was comparable to that of patients transplanted from MRD (93.1±1.5%) (P=not significant, NS), but it was significantly better than that of patients transplanted from haploidentical donors (66.7±15.7%, P=0.016) and MUD (79.0±2.9%, P=0.014). In the subgroup analysis of 1MMRD, no significant difference was observed between HLA class I-mismatched (n=32) and class II-mismatched patients (n=12) (5y OS; 93.8±4.3% vs 91.7±8.0%, P=NS). Comparison of the survival outcome based on the transplant period (1990–1999 vs 2000–2009) revealed that the probability of 5y OS of patients transplanted from 1MMRD was not significantly different [92.3±5.2% (n=26) vs 94.4±5.4% (n=18), P=NS], while that of patients transplanted from MUD significantly improved in the same period as we reported previously (67.1±5.5% (n=73) vs 86.1±3.1% (n=140), P=0.001).[11] In multivariate analysis, haploidentical donors (P<0.001), MUD (P<0.001), age ≥10 years (P<0.001), and transplant period (1990–1999 vs 2000–2009, P=0.006) were identified as independent covariates associated with unfavorable OS. Our analysis revealed that an HLA-mismatched related donor, especially 1MMRD, could be selected as a donor candidate for children with AA who need urgent transplantation.

Unrelated Donor BMT

Bone marrow transplantation from an unrelated donor (UBMT) is indicated as salvage therapy for patients with severe SAA who fail to respond to immunosuppressive therapy.[12] In several recent studies, the effect of HLA high-resolution matching on outcome of patients who received a UBMT has been elucidated.[13,14] However, results have been derived primarily from an analysis of patients with hematologic malignancies. Major obstacles for UBMT are different between patients with hematologic malignancies and patients with SAA. Relapse is a main cause of death for patients with hematologic malignancies, and Graft versus Leukemia effect may result in decrease in relapse rate. In contrast, graft failure is the main problem, and GVHD is the only negative effect for patients with SAA. Therefore, optimal HLA matching may be different between these two populations. Algorithms for donor selection derived from an analysis of patients with hematologic malignancies might not be useful for patients with SAA. However, a few studies have focused on the clinical significance of HLA-allele compatibility in patients with SAA.[15] In a previous study, we analyzed the clinical significance of HLA-allele mismatching in 142 patients with SAA, in whom data of high-resolution typing of HLA-A, -B, and -DRB1 were available. Mismatching of HLA-A or -B alleles between donor and recipient was a strong risk factor for acute and chronic GVHD and OS, whereas mismatching of the HLA-DRB1 allele did not have a significant effect on patient outcomes.[16] In the study from the National Marrow Donor Program, mismatching of HLA-DRB1 was the most crucial risk factor for OS.[17] On the contrary, restricting BMT to donor-recipient pairs perfectly matched at high-resolution typing reduces the chance of undergoing UBMT for many patients. Therefore, strategies for selecting a partially HLA-allele mismatched donor are required when a full matched donor cannot be identified. We report a detailed analysis of outcome in 301 patients with SAA who were typed for HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 by a molecular technique and underwent UBMT through the JMDP.[11] Of the 301 recipient/donor pairs, 101 (33.6%) were completely matched at 10 of 10 alleles, 69 (23%) were mismatched at one allele, and 131 (43.5%) were mismatched at ≥2 alleles. Subjects were classified into five subgroups: complete match group (group I); single-allele mismatch group (groups II and III); multiple alleles restricted to HLA-C, -DRB1, and -DQB1 mismatch group (group IV); and others (group V). Multivariate analysis indicated that only HLA disparity of group V was a significant risk factor for poor survival and grade II–IV acute GVHD. HLA-DPB1 mismatching was not associated with any clinical outcome. We recommend the use of an HLA 10 of 10 allele-matched unrelated donor. However, if such a donor is not available, any single-allele or multiple-allele (HLA-C, -DRB1, and -DQB1) mismatched donor is acceptable as an unrelated donor for patients with severe AA.

Conclusion

Treatment for childhood SAA has advanced in the past decade, most notably with the improvement in survival after HLA-matched UBMT and HLA-mismatched family donor BMT. Creation of an SAA outcomes registry might contribute to the improvement of outcome in children with SAA.

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Loss of heterozygosity in 7q myeloid disorders: clinical associations and genomic pathogenesis

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Loss of heterozygosity affecting chromosome 7q is common in acute myeloid leukemia and myelodysplastic syndromes, pointing toward the essential role of this region in disease phenotype and clonal evolution. The higher resolution offered by recently developed genomic platforms may be used to establish more precise clinical correlations and identify specific target genes. We analyzed a series of patients with myeloid disorders using recent genomic technologies (1458 by single-nucleotide polymorphism arrays [SNP-A], 226 by next-generation sequencing, and 183 by expression microarrays). Using SNP-A, we identified chromosome 7q loss of heterozygosity segments in 161 of 1458 patients (11%); 26% of chronic myelomonocytic leukemia patients harbored 7q uniparental disomy, of which 41% had a homozygous *EZH2* mutation. In addition, we describe an SNP-A–isolated deletion 7 hypocellular myelodysplastic syndrome subset, with a high rate of progression. Using direct and parallel sequencing, we found no recurrent mutations in typically large deletion 7q and monosomy 7 patients. In contrast, we detected a markedly decreased expression of genes included in our SNP-A defined minimally deleted regions. Although a 2-hit model is present in most patients with 7q uniparental disomy and a myeloproliferative phenotype, haploinsufficient expression of defined regions of 7q may underlie pathogenesis in patients with deletions and predominant dysplastic features. (*Blood.* 2012;119(25): 6109-6117)

Introduction

Complete loss of chromosome 7 (monosomy 7) or partial deletion involving its long arm [del(7q)] are highly recurrent chromosomal aberrations in myeloid disorders, including myelodysplastic syndrome (MDS), acute myeloid leukemia (AML), and juvenile myelomonocytic leukemia (JMML).[1,2] The International Prognostic Scoring System (IPSS), the most validated score for predicting the evolution of patients with MDS, does not discriminate among chromosome 7 anomalies, uniformly assigning these patients to the poor-risk karyotype group.[3] Other metaphase cytogenetic (MC) studies have consistently associated lesions involving the long arm of chromosome 7 with inferior survival in AML cases.[1,4] However, there is a contention that monosomy 7 and del(7q) are not equivalent in prognosis and disease phenotype spectrum.[5,6]

In the traditional genetic view, loss of heterozygosity (LOH) for 1 tumor suppressor gene (TSG) allele increases the chance of inactivation of the remaining allele and total loss of function for a cancer-protective locus. In accordance to this 2-hit model, we and other groups found loss-of-function hypomorphic homo- and hemizygous mutations in a variety of genes, including *TP53*, *CBL*, or *TET2*.[7-10] However, there is growing evidence that haploinsufficient TSGs also lead to hastened tumorigenesis, showing dramatic phenotypes with loss of only a single allele.[11,12] The haploinsufficient model is supported by recent studies in the context of myeloid disorders harboring a deletion of the long arms of chromosome 5 or chromosome 20,[13-15]and it is possible that monosomy 7/del(7q) cases are associated with a similar mechanism.

To better address the genomic and clinical complexity of myeloid malignancies associated with 7q abnormalities, we analyzed a large series of cases with single nucleotide polymorphism array (SNP-A)–based karyotyping, direct and next-generation sequencing (NGS), and microarray expression platforms to (1) examine the association of different SNP-A 7q lesions with certain clinical features and other genomic aberrations, (2) define a commonly deleted region or regions (CDRs) and search for recurrent tumor suppressor mutations, and (3) test the haploinsufficiency hypothesis.

Methods

Patients

Informed consent was obtained following the Declaration of Helsinki according to protocols approved by the review boards and ethics committees of the participating institutions. Presentation bone marrow (BM) aspirates from 1458 patients with myeloid malignancies were studied using SNP-A, including 200 AML cases analyzed by SNP-A and NGS 300 through The Cancer Genome Atlas project (TCGA; http://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp). Microarray expression data were available on a cohort of 183 patients with MDS and 17 healthy controls.[16]

Diagnosis of hypocellular myelodysplastic syndrome (hMDS) was made based on the presence of dysplastic features and the overall clinical presentation, including the presence of cytopenias, the absence of an excess of blasts (5% in BM or 2% in blood), and a decreased cellularity of the marrow of less than or equal to 20%. When indicated based on clinical suspicion, immunohistochemical staining for CD34 was performed to rule out, or find, collections of immature cells.

Metaphase cytogenetics

Chromosome preparations were G-banded using trypsin and Giemsa, and karyotypes were described according to the International System for Human Cytogenetic Nomenclature.[17]

SNP-A analysis

Genome-Wide Human SNP 6.0 and GeneChip Human Mapping 250K arrays (Affymetrix) were used for SNP-A analysis of bone marrow DNA as described previously.[18] Germ-line encoded copy number variants and nonclonal areas of uniparental disomy (UPD) were excluded from further analysis by a bioanalytic algorithm, based on lesions identified by SNP-A karyotyping in an internal control series (n = 1003) and reported in the Database of Genomic Variants (http://projects.tcag.ca/variation/). Size and location criteria (telomeric > 8.7 Mb and interstitial, > 25 Mb) were used for identification of somatic UPD. In 11 patients, a 7q microdeletion (median size, 0.3 Mb; range, 0.1-0.7 Mb) was detected but testing of germ line DNA was not possible because of lack of appropriate samples. These patients were excluded from analysis as the pathophysiologic significance of such small lesions is not clear. None of those 7q microdeletions was included in any of the CDRs described subsequently.

Direct sequencing

Sanger technique was used for sequencing all exons of 36 candidate genes, included in our SNP-A–defined CDRs, screening a subset of 50 7q LOH patients [UPD(7q), n = 7; del(7q), n = 31; monosomy 7, n = 12]. Samples from this cohort were not used for NGS.

Next-generation sequencing

Two NGS approaches were used in this study. We generated exome chromosome 7 libraries that were enriched for the content of chromosome 7 coding sequences using the SureSelect capture synthetic biotinylated RNA probes from Agilent Technologies, tiling all the coding regions from chromosome 7. Libraries were subjected to high-throughput sequencing on a Genome Analyzer IIx (Illumina) and applied to 11 7q LOH patients [del(7), n = 6; del(7q), n = 2; UPD(7q), n = 3].

The second approach involved the sequencing of 15 paired bone marrow mononuclear cells and $CD3^+$ lymphocytes (used as germ line controls) from 15 patients with different myeloid disorders and SNP-A findings. Among them, we included 2 patients with 7q LOH [UPD(7q) and del(7q)]. A rational bioanalytic algorithm was applied to identify candidate nonsynonymous alterations. First, nonredundantly mapped reads were used for whole exome assembly using the reference genome hg19. Next, the software algorithm called all the positions that vary from the reference genome. The candidate alterations were subtracted by the results of $CD3^+$ lymphocyte-derived DNA (double-checked by direct and simultaneous visualization using DNAnexus Site) and subsequently validated using Sanger sequencing. Moreover, gene mutations affecting 7q LOH were screened using whole exome sequencing results available through TCGA.

