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ORIGINAL ARTICLE

Ribosomal protein mutations in Korean patients with Diamond-Blackfan anemia

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Diamond-Blackfan anemia (DBA) is a congenital bone marrow failure syndrome characterized by hypoproliferative anemia, associated physical malformations and a predisposition to cancer. DBA has been associated with mutations and deletions in the large and small ribosomal protein genes, and genetic aberrations have been detected in ∼50–60% of patients. In this study, nine Korean DBA patients were screened for mutations in eight known DBA genes (*RPS19, RPS24, RPS17, RPS10, RPS26, RPL35A, RPL5* and *RPL11*) using the direct sequencing method. Mutations in *RPS19, RPS26* and *RPS17* were detected in four, two and one patient, respectively. Among the mutations detected in *RPS19*, two mutations were novel (c.26T>A, c.357-2A>G). For the mutation-negative cases, array-CGH analysis was performed to identify copy-number variations, and no deletions involving the known DBA gene regions were identified. The relative mRNA expression of *RPS19* estimated using real-time quantitative PCR analysis revealed two- to fourfold reductions in *RPS19* mRNA expression in three patients with *RPS19* mutations, and p53 protein expression analysis by immunohistochemistry showed variable but significant nuclear staining in the DBA patients. In conclusion, heterozygous mutations in the known DBA genes *RPS19, RPS26* and *RPS17* were detected in seven out of nine Korean DBA patients. Among these patients, *RPS19* was the most frequently mutated gene. In addition, decreased *RPS19* mRNA expression and p53 overexpression were observed in the Korean DBA patients, which supports the hypothesis that haploinsufficiency and p53 hyperactivation represent a central pathway underlying the pathogenesis of DBA.
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INTRODUCTION

Diamond-Blackfan anemia (DBA, MIM# 105650) is an inherited congenital bone marrow failure syndrome characterized by normochromic macrocytic anemia with reticulocytopenia and the absence or insufficiency of erythroid precursors in an otherwise normocellular bone marrow.[1] The disease is rare, with a reported incidence of seven cases per million live births,[2] and anemia usually occurs during early infancy, with more than 90% of the patients diagnosed before the age of 1 year. Although anemia is the most prominent feature, the disease is also associated with congenital malformations, mainly involving the head, upper limbs, heart and urogenital system, and growth retardation in ∼50% of patients.[1] Most DBA cases are sporadic, but the disease can be inherited through an autosomal dominant mode of inheritance.

Since the identification of mutations in the ribosomal protein (*RP*) *S19* gene, which was the first DBA gene to be reported, mutations have been reported in an increasing number of genes encoding RPs of both the small (RPS) and large (RPL) ribosomal subunits. At present, mutations in 10 genes encoding RPs of the small (*RPS24, RPS17, RPS19, RPS10, RPS26, RPS7*) and large (*RPL35A, RPL5, RPL11, RPL26*) ribosomal subunits have been described in DBA patients.[3,4] Together, mutations in these genes are present in ∼50% of all DBA patients.

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A clear genotype–phenotype correlation is not apparent in patients with *RPS19* mutations due to incomplete penetrance and widely variable expression, even within the same family.[5] In addition, much of our knowledge of the disease has been gained from patient registries in North America, the United Kingdom, Italy and France.[2,5–7] There have been only a few other genotype studies performed on Asian DBA patients.[8,9] Therefore, in this study, we screened nine Korean DBA patients for mutations in the known DBA genes, and the genotype–phenotype correlations were evaluated. In addition, RP haploinsufficiency was investigated as an underlying pathogenesis of DBA through *RPS19* mRNA quantification and p53 protein expression analyses in our cohort of Korean DBA patients.

