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Blood: Brief report

Identification of *TRIB1* R107L gain-of-function mutation in human acute megakaryocytic leukemia

Running title: TRIB1 mutation in human AMKL

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Scientific Heading: Myeloid neoplasia

Abstract

Trib1 has been identified as a myeloid oncogene in a murine leukemia model. Here we identified a *TRIB1* somatic mutation in a human case of Down syndrome-related acute megakaryocytic leukemia (DS-AMKL). The mutation was observed at well conserved arginine 107 residue in the pseudokinase domain. This R107L mutation remained in leukocytes of the remission stage in which *GATA1* mutation disappeared, suggesting the *TRIB1* mutation is an earlier genetic event in leukemogenesis. The bone marrow transfer experiment showed that AML development was accelerated by transducing murine bone marrow cells with the R107L mutant in which enhancement of ERK phosphorylation and C/EBPα degradation by Trib1 expression was even greater than in those expressing wild type. These results suggest that TRIB1 may be a novel important oncogene for DS-AMKL.

Introduction

The Down syndrome (DS) patients are predisposed to developing myeloid leukemia, and those patients frequently exhibit *GATA1* mutations.[1] However, it is proposed that the *GATA1* mutation is important for transient leukemia in DS but not sufficient for full blown leukemia, suggesting that additional genetic alterations are needed.[1] Therefore, it is important to search the subsequent genetic changes for DS-related leukemia (ML-DS) to predict malignant transformation and prognosis of the patients.

Trib1 has been identified as a myeloid oncogene that cooperates with *Hoxa9* and *Meis1* in murine acute myeloid leukemia (AML).[2] As a member of tribbles family of proteins, TRIB1 interacts with MEK1 and enhances ERK phosphorylation.[2,3] Moreover, TRIB1 promotes degradation of C/EBP family transcription factors including C/EBPα, an important tumor suppressor for AML, and we observed that degradation of C/EBPα by Trib1 is mediated by its interaction with MEK1.[4] Thus, TRIB1 plays an important role in the development of AML by modulating both the RAS/MAPK pathway and C/EBPα function together with Trib2 that has also been identified as a myeloid transforming gene.[5] Potential involvement of *TRIB1* in human leukemia has been reported in cases of AML with 8q34 amplification in which both *c-MYC* and *TRIB1* are included in the amplicon.[6] The enhancing effect of TRIB1 on the MAPK signaling suggests that TRIB1 alterations may be related to AML cases which do not show any mutations in the pathway members such as FLT3, c-Kit or Ras. In this report

we identified a novel somatic mutation of *TRIB1* in a case of human acute megakaryocytic leukemia developed in Down syndrome (DS-AMKL). Retrovirus-mediated gene transfer followed by bone marrow transfer indicated that the mutation enhanced leukemogenic activity and MAPK phosphorylation by TRIB1.

Methods

Patients

TRIB1 mutations have been investigated in 12 cases of transient leukemia (TL), 5 of DS-AMKL and 4 cell lines of DS-AML. Peripheral blood leukocytes of TL and bone marrow cells of DS-AMKL were used as sources for the molecular analysis. This study was approved by the Ethics Committee of Hirosaki University Graduate School of Medicine, and all clinical samples were obtained with informed consent from the parents of all patients, in accordance with the Declaration of Helsinki.

The patient #84 showed trisomy 21 and extensive leukocytosis at birth. Hematological findings revealed the white blood cell (WBC) count to be 148 x 10^9/l, including 87% myeloblasts, a hemoglobin level of 19.4 g/dl, and a platelet count of 259 x 10^9/l. Patent ductus arteriosus and atrial septal defect have been pointed out. Based on the hematological data and the chromosomal abnormality, the patient was diagnosed as Down syndrome-related TL. The hematological abnormality was then improved but 8 months later 3% of 6.9 x 10^9/l WBC became myeloblasts (Figure 1A). A karyotype analysis of bone marrow cells revealed 48,XY,+8,+21 in 3 of 20 cells. In addition, GATA1 mutation was detected at nt 113 from A to G, resulting in loss of the first methionine.[7] He was diagnosed as AMKL at this time, and his disease was in remission by subsequent chemotherapy.