Microarray data analysis

Previously published microarray expression data were obtained on a cohort of 183 MDS patients [monosomy 7/del(7q), n = 9].[16] Cell intensity calculation and scaling was performed using GeneChip Version 1.40 operating software (Affymetrix). Affymetrix CEL files were preprocessed using robust multiarray average. Data from 17 healthy controls were used to obtain patient and control expression ratios.

Statistical analysis

Comparisons of proportions and ranks of variables between groups were performed by the χ^2 test, Fisher exact test, Student t test or Mann-Whitney U test, as appropriate. We used the Kaplan-Meier and the Cox method to analyze overall survival (OS) and progression-free survival, with a 2-sided P less than or equal to .05 determining significance. In Cox models, examination of log (−log) survival plots and partial residuals was performed to assess that the underlying assumption of proportional hazards was met.

Results

Patient cohorts

Using SNP-A karyotyping, LOH segments involving 7q were identified in 161 of 1458 patients (11%), consisting of 9% MDS, 28% MDS/myeloproliferative neoplasms (MDS/MPN), 11% AML, 14% JMML, and 16% Fanconi anemia subsets (Figure 1A). MC identified 7q LOH in each of the cases detected by SNP-A except for 26 UPD cases and 11 patients in whom no interpretable metaphases were obtained (Figure 1B). In addition, in 7 cases a balanced translocation with 7q material was noted by MC; in all instances, SNP-A analyses detected a small deletion (> 1 and < 5 Mb) affecting the boundaries of the translocation. In 16 of 67 monosomy 7 cases by MC, SNP-A detected retained chromosome 7 material, probably contributing to marker chromosomes found by MC analysis. With increased resolution, there was a shift toward identification of more complex karyotypes and of additional lesions among the patients with isolated MC 7q aberrations (Figure 1C). By SNP-A, previously cryptic lesions were identified in 45% of the patients who otherwise showed a singular 7q LOH lesion by MC.

The 7q LOH cohort included men (57%) and women (43%) with a median age of 65 years (interquartile range, 59-73 years). The distribution of disease subsets and associated genomic lesions among the 3 classes of chromosome 7 lesions [UPD7q, del(7q) and monosomy 7] is shown in Figure 2, and Table 1 shows clinical characteristics at baseline.

Clinical and genomic correlates of monosomy 7/del(7q) patients

Compared with cases of partial deletions, those patients with del(7) were characterized by a lower number of genomic lesions per patient (1.2 vs 4.8; $P < .001$); the most remarkable the absence of 17p LOH cases among MDS patients.

Of 26 patients with monosomy 7 by SNP-A and a diagnosis of MDS, 20 (77%) fulfilled the diagnostic criteria of hMDS. Of note, these 20 patients had no other lesion detectable by SNP-A (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). That strong correlation between hMDS and the presence of an isolated monosomy 7 could not be established by MC, because no-growth was obtained in 8 hMDS-MC analyses and 4 high-risk MDS patients were described to harbor an isolated monosomy 7 by MC, whereas SNP-A found additional lesions in all of them. When patients with hMDS with or without monosomy 7 were compared, those with monosomy 7 showed a worse prognosis, with a higher transformation to leukemia ($P = .02$; hazard ratio, 3.4, 95%

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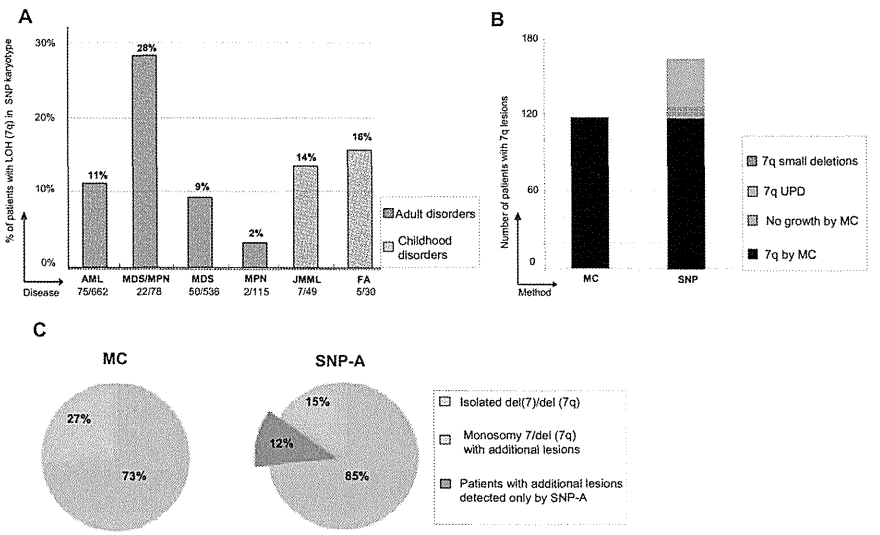


Figure 1. Frequency of detection of 7q and additional abnormalities by SNP-A. (A) Distribution of 7q LOH among the 1458 SNP-A–tested patients with myeloid malignancies, according to World Health Organization disease classification. (B) Number of patients with 7q LOH seen on MC and SNP-A. Lesions were observed in 117 of 1458 and 161 of 1458 patients when using MC and SNP-A, respectively. The additional 7q lesions found by SNP-A included those found in patients with no growth of MC cultures, small deletions affecting balanced translocation boundaries,[11] and UPD undetectable by MC.[26] (C) Percentage of patients with a sole 7q lesion versus accompanied by other abnormalities as identified by MC and SNP-A. SNP-A indicates single nucleotide polymorphism array;MDS/MPN, myelodysplastic syndrome/myeloproliferative neoplasm; AML, acute myeloid leukemia; MPN myeloproliferative neoplasms; JMML, juvenile myelomonocytic leukemia; FA, Fanconi anemia; UPD, uniparental disomy; MC, metaphase cytogenetics; monosomy 7, deletion of whole chromosome 7; and del(7q), partial deltion involving 7q.

confidence interval, 1.2%-9.7%; Figure 3A). Interestingly, 3 cases of monosomy 7 AML had a history of antecedent aplastic anemia. All the chromosome 7 lesions detected in 79 pediatric patients were monosomies; monosomy 7 was detected in 14% and 16% of patients with JMML and FA, respectively.

The subset of patients with del(7q) presented with an elevated percentage of high-risk disease (88% had AML or high-risk/intermediate-2 IPSS MDS) and a higher number of associated genomic lesions per patient. Del(5q) was the most common del(7q)-associated lesion found, occurring in 35 of 72 patients, all of which had AML or higher risk MDS. Of note, all del(5q) segments, except in 4 cases, involved either the centromeric or the telomeric extremes of the long arm of chromosome 5. The high frequency of del(5q) was followed closely by LOH 17p, seen in 14 of 72 patients, of which 5 of 14 were UPDs. Similar to patients with 5q, all patients had advanced stages of MDS or AML at diagnosis. All patients with LOH 17p spanned TP53; somatic mutations were present in 77% of cases tested.

The del(7q) MDS and MDS/MPN cohort had a shorter OS and time to leukemia transformation compared with patients with UPD(7q) or monosomy 7 (Figure 3B). In contrast, OS was similar in AML cases with monosomy 7 and del(7q) (Figure 3C), with both showing a significantly worse survival than in those patients with AML but without 7q LOH.

Clinical and genomic correlates of UPD(7q) patients

The UPD(7q) subset consisted of 26 patients, of which 17 were diagnosed with chronic myelomonocytic leukemia (CMML). Interestingly, 2 cases of AML and UPD(7q) also had history of antecedent CMML. The number of associated genomic lesions in the UPD(7q) cohort was lower than in the monosomy 7 and del(7q) subsets ($P = .03$ and $P < .001$, respectively), with a predominant presence of other regions of somatic copy neutral LOH rather than unbalanced defects. UPD(7q) was not associated with 5q or 17p LOH segments.

Comparing the 17 CMML patients to 55 CMML patients without UPD(7q) by SNP-A analysis, we found a trend toward worse survival. Those CMML patients with UPD(7q) showed a trend toward a shorter median OS (460 vs 730 days ($P = .2$; Figure 3D) and a higher rate of transformation to leukemia; whereas 26% of UPD(7q) patients progressed to higher-risk MDS or AML, advanced disease was observed in 13% of CMML patients without UPD(7q) ($P = .001$).

To test the prognostic validity and independence from known clinical variables of chromosome 7 SNP-A findings in patients' MDS and CMML, we developed a multivariate model for each cohort (Table 2). In the MDS model, the absence or presence of del(7q) or monosomy 7 kept the independent prognostic value when analyzed controlling for the clinical variables from the IPSS, ie, bone marrow blast percentage and number of cytopenias retained, whereas in the CMML model, the presence or absence of UPD 7q showed a trend toward statistical significance ($P = .1$) when tested together with the variables included in the score described by Onida et al,[19] that is, hemoglobin level below 12 g/dL, presence of circulating immature myeloid cells, absolute lymphocyte count greater than 2.5 × 10/L,[9] and marrow blasts greater than 10%.

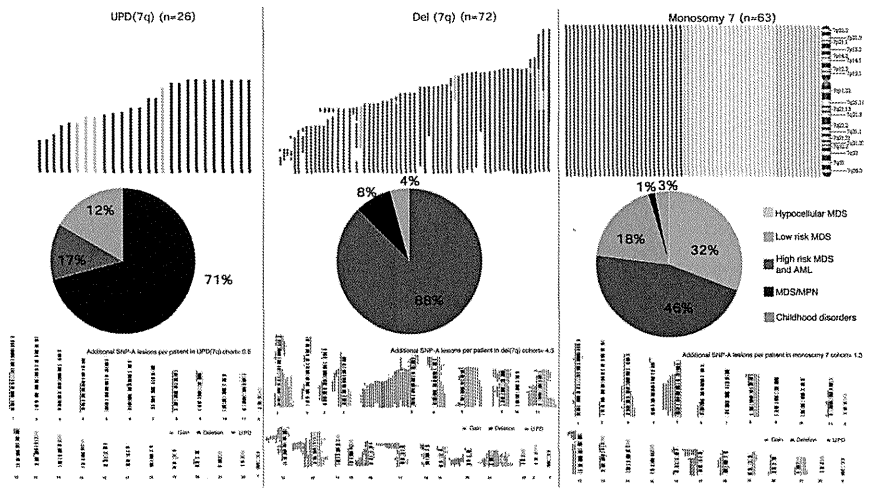


Figure 2. Distribution of disease subsets and associated genomic lesions among the 3 classes of chromosome 7 lesions. (Top) Distribution of LOH detected by SNP-A in the cohort, separated according to the nature of the lesion ([UPD(7q), del(7q), monosomy 7]. Patients have been grouped as follows: red, 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. (Middle) Distribution of disease status in patients with 7 LOH separated according the nature of the lesion. (Bottom) Additional SNP-A–detected genomic lesions separated according the same criteria as stated herein. MDS indicates myelodysplastic syndrome; AML, acute myeloid leukemia; UPD, uniparental disomy; monosomy 7, deletion of whole chromosome 7; del(7q), partial deletion involving 7q; and MDS/MPN, myelodysplastic syndrome/myeloproliferative neoplasm.