MATERIALS AND METHODS

Patients
Nine unrelated Korean DBA probands were referred for molecular diagnosis of DBA. The diagnosis of DBA was based on the following diagnostic criteria:[10] onset at an age of <1 year; macrocytic or normocytic anemia with no other significant cytopenias; reticulocytopenia; and normal marrow cellularity with a paucity of erythroid precursors. Patients older than 1 year at the time of onset were only included if they belonged to a DBA family with a dominant mode of inheritance. Informed consent was obtained from all patients and/or the participant's family members. Information on the phenotypic features was obtained from the available medical records and/or a detailed questionnaire, which was completed by the patient's physicians. This study followed the institutional review board guidelines of the Catholic Medical Center (IRB No. KC11EISI0836).

Mutation analysis of eight known DBA genes
Genomic DNA was isolated from the peripheral blood leukocytes using the QIAmp DNA Mini Kit (Qiagen, Hamburg, Germany). Polymerase chain reaction (PCR) was carried out using previously published primer sets for *RPS19*,[11] *RPL35A*,[12] *RPS26*,[13] *RPS10*[13] and *RPS17*[14] and newly designed primer sets for *RPL5*, *RPL11* and *RPS24*. All of the coding exons and the flanking intron/exon boundaries of the above genes were amplified. The PCR amplicons were bi-directionally sequenced using the Big Dye terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The chromatograms were analyzed using the Sequencher software version 5.0 (Gene Codes, Ann Arbor, MI, USA). Mutations were confirmed by sequencing two or more independent PCR reactions.

When a novel mutation was identified, the complete coding sequences of all other RP genes were determined to rule out the possibility of mutations in other RP genes. DNA and/or complementary DNA (cDNA) from the available family members were sequenced to determine whether the mutation cosegregated with the DBA phenotype within the pedigree. All mutations were described according to the Human Genome Variation Society nomenclature.[15] The following GenBank accession numbers were used: NM_001022.3 for *RPS19*, NM_000996.2 for *RPL35A*, NM_001029.3 for *RPS26*, NM_001014.4 for *RPS10*, NM_001021.3 for *RPS17*, NM_000969.3 for *RPL5*, NM_000975.3 for *RPL11* and NM_033022.3 for *RPS24*.

Reverse transcription–polymerase chain reaction and real-time quantitative reverse transcription–polymerase chain reaction
Total RNA was extracted from peripheral blood leukocytes using the High Pure RNA Isolation Kit (Roche Applied Science, Indianapolis, IN, USA), and the reverse transcriptase reaction was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) according to the manufacturer's protocol.

cDNA primers were designed to cover all coding exons of the *RPS19* gene to confirm the nucleotide changes and splicing effects, as well as for real-time quantitative reverse transcription–polymerase chain reaction (RT–qPCR). The reverse transcription–polymerase chain reaction (RT–PCR) product size was determined by electrophoresis. RT–qPCR was performed using iQ™ SYBR Green qPCR supermix (Bio-Rad Laboratories, Hercules, CA, USA) according to the protocol supplied by the manufacturer. The *RPS19* mRNA from three DBA patients and five normal individuals was reverse-transcribed into cDNA. The cDNA was amplified in duplicate in RT–qPCR reactions, and control reactions examining the expression of the human glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene were run in the same tube as an endogenous reference. The outcome of the amplification reactions was estimated as the value 2^{-ddCt}, where dCt is $Ct_{RPS19} - Ct_{GAPDH}$, and ddCt is $dCt_{sample} - mean\ dCt_{controls}$.

Detection of copy-number variants
For patients who did not have a mutation in the known DBA genes based on the sequencing analysis, array-CGH analysis was performed to detect the genomic rearrangements or deletions that may have occurred in the known DBA gene regions. The whole genome 135 K NimbleGen CGX cytogenetic array chip (Roche NimbelGen Inc., Madison, WI, USA), which includes 134 829 oligonucleotide probes per haploid genome, was used. For array-CGH analysis, 500 ng of test DNA and sex-matched reference DNA (Promega, Napeen, Canada) were random-prime labeled with Cy3 and Cy5, respectively, using the NimbleGen labeling kit. The samples were hybridized to the array at 42 °C, washed and scanned using the NimbleGen MS 200 Microarray Scanner. The data were analyzed using DEVA version 1.0.2 software (Roche NimbelGen).

