-binding sites of importance for FLT3-ITD-mediated proliferation and survival of hematopoietic cells *in vitro* as a result of STAT5 activation via Gab2.[13] However, there have been no reports regarding the *in vivo* effects of direct Grb2 binding by oncogenic FLT3 in leukemogenesis. Thus, we investigated the role of Grb2 binding in ETV6/FLT3-mediated leukemogenesis. Inspection of the ETV6 portion of the fusion protein revealed only two candidate tyrosines for direct binding of

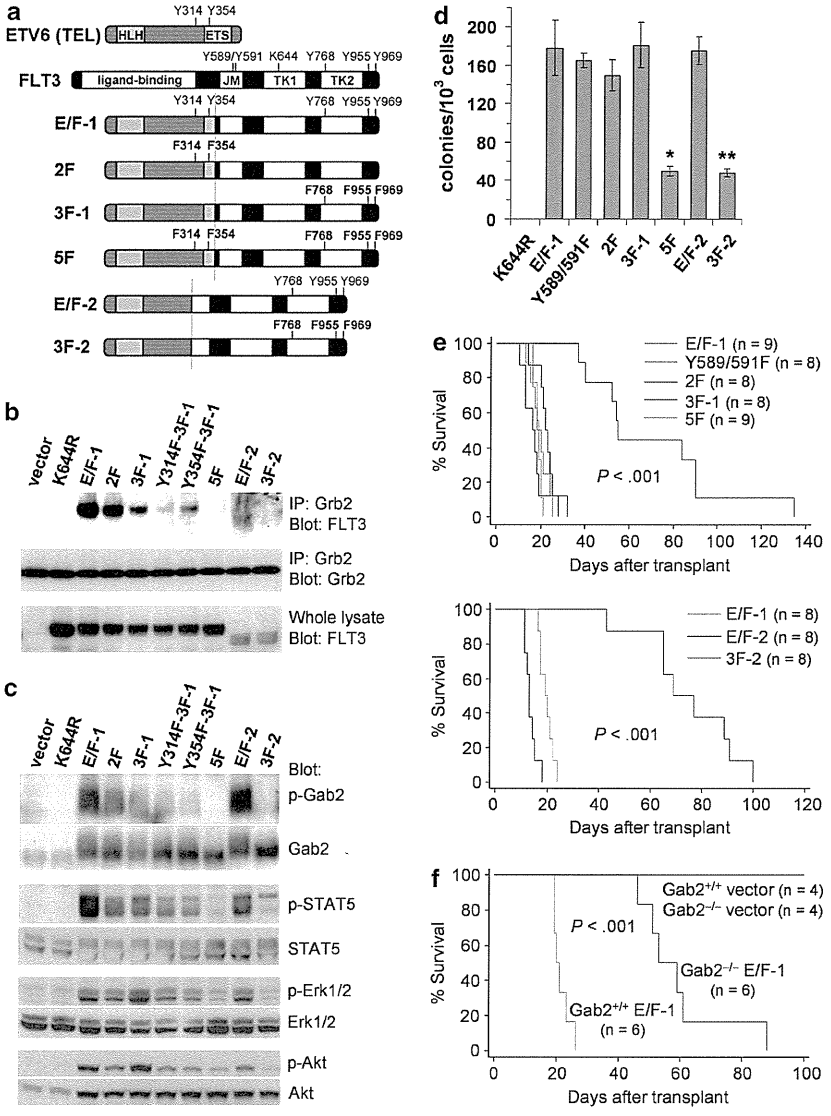

Figure 2. Both ETV6 and FLT3 portions contribute to ETV6/FLT3-mediated leukemogenesis via Grb2-Gab2 pathway. (**a**) Schematic representation of ETV6/FLT3 fusion proteins including the series of Grb2-binding site mutants. The point of fusion is indicated by a vertical dotted line. E/F-1 was cloned by us. E/F-2 was cloned previously (Vu *et al.*[5]). (**b**) Coimmunoprecipitation: lysates from Ba/F3 cells expressing the indicated ETV6/FLT3 proteins were immunoprecipitated using an anti-Grb2 antibody and blotted with anti-FLT3 (top) and anti-Grb2 (middle) antibodies. Whole-cell lysates were also blotted with the anti-FLT3 antibody (bottom). As a control, lysates from vector-transduced cells were included. Three independent experiments were performed and representative data are shown. (**c**) Activation of downstream targets was demonstrated by blotting the whole-cell lysates of Ba/F3 cells with the indicated phosphospecific antibodies. After stripping, the membranes were reprobed with the indicated total antibodies. Three independent experiments were performed and representative data are shown. (**d**) Cytokine-independent colony formation of whole bone marrow cells expressing ETV6/FLT3 wild-type or Grb2-binding mutants. The difference between E/F-1 and 5F (*) and between E/F-2 and 3F-2 (**) is statistically significant ($P<0.001$, unpaired *t*-test). Data are the mean ± s.d. of three independent experiments. (**e**) Survival curve for recipients of bone marrow transduced with ETV6/FLT3 and Grb2-binding mutants. Both E/F-1 and E/F-2 mice caused rapidly fatal MPD. 5F mutant mice and 3F-2 mutant mice developed MPD with a longer median survival of 55 and 73 days, respectively ($P<0.001$ vs E/F-1 and E/F-2, respectively). One of the 5F mutant mice died of severe anemia without showing any signs of MPD. Survival data are cumulative from two or three separate experiments for all retroviral constructs. (**f**) Survival curve for recipients of Gab2$^{-/-}$ and Gab2$^{+/+}$ bone marrow transduced with a vector control or E/F-1. The *P*-value represents a comparison of survival by E/F-1 on Gab2$^{-/-}$ vs Gab2$^{+/+}$ background.