Comparative analysis of SNP-A and MC

To provide a more detailed analysis about the added information that these karyotyping techniques could offer, Figure 4 illustrates how the distribution of disease subsets and outcome associations would be according to the lesion found by MC. Leaving aside those 44 patients with 7q LOH not detected by MC (26 UPD, 11 no growth, 7 small deletions in balanced translocations), the allocation of entities among MC del(7q) and monosomy 7 did not show a significant change. In fact, when only MC informative cases were considered, a multivariate model including both SNP-A and MC 7q lesions in MDS resulted in the variables cancelling each other ($P = .7$). No SNP-A–defined monosomy 7 was defined as a partial deletion by MC, because of which the strong association among monosomy 7 and hMDS and its high rate of transformation compared with hMDS without monosomy 7 remains unaltered. However, we must remark that this subgroup of MDS patients showed a higher frequency of no-growth MC analysis (in 8 hMDS patients, half of them harbored a monosomy 7 by SNP-A).

As a result of SNP findings, 16 patients were erroneously assigned to the monosomy 7 group by MC. SNP-A revealed that these samples instead had partial deletions and thus had been misclassified as high-risk patients. Probably, "relocation" of patients led to the lack of statistical difference noted in survival between MC-defined del(7q) and monosomy 7 patients (although a trend is still noted, $P = .07$).

Table 1. Comparative of clinical characteristics of patients at baseline according to the SNP-A–detected lesion nature

	Deletion(7), n = 63 (A)	Deletion(7q), n = 72 (B)	UPD(7q), n = 26 (C)	*P* (only significant comparisons reported)
Median age, y (range)	58 (27-70)	64 (56-72)	68 (63.2-77)	A vs B ($P = .01$) A vs C ($P = .009$)
Sex				
Male, %	59	56	57	
Female, %	41	44	43	
White blood cell count, × 10^9/L, median (IQR)	5.5 (2.7-25.7)	4.3 (2.1-13.5)	10.5 (8.1-37.9)	C vs A ($P = .028$) C vs B ($P = .01$)
Hemoglobin, g/dL, mean ± SD	9.1 ± 1.9	9.2 (8.9-10)	9.1 ± 2.1	
Mean corpuscular volume, median (IQR)	91 (85-99)	90 (86-104.8)	90 (85-103)	
Platelets, × 10^9/L, median (IQR)	47 (22-71)	50 (24-86)	46 (20.2-146.2)	
BM cellularity, median (%)	35 (22-61)	75 (45-90)	76(55-100)	A vs B ($P = .03$) A vs C ($P = .036$)

IQR indicates interquartile range; BM, bone marrow; UPD, uniparental disomy; and CMML, chronic myeloid leukemia..

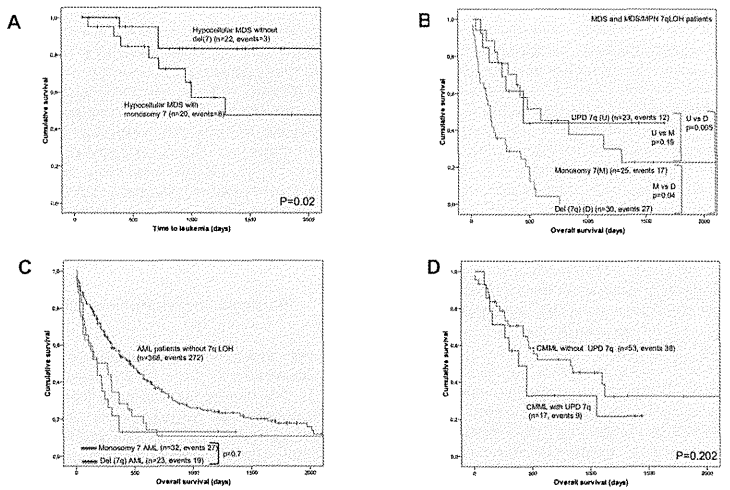


Figure 3. Differences in survival outcomes and progression-free survival of 7q LOH patients. *P* values presented correspond to the Cox regression between the groups indicated. AML indicates acute myeloid leukemia; Chr, chromosome, MDS/MPN, myelodysplastic syndrome/myeloproliferative neoplasm; UPD, uniparental 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 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]

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.

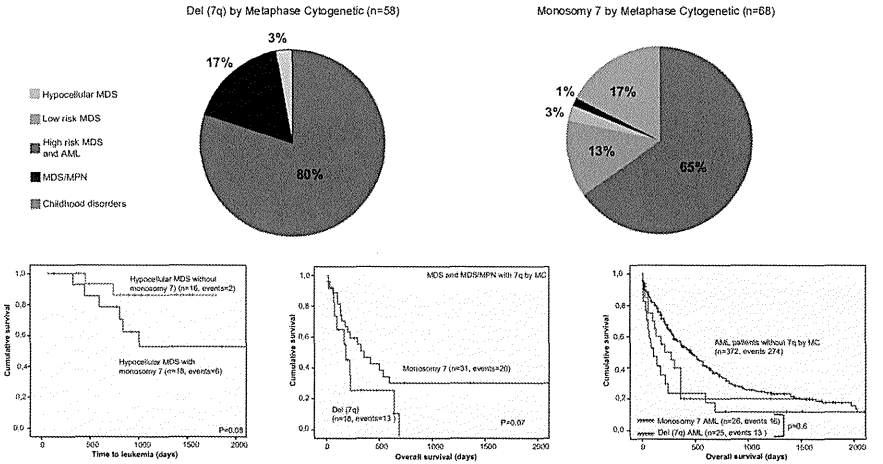


Figure 4. Illustration of how the distribution of disease subsets and outcome associations would be according to the lesion found by MC. (Top) Distribution of patients detected separated according to lesion detected by metaphase cytogenetics. Patients have been grouped as follows: red, AML + high risk 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.

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

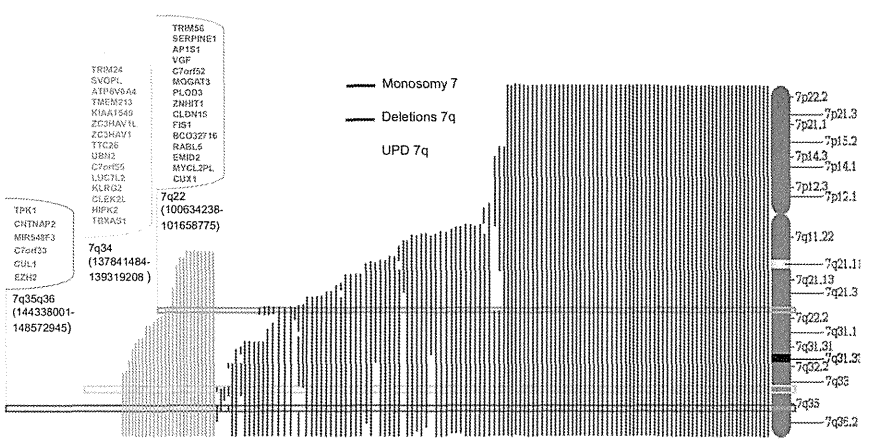


Figure 5. Identification of candidate genes on 7q by mapping CDRs by SNP-A. Three distinct CDRs, indicated by horizontal rectangles, were identified on 7q by mapping of SNP-A karyotyping. The connected keys show the candidate genes contained in each CDR; those genes sequenced in a test cohort of 50 patients 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	*CTAGE5*	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.

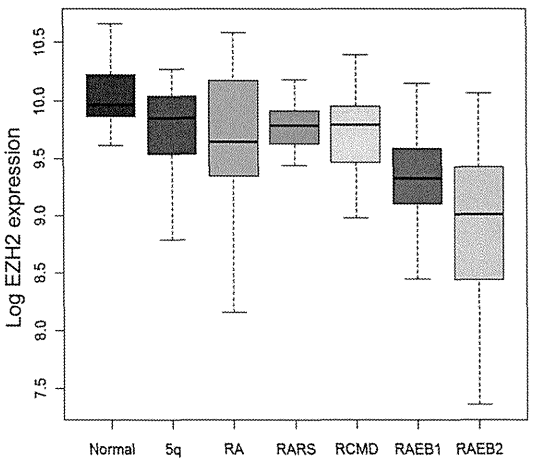
Testing the haploinsufficiency hypothesis: microarray expression data

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



	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

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.

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 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]).

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

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 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.

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)

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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 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).

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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.

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

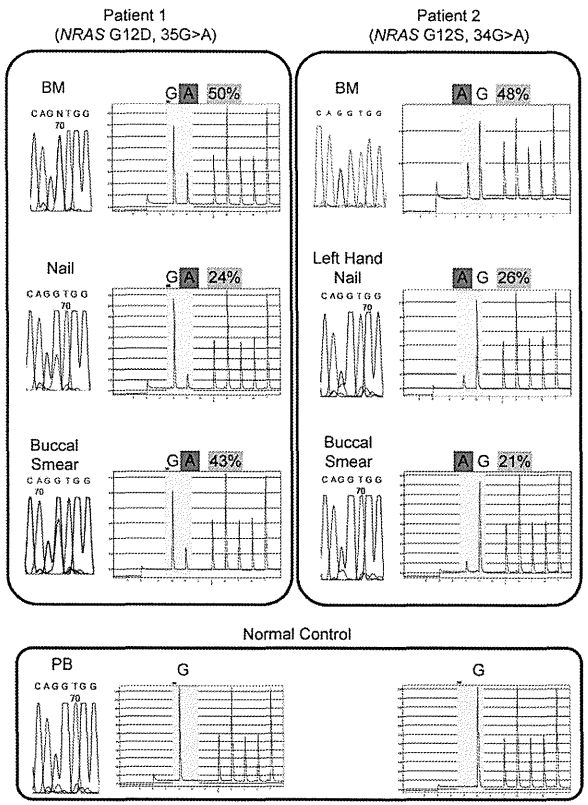

Figure 1. Direct sequencing and quantitative mutational analysis of *NRAS* in JMML patients. *NRAS* mutations are detected by direct sequencing and quantified by pyrosequencing. Direct sequencing identified oncogenic *NRAS* mutations: for Patient 1, G12D, 35G > A; for Patient 2, G12S, 34G > A) in BM-MNCs 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.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Extensive gene deletions in Japanese patients with Diamond-Blackfan anemia