p53 protein analysis in BM biopsies by immunohistochemistry
Immunohistochemistry for p53 was performed on bone marrow biopsy samples that were collected at the initial presentation from two DBA patients, and an age-matched normal bone marrow biopsy sample was simultaneously analyzed as a control. The formalin-fixed, paraffin-embedded tissue blocks were sliced at a 4-μm thickness, deparaffinized in xylene and rehydrated using a graded alcohol series, followed by incubation with 0.3% hydrogen peroxide for 10 min to block the endogenous peroxide activity. Antigen retrieval was performed using a pressure cooker at 121 °C in 10 mM citrate buffer (pH 6.0) for 15 min. The staining was processed using the Lab Vision Autostainer (Lab Vision Corporation, Fremont, CA, USA). The primary anti-p53 antibody (DO-1, Immunotech, Marseille, France) was diluted 1:50 in Thermo antibody diluents (Lab Vision Corporation), and the slides were incubated with the antibody for 40 min. After washing, the HRP polymer was applied and incubated for 15 min at room temperature. Lastly, the slides were covered with a mixed solution of DAB Plus Chromogen (Lab Vision Corporation) and DAB Plus Substrate (Lab Vision Corporation) to develop the color and were washed with deionized water, followed by hematoxylin counterstaining.

RESULTS

Characteristics of the patients

Of the nine Korean DBA patients, eight cases were sporadic, while one patient belonged to a family with unexplained anemia in a first-degree relative. All patients were Korean, and the male to female ratio was 1:1.25. The median age at the time of presentation was 2.0 months (range, 0 to 336 months). The patients' characteristics are summarized in Table 1. Anomalies associated with DBA were found in five patients (56%), and growth retardation was found in three patients (33%). Six patients (67%) responded to steroid therapy, while the remaining patients were steroid nonresponders.

Identification and characterization of mutations in known DBA genes

Sequencing analysis of eight known DBA genes revealed seven different mutations (Figure 1 and Table 1). Four different mutations were detected in the *RPS19* gene in four patients. These mutations included one deletion, two missense mutations (one of these cases was previously reported)[16] and one splice-site acceptor mutation. The deletion mutation, c.328delC in case I2, and one missense mutation, c.3G>A in case S1, have been previously reported.[6,17,18] The splice-site acceptor mutation in intron 4, c.357-2A>G in case S2, and the other missense mutation, c.26T>A resulting in Val9Glu in case I1, were novel mutations.

For the novel splice-site acceptor mutation identified in case S2, parental and sibling DNA was available, and the sequence change was confirmed to have occurred *de novo*. To demonstrate the effect of this splice-site acceptor mutation on mRNA splicing, we amplified the cDNA of the *RPS19* gene from the affected proband and performed bi-directional sequencing. The results demonstrated that the splice-site acceptor mutation resulted in an aberrantly spliced transcript lacking exon 5 (Supplementary Figure S1).

For the novel missense mutation, c.26T>A resulting in Val9Glu, *in silico* analyses using the SIFT program predicted that the protein function would be affected ($P<0.01$), and the Polyphen program predicted that the mutation was pathogenic, with a score of 2.680. Comparative evolutionary analysis showed that the valine residue at codon 9 is a highly conserved amino acid during evolution.[19]

Two different mutations in the *RPS26* gene were detected in two patients. In case S3, a previously reported donor splice-site mutation, c.3+1G>C, was identified in intron 1.[13] In case S4, a novel nonsense mutation, c.259C>T, which resulted in the substitution of the arginine residue at codon 87 with a stop codon, was identified. In case O1, a missense mutation, c.1A>G, which affects the translation initiation codon of the *RPS17* gene, was identified, and this proband has been previously reported.[20] No mutations were detected in *RPL35A*, *RPS10*, *RPL5*, *RPL11* and *RPS24*. Furthermore, all mutations were present only in a single allele and were heterozygous with the wild-type sequence. The novel

Table 1 Mutations identified in *RPS19*, *RPS26* and *RPS17* in Korean DBA patients