PCR and sequencing

The entire coding region of human *TRIB1* cDNA of patients' samples was amplified using Taq polymerase (Promega) and specific primer pairs (the sequences of the primers are available on request). The genomic DNA samples of patient #84 was also analyzed. The sequence analysis of *GATA1* was performed as described previously.[7] After checking the PCR products by agarose gel electrophoresis, the products were purified and directly sequenced.

Retroviral infection of murine bone marrow cells and bone marrow transfer

Bone marrow cells were prepared from eight-week-old female C57Bl/6J mice five days after injection of 150 mg/kg body weight of 5-fluorouracil (5-FU) (Kyowa Hakko Kogyo). Retroviral infection of bone marrow cells and bone marrow transfer experiments were performed as described.[2] Transduction efficiencies evaluated by flowcytometric techniques were comparable between wt (5.3%) and R107L (3.4%). Animals were housed, observed daily and handled in accordance with the guidelines of the animal care committee at the Japanese Foundation for Cancer Research. All the diseased mice were subjected to autopsy and analyzed morphologically and the blood was examined by flowcytometric techniques. The mice were diagnosed as positive for AML according to the classification of the Bethesda proposal.[9] The survival rate of each group was evaluated using the Kaplan-Meier method and differences between survival curves were compared using the log-rank test.

Immunoblotting

Immunoblotting was performed using cell lysates in RIPA buffer as described.[4] Anti-p44/42 ERK (Cell Signaling Technologies), anti-phospho-p44/42 ERK (Cell Signaling Technologies), anti-C/EBPα (Santa Cruz Biotechnology), anti-FLAG (Sigma) and anti-GAPDH (Hy Test Ltd.) antibodies were used.

Results and Discussion

The important role of TRIB1 on the MAPK signaling suggests that *TRIB1* alterations may occur in some AML cases which do not show overlapping mutations in the pathway members such as FLT3, KIT or RAS. Therefore, we tried to search mutations of *TRIB1* in cases of ML-DS and TL in which such mutations are infrequent.[8] In a case of DS-AMKL (#84) a nucleotide change from guanine to thymine has been identified at 902 that results in amino acid alteration from arginine107 (R107) to leucine (Figure 1B). The sequence changes were confirmed by subcloning the PCR product into the TA-type plasmid vector (data not shown). The nucleotide change was not observed in the DNA sample derived from nail of the same patient at all (Figure 1C), indicating that this change is a somatic mutation. Interestingly, the mutation was retained in the peripheral blood sample in the complete remission stage in which the GATA1 mutation completely disappeared (Figures 1C and 1D). These results indicate that the *TRIB1* mutation

precedes the onset of TL and the *GATA1* mutation, and suggest that *TRIB1* mutation occurred at the hematopoietic stem cell level and that the clone retaining the *TRIB1* mutation survived after chemotherapy. In the case #84 there was no mutation for *FLT3* exon 14, 15 and 20, PTPN11 exon 3 and 13, KRAS exon 2, 3 and 5, and KIT exon 8, 11 and 17 by the high resolution melt analysis (data not shown).

An additional mutation was found in a case of TL (#109) at the nucleotides 805 and 806 from GC to AT, which results in amino acid conversion from alanine (A75) to isoleucine (Supplemental Figure 1). *TRIB1* expression in DS-related and DS-unrelated leukemias was examined by real-time quantitative RT-PCR (Supplemental Figure 2).

R107 is located within a psuedokinase domain of TRIB1 that is considered as a functionally core domain of TRIB family proteins.[10] Sequence comparison among three TRIB family proteins as well as tribbles homologues in other organisms revealed that the R107 is well conserved in mammalian TRIB1 and TRIB2,[10] suggesting that this arginine residue is evolutionary conserved and may be related to an important function. On the other hand, A75 is located outside of the pseudokinase domain, not conserved between human and mouse, nor other tribbles homologues. Moreover, the N-terminal domain containing A75 is dispensable for the leukemogenic activity of Trib1.[4] Therefore, we tried to investigate whether the R107L mutation could affect the leukemogenic activity of *TRIB1*.