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Fifty percent of Diamond-Blackfan anemia (DBA) patients possess mutations in genes coding for ribosomal proteins (RPs). To identify new mutations, we investigated large deletions in the RP genes *RPL5*, *RPL11*, *RPL35A*, *RPS7*, *RPS10*, *RPS17*, *RPS19*, *RPS24*, and *RPS26*. We developed an easy method based on quantitative-PCR in which the threshold cycle correlates to gene copy number. Using this approach, we were able to diagnose 7 of 27 Japanese patients (25.9%) possessing mutations that were not detected by sequencing. Among these large deletions, similar results were obtained with 6 of 7 patients screened with a single nucleotide polymorphism array. We found an extensive intragenic deletion in *RPS19*, including exons 1-3. We also found 1 proband with an *RPL5* deletion, 1 patient with an *RPL35A* deletion, 3 with *RPS17* deletions, and 1 with an *RPS19* deletion. In particular, the large deletions in the *RPL5* and *RPS17* alleles are novel. All patients with a large deletion had a growth retardation phenotype. Our data suggest that large deletions in RP genes comprise a sizable fraction of DBA patients in Japan. In addition, our novel approach may become a useful tool for screening gene copy numbers of known DBA genes. (*Blood*. 2012;119(10): 2376-2384)

Introduction

Diamond-Blackfan anemia (DBA; MIN# 105650) is a rare congenital anemia that belongs to the inherited BM failure syndromes, generally presenting in the first year of life. Patients typically present with a decreased number of erythroid progenitors in their BM.[1] A main feature of the disease is red cell aplasia, but approximately half of patients show growth retardation and congenital malformations in the craniofacial, upper limb, cardiac, and urinary systems. Predisposition to cancer, in particular acute myeloid leukemia and osteogenic sarcoma, is also characteristic of the disease.[2]

Mutations in the *RPS19* gene were first reported in 25% of DBA patients by Draptchinskaia et al in 1999.[3] Since that initial finding, many genes that encode large (RPL) or small (RPS) ribosomal subunit proteins were found to be mutated in DBA patients, including *RPL5* (approximately 21%), *RPL11* (approximately 9.3%), *RPL35A* (3.5%), *RPS7* (1%), *RPS10* (6.4%), *RPS17* (1%), *RPS24* (2%), and *RPS26* (2.6%).[4-7] To date, approximately half of the DBA patients analyzed have had a mutation in one of these genes. Konno et al screened 49 Japanese patients and found that 30% (12 of 49) carried mutations.[8] In addition, our data showed that 22 of 68 DBA patients (32.4%) harbored a mutation in ribosomal protein (RP) genes (T.T., K.T., R.W., and E.I., unpublished observation, April 16, 2011). These abnormalities of RP genes cause defects in ribosomal RNA processing, formation of either the large or small ribosome subunit, and decreased levels of polysome formation,[4-6,9-12] which is thought to be one of the mechanisms for impairment of erythroid lineage differentiation.

Although sequence analyses of genes responsible for DBA are well established and have been used to identify new mutations, it is estimated that approximately half of the mutations remain to be determined. Because of the difficulty of investigating whole allele deletions, there have been few reports regarding allelic loss in DBA, and they have only been reported for *RPS19* and *RPL35A*.[3,6,13] However, a certain percentage of DBA patients are thought to have a large deletion in RP genes. Therefore, a detailed analysis of allelic loss mutations should be conducted to determine other RP genes that might be responsible for DBA.

In the present study, we investigated large deletions using our novel approach for gene copy number variation analysis based on quantitative-PCR and a single nucleotide polymorphism (SNP) array. We screened Japanese DBA patients and found 7 patients with a large deletion in an allele in *RPL5*, *RPL35A*, *RPS17*, or *RPS19*. Interestingly, all of these patients with a large deletion had a phenotype of growth retardation, including short stature and

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Table 1. Primers used for synchronized quantitative-PCR (s-q-PCR) of RPL proteins

Gene	Primer name	Sequence	Primer name	Sequence	Size, bp
RPL5	L5-02F	CTCCCAAAGTGCTTGAGATTACAG	L5-02R	CACCTTTTCCTAACAAATTCCCAAT	132
	L5-05F	AGCCCTCCAACCTAGGTGACA	L5-05R	GAATTGGGATGGGCAAGAACT	102
	L5-17F	TGAACCCTTGCCCTAAAACATG	L5-17R	TCTTGGTCAGGCCCTGCTTA	105
	L5-19F	ATTGTGCAAACTCGATCACTAGCT	L5-19R	GTGTCTGAGGCTAACACATTTCCAT	103
	L5-21F	GTGCCACTCTCTTGGACAAACTG	L5-21R	CATAGGGCCAAAAGTCAAATAGAAG	102
	L5-28F	TCCACTTTAGGTAGGCGAAACC	L5-28R	TCAGATTTGGCATGTACCTTTCA	102
RPL11	L11-06F	GCACCCACATGGCTTAAAGG	L11-6R	CAACCAACCCATAGGCCAAA	102
	L11-20F	GAGCCCCCTTTCTCAGATGATA	L11-20R	CATGAACTTGGGCTCTGAATCC	109
	L11-22F	TATGTGCAGATAAGAGGGCAGTCT	L11-22R	ATACAGATAAGGAAACTGAGGCAGATT	98
RPL19	L19-02F	TGGCCTCTCATAAAGGAAATCTCT	L19-02R	GGAATGCAGGCAAGTTACTCTGTT	103
	L19-08F	TTTGAAGGCAAGAAATAAGTTCCA	L19-08R	AGCACATCACAGAGTCCAAATAGG	107
	L19-16F	GGTTAGTTGAAGCAGGAGCCTTT	L19-16R	TGCTAGGGAGACAGAAGCACATC	102
	L19-19F	GGACCAGTAGTTGTGACATCAGTTAAG	L19-19R	CCCATTTGTAACCCCCACTTG	106
RPL26	L26-03F	TCCAAAGAGCTGAGACAGAAGTACA	L26-03R	TCCATCAAGACAACGAGAACAAGT	102
	L26-16F	TTTGAGAATGCTTGAGAGAAGGAA	L26-16R	TTCCAGCACATGTAAAATCAAGGA	102
	L26-18F	ATGTTTTAATAAGCCCTCCAGTTGA	L26-18R	GAGAACAGCAAGTTGAAAGGTTCA	102
	L26-20F	GGGCTTTGCTTGATCACTCTAGA	L26-20R	AGGGAGCCGAAAACATTTAC	104
RPL35A	L35A-01F	TGTGGCTTCTATTTTGCGTCAT	L35A-01R	GGAATTACCTCCTTTATTGCTTACAAG	121
	L35A-07F	TTTCCGTTCTGTCTATTGCTGTGT	L35A-07R	GAACCCTGAGTGGAGGATGTTC	113
	L35A-17F	GCCCACAACCTCCAGAGAATC	L35A-17R	GGATCACTTGAGGCCAGGAAT	104
	L35A-18F	TTAGGTGGGCTTTTCAGTCTCAA	L35A-18R	ATCTCCTGATTCCCCAACTTTGT	102
RPL36	L36-02F	CCGCTCTACAAGTGAAGAAATTCTG	L36-02R	CTCCCTCTGCCTGTGAAATGA	102
	L36-04F	TGCGTCCTGCCAGTGTTG	L36-04R	GGGTAGCTGTGAGAACCAAGGT	105
	L36-17F	CCCCTTGAAAGGACAGCAGTT	L36-17R	TTGGACACCAGGCACAGACTT	114

Table 2. Primers used for s-q-PCR of RPS proteins

Gene	Primer name	Sequence	Primer name	Sequence	Size, bps
RPS7	S7-11F	GCGCTGCCAGATAGGAAATC	S7-11R	TTAGGGAGCTGCCTTACATATGG	102
	S7-12F	ACTGGCAGTTCTGTGATGCTAAGT	S7-12R	ACTCTTGCTCATCTCCAAAACCA	102
	S7-16F	GTGTCTGTGCCAGAAAGCTTGA	S7-16R	GAACCATGCAAAAGTGCCAATAT	112
RPS10	S10-03F	CTACGGTTTTGTGTGGGTCACTT	S10-03R	CATCTGCAAGAAGGAGACGATTG	102
	S10-15F	GTTGGCCTGGAGTCGTGATTT	S10-15R	ATTCCAAGTGCACCATTTCCTT	101
	S10-17F	AATGGTGTTTAGGCCAACGTTAC	S10-17R	TTTGAACAGTGGTTTTTGTGCAT	100
RPS14	S14-03F	GAATTCCAAACCCTTCTGCAAA	S14-03R	TTGCTTCATTTACTCCTCAAGACATT	104
	S14-05F	ACAACCAGCCCTCTACCTCTTTT	S14-05R	GGAAGACGCCGGCATTATT	102
	S14-06F	CGCCTCTACCTCGCCAAAC	S14-06R	GGGATCGGTGCTATTGTTATTCC	102
	S14-09F	GCCATCATGCCGAAACATACT	S14-09R	AACGCGCCACAGGAGAGA	102
	S14-13F	ATCAGGTGGAGCACAGGAAAAC	S14-13R	GCGAGGGAGCTGCTTGATT	111
	S14-15F	AGAAGTTTTAGTGAGGCAGAAATGAGA	S14-15R	TCCCCTGGCTATTAAATGAAACC	102
	S14-19F	GATGAATTGTCCTTTCCTCCATTC	S14-19R	TAGGCGGAAACCAAAAATGCT	102
RPS15	S15-11F	CTCAGCTAATAAAGGCGCACATG	S15-11R	CCTCACACCACGAACCTGAAG	108
	S15-15F	GGTTGGAGAACATGGTGAGAACTA	S15-15R	CACATCCCTGGGCCACTCT	108
RPS17	S17-03F	ACTGCTGTCGTGGCTCGATT	S17-03R	GATGACCTGTTCTTCTGGCCTTA	121
	S17-05F	GAAAACAGATACAAATGGCATGGT	S17-05R	TGCCTCCCACTTTTCCAGAGT	114
	S17-12F	CTATGTGTAGGAGGTCCCAGGATAG	S17-12R	CCACCTGGTACTGAGCACATGT	102
	S17-16F	TAGCGGAAGTTGTGTGCATTG	S17-16R	CAAGAACAGAAGCAGCCAAGAG	102
	S17-18F	TGGCTGAATCTGCCTGCTT	S17-18R	GCCTTGTATGTACCTGGAAATGG	103
	S17-20F	GGGCCCTTCACAAATGTTGA	S17-20R	GCAAAACTCTGTCCCTTTGAGAA	101
RPS19	S19-24F	CCATCCCAAGAATGCACACA	S19-24R	CGCCGTAGCTGGTACTCATG	120
	S19-28F	GACACACCTGTTGAGTCCTCAGAGT	S19-28R	GCTTCTATTAACTGGAGCACACATCT	114
	S19-36F	CTCTTGAGGGTGGTCTGGAAAT	S19-36R	GTCTTTGCGGGTTCTTCCTCTAC	102
	S19-40F	GGAACGGTGTCAGGATTCAAG	S19-40R	AGCGGCTGTACACCAGAAATG	101
	S19-44F	CTGAGGTTGAGTGTCCCATTTCT	S19-44R	GCACCGGGCCTCTGTTATC	104
	S19-57F	CAGGGACACAGTGCTGAGAAACT	S19-57R	TGAGATGTCCCATTTTCACTATTGTT	101
	S19-58F	CATGATGTTAGCTCCGTTGCATA	S19-58R	ATTTTGGGAAGAGTGAAGCTTAGGT	102
	S19-62F	GCAACAGAGCGAGACTCCATTT	S19-62R	AGCACTTTTCGGCACTTACTTCA	102
	S19-65F	ACATTTCCCAGAGCTGACATGA	S19-65R	TCGGGACACCTAGACCTTGCT	102
RPS24	S24-17F	CGACCACGTCTGGCTTAGAGT	S24-17R	CCTTCATGCCCAACCAAGTC	101
	S24-20F	ACAAGTAAGCATCATCACCTCGAA	S24-20R	TTTCCCTCACAGCTATCGTATGG	105
	S24-32F	GGGAAATGCTGTGTCCACATACT	S24-32R	CTGGTTTCATGGCTCCAGAGA	105
RPS26	S26-03F	CGCAGCAGTCAGGGACATTT	S26-03R	AAGTTGGGCGAAGGCTTTAAG	104
	S26-05F	ATGGAGGCCGTCTAGTTTGGT	S26-05R	TGCCTACCCTGAACCTTGCT	102
RPS27A	S27A-09F	GCTGGAGTGCATTCGCTTGT	S27A-09R	CACGCCTGTAATCCCCACTAA	102
	S27A-12F	CAGGCTTGGTGTGCTGTGACT	S27A-12R	ACGTCCATCTTCCAGCTGCTT	103
	S27A-18F	GGGTTTTTCCTGTTTGGTATTTGA	S27A-18R	AAAGGCCAGCTTTGCAAGTG	111
	S27A-22F	TTACCATATTGCCAGTCTTTCCATT	S27A-22R	TTCATATGCATTTGCACAAACTGT	106