Grb2 at positions 314 and 354. To test whether Grb2 binds directly to ETV6/FLT3, we made a series of Grb2-binding mutants of ETV6/FLT3 (Figure 2a). The Y314/354F double point mutant (2F) and the Y768/955/969F triple mutant (3F-1) of E/F-1 showed a reduced ability to bind Grb2 as compared with the E/F-1. Furthermore, when we mutated tyrosines 314 or 354 to phenylalanine in the context of the Y768/955/969F triple mutant, the association of ETV6/FLT3 with Grb2 was significantly reduced as compared with the Y768/955/969F triple mutant. Finally, when we mutated all five specific tyrosines (Y314/354/768/955/969) to phenylalanine, we observed that ETV6/FLT3 was no longer able to bind Grb2 (Figure 2b). Simultaneous mutation of these five tyrosine residues resulted in an absence of Gab2 phosphorylation, and impaired activation of STAT5, Erk1/2 and Akt in Ba/F3 cells (Figure 2c). ETV6/FLT3 variant E/F-2, which lacked the Grb2-binding sites of ETV6, was unable to bind to Grb2 when all three Grb2-binding sites of

FLT3 were mutated (Figure 2b). This Y768/955/969F triple mutant of E/F-2 (3F-2) was also unable to phosphorylate Gab2 and showed weaker activation of STAT5, Erk1/2 and Akt as compared with E/F-2 in Ba/F3 cells (Figure 2c). Both E/F-1 and E/F-2 transformed bone marrow cells to be capable of cytokine-independent growth in methylcellulose medium. Transformation was significantly decreased in the 5F and 3F-2 mutants, which were unable to bind Grb2, but not in 2F and 3F-1 mutants (Figure 2d). To examine the contribution of Grb2 binding to ETV6/FLT3-induced MPD *in vivo*, we performed BMT experiments. White blood cell (WBC) counts and spleen weights of the mice receiving 5F-transduced bone marrow were significantly lower than those receiving E/F-1-transduced bone marrow (Supplementary Table S1). Flow cytometric analyses of spleen cells showed that 5F mice had a reduced fraction of Mac-1^+/Gr-1^+ cells compared with E/F-1 mice (Supplementary Figure S5A). 5F mice showed significantly less infiltrate in the hepatic lobules or periportal areas than the E/F-1 mice (Supplementary Figure S5B). Recipients of both 2F and 3F-1 developed rapidly fatal MPD with a comparable latency to those of E/F-1. Survival of 5F and 3F-2 mice was significantly prolonged compared with that of E/F-1 and E/F-2 mice, respectively (55 days vs 18 days, 73 days vs 13 days, respectively; $P<0.001$; Figure 2e), although most 5F and 3F-2 recipient mice eventually succumbed to MPD.