R107L was introduced into the murine *Trib1* cDNA by site-directed mutagenesis. Both wild type (wt) and R107L cDNAs were subcloned into the pMYs-IRES-GFP (pMIG) retroviral vector, and were used for retrovirus-mediated gene transfer followed by bone marrow (BM) transfer according to the method previously described.[1] All the mice transplanted with BM cells expressing wt (n=15) or R107L (n=12) developed AML (Figure 2A). The mean survival time was shorter in the recipients with R107L-expressing BM cells (110 d) than those with wt (136 d) (Figure 2A). The difference was significant, the p value being 0.0111 as calculated by the log-rank test. The result indicates that the R107L mutation enhances the leukemogenic activity of TRIB1. These results also suggest that *TRIB1* mutation might cooperate with *GATA1* mutation in the genesis of DS-AMKL, and that trisomy 21, *TRIB1* and *GATA1* mutations occurred consecutively, which contributed to the multi-step leukemogenic process.

We have shown that TRIB1 interacts with MEK1 and enhances phosphorylation

of ERK.[2] The R107L mutant enhanced ERK phosphorylation more extensively than wt (Figure 2B) in AML cells derived from bone marrow of recipient mice, and more significant degradation of C/EBPα was induced by the R107L mutant (Figure 2C). These findings might be correlated to the enhanced leukemogenic activity of the mutant. Both R107L and wt proteins could interact with MEK1, having the binding motif in their C-termini. The residue 107 is located at subdomain II of the pseudokinase domain.[11] The mutation may affect conformation of the domain and may promote the MEK1 function on ERK, though additional studies are required to address the possibility. A recent study demonstrates that Trib1 and Trib2 failed to show ERK phosphorylation in 32D cells.[12] The different response to Trib1 between primary leukemic cells and the cell line might depend on the cellular context and/or combination of additional mutations. The AML phenotypes were somewhat varied in each cases and Mac-1-positive/Gr-1-negative AMLs were more remarkable in wt than in R107L, though the difference was not statistically significant (Supplemental Figures 3 and 4, and Supplemental Table 1). The current study underscores the role of TRIB1 in human leukemogenesis and the significance of the R107L mutation in its function. Further sequence analysis of tribbles family genes in a larger cohort will emphasize the importance of R107L and/or additional mutations of *TRIB1* in leukemic patients.

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Authorship

Contribution: T.Y., E.I., Y.H., and T. N. designed and performed the research and wrote the manuscript; T.To., Y.A., R.K., and M.P. performed the research; Y.K., T.Ta., and Y.Y. contributed to the BMT analysis. The authors declare no competing financial interests.

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Figure legends

Figure 1. *TRIB1* R107L mutation identified in Down syndrome-related leukemias. (A) Giemsa-staining of the case #84 peripheral blood smear diagnosed as AMKL. The image was acquired using a BX40 microscope equipped with a 100X/1.30 NA oil objective (Olympus, Tokyo, Japan) and a C-4040 digital camera (Olympus). (B) Fluorescent dye sequencing chromatographs of *TRIB1* genotyping by direct sequencing of the case #84 using a cDNA sample as a template. The vertical arrow indicates mixed G and T signals at codon 107. (C) Fluorescent dye sequencing chromatographs of *TRIB1* of peripheral blood leukocytes (top) or nail (bottom) in the same case at the complete remission stage. The red arrows indicate the mutation remains in leukocytes but not in nail. The reverse strand sequences are shown. (D) GATA1 sequence. The start codon that was mutated in AMKL[7] is normal in the peripheral blood leukocytes at the remission stage.

Figure 2. AML development by bone marrow transfer using *Trib1* wt and R107L. (A) Kaplan-Meier survival curves are shown. The P value was calculated with the log rank test. (B) Immunoblot analysis of *Trib1* wt AML (Mac-1 56.2%, Gr-1 52.5%, $CD34^{lo}$, c-kit^-, Sca-1^-) and R107L AML (Mac-1 41.4%, Gr-1 25.2%, $Cd34^{lo}$, c-kit^{lo}, Sca-1^-) derived from bone marrow of recipient mice (wt #T73 and R107L #T151 in Supplemental Table 1). Enhancement of ERK phosphorylation is more significant in R107L. Relative values of ERK phosphorylation were calculated by densitometric analysis. (C) Immunoblot analysis for C/EBPα of the same AML samples as B. Relative expression level of C/EBPα is quantitated (right).