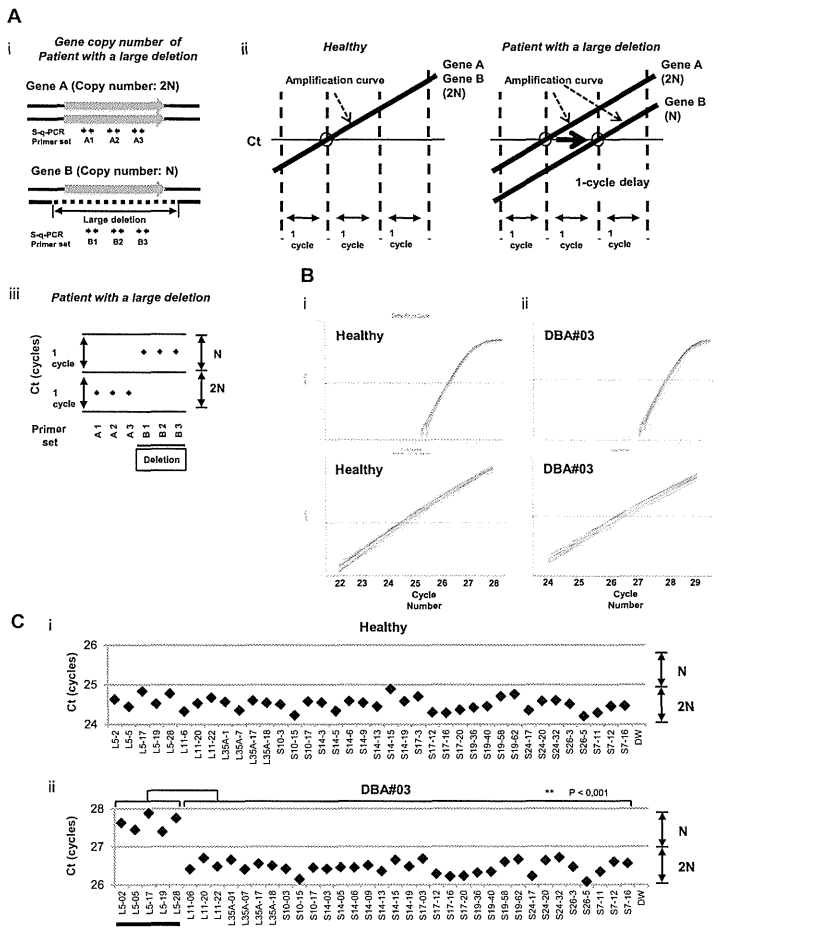

Figure 1. s-q-PCR can determine a large gene deletion in DBA. (A) Concept of the DBA s-q-PCR assay. The difference in gene copy number between a healthy sample and that with a large deletion is 2-fold (i). When all genomic s-q-PCR for genes of interest synchronously amplify DNA fragments, a 2-fold difference in the gene copy number is detected by a 1-cycle difference of the Ct scores of the s-q-PCR amplification curves (ii). Also shown is a dot plot of the Ct scores (iii). (B) Results of the amplification curves of s-q-PCR performed with a healthy person (i) and a DBA patient (patient 3; ii). The top panel shows the results of PCR cycles; the bottom panel is an extended graph of the PCR cycles at logarithmic amplification. (C) Graph showing Ct scores of s-q-PCR. If all specific primer sets for DBA genes show a 1-cycle delay relative to each other, this indicates a large deletion in the gene. Gene primer sets with a large deletion are underlined in the graph. $^{**}P < .001$.

small-for-gestational age (SGA), which suggests that this is a characteristic of DBA patients with a large gene deletion in Japan.

Methods

Patient samples

Genomic DNA was extracted using the GenElute Blood Genomic DNA Kit (Sigma-Aldrich) according to the manufacturer's protocol. Clinical manifestation of patients from a Japanese DBA genomic library are listed elsewhere or are as reported by Kouno et al.[8] The study was approved by the institutional review board at the National Institute of Infectious Diseases and Hirosaki University.

DBA gene copy number assay by s-q-PCR

For s-q-PCR, primers were designed using Primer Express Version 3.0 software (Applied Biosystems). Primers are listed in Tables 1 and 2. Genomic DNA in water was denatured at 95°C for 5 minutes and immediately cooled on ice. The composition of the s-q-PCR mixture was as follows: 5 ng of denatured genomic DNA, 0.4mM forward and reverse primers, 1× SYBR Premix Ex Taq II (Takara), and 1× ROX reference dye II (Takara) in a total volume of 20 μL (all experiments were performed in duplicate). Thermal cycling was performed using the Applied Biosystems 7500 fast real-time PCR system. Briefly, the PCR mixture was denatured at 95°C for 30 seconds, followed by 35 cycles of 95°C for 5 seconds, 60°C for 34 seconds, and then dissociation curve measurement. Threshold cycle (Ct) scores were determined as the average of duplicate samples. The technical errors of Ct scores in the triplicate analysis were within 0.2 cycles (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). The sensitivity and specificity of this method was evaluated with 15 healthy samples. Any false positive was not observed in all primer sets in all healthy samples (supplemental Figure 2). We performed direct sequencing of the s-q-PCR products. The results of the sequence analysis were searched for using BLAST to confirm uniqueness. Sequence data were obtained from GenBank (http://www.ncbi.nlm.nih.gov/gene/) and Ensemble Genome Browser (http://uswest.ensembl.org).

Genomic PCR

Genomic PCR was performed using KOD FX (Toyobo) according to the manufacturer's step-down PCR protocol. Briefly, the PCR mixture contained 20 ng of genomic DNA, 0.4mM forward and reverse primers, 1mM dNTP, 1× KOD FX buffer, and 0.5 U KOD FX in a total volume of 25 μL in duplicate. Primers are given in supplemental Figure 3 and Table 2. PCR mixtures were denatured at 94°C for 2 minutes, followed by 4 cycles of 98°C for 10 seconds, 74°C for 12 minutes, followed by 4 cycles of 98°C for 10 seconds, 72°C for 12 minutes followed by 4 cycles of 98°C for 10 seconds, 70°C for 12 minutes, followed by 23 cycles of 98°C for 10 seconds and 68°C for 12 minutes. PCR products were loaded on 0.8% agarose gels and detected by LAS-3000 (Fujifilm).

DNA sequencing analysis

The genomic PCR product was purified by the GenElute PCR clean-up kit (Sigma-Aldrich) according to the manufacturer's instructions. Direct sequencing was performed using the BigDye Version 3 sequencing kit. Sequences were read and analyzed using a 3120x genetic analyzer (Applied Biosystems).

SNP array-based copy number analysis

SNP array experiments were performed according to the standard protocol of GeneChip Human Mapping 250K Nsp arrays (Affymetrix). Microarray data were analyzed for determination of the allelic-specific copy number using the CNAG program, as described previously.[14] All microarray data are available at the EGA database (www.ebi.ac.uk/ega) under accession number EGAS00000000105.

Results

Construction of a convenient method for RP gene copy number analysis based on s-q-PCR

We focused on the heterozygous large deletions in DBA-responsible gene. The difference in copy number of genes between a mutated DBA allele and the intact allele was 2-fold (N and 2N; Figure 1Ai). If each PCR can synchronously amplify DNA fragments when the template genomic DNA used is of normal karyotype, it is possible to conveniently detect a gene deletion with a 1-cycle delay in s-q-PCR analysis (Figure 1Aii-iii).