Finally, we compared the ability of ETV6/FLT3 to transform primary myeloid cells from the bone marrow of Gab2$^{-/-}$ and Gab2$^{+/+}$ mice. Expression of E/F-1 in Gab2$^{-/-}$ cells resulted in an approximately threefold lower number of cytokine-independent CFU-C (Supplementary Figure S6A). We assessed the relative contribution of the *Gab2* gene to ETV6/FLT3-mediated leukemogenesis in BMT experiments using Gab2$^{-/-}$ and Gab2$^{+/+}$ donor mice. All mice transplanted with Gab2$^{+/+}$ bone marrow cells expressing ETV6/FLT3 developed severe MPD (median WBC, $236\times10^3/\mu l$; spleen weight, 561 mg), as expected (Supplementary Figure S6B). ETV6/FLT3-induced myeloproliferation was attenuated in mice transplanted with Gab2$^{-/-}$ bone marrow cells expressing ETV6/FLT3 (median WBC, $31\times10^3/\mu l$; spleen weight, 350 mg). Survival of mice injected with Gab2$^{-/-}$ bone marrow cells expressing E/F-1 was significantly prolonged in comparison with those injected with Gab2$^{+/+}$ cells (56 days vs 21 days, $P<0.001$; Figure 2f), although all recipients of Gab2$^{-/-}$ background bone marrow cells eventually succumbed to MPD.

Our results suggest that ETV6/FLT3 has more potent oncogenic activity than FLT3-ITDs and can transform progenitor cells with the capacity to differentiate into myeloid and lymphoid progeny, supporting the contention that human *ETV6/FLT3*-positive MLN-eo is a stem cell disorder. Unlike FLT3-ITDs, mice that received the Y589/591F mutant of ETV6/FLT3 also developed a lethal MPD with a short latency. The reason for the discrepancy between ETV6/FLT3 and FLT3-ITDs is not clear. This may be due to altered structural conformation of ETV6/FLT3 relative to wild-type FLT3 or alternatively, it may be due to different subcellular localization of the fusion protein and FLT3-ITDs.[14,15] Recently it was reported that sunitinib and sorafenib, tyrosine kinase inhibitors with multiple targets including FLT3, had therapeutic efficacy in two patients with *ETV6/FLT3*-positive MLN-eo.[6] Unfortunately, similar to most of the patients with *FLT3-ITD*-positive AML, relapse and resistance occurred in both patients. Although clinical application of Grb2 inhibitors remains limited to just a phase I trial of a liposomal antisense for hematological malignancies, the results of the current study indicate therapeutic potential against Grb2 in patients with *ETV6/FLT3*-positive MLN-eo. In addition, previous studies have shown that the Grb2-Gab2 pathway also has an important role in FLT3-ITD-mediated cell proliferation and survival.[13,15] These findings suggest that inhibition of this pathway may be useful in the treatment of FLT3-associated leukemia.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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bjh short report

Outcome of children with refractory anaemia with excess of blast (RAEB) and RAEB in Transformation (RAEB-T) in the Japanese MDS99 study

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Summary

We report the outcome of 16 children with refractory anaemia with excess of blasts (RAEB; $n = 4$) and RAEB in transformation (RAEB-T; $n = 12$) following induction therapy with etoposide, cytarabine and mitoxantrone (ECM) prior to haematopoietic stem cell transplantation (HSCT). The median observation period was 77 months (range 5–123). Complete remission rate was 81% following induction; no toxic deaths occurred. Eight-year event-free survival and overall survival was 50% and 56%, respectively. None of the three patients with a complex karyotype survived, suggesting karyotype is a crucial prognostic factor for survival. This study indicates the safety and high remission rate of ECM and high survival rates after HSCT for paediatric RAEB and RAEB-T.

