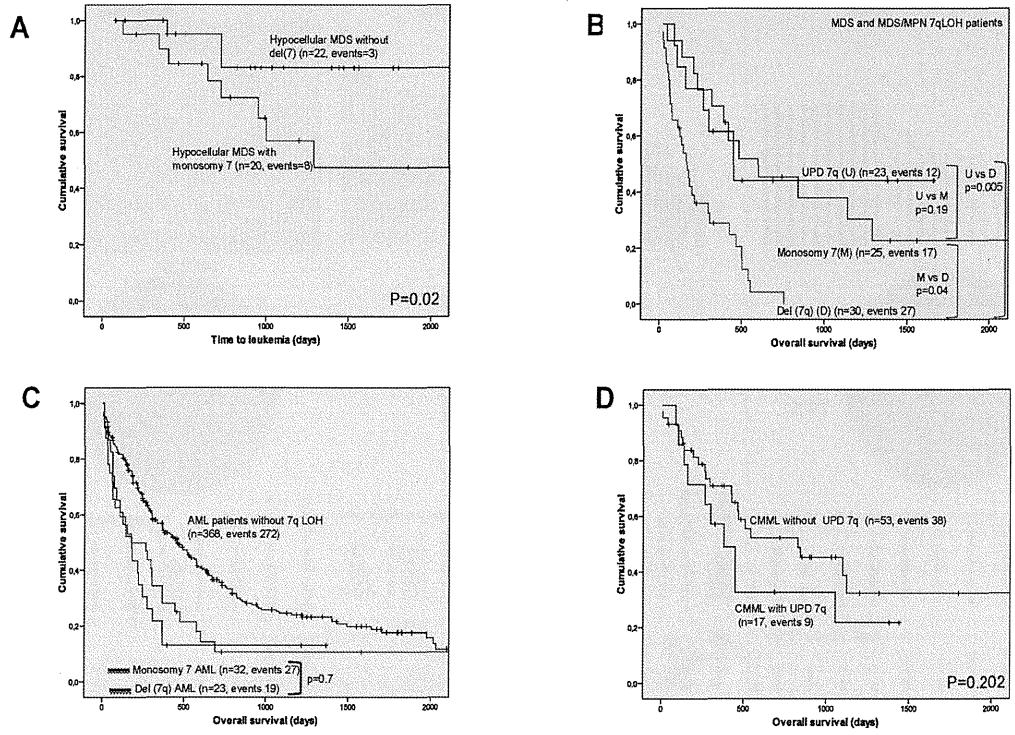

Figure 3. Differences in survival outcomes and progression-free survival of 7q LOH patients. *P* values presented correspond to the Cox regression between the groups indicated. AML indicates acute myeloid leukemia; Chr, chromosome, MDS/MPN, myelodysplastic syndrome/myeloproliferative neoplasm; UPD, uniparental disomy; monosomy 7, deletion of whole chromosome 7; del(7q), partial deletion involving 7q; and CMML, chronic myeloid leukemia.

There are 2 main limitations of SNP-A relative to MC: SNP-A does not detect balanced translocations and SNP-A cannot distinguish whether multiple abnormalities exist in a single clone. We tested whether both techniques could complement each other to solve these shortcomings. We grouped patients based on the presence of monosomy 7/del(7q) in less than 100% or in 100% of the metaphases analyzed. Patients with a clone burden of 100% had a lower median OS (175 vs 235 days; $P = .150$), probably because of the presence of more patients with MDS-derived AML (12 vs 6 cases; $P = .103$), although neither of these findings reached statistical significance. Regarding balanced rearrangements, they were present in 28% of patients, included in complex karyotypes with 5 or more abnormalities in all cases except for 1 patient with a del(7q) and an inv(3)(q21q26). The latter was the only recurrent balanced rearrangement, present in a second patient. The accumulation of this balanced aberrations patients with complex karyotypes and more than 10% of blasts explains partly why the presence of balanced rearrangements did not add independent prognostic value when 1 of those 2 variables was tested simultaneously

Exploring the 2-hit model: SNP-A definition of CDRs and NGS approach

To determine the location of genes on 7q that may be involved in clonal hematopoiesis, we analyzed the SNP-A karyotyping results from 161 patients and defined 3 CDRs, localized in bands 7q22 (100634238-101658775), 7q34 (137841484-139319208), and between bands 7q35 and 7q36.1 (144338001-148572945, Figure 4A). Genomic annotation of the CDRs was performed, and several candidate genes mapping within the CDR were noted (Figure 5); these genes were Sanger sequenced in a cohort of 50 cases with 7q LOH. The third CDR was defined by a single patient with a small deletion containing 6 genes. We sequenced all exons of these genes and detected a mutation in *EZH2*, located in exon 19 involving position Ile715, that produced a frameshift mutation. We found no

Table 2. Multivariate Cox proportional hazards regression models testing the prognostic value of SNP-A chromosome 7 findings in MDS and CMML

MDS multivariate Cox model (n = 274)			CMML multivariate Cox model (n = 70)		
	P	HR (95% CI)		*P*	HR (95% CI)
BM blasts*	≤ .001	1.8 (1.3-2.4)	BM blasts > 10%	.01	10.4 (2.6-41.4)
Presence of blasts in PB	.01	10.3 (2.5-42.2)			
No. of cytopenias†	.13	1.7 (0.8-3.4)	Lymphocyte count > 2.5 × 10^9/L	.6	0.7 (0.2-2.7)
7q LOH SNP-A category‡	≤ .001	4.5 (3.1-6.7)	Hemoglobin level < 12 g/dL	.4	1.7 (0.4-8)
Presence of a UPD(7q)	.1	4.4 (0.8-16)			

HR indicates hazard ratio; BM, bone marrow; PB, peripheral blood; SNP-A, single nucleotide polymorphism array; and CI, confidence interval.

*Three BM blasts categories according to the percentage described: < 5; 5-10; and 11-20.

†Number of cytopenias categories defined as good (0-1) and poor (2-3). Cytopenias defined as hemoglobin less than 10 g/dL, absolute neutrophil count less than 1.8 × 10^9/L, and platelets less than 100 × 10^9/L.

‡7q LOH SNP-A category defined as good, no deletion; intermediate, monosomy 7; and poor, partial deletion involving 7q.

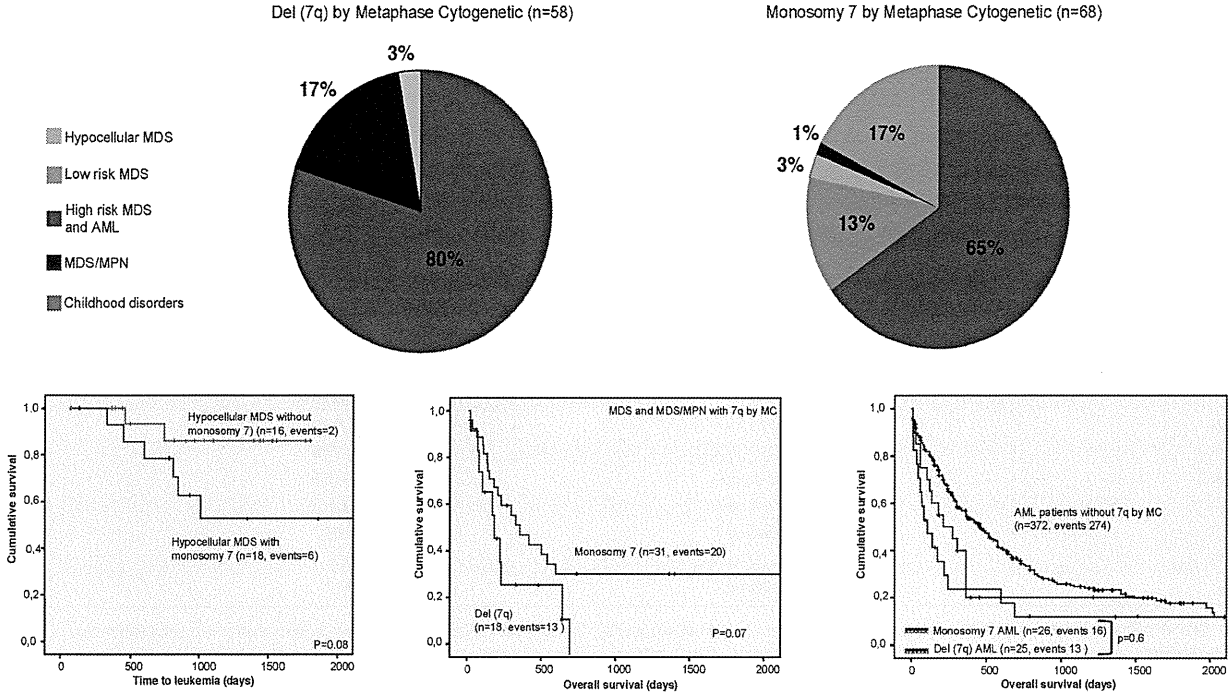

Figure 4. Illustration of how the distribution of disease subsets and outcome associations would be according to the lesion found by MC. (Top) Distribution of patients detected separated according to lesion detected by metaphase cytogenetics. Patients have been grouped as follows: red, AML + high risk and intermediate-2 MDS; gray, low risk and intermediate-1 MDS; blue, hypocellular MDS; black, MDS/MPN; and green, Fanconi anemia and JMML. (Bottom) Differences in survival outcomes and progression-free survival according to MC findings. *P* values presented correspond to the Cox regression between the groups indicated. AML indicates acute myeloid leukemia; Chr, chromosome, MDS/MPN, myelodysplastic syndrome/myeloproliferative neoplasm; UPD, uniparental disomy; monosomy 7, deletion of whole chromosome 7; del(7q), partial deletion involving 7q; and CMML, chronic myeloid leukemia.