Table 3. Summary of mutations and the mutation rate observed in Japanese DBA patients

Gene	Sequencing analysis
RPS19	10
RPL5	6
RPL11	3
RPS17	1
RPS10	1
RPS26	1
RPL35A	0
RPS24	0
RPS14	0
Mutations, n (%)	22 (32.4%)
Total analyzed, N	68

To apply this strategy for allelic analysis of DBA, we prepared primers for 16 target genes, *RPL5*, *RPL11*, *RPL35A*, *RPS10*, *RPS19*, *RPS26*, *RPS7*, *RPS17*, *RPS24*, *RPL9*, *RPL19*, *RPL26*, *RPL36*, *RPS14*, *RPS15*, and *RPS27A*, under conditions in which the Ct of s-q-PCR would occur within 1 cycle of that of the other primer sets (Tables 1 and 2). At the same time, we defined the criteria of a large deletion in our assay as follows. If multiple primer sets for one gene showed a 1-cycle delay from the other gene-specific primer set at the Ct score, we assumed that this represented a large deletion. As shown in Figure 1Bii and 1Cii, the specific primer sets for *RPL5* (L5-02, L5-05, L5-17, L5-19, and L5-28) detected a 1-cycle delay with respect to the mutated allele of patient 3. This assessment could be verified by simply confirming the difference of the cycles with the s-q-PCR amplification curves.

Study of large gene deletions in a Japanese DBA genomic DNA library

Sixty-eight Japanese DBA patients were registered and blood genomic DNA was collected at Hirosaki University. All samples were first screened for mutations in *RPL5*, *L11*, *L35A*, *S10*, *S14*, *S17*, *S19*, and *S26* by sequencing. Among these patients, 32.4% (22 of 68) had specific DBA mutations (Table 3 and data not shown). We then screened for large gene deletions in 27 patients from the remaining 46 patients who did not possess mutations as determined by sequencing (Table 4).

When we performed the s-q-PCR DBA gene copy number assay, 7 of 27 samples displayed a 1-cycle delay of Ct scores: 1 patient had *RPL5* (patient 14), 1 had *RPL35A* (patient 71), 3 had *RPS17* (patients 3, 60, 62), and 2 had *RPS19* (patients 24 and 72; Figure 2 and Table 4). Among these patients, the large deletions in the *RPL5* and *RPS17* genes are the first reported cases of allelic deletions in DBA. From these results, we estimate that a sizable number of Japanese DBA patients have a large deletion.

Based on our findings, the rate of large deletions was approximately 25.9% (7 of 27) in a category of unspecified gene mutations. Such mutations have typically gone undetected by conventional sequence analysis. We could not find any additional gene deletions in the analyzed samples.

Confirmation of the gene copy number for DBA genes by genome-wide SNP array

We performed genome-wide copy number analysis of the 27 DBA patients with a SNP array to confirm our s-q-PCR results. SNP array showed that patient 3 had a large deletion in

Table 4. Characteristics of DBA patients tested

Patient no.	Age at diagnosis	Sex	Hb, g/dL	Large deletion by s-q-PCR	Large deletion by SNP array	Inheritance	Malformations	Response to first steroid therapy
Patients with a large deletion in RP genes								
3*†	1 y	M		RPL5	RPL5	Sporadic	Short stature, thumb anomalies	Response
14*	5 y	M	5.5	RPS17	RPS17	Sporadic	White spots, short stature	Response
24*†	1 mo	F	5.5	RPS19	ND	Sporadic	Short stature, SGA	Response
60*†	2 mo	F	2.4	RPS17	RPS17	Sporadic	SGA	NT
62*†	1 mo	F	6.2	RPS17	RPS17	Sporadic	Small ASD, short stature, SGA	Response
71	0 y	M	5.3	RPL35A	RPL35A	Sporadic	Thumb anomalies, synostosis of radius and ulna, Cohelia Lange-like face, cleft palate, underdescended testis, short stature, cerebellar hypoplasia, fetal hydrops	NT
72†	0 y	M	2	RPS19	RPS19	Sporadic	Thumb anomalies, flat thenar, testicular hypoplasia, fetal hydrops, short stature, learning disability	No
Patients without a large deletion in RP genes								
5*	1 y	F	3.1	ND	ND	Sporadic	ND	Response
15*	1 mo	F	1.6	ND	ND	Sporadic	ND	Response
21*	1 y	F	2.6	ND	ND	Sporadic	ND	Response
26*	1 y 1 mo	F	8	ND	ND	Sporadic	Congenital hip dislocation, spastic quadriplegia, hypertelorism, nystagmus, short stature, learning disability	Response
33*	2 mo	F	1.3	ND	ND	Sporadic	ND	Response
36*	0 y	M	8.2	ND	ND	Familial	ND	Response
37*	4 y	M	6.1	ND	ND	Sporadic	Hypospadias, underdescended testis, SGA	NT
45*	5 d	M	5.1	ND	ND	Sporadic	Short stature, microcephaly, mental retardation, hypogammaglobulinemia	Poor
50*	2 m	F	3.4	ND	ND	Familial	ND	Response
61*	9 m	M	4	ND	ND	Sporadic	ND	Response
63*	0 y	M	6.8	ND	ND	Sporadic	Micrognathia, hypertelorism, short stature	Response
68	1 y 4 mo	M	5.9	ND	ND	Sporadic	ND	NT (CR)
69	1 y	M	9.3	ND	ND	Sporadic	ND	Response
76	0 y	M	4	ND	ND	Sporadic	ND	Response
77	0 y	M	7.8	ND	ND	Familial	Short stature	No
83	9 mo	F	3	ND	ND	Sporadic	ND	NT
90	10 mo	M	9	ND	ND	Sporadic	ND	No
91	0 y	F	3.8	ND	ND	Sporadic	ND	Response
92	2 mo	M	3.7	ND	ND	Sporadic	ASD, PFO, melanosis, underdescended testis, SGA, short stature	Response
93	11 mo	M	2.2	ND	ND	Sporadic	White spots, senile face, corneal opacity, underdescended testis, syndactyly, ectrodactyly, flexion contracture, extension contracture	Response

ND indicates not detected; NT, not tested; CR, complete remission; ASD, atrial septal defect; and PFO, persistent foramen ovale.
*Status data of Japanese probands 3 to 63 is from a report by Konno et al.[8]
†Large deletions of the parents of 5 DBA patients (3, 24, 60, 62, and 72) were analyzed by s-q-PCR, but there were no deletions in DBA genes in any of the 5 pairs of parents.

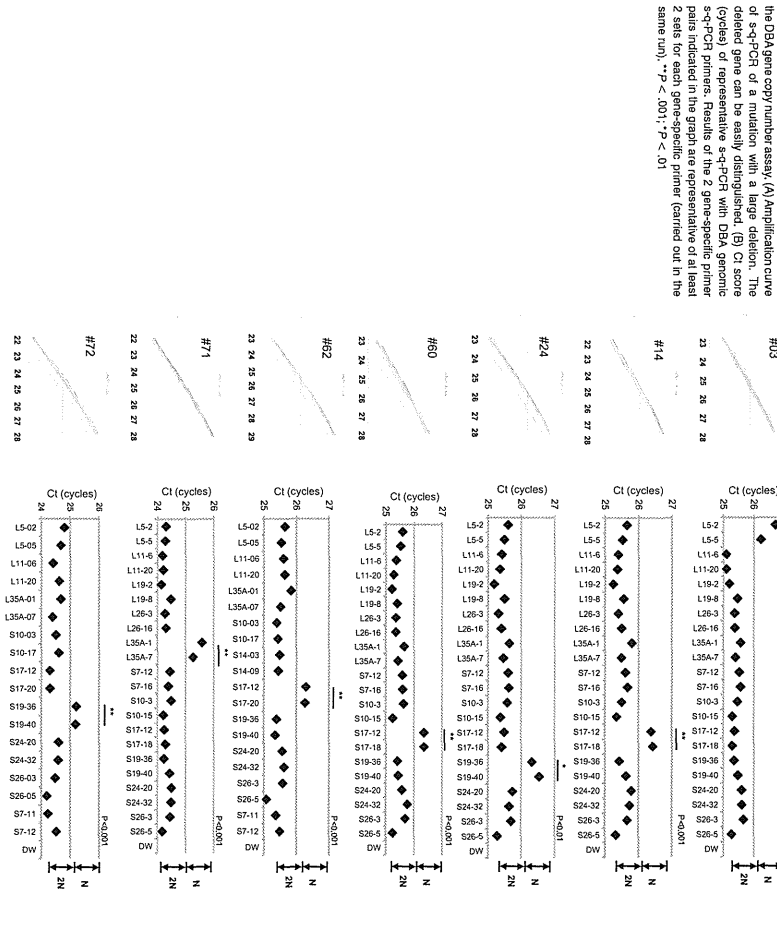

Figure 2. Detection of 7 mutations with a large deletion in DBA patients. Genomic DNA of 27 Japanese DBA patients with unknown mutations were subjected to the DBA gene copy number assay. (A) Amplification curve of s-q-PCR of a mutation with a large deletion. The deleted gene can be easily distinguished. (B) Ct score (cycles) of representative s-q-PCR with DBA genomic s-q-PCR primers. Results of the 2 gene-specific primer pairs indicated in the graph are representative of at least 2 sets for each gene-specific primer (carried out in the same run). **$P < .001$; *$P < .01$

chromosome 1 (ch1) spanning 858 kb (Figure 3A); patient 71 had a large deletion in ch3 spanning 786 kb (Figure 3B); patients 14, 60, and 62 had a large deletion in ch15 spanning 270 kb, 260 kb, and 330 kb, respectively (Figure 3C); and patient 72 had a large deletion in ch19 spanning 824 kb (Figure 3D). However, there were no deletions detected in ch19 in patient 24 (Figure 3D). Genes estimated to reside within a large deletion are listed in supplemental Table 1. Consistent with these s-q-PCR results, 6 of 7 large deletions were detected and confirmed as deleted regions, and these large deletions contained *RPL5*, *RPL35A*, *RPS17*, and *RPS19* (Table 4 and supplemental Table 1). Other large deletions in RP genes were not detected by this analysis. From these results, we conclude that the synchronized multiple PCR amplification method has a detection sensitivity comparable to that of SNP arrays.