Keywords: refractory anaemia with excess of blast, refractory anaemia with excess of blast in transformation, childhood, haematopoietic stem cell transplantation.

Myelodysplastic syndrome (MDS) is a rare haematological disorder in childhood, accounting for <5% of all paediatric haematopoietic neoplasias (Passmore *et al*, 2003; Niemeyer & Kratz, 2008) . In the French–American–British (FAB) Co-operative Group classification, refractory anaemia with excess blasts (RAEB) is defined by dysplastic morphology in two or more cell lineages with more than 5% blasts and RAEB in transformation (RAEB-T) is defined as RAEB with 20–29% blasts.

There is no standardized therapy for paediatric RAEB and RAEB-T. Conventional acute myeloid leukaemia (AML)-type chemotherapy without haematopoietic stem cell transplantation (HSCT) resulted in survival rates below 30% (Sasaki *et al*, 2001; Woods *et al*, 2002). Although the only curative treatment strategy is considered to be allogeneic HSCT, the therapeutic utility of AML-type remission induction therapy before HSCT remains unclear.

To evaluate safety and efficacy of AML-type remission induction therapy before allogeneic HSCT, we conducted the MDS99 study for paediatric RAEB and RAEB-T and report patient outcomes.

Patients and methods

Nineteen patients with paediatric RAEB and RAEB-T who were enrolled in the observational study MDS99 between October 1999 and June 2004 were treated according to the study protocol. Diagnosis was confirmed by central review of morphology performed by the MDS committee of the Japanese Society of Paediatric Haematology (JSPH) according to the FAB classification (Hasegawa *et al*, 2009). None of the patients had undergone previous chemotherapy or radiotherapy, or had a history of inherited bone marrow failure syndrome or aplastic anaemia. Remission induction therapy

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consisted of etoposide 150 mg/m^2 2-h infusion (days 1–5), cytarabine 200 mg/m^2 12-h infusion (days 6–12), mitoxantrone 5 mg/m^2 intravenous injection (days 6–10) and intrathecal injection of methotrexate, cytarabine and hydrocortisone (day 6). This ECM regimen was part of the previously reported Japanese AML treatment regimen (Tomizawa *et al*, 2007; Tsukimoto *et al*, 2009). Complete remission (CR) was defined morphologically as <5% blasts and recovery of normal haematopoiesis. After remission induction therapy, HSCT was performed without consolidation therapy. Conditioning regimens and stem cell sources were determined by each physician. All treatments were performed with informed consent from the patients' parents or guardians according to the Declaration of Helsinki. This study was approved by the Institutional Review Board of each participating institution. Survival curves were calculated by the Kaplan–Meier method.

Results

The characteristics of 19 study patients are shown in Tables I and II. Survival status was as of April 2011. In further analysis, we focused on 16 patients who underwent ECM regimen as remission induction therapy. Of these patients, 13 patients achieved complete remission and CR rate was estimated to be 81%. Median time between initiation of chemotherapy and remission evaluation (remission induction duration) was 41 d (range 29–155). Grade 3 or 4 non-haematological toxicities according to the National Cancer Institute-Common Toxicity Criteria were reported in nine patients (infection 4, allergic reaction 4 and mucositis 1), but no toxic death was observed. One patient [unique patient number (UPN) 19] who underwent another induction regimen did not achieve CR. Among 16 patients, one patient (UPN 8) who achieved CR after ECM regimen did not receive HSCT because of parental refusal and she died of primary disease (DOD). The other 15 patients underwent allogeneic HSCT (12 in CR and 3 in non-CR). Outcomes of all patients are shown in Figure S1. All preconditioning regimens were myeloablative [total body irradiation (TBI) regimen, $n = 15$; non-TBI, $n = 3$]. Cyclosporine or tacrolimus plus methotrexate were used for graft-*versus*-host- disease (GVHD) prophylaxis.