other somatic mutations by this strategy. *EZH2* proved to be recurrent in patients with a myelodysplastic/myeloproliferative component and UPD(7q). Supplemental Table 1 summarizes EZH2 mutations found in our 7q LOH patients that we reported previously in part.[20]

In an effort to overcome the limitations inherent to the classic screening method, which was limited to the genes located in the CDRs, we applied 2 NGS approaches. First, we generated exome chromosome 7 libraries from 11 cases with LOH 7q (monosomy 7, n = 6; del(7q), n = 2; UPD(7q), n = 3) and subjected them to

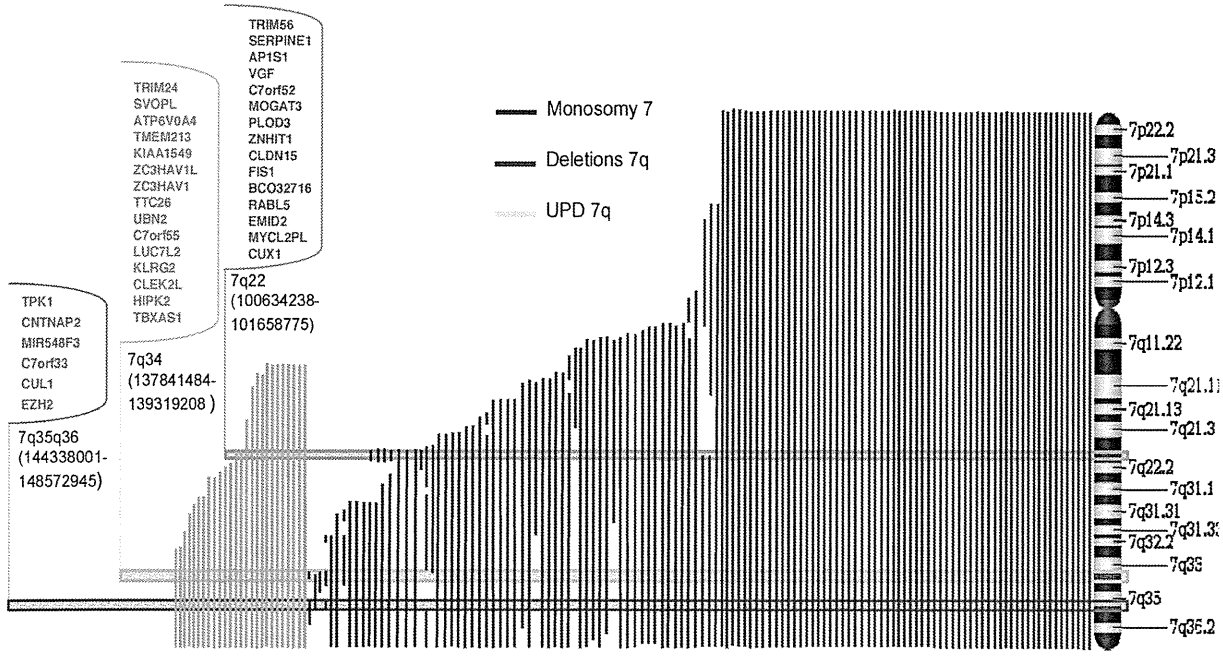

Figure 5. Identification of candidate genes on 7q by mapping CDRs by SNP-A. Three distinct CDRs, indicated by horizontal rectangles, were identified on 7q by mapping of SNP-A karyotyping. The connected keys show the candidate genes contained in each CDR; those genes sequenced in a test cohort of 50 patients with LOH 7q are in bold. CDR indicates commonly deleted region; SNP-A, single nucleotide polymorphism array; and LOH, loss of heterozygosity.

Table 3. Somatic mutations found in regions of 7q LOH patients by NGS

Diagnosis	SNP-A LOH on chromosome 7	Gene	Mutation
MDS/MPN (CMML)	UPD 7q11.21-qter	*EZH2*	R690H
MDS/MPN (aCML)	UPD 7q32.1-qter	*EZH2*	R690H
MDS (RCMD)	Del 7q21.3-qter	*LMTK2A*	A1147T
MDS (RCMD)	Monosomy 7	*NRCAM*	Q1040K
AML	Del 7q21.12q36.3	*ZAN*	N1098Del
AML	Monosomy 7	*GRM8*	A686V
AML	Monosomy 7	*ENSG00000133375*	R68Q
AML	Del 7q31.31-qter	*LOC641808*	V162fs
AML	Monosomy 7	*SEMA3A*	R613Q
AML	Del 7q31.1q36.3	*DYNC1I1*	R239W
AML	Monosomy 7	*HYAL4*	N253K
AML	Monosomy 7	*FAM40B*	C182R
AML	Monosomy 7	*LOC100128744*	P354L
AML	Monosomy 7	*LUC7L2*	R252fs
AML	Del 7q21.11q36.3	*CTAGE6*	T288M
AML	Monosomy 7	*FAM115A*	F193S
AML	Del 7q35-qter	*CUL1*	E241D
AML	Monosomy 7	*EZH2*	E745fs
AML	Del 7q11.21q36.3	*EZH2*	R690H
AML	Monosomy 7	*SSPO*	T426R

SNP-A indicates single nucleotide polymorphism array; LOH, loss of heterozygosity; Del, deletion; MDS/MPN, myelodysplastic syndrome/myeloproliferative neoplasm; CMML, chronic myelomonocytic leukemia; UPD, uniparental disomy; RCMD, refractory cytopenia with multilineage dysplasia; AML, acute myeloid leukemia; and aCML, atypical chronic myelogenous leukemia.

high-throughput sequencing on a Genome Analyzer (Illumina). Second, paired (bone marrow and $CD3^+$) samples from 15 myeloid neoplasms were subjected to whole exome sequencing using HiSeq 2000 (Illumina), including 3 patients with 7q LOH (UPD, deletion, and monosomy). Finally, we used publically available NGS data from TCGA for 74 AML patients. Supplemental Figure 2 shows the somatic mutations found in regions of 7q LOH: *NRCAM* (Q1040K) in a patient with refractory cytopenia with multilineage dysplasia with monosomy 7, *LMTK2* (A1147T) in a refractory cytopenia with multilineage dysplasia patient with del(7q), and *EZH2* (R690H) in a third MDS/MPN patient with UPD(7q). Of note, only mutations of *EZH2* proved to be recurrent [10/19 CMML patients with UPD(7q)], when sequencing by Sanger technique a confirmatory cohort of 50 cases with 7q LOH. Table 3 shows somatic mutations, localized on chromosome 7q, in patients with 7q LOH found using NGS in our patients and in the TCGA project.

Testing the haploinsufficiency hypothesis: microarray expression data

We examined the gene expression profiles of the $CD34^+$ cells of 183 MDS patients, of which 9 cases had monosomy 7 or del(7q). We found that expression of 40% of the genes included in our SNP-A–defined CDRs were significantly reduced in those monosomy 7/del(7q) patients. These genes included *LUC7L2*, *ZNHIT1*, *TTC26*, *RABL5*, *TRIM24*, *EZH2*, *ZC3HAV1L*, *CNTNAP2*, *TRIM24*, *CUX1*, *FIS1*, *RABL5*, *ZC3HAV1*, and *TBXAS1* (supplemental Figure 3). The mean decrease in expression levels was 42% to 33% of that in healthy controls. We also determined the expression of these genes in the 174 cases of MDS that did not have any chromosome 7 deletions, and most interestingly, we found that *EZH2* and *RABL5* were significantly down-regulated even in samples diploid for chromosome 7. Of note, we found that down-regulation of EZH2 was significantly reduced in patients with excess of blasts (Figures 5-6 and supplemental Figure 4).

Discussion

Unlike myeloid disorders harboring an isolated chromosome 5q deletion, a clear genotype-phenotype relationship has not been described in cases with 7q LOH, and underlying pathogenetic mechanisms remain unclear. Here, we applied high-resolution genomic technologies to accurately define the extent and nature of chromosomal lesions and to explore relevant clinical associations of inactivating mutations or insufficient gene dosage in a large cohort of patients with myeloid malignancies involving LOH of the long arm of chromosome 7. Our analyses demonstrate that in those subsets with isolated 7q LOH or accompanied by a very low number of additional lesions, the genotype-phenotype relation is clearly discernible. We found a correlation between an isolated deletion of the long arm of chromosome 7 and MDS with hypoplastic features, and between the presence of UPD(7q) in diploid MDS/MPN patients. In the latter group, the predominant driving genomic event was the presence of inactivating mutations involving *EZH2*, a finding supported by previous studies,[21] whereas gene dosage effect seems to be paramount in typically large monosomy 7/del(7q) cases.