Figure 1

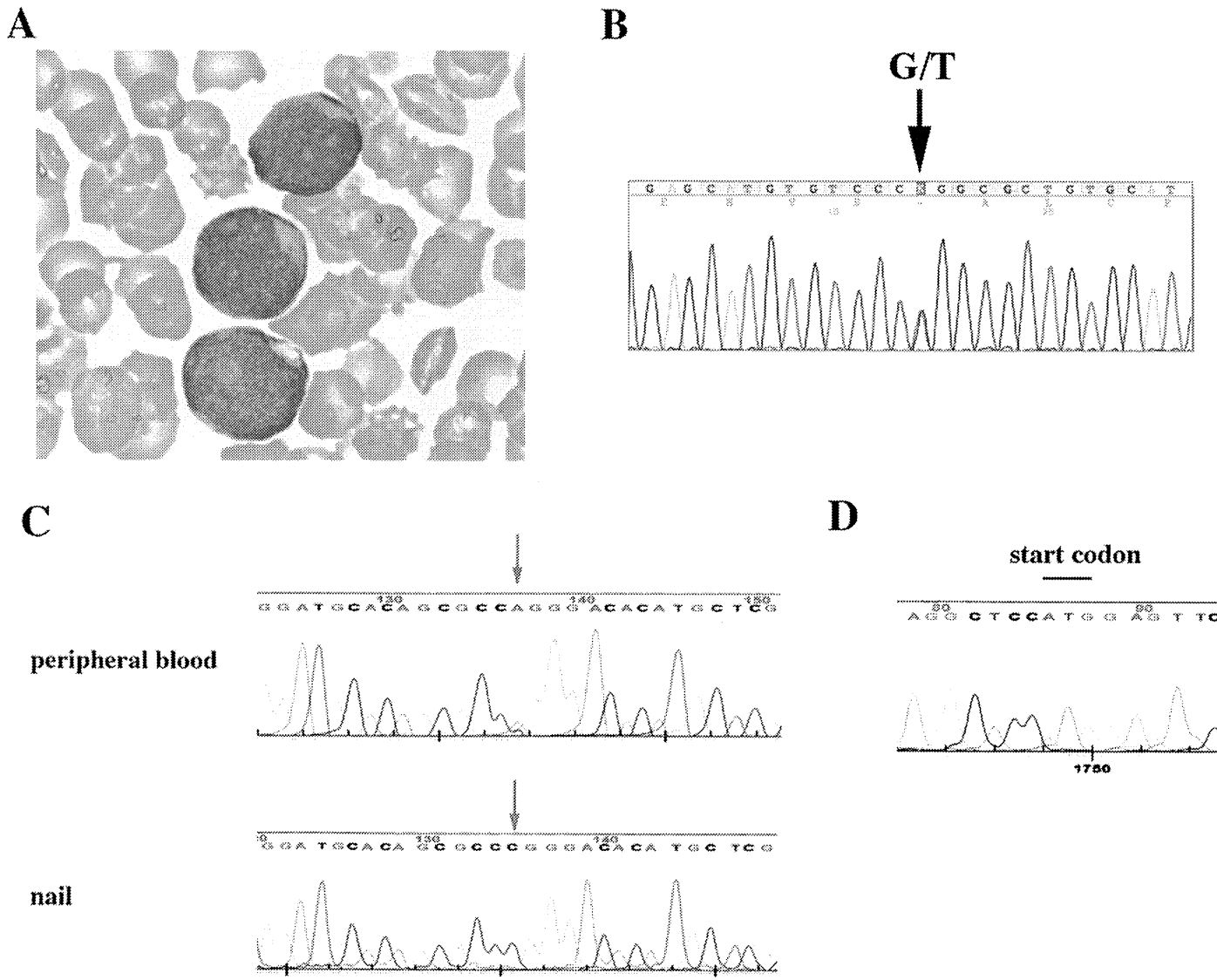