Table I. Details of patients with RAEB/RAEB-T in the MDS99 study.

Number of patients	19
Gender: male/female	10/9
Age at diagnosis (years): median (range)	6 (1–14)
Disease type: RAEB/RAEB-T	7/12
Remission induction: ECM/other/none	16/1/2
HSCT type	
MSD-BMT	2
MSD-PBSCT	1
MFD-BMT	2
UD-BMT	5
UD-CBT	8
Interval between diagnosis and HSCT (months): median (range)	5 (2–8)
Observation period in months: median (range)	77 (5–123)
Outcome: alive/dead	11/8

RAEB, refractory anaemia with excess blasts; RAEB-T, RAEB in transformation; HSCT, haematopoietic stem cell transplantation; ECM, etoposide, cytarabine, mitoxantrone; MSD, human leucocyte antigen (HLA)-matched sibling donor; BMT, bone marrow transplantation; PBSCT, peripheral blood stem cell transplantation; MFD, HLA-matched family donor; UD, unrelated donor; CBT, cord blood transplantation.

As for the 12 patients who underwent HSCT in CR after ECM regimen, eight patients achieved continuous CR (CCR), three suffered transplantation-related mortality (TRM) [invasive aspergillosis (IA), sepsis and interstitial pneumonitis (IP)] and one suffered DOD. One of the three patients who failed to attain CR after ECM regimen failed to engraft but achieved CCR after the second HSCT, whereas the other two succumbed to disease after HSCT.

Cytogenetic abnormalities were observed in 12 of 19 patients. The 19 patients were classified into four groups as follows; structurally complex karyotype [defined as more than or equal to three chromosomal aberrations in the presence of at least one structural aberration (Gohring *et al*, 2010); $n = 3$], monosomy 7 ($n = 2$), other abnormal karyotype ($n = 7$) and normal karyotype ($n = 7$). Two of the three patients with structurally complex karyotype achieved CR after ECM regimen but the other did not. All these patients died after HSCT (2 DOD, 1 TRM). One of the two patients with monosomy 7 DOD without HSCT due to parental refusal and the other remains in CCR after HSCT without remission induction therapy. Of seven patients with other abnormal karyotypes, six achieved CR with ECM regimen and are alive without disease after HSCT, whereas the other patient failed to achieve CR with ECM regimen and suffered DOD after HSCT. Of seven patients who showed normal karyotype and underwent HSCT, four remain CCR and three died (2 DOD, 1 TRM).

Acute GVHD II–IV was seen in 13 patients and chronic GVHD in 8. No influence of GVHD was observed on survival rate.

The 8-year event-free survival (EFS) and overall survival (OS) of 16 patients who underwent ECM regimen was 50 ± 13% and 56 ± 12%, respectively (Fig 1). No serious late effect was observed among long-term survivors other than secondary hypogonadism due to HSCT.

Discussion

This study aimed to evaluate the safety and efficacy of the ECM regimen, which is an AML-type remission induction therapy, before allogeneic HSCT for paediatric RAEB and RAEB-T. This regimen has been used for AML remission induction and its safety and efficacy have already been

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Table II. Clinical characteristics of 19 patients in the MDS 99 study for RAEB/RAEB- T.