As expected, the spectrum of entities in each 7q LOH subgroup was relatively heterogeneous. Nevertheless, we found strong associations of SNP-A abnormalities with particular clinical entities that merit emphasis. We found a significant association of large MC-cryptic UPD(7q) segments among CMML patients (26%). In our experience, CMML shows a strikingly elevated frequency of somatic UPD compared with other myeloid disorders (data not shown). Particularly high frequencies of somatic UPD have been described for some neoplasms, suggesting that this specific type of chromosomal instability may be related to pathologic pathways that are common in some malignancies but absent in others.[22] In addition, we observed a trend toward worse median survival of CMML patients harboring UPD(7q). The lack of MC lesions in a significant proportion of CMML patients and their controversial

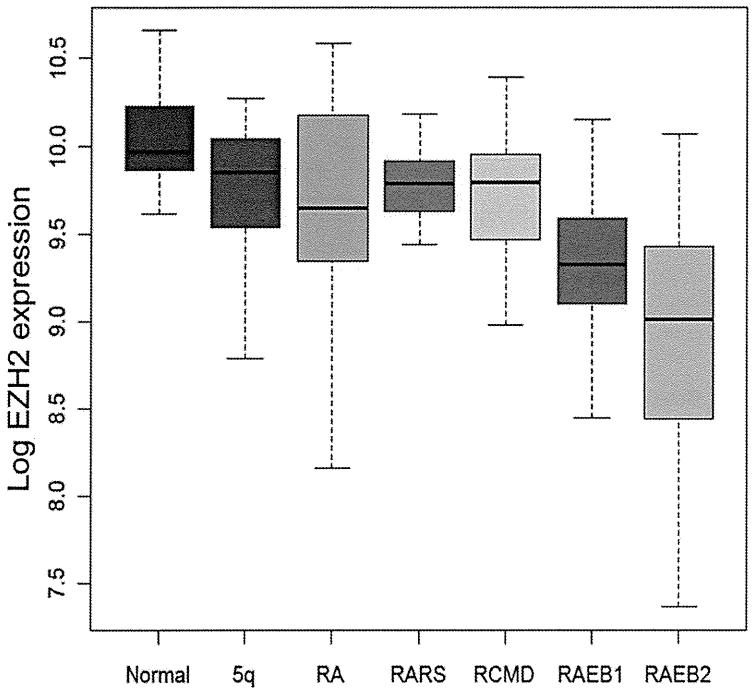

Figure 6. Box plots showing the *EZH2* expression ratios obtained in CD34⁺ cells of 174 MDS cases without 7q LOH and 17 healthy controls. A significant down-regulation of expression was identified for EZH2 in excess of blasts subgroups. NML indicates normal controls; 5q-, 5q-syndrome; RA, refractory anemia; RARS, refractory anemia with ring sideroblasts; RCMD, refractory anemia with multilineage dysplasia; and RAEB, refractory anemia with excess of blasts.

	NML	5q-	RA	RARS	RCMD	RAEB1	RAEB2
Mean	870.668741	839.9296	911.6609	858.685444	880.8111386	654.454716	557.388956
Ttest		0.019892	0.151551	0.00488668	0.004296769	1.0936E-07	2.5564E-09
N	17	17	15	23	43	37	39

impact on survival,[19,23] increases the value of a possible prognostic significance of UPD(7q).

Hypocellular MDS is a relatively uncommon entity among myeloid disorders, with a possible immune-related pathogenetic component, and a diagnosis that frequently overlaps with aplastic anemia.[24] Within our cohort, 8% of patients had hypocellular MDS, a slightly lower percentage than what has been described in other studies.[25,26] Of note, half of our hMDS cohort harbored monosomy 7 as the sole SNP-A lesion detected, conferring on them a higher rate of leukemia transformation. This finding may be helpful for distinguishing hMDS from other MDS.

The risk group assignment of MDS patients with monosomy 7 has been investigated in several studies.[5,6] These studies reported dissimilar results that could be driven by the difficulty of dissecting, in a highly precise and reproducible way, the karyotype defects by conventional chromosome banding techniques. We and others, using more accurate karyotyping means, showed discrepancies in the context of 5q lesions.[27,28] In our cohort, MDS patients harboring monosomy 7 presented a longer median OS than patients with partial deletions, more closely approximating that reported for the intermediate cytogenetic group in the IPSS.[3] We also showed that the wrong assignment to the monosomy 7 subgroup by MC of a significant number of cases with partial deletions by SNP-A seems to be the responsible of a loss in the prognostic value of the conventional karyotyping technique. The better survival of those patients with a wider loss of genes in chromosome 7 (monosomy 7) than those with partial deletions could be presented as paradoxical. The frequent presence of monosomy 7 either in childhood disorders and not accompanied by other chromosomal lesions on one hand, and the common association of partial deletions of 7 with other chromosomal abnormalities shown to be early events in the genesis of dysplasia [del(5q)] on the other hand,[29,30] prompt us to speculate that monosomy 7 might be a founding genomic aberration and that partial deletions of 7 might represent a secondary event in the context of preexisting genomic instability and therefore within a more aggressive clone.

The large size of the typical chromosomal LOH involving the long arm of chromosome 7 in myeloid disorders has complicated the search for a mutated TSG in this region. In this study, we used 2 approaches: (1) a classic approach with the definition of commonly deleted regions and direct sequencing of candidate genes and (2) a next-generation whole exome strategy. Three SNP-A–defined CDRs were described encompassing, with slight differences, those described previously.[31,32] NGS technology allowed us to cover all coding exons, and because no recurrent mutation other than *EZH2* was found, led us to conclude that the absence of recurrent somatic mutations in patients with monosomy 7/del(7q) is a hallmark of the disease pathogenesis in this unique category of myeloid neoplasms

The lack of recurrent mutations in any of the genes mapping to the segment of LOH in most of the patients with large monosomy 7/del(7q) prompted us to test the haploinsufficiency hypothesis by analyzing the expression profiles of patients with that kind of lesions. The dosage effect resulting from the loss of the whole q arm of chromosome 7 particularly affected genes localized in our 3 CDRs; 14 genes included in our SNP-A–defined minimally deleted regions had a mean decreased expression between 42% and 33%. In addition, 2 of these genes, *EZH2* and *RABL5*, were significantly down-regulated even in samples that did not have monosomy 7/del(7q). This current study showed that down-regulation of EZH2 in the absence of LOH is common in advanced MDS. These results point to the importance of haploinsufficiency of the genes located in the 7q CDRs in the pathobiology of MDS and suggests that other genetic or epigenetic mechanisms may silence these genes in cases without 7q LOH.

In summary, the present study of 7q disorders, gathering data from a large series of patients using recent genomics technologies, shows that SNP-A complements traditional MC not only by detection of cryptic abnormalities but also by precisely defining the extent and nature of the lesions with strong clinical associations. Although a 2-hit model is supported for most patients with UPD(7q) and a overlapping MDS/MPN phenotype, our results suggest that haploinsufficient expression of select regions of 7q is the driving pathogenetic mechanism in those patients with predominant dysplastic features and loss of chromosome 7 material.

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Authorship

Contribution: A.J., Y.S., and J.P.M. were responsible for overall design, data collection, analysis, and interpretation, statistical analysis, manuscript preparation, and writing and completion of the manuscript; H.M., A.V., A.M.J., B.P., V.V., R.V.T., C.L.O., A.M.M., and A.P. analyzed data and edited the manuscript; A.G.K., K.M., H.M., A.R.M., M.A.S., M.A.M., S.K., A.L., J.B., and G.J.M. gathered data and edited the manuscript; and all authors approved the final version of the manuscript and its submission.

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ORIGINAL ARTICLE

Diagnosis of acquired bone marrow failure syndrome during childhood using the 2008 World Health Organization classification system

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Abstract Distinguishing hypoplastic myelodysplastic syndrome from aplastic anemia (AA) is challenging. In the present study, Japanese and Chinese pediatric hematologists and pathologists conducted a joint review of bone marrow (BM) smears and trephine biopsies in 100 children with acquired BM failure syndrome, using the criteria proposed in the 2008 edition of the World Health Organization classification of hematopoietic and lymphoid tissues. The final consensus for the diagnoses of 100 children was AA in 29 patients, refractory cytopenia of childhood (RCC) in 58 patients, and refractory cytopenia with multilineage dysplasia (RCMD) in 13 patients. No significant differences between Japanese and Chinese children were found with regards to clinical and laboratory findings, or the distribution of diagnoses. Patients with RCC/RCMD showed milder disease severity and BM hypocellularity than those with AA. To establish the provisional entities for RCC, it is essential to prospectively compare the clinical outcomes between AA and RCC groups in a large number of patients.

Keywords Diagnosis · Bone marrow failure syndrome · Childhood · Classification system · 2008 world health organization

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Introduction

The incidence of aplastic anemia (AA) is approximately 3-fold more common in East Asia than in Europe and United States where yearly incidence rates are approximately $2/10^6$. Geographical variations may play a role in this discrepancy, which partly may be due to genetic disposition and/or environmental factors [1]. In addition, the difference in diagnostic criteria between Eastern and Western countries may be responsible for the variation in outcomes. Previously, bone marrow (BM) trephine biopsies were not common in East Asia. Moreover, most patients with relative erythroid hyperplasia and dysplasia were diagnosed as AA [2].