Detailed examination of a patient with intragenic deletion in the RPS19 allele (patient 24)

Interestingly, for patient 24, in whom we could not detect a large deletion by SNP array at s-q-PCR gene copy number analysis, 2 primer sets for *RPS19* showed a 1-cycle delay (RPS19-36 and RPS19-40), but 2 other primer pairs (RPS19-58 and RPS19-62) did not show this delay (Figure 4A). We attempted to determine the deleted region in detail by testing more primer sets on *RPS19*. We tested a total of 9 primer sets for *RPS19* (Figure 4B) and examined the gene copy numbers. Surprisingly, 4 primer sets (S19-24, S19-36, S19-40, and S19-44) for intron 3 of *RPS19* indicated a 1-cycle delay, but the other primers for *RPS19* located on the 5′untranslated region (5′UTR), intron 3, or 3′UTR did not show this delay (S19-57, S19-58, S19-28, S19-62, and S19-65; Figure 4B-C). These results suggest that the intragenic deletion occurred in the *RPS19* allele. To confirm this deleted region precisely, we performed genomic PCR on *RPS19*, amplifying a region from the 5′UTR to intron 3 (Figure

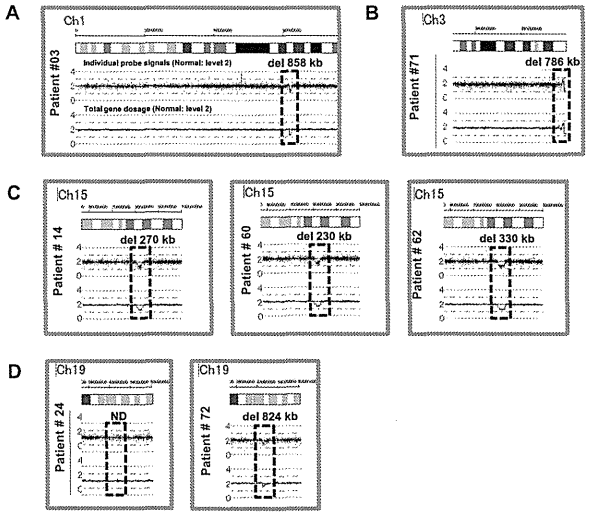

Figure 3. Results of SNP genomic microarray (SNP-chip) analysis. Genomic DNA of 27 Japanese DBA patients with unknown mutations was examined using a SNP array. Six patients had large deletions in their chromosome (ch), which included one DBA-responsible gene. Patient 3 has a large deletion in ch1 (A), patient 71 has a deletion in ch3 (B), patients 14, 60, and 62 have deletions in ch15 (C), and patient 72 has a deletion in ch19 (D).

4B). In patient 24, we observed an abnormally sized PCR product at a low molecular weight by agarose gel electrophoresis (Figure 4D). We did not detect a wild-type PCR product from the genomic PCR. This finding is probably because PCR tends to amplify smaller molecules more easily. However, we did detect a PCR fragment at the correct size using primers located in the supposedly deleted region. These bands were thought to be from the products of a wild-type allele. Sequencing of the mutant band revealed that intragenic recombination occurred at a homologous region of 27 nucleotides, from −1400 to −1374 in the 5′ region, to +5758 and +5784 in intron 3, which resulted in the loss of 7157 base pairs in the *RPS19* gene (Figure 4E). The deleted region contains exons 1, 2, and 3, and therefore the correct *RPS19* mRNA could not be transcribed.

Genotype-phenotype analysis and DBA mutations in Japan

Patients with a large deletion in DBA genes had common phenotypes (Table 4). Malformation with growth retardation (GR), including short stature or SGA, were observed in all 7 patients. In patients who had a mutation found by sequencing, half had GR (11 of 22; status data of DBA patients with mutations found by sequencing are not shown). GR may be a distinct phenotypic feature of large deletion mutations in Japanese DBA patients. Familial mutations were analyzed for parents for 5 DBA patients with a large deletion (patients 3, 24, 60, 62, and 72) by s-q-PCR. There are no large deletions in all 5 pairs of parents in DBA-responsible genes. Four of the 7 patients responded to steroid therapy. We have not observed significant phenotypic differences between patients with extensive deletions and other patients with regard to blood counts, responsiveness to treatment, or other malformations.

Discussion

Many studies have reported RP genes to be responsible for DBA. However, mutations have not been determined for approximately half of DBA patients analyzed. There are 2 possible reasons for this finding. One possibility is that patients have other genes responsible for DBA, and the other is that patients have a complicated set of mutations in RP genes that are difficult to detect. In the present study, we focused on the latter possibility because we have found fewer Japanese DBA patients with RP gene mutations (32.4%) compared with another cohort study of 117 DBA patients and 9 RP genes (approximately 52.9%).[4] With our newly developed method, we identified 7 new mutations with a large deletion in *RPL5*, *RPL35A*, *RPS17*, and *RPS19*.

The frequency of a large deletion was approximately 25.9% (7 of 27) in our group of patients who were not found to have mutations by genomic sequencing. Therefore, total RP gene mutations were confirmed in 42.6% of these Japanese patients (Table 5). Interestingly, mutations in *RPS17* have been observed at a high rate (5.9%) in Japan relative to that in other countries (1%).[5,15,16] Although the percentage of DBA mutations differs among different ethnic groups,[8,17-19] a certain portion of large deletions in DBA-responsible genes are likely to be determined in other countries by new strategies.

In the present study, we analyzed patient data to determine genotype-phenotype relations. To date, large deletions have been reported with RPS19 and RPL35A in DBA patients.[3,6,13] *RPS19* large deletions/translocations have been reported in 12 patients, and *RPL35A* large deletions have been reported in 2 patients.[19] GR in patients with a large deletion has been observed previously with *RPS19* translocations,[3,19-21] but it was not found in 2 patients with *RPL35A* deletion.[6] Interestingly, all of our patients with a large deletion had a phenotype

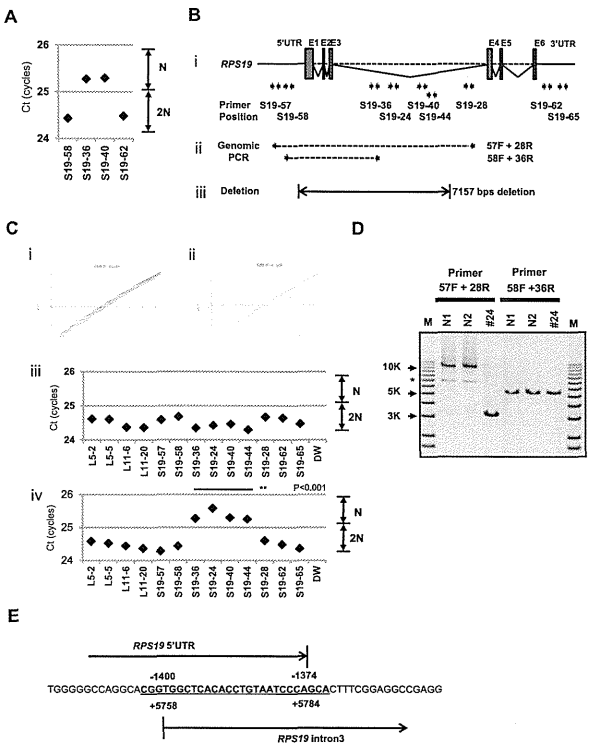

Figure 4. Result of s-q-PCR gene copy number assay for patient 24. (A) Results of s-q-PCR gene copy number assay for *RPS19* with 4 primer sets. (Bi) The *RPS19* gene copy number was analyzed with 9 specific primer sets for *RPS19* that span from the 5′UTR to the 3′UTR. (ii) Primer positions of genomic PCR for *RPS19*. (iii) Region determined to be an intragenic deletion in *RPS19*. (C) Results of gene copy number assay for *RPS19* show a healthy person (i,iii) and a DBA patient (ii,iv), and Ct results are shown (iii-iv). Patient 24 showed a "1-cycle delay" with primers located in the intron 3 region, but other primer sets were normal. (D) Results of genomic PCR amplification visualized by agarose gel electrophoresis to determine the region of deletion. N1 and N2 are healthy samples. *Nonspecific band. (E) Results from the genomic sequence of the 3-kb DNA band from genomic PCR on patient 24 showing an intragenic recombination from −1400 to 5784 (7157 nt) in *RPS19*. **$P < .001$.

of GR, including short stature and SGA, which suggests that this is a characteristic of DBA with a large gene deletion in Japan. Our study results suggest the possibility that GR is associated with extensive deletion in Japanese patients. Although further case studies will be needed to confirm this possibility, screening of DBA samples using our newly developed method will help to advance our understanding of the broader implications of the mutations and the correlation with the DBA genotype-phenotype.

Table 5. Total mutations in Japanese DBA patients, including large gene deletions

Gene	Mutation rate
RPS19	12(17.6%)
RPL5	7(10.3%)
RPL11	3 (4.4%)
RPS17	4 (5.9%)
RPS10	1 (1.5%)
RPS26	1 (1.5%)
RPL35A	1 (1.5%)
RPS24	0
RPS14	0
Mutations, n (%)	29(42.6%)
Total analyzed, N	68

Copy number variation analysis of DBA has been performed by linkage analysis, and the *RPS19* gene was first identified as a DBA-susceptibility gene. Comparative genomic hybridization array technology has also been used to detect DBA mutations in *RPL35A*, and multiplex ligation-dependent probe amplification has been used for *RPS19* gene deletion analysis.[3,6,13,22] However, these analyzing systems have problems in mutation screening. Linkage analysis is not a convenient tool to screen for multiple genetic mutations, such as those in DBA, because it requires a high level of proficiency. Although comparative genomic hybridization technology is a powerful tool with which to analyze copy number comprehensively, this method requires highly specialized equipment and analyzing software, which limits accessibility for researchers. Whereas quantitative PCR–based methods for copy number variation analysis are commercially available (TaqMan), they require a standard curve for each primer set, which limits the number of genes that can be loaded on a PCR plate. To address this issue, a new method of analysis is needed. By stringent selection of PCR primers, the s-q-PCR method enables analysis of many DBA genes in 1 PCR plate and the ability to immediately distinguish a large deletion using the s-q-PCR amplification curve. In our study, 6 of 7 large deletions in the RP gene detected by s-q-PCR were confirmed by SNP arrays (Figure 3). Interestingly, we detected

1 large intragenic deletion in *RPS19*, which was not detected by the SNP array. This agreement between detection results suggests that the s-q-PCR copy number assay could be useful for detecting large RP gene deletions.

In the present study, 7 DBA patients carried a large deletion in the RP genes. This type of mutation could be underrepresented by sequencing analysis, although in the future, genome sequencing might provide a universal platform for mutation and deletion detection. We propose that gene copy number analysis for known DBA genes, in addition to direct sequencing, should be performed to search for a novel responsible gene for DBA. Although at present, it may be difficult to observe copy numbers on all 80 ribosomal protein genes in one s-q-PCR assay, our method allows execution of gene copy number assays for several target genes in 1 plate. Because our method is quick, easy, and low cost, it could become a conventional tool for detecting DBA mutations.