UPN	Age (years)	Diagnosis	Cytogenetics	Response to ECM	Interval between diagnosis and HSCT (months)	Preconditioning	HSCT type	aGVHD (Grade)	cGVHD	Outcome	Survival (months)
1	13	RAEB-T	Other	CR	6	TBI	UD-CBT	II	No	CCR	106+
2	4	RAEB-T	Complex	Non-CR	4	Non-TBI	UD-CBT	II	No	DOD	11
3	10	RAEB-T	Other	CR	5	TBI	UD-CBT	III	No	CCR	119+
4	10	RAEB	Other	CR	3	TBI	UD-CBT	III	Yes	CCR	123+
5	10	RAEB	Normal	Non-CR	5	TBI	UD-BMT	II	No	DOD	10
6	6	RAEB-T	Normal	CR	5	TBI	UD-CBT	II	No	TRM (sepsis)	8
7	4	RAEB-T	Normal	CR	3	TBI	MFD-BMT	II	Yes	CCR	77+
8	11	RAEB-T	Monosomy 7	CR	NA	NA	NA	NA	NA	DOD	5
9	11	RAEB-T	Other	CR	4	TBI	MFD-BMT	III	Yes	CCR	106+
10	1	RAEB-T	Complex	CR	8	TBI	UD-CBT	II	No	TRM (IP)	11
11	3	RAEB-T	Normal	Non-CR	2	TBI	UD-CBT	I	Yes	CCR	107+
12	3	RAEB	Normal	CR	5	Non-TBI	MSD-BMT	I	Yes	CCR	107+
13	4	RAEB	Normal	CR	6	TBI	UD-BMT	IV	Yes	TRM (IA)	16
14	14	RAEB-T	Other	CR	7	TBI	UD-BMT	II	No	CCR	104+
15	13	RAEB-T	Other	CR	5	TBI	MSD-BMT	I	No	CCR	102+
16	6	RAEB-T	Complex	CR	5	TBI	UD-BMT	I	Yes	DOD	21
17	5	RAEB*	Monosomy 7	NA	2	Non-TBI	MSD-PBSCT	III	Yes	CCR	72+
18	6	RAEB*	Normal	NA	5	TBI	UD-BMT	I	No	CCR	92+
19	3	RAEB†	Other	NA	4	TBI	UD-CBT	II	No	DOD	7

UPN, unique patient number; ECM, etoposide, cytarabine, mitoxantrone; HSCT, haematopoietic stem cell transplantation; aGVHD, acute GVHD; cGVHD, chronic GVHD; RAEB, refractory anaemia with excess blasts; RAEB-T, RAEB in transformation; CR, complete remission; NA, not applicable; TBI, total body irradiation; MSD, human leucocyte antigen (HLA)-matched sibling donor; MFD, HLA-matched family donor; UD, unrelated donor; CBT, cord blood transplantation; BMT, bone marrow transplantation; PBSCT, peripheral blood stem cell transplantation; +, patient is alive.

*Patients who did not receive any pre-transplant chemotherapy.

†Patient who underwent pre-transplant chemotherapy other than ECM regimen.

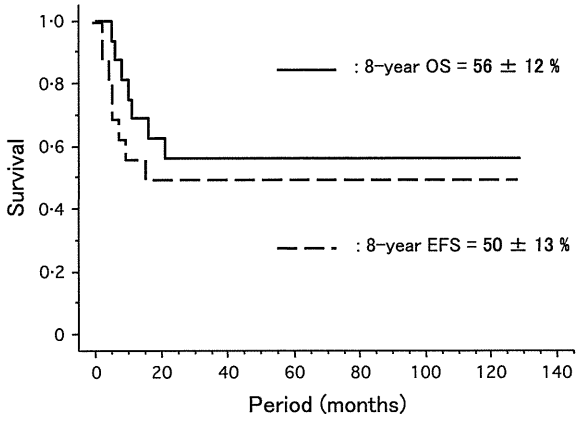

Fig 1. Survival curves of children with RAEB and RAEB-T enrolled in the MDS99 study.

reported in previous Japanese AML studies (Tomizawa *et al*, 2007; Tsukimoto *et al*, 2009). In the present study, remission induction rate with ECM regimen was as high as 81%, which was comparable with that of previous AML studies (Tomizawa *et al*, 2007; Tsukimoto *et al*, 2009). The duration of remission induction (median 41 d) was also acceptable although more than 60 d were required in two patients. Grade 3 or 4 non-haematological toxicities were reported in nine out of 16 patients, however, no toxic death was observed. Although there are several reports regarding the safety and efficacy of AML-type remission induction therapy for paediatric RAEB and RAEB-T, the results are not conclusive (Hasle *et al*, 1996; Chan *et al*, 1997; Creutzig *et al*, 1998; Webb *et al*, 2002; Woods *et al*, 2002). Considering the results of this study, we conclude that the ECM regimen is safe, with a high remission induction rate, for paediatric RAEB and RAEB-T. One might speculate that the advantage of AML-type remission induction therapy was that children with RAEB and RAEB-T became transfusion-independent and less susceptible to infection after remission was obtained and consequently they would be in a much more stable state than non-CR patients.