Childhood myelodysplastic syndrome (MDS) is very rare. In addition, hypocellularity of the BM is more common in childhood MDS. Thus, it is often difficult to distinguish hypoplastic MDS from AA, especially in cases without cytogenetic abnormalities. The new edition of the World Health Organization (WHO) classification for myeloid neoplasms outlines a provisional entity for refractory cytopenia for childhood (RCC) in which the diagnostic criteria for distinguishing RCC from AA are proposed [3].

The present study reviewed and classified the slides of BM smears and trephine biopsies in 100 children with acquired bone marrow failure syndrome(BMFS) in Japan

and China according to the strict criteria proposed by the WHO classification system (2008 edition).

Design and methods

A total of 100 children with cytopenia and hypocellular BM (50 cases from Japan and 50 cases from China between 2009 and 2011) were included in our study. Chinese cases were diagnosed at the Blood Disease Hospital, Chinese Academy of Medical Sciences (CAMS). Japanese cases were registered to the central review system of the Japanese Society of Pediatric Hematology (JSPH).

Individuals were eligible if the following criteria were satisfied: patients had to be 18 years old or younger, and with hypocellular BM. To obtain a diagnosis for cytopenia, at least two of the following must be present: (1) neutrophil $<1.5 \times 10^9$/l; (2) hemoglobin <10 g/dl; and (3) platelet $<50 \times 10^9$/l. Patients with clinical signs of inherited BMFS and/or positive chromosome fragility tests were excluded. Also, patients who had previously been treated with anticancer drugs and radiation were not eligible to participate. All patients had both bone marrow aspirate cytology and trephine biopsy samples.

The severity of the disease was classified according to internationally accepted criteria [4, 5]. AA patients exhibited no morphological changes in their hematopoietic cell lineages. RCC was defined as persistent cytopenia with <5 % blasts in the BM and <2 % blasts in the peripheral blood (PB). In addition, RCC patients had <10 % dysplastic changes in more than two cell lineages, or >10 % in one cell lineage. Refractory cytopenia with multilineage dysplasia (RCMD) exhibited >10 % of the dysplastic changes in more than two cell lineages. Dysplastic features of BM aspirate cytology and trephine biopsies sampled were evaluated according to recommendations by the French–American–British (FAB) Cooperative Leukemia Working Group and the morphology group of the European Working Group MDS in children (EWOG-MDS) [6, 7].

Bone marrow hypocellularity was classified as mild to moderate (5–50 % of the normal age-matched controls) and as severe (<5 % of the normal age-matched controls) according to the results obtained from the trephine biopsies. Cytogenetic examinations were performed with trypsin–Giemsa banding techniques. Twenty metaphases were analyzed at each examination. A cytogenetic clone was thought to exist when two or more cells had the same structural chromosome changes or extra chromosome. At least three cells with the same missing chromosome were considered to constitute a clone.

Before the joint meeting between Japan and China, slides from 50 cases were reviewed by two pediatric

Table 1 Comparison of patients' characteristics and laboratory findings in Japanese and Chinese children with bone marrow failure syndrome

	Japan	China	*p* value
No. of patients	50	50	
Median age at diagnosis, years (range)	10 (1–18)	9 (3–16)	0.422
Gender, male/female	25/25	20/30	0.315
Severity of cytopenia			
Very severe	6	4	
Severe	17	9	0.109
Non-severe	27	37	
Peripheral blood data at diagnosis			
Median			
WBC, $\times 10^9$/l (range)	2.8 (0.4–5.0)	3.9 (0.17–7.0)	0.009
Neutrophil, $\times 10^9$/l (range)	0.58 (0–2.7)	0.68 (0–2.1)	0.269
Platelet, $\times 10^9$/l (range)	20 (2–83)	25 (4–64)	0.423
Hemoglobin, g/dl (range)	7.4 (4.1–14.7)	7.8 (4.5–12.0)	0.689
Reticulocyte, $\times 10^9$/l (range)	31.8 (1.5–70.7)	42.8 (1.2–123.2)	0.920
Mean corpuscular volume, fl (range)	98 (76–112)	99 (75–118)	0.764
Days from onset to diagnosis			
≤30	25	24	
30–180	11	8	0.608
≥180	14	18	
Final diagnosis			
AA	12	17	
RCC	33	25	0.265
RCMD	5	8	

AA aplastic anemia, *RCC* refractory cytopenia of childhood, *RCMD* refractory cytopenia with multilineage dysplasia, *WBC* white blood cell count

hematologists and one pathologist from each country. The joint review meeting was held for 3 days in March 2011 at Blood Disease Hospital, CAMS in China. In the first step, the slides of each country were exchanged and reviewed by observers separately. In the second step, the consensus review for all cases was performed by four hematologists and two pathologists from both countries. The materials included PB, BM aspirate smears and BM trephine biopsies. The present study was approved by the ethics committee of Blood Disease Hospital, CAMS and Nagoya University Hospital. Chi-square tests and independent-samples T tests were used to compare the difference between two groups. p values of <0.05 were considered to be statistically significant.

Results

Comparisons of patient characteristics and laboratory findings at diagnosis between China and Japan are presented in Table 1. Patient characteristics were comparable between the two countries. Although the median WBC count was significantly higher in Chinese children than Japanese children ($p = 0.009$), other PB counts at diagnosis were not different. The days from onset to diagnosis widely varied from 1 to 4165 days. 32 patients required more than 180 days. 7 patients presented with isolated thrombocytopenia, which had gradually proceeded to pancytopenia. They required 47–3040 days from onset to diagnosis.

There were 9 cases whose status of diagnosis was in question between hematologists and pathologists. 4 cases were diagnosed as AA by hematologists, but as RCC by pathologists, the final diagnoses were all RCC. In contrast, 2 cases were diagnosed as RCC by hematologists, but as AA by pathologists, the final diagnoses were RCC. Among the 3 cases who were diagnosed as RCMD by hematologists, 2 cases were diagnosed as RCC and one case was diagnosed as AA by pathologists, by the joint review committee, the diagnoses of 2 cases were consistent with pathologists, the other one was diagnosed RCMD.

Final consensus for the diagnoses of 100 patients was as follows: AA in 29 cases, RCC in 58 cases and RCMD in 13 cases. The distribution of diagnoses was not different between Japanese and Chinese: 12:17 in AA, 33:25 in RCC and 5:8 in RCMD, respectively.

Table 2 displays patient characteristics and laboratory data for the AA, RCC and RCMD groups. Among the three

Table 2 Comparison of patients' characteristics and laboratory findings among children with AA, RCC and RCMD

	AA	RCC	RCMD	p value
No. of patients	29	58	13	
Median age at diagnosis, years (range)	9 (3–14)	9 (1–18)	10 (5–16)	0.749
Gender, male/female	11/18	27/21	7/6	0.591
Severity of cytopenia				
Very severe	8	2	0	
Severe	12	11	3	<0.001
Non severe	9	45	10	
Peripheral blood data at diagnosis				
Median				
WBC, $\times 10^9$/l (range)	3.1 (0.17–6.3)	3.7 (1.1–7.0)	3.2 (1.4–5.5)	0.075
Neutrophil, $\times 10^9$/l (range)	0.48 (0–2.1)	0.67 (0–2.7)	0.76 (0.36–1.07)	0.164
Platelet, $\times 10^9$/l (range)	16 (2–57)	27 (6–83)	28 (2–64)	0.224
Hemoglobin, g/dl (range)	7.4 (4.1–12.0)	8.0 (4.0–14.7)	7.5 (4.2–11.6)	0.327
Reticulocyte, $\times 10^9$/l (range)	13 (1.2–42)	43 (1.5–123)	48 (10–94)	0.003
Mean corpuscular volume, fl (range)	94 (75–112)	99 (80–118)	97 (88–110)	0.061
Cellularity in the bone marrow				
Mild–moderate hypocellularity	13	54	13	
Severe hypocellularity	16	4	0	<0.001
Chromosomal abnormalities	1	3	0	0.68
Days from onset to diagnosis				
≤30	17	28	4	
30–180	4	12	3	0.537
≥180	8	18	6	

AA aplastic anemia, *RCC* refractory cytopenia of childhood, *RCMD* refractory cytopenia with multilineage dysplasia, *WBC* white blood cell count

groups, there were no significant differences with regards to median age at diagnosis, sex, or days from onset to diagnosis. While 8 out of 29 (28 %) patients in the AA group had very severe cytopenia, only 2 of the 58 patients (3 %) in the RCC group and none of the 13 patients in the RCMD group had very severe cytopenia. On the other hand, 45 of the 58 patients (78 %) in the RCC group and 10 of the 13 patients (77 %) of the RCMD group had non severe cytopenia ($p < 0.001$). In addition, 16 out of 29 AA patients (55 %) exhibited severe hypoplastic of BM cellularity, while only 4 out of 58 RCC patients (7 %) and none of the RCMD patients had severe hypoplastic BM. A number of the RCC/RCMD patients exhibited mild to moderate hypocellularity ($p < 0.001$).