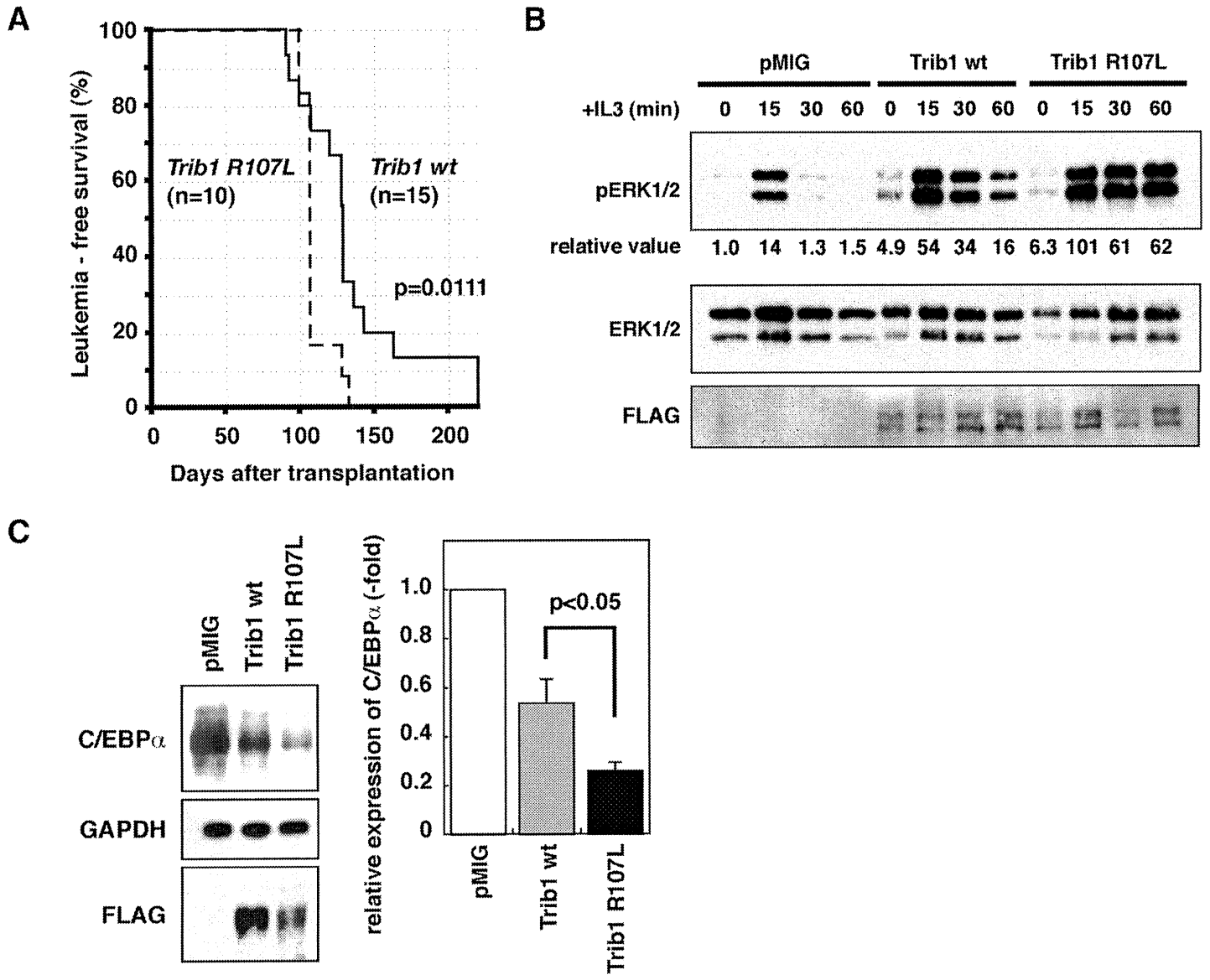