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Authorship

Contribution: M.K. designed and performed the research, analyzed the data, and wrote the manuscript; A.S-O. and S. Ogawa performed the SNP array analysis; T.M., M.T., and M.O designed the study; T.T, K. Terui, and R.W. analyzed the mutations and status data; H.K., S. Ohga, A.O., S.K., T.K., K.G., K.K., T.M., and N.M. analyzed the status data; A.M., H.M., K. Takizawa, T.M., and K.Y., performed the research and analyzed the data; E.I. and I.H. designed the study and analyzed the data; and all authors wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Frequent somatic mosaicism of *NEMO* in T cells of patients with X-linked anhidrotic ectodermal dysplasia with immunodeficiency

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Somatic mosaicism has been described in several primary immunodeficiency diseases and causes modified phenotypes in affected patients. X-linked anhidrotic ectodermal dysplasia with immunodeficiency (XL-EDA-ID) is caused by hypomorphic mutations in the *NF-κB essential modulator (NEMO)* gene and manifests clinically in various ways. We have previously reported a case of XL-EDA-ID with somatic mosaicism caused by a duplication mutation of the *NEMO* gene, but the frequency of somatic mosaicism of *NEMO* and its clinical impact on XL-EDA-ID is not fully understood. In this study, somatic mosaicism of *NEMO* was evaluated in XL-EDA-ID patients in Japan. Cells expressing wild-type NEMO, most of which were derived from the T-cell lineage, were detected in 9 of 10 XL-EDA-ID patients. These data indicate that the frequency of somatic mosaicism of *NEMO* is high in XL-ED-ID patients and that the presence of somatic mosaicism of *NEMO* could have an impact on the diagnosis and treatment of XL-ED-ID patients. (*Blood*. 2012;119(23):5458-5466)

Introduction

X-linked anhidrotic ectodermal dysplasia with immunodeficiency (XL-EDA-ID) is a disease with clinical features including hypohidrosis, delayed eruption of teeth, coarse hair, and immunodeficiency associated with frequent bacterial infections.[1-5] The gene responsible for XL-EDA-ID has been identified as *NF-κB essential modulator (NEMO)*.[6-8] NEMO is necessary for the function of IκB kinase, which phosphorylates and degrades IκB to activate NF-κB.[9-10] Defects in NEMO cause various abnormalities in signal transduction pathways involving NF-κB, and affect factors such as the IL-1 family protein receptors, the TLRs, VEGFR-3, receptor activator of nuclear factor κB (RANK), the ectodysplasin-A receptor, CD40, and the TNF receptor I.[7] Whereas a complete loss of NEMO function in humans is believed to cause embryonic lethality,[11] *NEMO* mutations in XL-EDA-ID patients are hypomorphic,[8] causing a partial loss of NEMO functions.

In XL-EDA-ID, NEMO defects lead to diverse immunologic features including susceptibility to pathogens, impaired Ab response to polysaccharides,[2,4,12] hypogammaglobulinemia,[13] hyper IgM syndrome,[14] and impaired NK-cell activity,[15] with a large degree of variability in phenotypes among the patients. For example, approximately one-tenth of XL-EDA-ID patients exhibit reduced mitogen-induced proliferation of T lymphocytes.[12] Moreover, one-fourth suffer from inflammatory disorders such as inflammatory bowel disease and rheumatoid arthritis,[12] although the inflammatory process usually relies on NF-κB activation.[16] One explanation for this clinical variability is that the XL-EDA-ID phenotype is *NEMO* genotype-specific. Although the XL-EDA-ID database reported by Hanson et al succeeds to some extent in linking the specific clinical features to *NEMO* genotype,[12] the penetrance of some clinical features is not high and the mechanism accounting for this variability is unknown.

Recently, we have reported a case of spontaneous reversion mosaicism of the *NEMO* gene in XL-EDA-ID, which showed an atypical phenotype involving decreased mitogen-induced T-cell proliferation along with decreased CD4 T cells (patient 1).[17] There have been no subsequent reports on somatic mosaicism in XL-EDA-ID, and its prevalence and impact on the clinical features of the disease is unknown. In this study, we describe the younger brother of patient 1, who suffered from XL-EDA-ID with the same mutation and somatic reversion mosaicism of *NEMO*. Patient 2 showed intriguing laboratory findings in that mitogen-induced T-cell proliferation varied in accordance with the rate of detected reversion in the peripheral blood. These 2 cases led us to perform a nationwide study of XL-EDA-ID patients in Japan that revealed a high incidence of somatic mosaicism of *NEMO*.

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Table 1. Clinical and genetic features of XL-EDA-ID patients

Patient	Mutation	Ectodermal dysplasia	Mitogen-induced proliferation	Infections	Complications	Therapy	Sex chromosome chimerism
1	Duplication	+	Reduced	Sepsis (S.P. and P.A.)	Chronic diarrhea	IVIG	100% XY
				Disseminated M.A.C.	Failure to thrive	RFP, CAM, AMK, EB	
				Skin abscess (S.A.)	Small intestinal stenosis	Rifabutin	
				Invasive *Aspergillus*	Lymphedema		
2	Duplication	+	Reduced	Sepsis (*E coli*)	Failure to thrive	IVIG, ST, EB, CAM	99.8% XY 0.2% X
				Disseminated M.S.		Rifabutin, SCT	
3	D311E	–	Normal	Disseminated B.C.G.		IVIG, INH	100% XY
				Sepsis (S.P.)		RFP, SCT	
4	A169P	+	Normal	Meningitis (S.P.)	IBD	IVIG, ST, PSL	99% XY
					Interstitial pneumonia	CyA, MTX, Infliximab	
					Rheumatoid arthritis		
5	L227P	+	Normal	Recurrent pneumonia	IBD	ST, mesalazine	Not done
				Pyogenic coxitis		Infliximab	
				Recurrent otitis media			
6	R182P	+	Not done	Recurrent otitis media	IBD	ST, mesalazine	99.8% XY 0.2% X
				UTI, Recurrent stomatitis			
				Subepidermal abscess			
7	R175P	+	Normal	Recurrent sepsis (S.P.)		IVIG	100% XY
8	Q348X	+	Normal	Disseminated B.C.G.	IBD	IVIG, ST	100% XY
9	R175P	+	Normal	Recurrent pneumonia	IBD	IVIG	100% XY
				Recurrent otitis media		5-aminosalicylic acid	
				Kaposi varicelliform eruption			
10	1167 ins C	+	Normal	Sepsis and Enteritis (E.A)	Failure to thrive	IVIG, SCT	Not done
				Sepsis (C.G.)	Pyloric stenosis, colon polyps		
				UTI (K.P.)			

S.P. indicates *Streptococcus pneumoniae*; P.A., *Pseudomonas aeruginosa*; IVIG, intravascular immunoglobulin infusion; M.A.C., *Mycobacterium avium* complex; S.A., *Staphylococcus aureus*; *E coli*, *Escherichia coli*; ST, trimethoprim-sulfamethoxazole; M.S., *Mycobacterium szulgai*; AMK, amikacin; EB, ethambutol; CAM, clarithromycin; SCT, stem cell transplantation; B.C.G., Bacille de Calmette et Guerin; INH, isoniazid; RFP, rifampicin; IBD, inflammatory bowel disease; PSL, prednisolone; CyA, cyclosporine A; MTX, methotrexate; UTI, urinary tract infection; E.A., *Enterobacter aerogenes*; C.G., *Candida glabrata*; and K.P., *Klebsiella* pneumonia.

Methods

Informed consent

Informed consent was obtained from the patients and their families following the Declaration of Helsinki according to the protocol of the Internal Review Board of Kyoto University, which approved this study.

Patients

Patient 1 was an XL-EDA-ID patient with a duplication mutation of the *NEMO* gene spanning intron 3 to exon 6. This patient has been reported previously[17] and died from an *Aspergillus* infection at the age of 4. Patient 2, born at term, was the younger brother of patient 1. This patient was also diagnosed as XL-EDA-ID with the same duplication mutation as patient 1 by genetic study. He received trimethoprim-sulfamethoxazole prophylaxis and a monthly infusion of immunoglobulin from the age of 1 month. The patient maintained good health and had a body weight of 7899*g* at 6 months when he started to fail to thrive. Except for poor weight gain, patient 2 appeared active with a good appetite, negative C-reactive protein, normal white blood cell counts, and no apparent symptoms. At 19 months of age, *Mycobacterium szulgai* was detected by venous blood culture, and the patient was treated with multidrug regimens including ethambutol, rifabutin, and clarithromycin based on the treatment of systemic *Mycobacterium avium* complex infection. The patient responded well to the treatment and his weight increased from 7830g to 9165g within a month after the treatment was initiated. Patient 2 received an unrelated cord blood cell transplantation at 26 months of age, containing 8.5×10^7 nucleated cells/kg (4.4×10^5 $CD34^+$ cells/kg), which was matched at 5 of 8 loci: mismatches occurred at 1 HLA-B and 1 HLA-C allele (according to serology), and at 1 HLA-A, 1 HLA-B, and 1 HLA-C allele (according to DNA typing). The preconditioning regimen consisted of fludarabine (30 mg/m^2/d) on days −7 to −3, melphalan (70 mg/m^2/d) on days −6 to −5, and rabbit anti-thymocyte globulin (2.5 mg/kg/d) on days −6 to −2. At first, Tacrolimus (0.024 mg/kg/d) was used to prevent GVHD, but this was switched to cyclosporin A (3 mg/kg/d) on day 9 because of drug-induced encephalopathy. Neutrophil ($> 0.5 \times 10^9$/L) and platelet ($> 50 \times 10^9$/L) engraftment were examined on days 13 and 40, respectively. Although $CD19^+$ cells (2042/μL, 94% donor chimerism), $CD56^+$ cells (242/μL, 97% donor chimerism), and monocytes (557/μL, 69% donor chimerism) were successfully generated, $CD3^+$ cells were not detected in the peripheral blood by day 54. The patient suffered from septic shock and died on day 60. Patients 3 to 10 were XL-EDA-ID patients recruited nationwide in Japan. Clinical details of patients 3, 4, and 10 have been reported previously.[18-20] These patients had clinical phenotypes characteristic of XL-EDA-ID such as ectodermal dysplasia, innate and/or acquired immunity defects, and susceptibility to pyogenic bacteria and *Mycobacterium* infection. Every patient had a mutation in the *NEMO* gene that caused reduced NF-κB activation in a NEMO reconstitution assay, as described in "Proliferation of $NEMO^{normal}$ and $NEMO^{low}$ T cells." Patient profiles are listed in Table 1.

Flow cytometric analysis

NEMO intracellular staining was performed as previously described.[17] The cells were stained for the following lineage markers before staining for NEMO: CD4, CD8, CD14, CD15, CD19, CD56, CD45RA (BD Biosciences/BD Pharmingen), and CCR7 (R&D Systems Inc). Intracellular staining of human IFN-γ, TNF-α, and NEMO was performed as previously described.[18] The stained cells were collected using a FACSCalibur flow cytometer (BD Biosciences) and analyzed using the FlowJo software (TreeStar).

Reporter assay

Wild-type and mutant *NEMO* cDNAs were generated from a healthy volunteer and the recruited XL-EDA-ID patients by RT-PCR; the cDNAs were subcloned into the p3xFLAG-CMV14 vector (Sigma-Aldrich). NEMO null rat fibroblast cells (kindly provided by Dr S. Yamaoka, Department of Molecular Virology, Graduate School of Medicine, Tokyo Medical and Dental University, Tokyo, Japan) were plated at a density of