Data for cytogenetic analyses were available from 75 patients. Abnormal karyotypes were detected in one patient from the AA group (47,XX,+8[10]/46,XX[10]) and in 3 patients in the RCC group (47,XX,+8[10]; 46,Y, t(x:3)(p11.2;q13)[10]; 47, XY,+8[1]/49, idem, +6,+21[3]/46, XY[16]).

Discussion

It is the first project to have a joint meeting to review BM samples from children with BMFS between Japanese and Chinese hematologists and pathologists. Our results demonstrated that the clinical and laboratory findings and the distribution of diagnosis was not different in Japanese and Chinese children. Patients with RCC/RCMD were milder in disease severity and BM hypocellularity, compared to those with AA.

According to the FAB classification, the annual report from JSPH indicated that the number of AA and RA was 71:9 in 2006 and 63:6 in 2007, respectively. The annual incidence of childhood AA in Japan was $3.7/10^6$. Using the new criteria, it is may be $<2.0/10^6$, which is comparable with the incidence of AA in the Western countries.

According to 2008 WHO classification system, the ratio of AA and RCC/RCMD in Germany is unknown, but the proportion of very severe AA among severe AA (64 %) was much higher than that of Japanese children [7]. Among 1002 Japanese children with AA, the distribution of disease severity was as follows: very severe in 246 (24.6 %) children; severe in 305 (30.4 %) children; and non-severe in 451 (45.0 %) children, respectively. These figures suggest that a considerable number of children with AA in Asia may be diagnosed as RCC using the criteria set forth from the German group. Thus, the difference in diagnostic criteria may be responsible for the high incidence of AA in Asian countries.

WHO classification system recommended that children who satisfy the criteria for RCMD should be considered as RCC until the numbers of lineages involved are fully evaluated whether it is an important prognostic discriminator in childhood MDS [3]. In our study, 13 of the 71 MDS children (18 %) were classified as RCMD. The BM samples were more cellular, and dysplasia of cell morphology was more prominent than those in RCC.

The most important aspect of the new proposal from the WHO classification system is whether the diagnosis has an impact on clinical outcomes including, response to treatment and incidence of late clonal diseases. From the German group, results from immunosuppressive therapy (IST) with anti-thymocyte globulin and cyclosporine for children with RCC were reported [8]. The response rate and 3-year overall survival rate in children with RCC were comparable to those with severe AA who received the same IST [9]. Unfortunately, due to a short follow-up period and variety of treatments, we could not define this issue. It is very important to collaborate with all Asian countries to compare the frequence of AA and RCC in children between Asian and Western countries. To establish the new entity of RCC, future studies should unravel the etiology and biological nature of both AA and RCC.

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Author contributions

AJS wrote the paper, collected, and analysed data. RT edited the paper and created the figures. HMR designed the research and wrote the paper. BRS designed the research and edited the paper. JGH performed the research. CAT designed the research and wrote the paper.

Conflicts of interest

None of the authors (AJS, RT, HMR, BRS, JGH, or CAT) report any conflict of interest with the data presented herein.

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Mutation in the *THPO* gene is not associated with aplastic anaemia in Japanese children

Aplastic anaemia (AA) is a rare heterogeneous disorder characterized by pancytopenia (Young *et al*, 2008), affecting two people per million per year in Western countries and double or triple that in East Asia (Marsh *et al*, 2009). Age distribution shows peaks in children, young adults and older adults (aged > 60 years) (Passweg & Marsh, 2010). Although different AA-related genetic backgrounds may account for regional disparities, the aetiology remains unidentified in more than 80% of cases (Marsh *et al*, 2009; Pulsipher *et al*, 2011). Some inherited cases have been linked to various syndromes, such

Table I. Summary of SNPs in *THPO*.

Exon	Nucleotide changes	Protein changes	AA patients	Normal controls
Exon 2 (rs6087)	c.173 C>G	Ala58Val	1/83 (Patient 34)	0/48
Exon 5 (rs117656396)	c.793 C>T	Leu265Phe	1/83 (Patient 31)	1/48 (Control 5)
Exon 7 (not reported)	c.1120 A>G	Thr374Ala	1/83 (Patient 23)	1/48 (Control 47)

as Fanconi anaemia (FA) and dyskeratosis congenita (DC), but other unidentified genetic backgrounds are presumably associated with the development of pancytopenia in many of the remaining patients (Dokal & Vulliamy, 2010).

The *THPO* gene (previously termed *c-MPL*) encodes thrombopoietin (THPO). The interaction of THPO with its receptor is responsible for megakaryopoiesis and platelet activation, as well as the maintenance of haematopoietic stem cells (HSCs). Biallelic mutations in *THPO* have been described in congenital amegakaryocytic thrombocytopenia (CAMT), which causes thrombocytopenia associated with bone marrow hypocellularity within the first few months of life (Ihara *et al*, 1999; Geddis, 2011), and *THPO* is considered as the only causative gene for CAMT (Savoia *et al*, 2007; Chung *et al*, 2011). A recent whole-exome sequence study showed that *THPO* mutations were responsible in two pedigrees of familial AA (Walne *et al*, 2012). However, no studies have clarified the incidence of *THPO* mutations among East Asian patients with AA.

To determine whether *THPO* is associated with AA in Japanese children, we analysed *THPO* gene sequences, comprising 12 exons over a 17-kb genomic region on 1p34, for 83 Japanese children (<18 years old) with AA who had no family history of bone marrow failure syndrome, and 48 Japanese healthy individuals as controls. All coding exons in *THPO* were amplified by polymerase chain reaction of genomic DNA from patients with AA (Table SI) using a BigDye terminator cycle sequencing kit (Life Technologies, Carlsbad, CA, USA), and the products were analysed in an ABI/PRISM 3130xl Genetic Analyser (Life Technologies). The ethics committee at Nagoya University Graduate School of Medicine approved this study.

Although we did not find any *THPO* mutations in the 83 AA patients, we identified three single nucleotide alterations, including an unreported non-synonymous nucleotide change in one patient (Patient 23; exon 7, c.1120 A>G, Thr374Ala); the same genetic alteration was also found in one of the 48 normal controls (Control 47) (Figure S1). We concluded that this single nucleotide change represents an unreported rare single nucleotide polymorphism (SNP). In addition, Patient 34 (exon 2, c.173 C>G, p.Ala58Val) and Patient 31 (exon 5, c.793 C>T, p.Leu265Phe) showed rare, previously reported SNPs in the *THPO* gene. An SNP in exon 5 was also found in one of the 48 normal controls (Control 5) (Table I).

Very recently, Walne *et al* (2012) identified *THPO* as a causative gene in familial AA using an exome-sequencing technique. They provided the first report of a link between homozygous *THPO* mutations and familial AA in two families (from Tunisia and Pakistan) (Walne *et al*, 2012). Our study found no significant *THPO* mutations in Japanese children with AA. In addition, the incidence of SNPs did not differ significantly between AA patients (3·6%; 3 of 83) and normal individuals (4·2%; 2 of 48). We assume that the presence of these rare SNPs in *THPO* is not associated with the pathogenesis of AA in Japanese children.

In summary, we screened for genetic alterations in the *THPO* gene in 83 Japanese AA patients, and found that neither *THPO* mutations nor SNPs are pathognomonic for most Japanese AA children.

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Supporting Information

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

Fig S1. Identification of the unreported SNP in the *THPO* gene

Table SI. PCR primers and setting.

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Use of Sorafenib as an effective treatment in an AML patient carrying a new point mutation affecting the Juxtamembrane domain of *FLT3*

In acute myeloid leukaemia (AML), mutations of the FMS-like tyrosine kinase-3 gene (*FLT3*) are found in about one-third of patients. The most common mutations are the internal tandem duplication (*FLT3*-ITD) within exon 14, encoding the juxtamembrane (JM) domain (Nakao *et al*, 1996), and the point mutations involving exon 20, coding the second tyrosine-kinase domain (FLT3-TKD) (Abu-Duhier *et al*, 2001). A third class of activating point mutations in the JM (*FLT3*-JM-PM) has been identified in AML patients, however its biological and clinical significance remain to be clarified (Reindl *et al*, 2006; Gianfelici *et al*, 2011).

Sorafenib (Nexavar, Bayer HealthCare Pharmaceuticals, Wayne, NJ, USA) is a multi-targeted tyrosine kinase inhibitor (TKI), which acts against RAF kinases, KDR receptors, both wild type and mutated FLT3, PDGFR receptors, KIT, and RET kinase (Wilhelm *et al*, 2004). Given the efficiency of Sorafenib at inducing remissions in relapsed/refractory FLT3-ITD mutated AML (Metzelder *et al*, 2009), it is anticipated that its use will continue to increase in both relapsed and frontline settings.

A new mutation within exon 14 of *FLT3* (FLT3-JM-PM), which was responsive to Sorafenib, is described here in a refractory AML patient.

In December 2008, a 68-year-old man was diagnosed with AML, French-American-British classification M1 with high-risk cytogenetic (complex karyotype with monosomy of chromosome 7 and trisomy 8) and a point mutation in exon 14 of the *FLT3* gene, which encodes the JM domain (L576Q). At diagnosis, this mutation was detected by polymerase chain reaction (PCR) in real time and high resolution melting analysis (Tan *et al*, 2008) (Fig 1A). Bidirectional sequencing analysis of exon 14 showed a heterozygous missense mutation (c.1727 T > A; p.L576Q; ENST00000241453 (Fig 1B). Since 2009, 173 AML patients have been studied at the point of diagnosis. High resolution melting assay was able to detect two cases of missense *FLT3*-JM mutations (representing an incidence of 1·15%) and two well-known polymorphisms of *FLT3*-JM.