Figure 2


A
Leukemia - free survival (%)
100
80
60
40
20
0
Trib1 R107L
(n=10)
Trib1 wt
(n=15)
p=0.0111
0
50
100
150
200
Days after transplantation
B
pMIG
Trib1 wt
Trib1 R107L
+IL3 (min)
0 15 30 60 0 15 30 60 0 15 30 60
pERK1/2
relative value
1.0 14 1.3 1.5 4.9 54 34 16 6.3 101 61 62
ERK1/2
FLAG
C
pMIG
Trib1 wt
Trib1 R107L
C/EBPα
GAPDH
FLAG
relative expression of C/EBPα (-fold)
1.0
0.8
0.6
0.4
0.2
0
p<0.05
pMIG
Trib1 wt
Trib1 R107L

Extensive gene deletions in Japanese patients with Diamond–Blackfan anemia

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Running title: Large deletions in DBA responsible genes

Abstract

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 to 3. Other cases included a proband with an *RPL5* deletion, 1 had an *RPL35A* deletion, 3 had *RPS17* deletions, and 1 had 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 cases in Japan. In addition, our novel approach may become a useful tool for screening gene copy numbers of known DBA genes.

Introduction

Diamond–Blackfan anemia (DBA, MIN# 105650) is a rare congenital anemia that belongs to the inherited bone marrow failure syndromes, generally presenting in the first year of life. Patients typically present with a decreased number of erythroid progenitors in their bone marrow[1]. A main feature of the disease is red cell aplasia, but approximately half of the 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, such as *RPL5* (~21%), *RPL11* (~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 patients have had a mutation in one of these genes. Konno et al screened 49 Japanese patients and found that 30% (12/49) carried mutations[8]. Additionally, our data showed that 22 out of 68 DBA patients (32.4%) harbored a mutation in ribosomal protein (RP) genes (unpublished observation). 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 is 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 this report, 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 small-for-date, which suggests that this is a characteristic of DBA with a large gene deletion in Japan.

Methods

Patient samples

Genomic DNA was extracted using the GenElute Blood Genomic DNA Kit (Sigma, St. Louis, MO, USA) according to the manufacturer's protocol. Clinical manifestation of patients from a Japanese DBA genomic library are listed elsewhere or reported by Konno 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 synchronized quantitative-polymerase chain reaction (s-q-PCR)

Primers were designed using Primer Express 3.0 Software (Applied Biosystems, Foster City, CA, USA). 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 q-PCR mixture was as follows: 5 ng denatured genomic DNA, 0.4 mM forward and reverse primers, 1x SYBR Premix Ex Taq™ II (Takara, Shiga, Japan), and 1x ROX reference dye II (Takara) in a total volume of 20 μL (all samples 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, followed by 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). The sensitivity and specificity of this method was evaluated with fifteen 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 q-PCR products. The results of 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, Osaka, Japan) according to the manufacturer's step down PCR protocol. Briefly, the PCR mixture contained 20 ng of

genomic DNA, 0.4 mM forward and reverse primers, 1 mM dNTP, 1x 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, Tokyo, Japan).

DNA sequencing analysis

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

SNP array based copy number analysis

SNP array experiments were performed according to the standard protocol of Affymetrix GeneChip Human Mapping 250K Nsp arrays (Affymetrix, Santa Clara, CA). Microarray data were analyzed for determination of allelic-specific copy number using the CNAG program as previously described[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 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 normal karyotype, it is possible to conveniently detect a gene deletion with a 1-cycle delay in q-PCR analysis (**Figure 1Aii and 1Aiii**).

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 that the Ct of 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 **Figures 1Bii** and **1Cii**, the specific primer sets for *RPL5* (L5-02, -05, -17, -19, and -28) detected a 1-cycle delay with respect to the mutated allele of patient #03. 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% (22/68) had specific DBA mutations (**Table 3** and data not shown individually). 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, 7/27 samples displayed a 1-cycle delay of Ct scores: 1 patient had *RPL5* (#14), 1 had *RPL35A* (#71), 3 had *RPS17* (#03, #60, and #62), and 2 had *RPS19* (#24 and #72) (**Figure 2** and **Table 4**). Among these cases, 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 has a large deletion.

Based on our findings, the rate of large deletions was approximately 25.9% (7/27) in a category of unspecified gene mutation. 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 #03 had a large deletion in chromosome (ch) 1 spanning 858 kb (**Figure 3A**), #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 #72 had a