In spite of the high remission induction rate of the ECM regimen, EFS was not satisfactory. In our study, post-transplantation events were relapse in four, TRM in three and rejection in one. Recently, the European Working Group on Childhood MDS reported results of HSCT for advanced MDS in children using a uniform transplantation regimen (Strahm *et al*, 2011). In their report, EFS was reported as 59% and the cumulative incidence of TRM and relapse incidence were 21% each (Strahm *et al*, 2011). Although Strahm *et al* (2011) did not recommend specific pre-transplant therapy, they concluded the efficacy of AML-type remission induction therapy was restricted to more advanced MDS (i.e. MDS related AML). The major differences between the study reported by Strahm *et al* (2011) and ours included preparative regimens and stem cell sources. The earlier study used a uniform regimen consisting of busulfan, cyclophosphamide and melphalan and their stem cell sources were almost exclusively bone marrow [MSD in 40% and unrelated donor (UD) 60%] (Strahm *et al*, 2011). We mainly used TBI regimen and UD-cord blood was predominantly used as stem cell source. Recently, Madureira *et al* (2011) described the outcome of childhood MDS patients after cord blood transplantation (Madureira *et al*, 2011) with a reported OS of 42%, which is comparable to our study.

A structurally complex karyotype has been reported to be the strongest prognostic factor in advanced childhood MDS (Gohring *et al*, 2010). In the present study, all three patients with a structurally complex karyotype did not achieve CCR, regardless of haematological status at the time of HSCT. Children with a structurally complex karyotype will need novel approaches in addition AML-type remission induction therapy and myeloablative HSCT, whereas a current treatment scheme may be adequate for those with normal karyotype and other abnormal karyotypes. Considering the rarity of the disease, an international collaboration is warranted to conduct a prospective trial in order to explore an appropriate therapeutic strategy.

Conflicts of interest

The authors declare no conflict of interest.

Author contributions

In this study, A.K., D.H. and A.M. wrote the paper. A.K., D. H. and Y.O. analysed the data. D.H., Y.O., K.H. and S.K. performed the research. J.O., T.N. and A.M. designed the research study.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Schema of therapeutic procedure in the MDS 99 study.

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Missense mutations in *PML-RARA* are critical for the lack of responsiveness to arsenic trioxide treatment

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Missense mutations in *PML-RARA* are critical for the lack of responsiveness to arsenic trioxide treatment

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Arsenic trioxide (As_2O_3) is a highly effective treatment for patients with refractory/relapsed acute promyelocytic leukemia (APL), but resistance to As_2O_3 has recently been seen. In the present study, we report the findings that 2 of 15 patients with refractory/relapsed APL treated with As_2O_3 were clinically As_2O_3 resistant. Leukemia cells from these 2 patients harbored missense mutations in promyelocytic leukemia gene–retinoic acid receptor-α gene (*PML-RARA*) transcripts, resulting in amino acid substitutions of A216V and L218P in the PML B2 domain. When wild-type or mutated PML-RARA (PR-WT and PR-B/L-mut, respectively) were overexpressed in HeLa cells, immunoblotting showed SUMOylated and/or oligomerized protein bands in PR-WT but not in PR-B/L-mut after As_2O_3 treatment. Protein-localization analysis indicated that PR-WT in the soluble fraction was transferred to the insoluble fraction after treatment with As_2O_3, but PR-B/L-mut was stably detected in fractions both with and without As_2O_3. Immunofluorescent microscopy analysis showed PR-WT localization as a microgranular pattern in the cytoplasm without As_2O_3 and as a macrogranular pattern with As_2O_3. PR-B/L-mut was diffusely observed in the cytoplasm with and without As_2O_3. Nearly identical localization patterns were observed in patients' primary cells. Therefore, B2 domain mutations may play an important role in aberrant molecular responses to As_2O_3 and may be critical for As_2O_3 resistance in APL. (*Blood.* 2011;118(6):1600-1609)