Induction therapy was initiated with idarubicin (12 mg/m^2) i.v. days 1–3 and cytarabine (200 mg/m^2) i.v. days 1–7. Disease progression was noted 12 d later with a peripheral blood increment in the absolute blast count. On January 2009, fludarabine, idarubicin and bortezomib were administered in a clinical trial (IIS-ELEU0070/26866138CAN2015/EUDRAFAFT) for refractory AML. Persistent pancytopenia appeared and a bone marrow (BM) sample obtained after 1 month showed a hypocelular marrow with 70% blasts. On February 2009, therapy with clofarabin 40 mg/m^2, cyclophosphamide 440 mg/m^2 and etoposide 100 mg/m^2 was initiated, but was discontinued due to liver toxicity. BM obtained after 22 d showed <50% blast reduction. Therefore, low-dose

Brief report

Somatic mosaicism for oncogenic *NRAS* mutations in juvenile myelomonocytic leukemia

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Juvenile myelomonocytic leukemia (JMML) is a rare pediatric myeloid neoplasm characterized by excessive proliferation of myelomonocytic cells. Somatic mutations in genes involved in GM-CSF signal transduction, such as *NRAS*, *KRAS*, *PTPN11*, *NF1*, and *CBL*, have been identified in more than 70% of children with JMML. In the present study, we report 2 patients with somatic mosaicism for oncogenic *NRAS* mutations (G12D and G12S) associated with the development of JMML. The mutated allele frequencies quantified by pyrosequencing were various and ranged from 3%-50% in BM and other somatic cells (ie, buccal smear cells, hair bulbs, or nails). Both patients experienced spontaneous improvement of clinical symptoms and leukocytosis due to JMML without hematopoietic stem cell transplantation. These patients are the first reported to have somatic mosaicism for oncogenic *NRAS* mutations. The clinical course of these patients suggests that *NRAS* mosaicism may be associated with a mild disease phenotype in JMML. (*Blood*. 2012;120(7):1485-1488)

Introduction

Juvenile myelomonocytic leukemia (JMML) is a rare myeloid neoplasm characterized by excessive proliferation of myelomonocytic cells. Somatic mutations in genes involved in GM-CSF signal transduction, such as *NRAS*, *KRAS*, *PTPN11*, *NF1*, and *CBL*, have been identified in more than 70% of children with JMML.[1-3] The term "somatic mosaicism" is defined as the presence of multiple populations of cells with distinct genotypes in one person whose developmental lineages trace back to a single fertilized egg.[4] Somatic mosaicism of various genes, including some oncogenes, has been implicated in many diseases. For example, somatic mosaicism for *HRAS* mutations is found in patients with Costello syndrome.[5-7] Whereas germline mutations in causative genes (ie, *PTPN11*, *NRAS*, *NF1*, and *CBL*) are found in JMML patients,[3,8-11] the presence of somatic mosaicism for these genes has never been reported. In the present study, we describe 2 cases of JMML in which the patients display somatic mosaicism for oncogenic *NRAS* mutations (G12D and G12S).

Study design

Written informed consent for sample collection was obtained from the patients' parents in accordance with the Declaration of Helsinki, and molecular analysis of the mutational status was approved by the ethics committee of the Nagoya University Graduate School of Medicine (Nagoya, Japan).

Patient 1. A 10-month-old boy had hepatosplenomegaly and leukocytosis (72.1 × 10^9/L) with monocytosis (13.3 × 10^9/L; Table 1). The patient's BM contained 7% blasts with myeloid hyperplasia. Cytogenetic analysis revealed a normal karyotype and colony assay of BM mononuclear cells (BM-MNCs) showed spontaneous colony formation but GM-CSF hypersensitivity assay was not tested. The diagnostic criteria for JMML, as developed by the European Working Group on Myelodysplastic Syndrome in Childhood, was fulfilled,[12] and the patient was treated with IFN-α and 6-mercaptopurine. His clinical and laboratory findings gradually resolved without hematopoietic stem cell transplantation. However, 11 years after the diagnosis of JMML, the patient developed thrombocytopenia (7.6 × 10^9/L) and BM findings showed trilineage dysplasia with low blast count compatible with refractory anemia. The patient did not have any physiologic abnormalities, such as facial deformity, and there was no family history of malignancy or congenital abnormalities.

Patient 2. A 10-month-old boy had anemia, hepatosplenomegaly, and leukocytosis (31.8 × 10^9/L) with monocytosis (6.4 × 10^9/L; Table 1). The patient's BM exhibited myeloid hyperplasia and granulocytic dysplasia with 5% blasts. Cytogenetic

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Table 1. Patient characteristics

	Patient 1	Patient 2
Age, mo	10	10
Sex	Male	Male
Liver, cm	12	5
Spleen, cm	8	10
WBCs, $\times 10^9$/L	72.1	31.8
Monocytes, %	18.5	20
Blasts, %	4	2
Hb, g/dL	8.9	5.4
Platelets, $\times 10^9$/L	59	100
HbF, %	2.1	1.7
BM blasts, %	7	5
Karyotype	46,XY [20/20]	46,XY [20/20]
Monosomy 7 (FISH)	Negative	Negative
Spontaneous colony formation	Positive	Positive
Gene mutation	*NRAS*, G12D 35G > A	*NRAS*, G12S 34G > A
Treatment	IFN-α-2b, 6-MP	None
Observation period, mo	231	103
Outcome	Alive	Alive
Fraction of mutant alleles, % (pyrosequencing)		
Nail (whole)	24	12.5 (average)
Nail (left hand)	ND	26
Nail (right hand)	ND	13
Nail (left foot)	ND	8
Nail (right foot)	ND	3
Buccal smear cells	43	21
Hair bulbs	5	ND
Family studies		
Father	Wild-type	Wild-type
Mother	Wild-type	Wild-type
Sibling	ND	Wild-type

Hb indicates hemoglobin; 6-MP, 6-mercaptopurine; and ND, not done.

analysis revealed a normal karyotype. Colony assay of BM-MNCs showed spontaneous colony formation and GM-CSF hypersensitivity. Although the diagnostic criteria for JMML were fulfilled,[12] the patient's clinical symptoms and leukocytosis improved spontaneously within a few months without cytotoxic therapy or hematopoietic stem cell transplantation. The patient has remained healthy and has experienced no hematologic or physiologic abnormalities. The most recent follow-up examination was conducted when the patient was 8 years of age.

Detailed methods for experiments are described in supplemental Methods (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

Results and discussion

DNA sequencing for JMML-associated genes (ie, *NRAS*, *KRAS*, *PTPN11*, and *CBL*) was performed (Figure 1 and Table 1). In Patient 1, the *NRAS* G12D mutation was identified in BM-MNCs at the time of diagnosis of both JMML and MDS. We identified the same G12D mutation in DNA derived from buccal smear cells and nails of both hands; however, the sequence profile of the nails showed a low signal for the mutant allele compared with signal of blood cells. In Patient 2, the *NRAS* G12S mutation was identified in DNA from BM-MNCs, buccal smear cells, and nails of the left hand. However, the sequence profiles of buccal smear cells and nails of the left hand showed a low signal for the mutant variant. No mutation was detected in DNA from the PB-MNCs of the patient's parents or sibling.

We used pyrosequencing to quantify the fraction of mutated alleles in DNA samples from different somatic tissues (Figure 1 and Table 1). The frequency of mutated alleles varied by tissue type as follows. For Patient 1: BM-MNCs, 50%; nails, 24%; buccal smear cells, 43%; and hair bulbs, 5%. For Patient 2: buccal smear cells, 21%; nails of left hand, 26%; nails of right hand, 13%; nails of left foot, 8%; and nails of right foot, 3%. We cloned the PCR product of *NRAS* exon 2 from the nails of Patient 1 and picked up 15 clones. The clones were sequenced. Four of the 15 clones (27%) contained the mutant allele, which is consistent with the results of pyrosequencing analysis (24% mutant allele). Because the confirmed detection level by pyrosequencing technique was above 5%, results with a low percentage (< 5%) of mutant allele (ie, hair bulbs in Patient 1) should be interpreted with caution.[13,14]

We diagnosed 2 JMML patients as having somatic mosaicism of *NRAS* mutations: G12D for Patient 1 and G12S for Patient 2. The diagnoses were based on negative familial studies and mutational allele quantification analyses that showed diversity in the chimeric mutational status of different somatic tissues. Although DNA from buccal smear cells might be contaminated with WBCs, we also identified mutations in DNA from the nail tissue, which is known to be a good biologic material without contamination from hematopoietic cells, in both patients. These data suggest that a portion of the *NRAS*-mutated somatic cells were derived from one cell that acquired the mutation at a very early developmental stage. Although both somatic and germline mutations of RAS pathway genes (ie, *PTPN11*, *NRAS*, *NF1*, and *CBL*) are found in some JMML patients,[3,8-11] somatic mosaicism for these genes has never been reported. To the best of our knowledge, the present study is