Introduction

Acute promyelocytic leukemia (APL) is characterized by the reciprocal chromosomal translocation t(15;17)(q22;q21), leading to fusion of the promyelocytic leukemia gene (*PML*) on chromosome 15 and the retinoic acid receptor-α gene (*RARA*) on chromosome 17.[1] *PML-RARA* fusions are detectable in > 95% of patients with APL. In 1985, *all-trans* retinoic acid (ATRA) was introduced for the treatment of APL as a differentiation therapy, and a dramatic improvement in the overall survival of patients with APL has been obtained.[2-4] However, approximately 10%-30% of patients eventually relapse after treatment with combination chemotherapies with ATRA.[5-7]

Arsenic trioxide (As_2O_3) is a critical drug for the treatment of APL and is clinically effective even in ATRA-resistant patients.[8] As_2O_3 is a natural substance that has been used medically for over 2400 years. In the 1970s, a group in China identified As_2O_3 as a component of an anticancer reagent.[9] Over the last 18 years, clinical trials conducted worldwide have demonstrated the efficacy of As_2O_3 for the treatment of relapsed patients with APL.[10,11] Recently, it was also reported that As_2O_3 improves event-free survival and overall survival of adult APL when As_2O_3 is used as a consolidation treatment after obtaining the first remission.[12] Currently, the role of As_2O_3 in frontline therapy is under investigation.[10,13]

Rapid degradation of PML-RARA via targeting of PML has been reported as a molecular mechanism for the effectiveness of As_2O_3.[14] Furthermore, As_2O_3 induces posttranslational modifications of PML-RARA with small ubiquitin-related modifier (SUMO) and ubiquitin, resulting in the transfer of PML-RARA from the soluble fraction to the insoluble nuclear matrix[14] and the degradation of both PML and PML-RARA.[14-17] In addition to the significant clinical effectiveness of As_2O_3 for patients with APL, acquired resistance to As_2O_3 therapy has been recognized in clinical practice.[18] Several studies have indicated that arsenic-resistant NB4 cells in vitro show higher glutathione levels than in parental cells.[19-21] However, the detailed molecular mechanisms of resistance to As_2O_3 remain unclear.

Very recently, 2 studies reported that As_2O_3 binds directly to cysteine residues in zinc fingers located within the RBCC motif that contains 3 cysteine-rich zinc-binding domains, a RING-finger (R), 2 B-box motifs (B1 and B2), and a coiled-coil (CC) domain,[22,23] in PML-RARA and PML.[24,25] An intriguing hypothesis is that impairment of As_2O_3 binding to PML-RARA due to conformational changes may result from genetic mutations and/or abnormal posttranslational modifications. These events may be related to resistance to As_2O_3 therapy.

We report the clinical significance and frequency of As_2O_3 resistance in patients with APL. Fifteen patients with APL were treated with As_2O_3 after combination chemotherapy with ATRA, and 2 patients showed clinical As_2O_3 resistance. Interestingly, in both of these As_2O_3-resistant patients, missense genetic mutations in the *PML-RARA* fusion transcript were observed in the leukemia cells. We demonstrated that the mutations, which were located in the PML RBCC region, were critical for PML localization and As_2O_3 responsiveness in vitro. Our observations suggest that acquired genetic mutations in the *PML-RARA* transcript may be a critical molecular mechanism of resistance to As_2O_3 therapy.

Methods

Patients

From January 2000 to December 2008 at Nagoya University Hospital, Japan, 15 patients with APL who showed relapse or disease progression

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