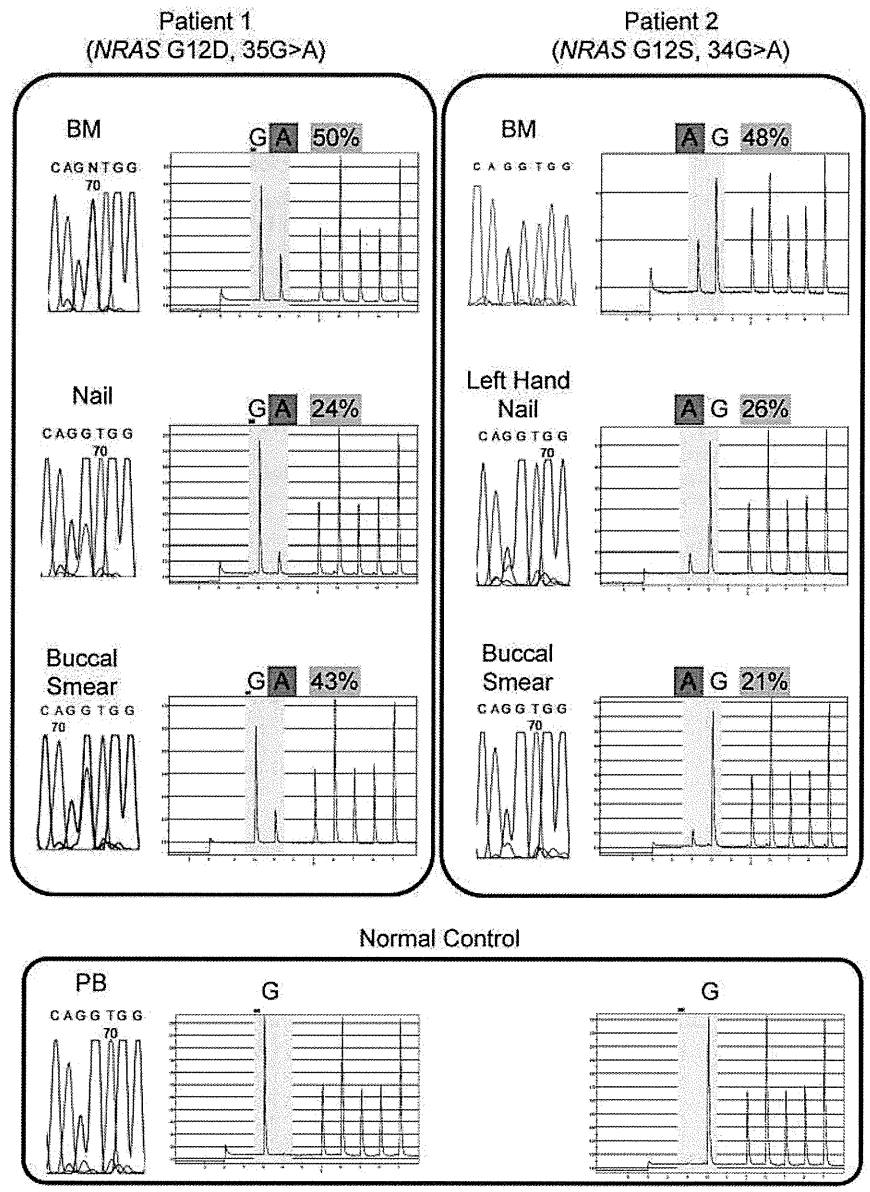

Figure 1. Direct sequencing and quantitative mutational analysis of *NRAS* in JMML patients. *NRAS* mutations are detected by direct sequencing and quantified by pyrosequencing. Direct sequencing identified oncogenic *NRAS* mutations: for Patient 1, G12D, 35G > A; for Patient 2, G12S, 34G > A) in BM-MNCs at diagnosis of JMML and in the nails and buccal smear cells. Quantification by pyrosequencing revealed that the fractions of mutated allele varied among different tissue types. For Patient 1: BM, 50%; nail, 24%; and buccal smear, 43%. For Patient 2: BM, 48%; left-hand nail, 26%; and buccal smear, 21%.

the first report of JMML patients with somatic mosaicism of mutations in RAS pathway genes.

Germline RAS pathway mutations are often associated with dysmorphic features similar to Noonan syndrome or its associated diseases. Correspondingly, JMML patients with germline *NRAS* or *CBL* mutations exhibit characteristic dysmorphic features.[3,10] Although our patients did not show any dysmorphic or developmental abnormalities, they should receive careful medical follow-up, especially for the occurrence of other cancers, because of the oncogenic nature of the mutations.

In general, JMML is a rapidly fatal disorder if left untreated.[8] However, recent clinical genotype-phenotype analyses have revealed heterogeneity in their clinical course. We and other researchers have reported that patients with *PTPN11* mutations have a worse prognosis than patients with other gene mutations, including *NRAS* and *KRAS*.[15,16] Both of the JMML patients in the present study with somatic mosaicism of oncogenic *NRAS* mutations have had a mild and self-limiting clinical course. We analyzed nails of other 3 JMML patients with RAS mutations who experienced aggressive clinical course and none showed somatic mosaicism (data not shown). In analogy to the mild phenotype of JMML patients with germline mutations in *PTPN11*, we speculate that JMML patients with somatic mosaicism of RAS genes might have a mild clinical course. We are planning to confirm these observations in larger cohort.

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Authorship

Contribution: S.D. and H.M. designed and conducted the research, analyzed the data, and wrote the manuscript; A.S., M.M.-E., M. Sato, H.K., A.K., M. Sotomatsu, and Y.H. treated the patients; Y.T., Y.F.-H., K.Y., H.H., H.K., N.Y., H.S., A.N., X.W., O.I., Y.X.,

N.N., M.T., A.H., and K.K. conducted the research; and S.K. designed the research, analyzed the data, and wrote the manuscript.

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Inherited bone marrow failure syndromes in 2012

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Abstract Inherited bone marrow failure syndromes (CBMFS) are a heterogeneous group of genetic disorders characterized by bone marrow failure, congenital anomalies, and an increased risk of malignant disease. The representative diseases with trilineage involvement are Fanconi anemia and dyskeratosis congenita, while the disease with the single lineage cytopenia is Diamond–Blackfan anemia. Recent advances in our understanding of these diseases have come from the identification of genetic lesions responsible for the disease and their pathways. Although recent studies have identified many causative genes, mutations of these genes have only been found in less than half of the patients. Next-generation sequencing technologies may reveal new causative genes in these patients. Also, induced pluripotent stem cells derived from patients with CBMFS will be useful to study the pathophysiology of the diseases. The only long-term curative treatment for bone marrow failure in patients with inherited bone marrow failure syndromes is allogeneic hematopoietic stem cell transplantation, although this procedure has a risk of severe adverse effects. Multicenter prospective studies are warranted to establish appropriate conditioning regimens aimed at reducing transplant-related mortality.

Keywords Inherited bone marrow failure syndrome · Fanconi anemia · Diamond–Blackfan anemia · Dyskeratosis congenita

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Introduction

Inherited bone marrow failure syndromes (IBMFS) are a heterogeneous group of genetic disorders characterized by bone marrow failure, congenital anomalies, and increased risk of malignant disease. Such bone marrow failure may affect all three hematopoietic cell lineages or single cell lineages individually. Diseases characterized by trilineage involvement include Fanconi anemia and dyskeratosis congenita, while Diamond–Blackfan anemia results in single-lineage cytopenia. Recent advances in our understanding of these diseases have arisen from the identification of genetic lesions responsible for such diseases and their pathogenic pathways. These investigations have further clarified both normal and pathological hematopoiesis. In this current review, we describe recent insights into three IBMFS: Fanconi anemia, Diamond–Blackfan anemia, and dyskeratosis congenita.

Fanconi anemia

Fanconi anemia (FA) is a rare autosomal recessive disease characterized by congenital abnormalities, progressive bone marrow failure, and cancer susceptibility. FA, which has an incidence of less than 10 per million live births, is the most frequent inherited cause of aplastic anemia [1]. FA is a genetically heterogeneous disease defined by complementation groups. To date, 15 genes have been identified as playing a causative in FA and these genes, FANCA to FANCP, have been cloned [2]. Children with FA often develop aplastic anemia during the first decade of life, with death often resulting from complications of bone marrow failure, such as severe infection or bleeding. FA

patients also develop clonal chromosomal abnormalities in bone-marrow progenitor cells, such as monosomy 7, which are associated with myelodysplastic syndrome (MDS) and acute myeloblastic leukemia (AML) [3]. The gene FANCD1, which is responsible for complementation group FA-D1, is identical to the hereditary breast cancer susceptibility gene, BRCA2, and has been reported as affected in 3 % of patients with Fanconi anemia. As compared with children from other FA groups, more severe phenotypes are seen in FA-D1 patients, such as co-occurrence of multiple anomalies, development of multiple malignancies with earlier onset, and increased incidence of leukemia and solid tumors [4], including Wilms tumor, neuroblastoma, and brain tumors.

While the treatment of choice for FA patients remains allogeneic stem cell transplantation (SCT) from an HLA-matched sibling or unrelated donor, older patients may develop squamous-cell carcinomas (SCCs) of the head and neck or gynecological system. In particular, some studies have demonstrated there is a high incidence of SCC, such as esophageal cancer, in FA patients who have received SCT. The age-specific hazard of SCC has been shown to be 4.4-fold higher in patients who receive transplants and, in addition, SCCs occurred at significantly younger ages in the transplant group [5]. Thus, further investigations of the complete care of FA patients need to be undertaken.

Complementation groups and genes of FA

Since cells derived from FA patients are hypersensitive to DNA interstrand cross-linking (ICL) agents, such as diepoxybutane (DEB), mitomycin C (MMC), and cisplatin, it is expected that FA genes are involved in ICL repair. In 1993, FANCC was the first FA gene to be cloned by expression cloning [6, 7]. Subsequently, 15 other genes have been cloned (Table 1). At present, the FANC genes, which range from FANC"A" to FANC"P", and the FA pathway have been shown to resolve ICLs encountered during DNA replication. There are three primary groups of FA proteins, which include the FA core complex, the ID (FANCD2/I) complex, and the BRCA complex (Fig. 1). In these groups, there are eight FA proteins (FANCA/B/C/E/F/G/L/M) that form a multi-subunit ubiquitin E3 ligase complex, the FA core complex, which activates the monoubiquitination of the ID complex after genotoxic stress, such as ICL, or during the S phase (Fig. 1) [8, 9]. The monoubiquitinated ID complex forms foci on damaged DNA. FANCM is also a crucial gene, as it is a sensor for detecting stalled DNA replication. The BRCA complex, which is also referred to as homologous recombination (HR), consists of FANCD1, FANCJ, FANCN, and FANCO, and is located downstream of the DI complex on the ICL repair pathway.

Table 1 Genes mutated in patients with Fanconi anemia

	Other names	Chromosomal Locus	Population in FA patients (%)
FANCA		16q24.3	60–70
FANCB		Xp22.2	2
FANCC		9q22.3	14
FANCD1	BRCA2	13q13.1	3
FANCD2		3p25.3	3
FANCE		6p22-p21	3
FANCF		11p15	2
FANCG		9p13	10
FANCI		15q25-q26	1
FANCJ	BACH1/BRIP1	17q22	2
FANCL		2p16.1	0.20
FANCM		14q21.3	0.20
FANCN	PALB2	16p12.3-p12.2	0.70
FANCO	RAD51C	17q22	0.20
FANCP	SLX4	16p13.3	0.20

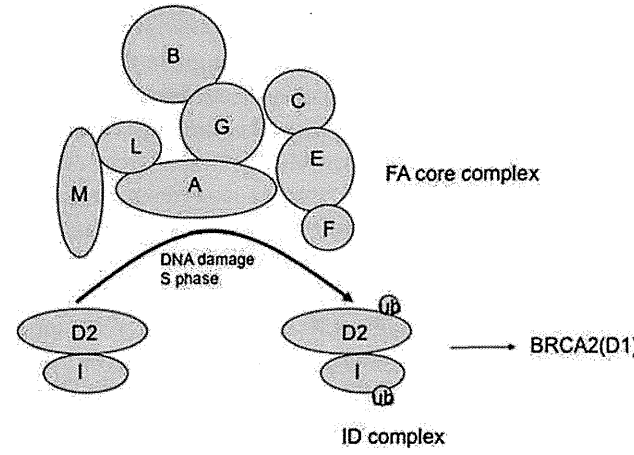

Fig. 1 Simplified scheme of the FA pathway. Depending on the FA core complex, FANCD2 and FANCI are monoubiquitinated after DNA damage or during the S-phase

Prognosis factors in FA patients

Although FANC gene knockout mice models have been established, they differ from the hematological phenotypes of human FA patients [10]. With the exception of the lethal phenotype of the BRCA2/FANCD1 knockout mouse, the hematological parameters of the other FA groups show only a slightly decreased platelet count and a slightly increased erythrocyte mean cell volume in mice at a young age, which did not progress to aplastic anemia or leukemia. However, both male and female mice showed hypogonadism and impaired fertility, which is consistent with the human FA patient phenotypes.

Recent studies have revealed a relationship between the acetaldehyde and FA pathways. Acetaldehyde is an organic chemical compound that is naturally present in coffee, bread, and ripe fruit, which is produced as product of a plant's normal metabolism. It is also produced by the oxidation of ethylene. In the liver, the enzyme alcohol dehydrogenase (ADH) oxidizes ethanol into acetaldehyde, which is then further oxidized into harmless acetic acid by acetaldehyde dehydrogenase (ALDH). These oxidation reactions are coupled with the reduction of NAD^+ to NADH. ALDH2, an isozyme of ALDH, contains the functional polymorphism, *ALDH2* Glu487Lys. An association between this polymorphism and squamous cell carcinomas, such as esophageal cancer in alcoholics, has been reported. A recent study reported that exposure of cells to acetaldehyde results in a concentration-dependent increase in FANCD2 monoubiquitination [11]. Acetaldehyde also stimulates BRCA1 phosphorylation at Ser1524 and increases the level of H2AX, a marker of homologous recombination. Both modifications occur in a dose-dependent manner.

Another report showed that ALDH2 is essential for the development of FANCD 2(−/−) embryos [12]. Nevertheless, mothers with AA enzyme (ALDH 2(+/−)) can support the development of double-mutant (ALDH2 (−/−)FANCD2(−/−)) mice. These embryos are unusually sensitive to ethanol exposure in utero, with ethanol consumption by postnatal double-deficient mice rapidly precipitating bone marrow failure. ALDH2 (−/−)FANCD2(−/−) mice also spontaneously develop acute leukemia.

This previous study also provided the first evidence of the factors responsible for driving the FA hematological phenotype in mice. DNA damage caused by acetaldehyde may contribute critically to the genesis of fetal alcohol syndrome in fetuses, as well as to abnormal development, bone marrow failure, and cancer predisposition in FA patients. This research group also focused on hematopoietic stem cells (HSCs) in another study [13]. They reported finding that some aged ALDH2(−/−)FANCD2(−/−) mutant mice that did not develop leukemia spontaneously developed aplastic anemia, with a concomitant accumulation of damaged DNA within the hematopoietic stem and progenitor cell (HSPC) pool. Only HSPCs and not the more mature blood precursors require Aldh2 for protection against acetaldehyde toxicity. There is more than a 600-fold reduction in the HSC pool of mice deficient in both FA pathway-mediated DNA repair and acetaldehyde detoxification. This study data indicated that the emergence of bone marrow failure in FA was probably due to aldehyde-mediated genotoxicity restricted to the HSPC pool.

All of the ALDH data suggest that ALDH2 polymorphism is critical to the prognosis of FA patients.

Intercrosslink repair

DNA ICLs are toxic to dividing cells, as they induce mutations, chromosomal rearrangements, and cell death. In order to survive, organisms have developed strategies for dealing with DNA damage. As such, specialized repair pathways have evolved for specific kinds of DNA damage, including double-strand break (DSB) and ICL. Inducers of ICLs are important drugs in cancer treatment and include the well-known chemotherapeutic agents mitomycin C, cisplatin, cyclophosphamide, and their respective derivatives. While cells derived from most individuals with FA are hypersensitive to ICLs, they are generally not hypersensitive to inducers of DSBs such as ionizing radiation, indicating that the ICL repair pathway is distinct from that of DSB.

Homologous recombination is a DNA repair pathway that utilizes strand exchange in a gene conversion reaction involving a single-strand and a DNA duplex. In mammalian cells, this is a major repair pathway for DNA damage such as DSBs. The strand exchange protein RAD51 and the products of the hereditary breast cancer susceptibility genes BRCA1 and BRCA2 [14, 15] are critical proteins in HR in mammalian cells.

In 2005, a cellular study in humans showed that mutation of either the FA core complex members or the FANCD2 monoubiquitination site resulted in HR defects [16]. These defects, however, are mild compared with those resulting from a BRCA2 deficiency. HR measurements in these previous studies were performed with the widely used DR-GFP reporter system, in which a DSB formed by I-SceI endonuclease results in green fluorescent protein-expressing (GFP+) cells repaired by HR (Fig. 2). Further studies have reported on the mechanisms of ICL repair, particularly in terms of the replication-coupled manner. A 2008 study using a cell-free system based on *Xenopus* egg extracts found that ICL is repaired in a replication-dependent manner [17]. Another study in the *Xenopus* egg showed that ubiquitinated FANCI-FANCD2 is essential for replication-dependent ICL repair and that it is able to control the incision step [18]. Development and use of a TR-GFP assay, a modified version of the DR-GFP HR assay system, demonstrated that ICL repair in mammalian cells is dependent on DNA replication. The TR-GFP assay uses a DNA template with a site-specific ICL at sequences that are complemented to triplex-forming oligonucleotide conjugated with psoralen (pso-TFO) [19]. The construct also contains an origin of replication from the Epstein–Barr virus (EBV), enabling replication in human cells. Their results showed that ICL-induced HR was substantially compromised in the absence of FA proteins, suggesting that the FA pathway is specifically involved in replication-coupled HR repair. Use of direct assays for ICL-induced HR in vivo, along with studies that