Patient (gender)	Inheritance	DNA change	Exon/intron	Predicted protein change	Presenting age, months	Hb/reticulo-cyte (%)	Associated anomalies	Growth retardation	Steroid responsive	Reference
Mutations in the RPS19 gene										
S1 (F)	Sporadic	c.3G>A	Ex 2	p.Met1?	At birth	5.0/0.36	Hydronephrosis	Yes	No	16
I1 (M)	Sporadic	c.26T>A[a]	Ex 2	p.Val9Glu	5	2.8/2.45	Polydactyly	No	Yes	
I2 (F)	Sporadic	c.328delC	Ex 4	p.Leu110*	3	6.1/0.25	None	No	Yes	7
S2 (F)	*De novo* (f,m,b normal sequence)	c.357−2A>G[a]	Int 4	Splicing defect	45 days	3.7/0.85	None	Yes	Yes	
Mutations in the RPS26 gene										
S3 (M)	Sporadic	c.3+1G>C	Int 1	—	1	2.3/NA	Strabismus, SNHL	No	Yes	13
S4 (M)	Sporadic	c.259C>T[a]	Ex 3	p.Arg87*	2	4.0/0.92	None	No	No	
Mutations in the RPS17 gene										
O1 (M)	Sporadic	c.1A>G	Ex 1	p.Met1Val	2	1.9/NA	Infantile spasm	Yes	Yes	20
Patients without mutation of eight RP genes										
S5 (F)	Sporadic				21 days	4.3/0.3	Small ASD, strabismus	No	Yes	
S6 (F)	Familial				28 years	4.4/2.84	None	No	No	

Abbreviations: ASD, atrial septal defect; b, brother; F, female; f, father; M, male; m, mother; SNHL, sensorineural hearing loss.
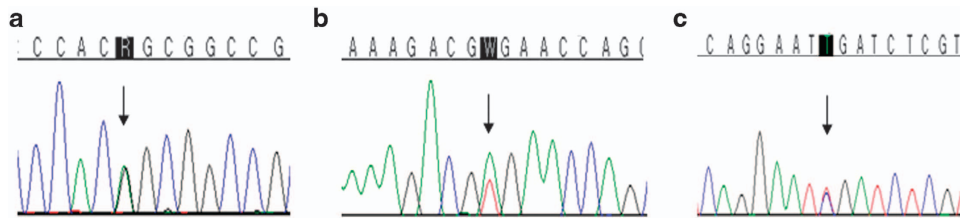[a]Represents novel mutations.

Figure 1 Three novel mutations were identified in this study. Panels **a**–**c** show the sequencing chromatograms of (**a**) *RPS19* c.357 −2A>G, (**b**) *RPS19* c.26T>A and (**c**) *RPS26* c.259C>T.

mutations were submitted to the DBA Mutation Database (http://www.dbagenes.unito.it), the Locus Specific Database.

Results of array-CGH analysis

Deletions involving the RP genes are estimated to be present in 4–10% of all DBA patients and in ∼17% (95% confidence interval, 9–30%) of the previously sequenced mutation-negative DBA patients.[12] However, in our cohort, two probands were found to have no mutations in the known DBA genes according to sequencing analysis, and array-CGH analysis did not identify any deletions in the known DBA gene regions. In addition, we were unable to identify a copy-number variant region after excluding the copy-number variant regions reported in the Database of Genomic Variants (October 2011, http://projects.tcag.ca/variation/project.html).

Quantitation of *RPS19* mRNA in patients with *RPS19* mutations

We determined the quantity of *RPS19* mRNA, normalized to the *GAPDH* expression level, in three patients with *RPS19* mutations, including a patient with an initiation codon mutation (S1), a deletion mutation that resulted in a premature stop codon (I2) and a splice-site mutation (S2). We found a two- to fourfold decrease in the *RPS19* mRNA levels in all three patients with *RPS19* mutations compared with the control individuals (Figure 2).

p53 protein analysis in BM biopsies by immunohistochemistry

Immunohistochemical analysis of the p53 protein expression in the bone marrow biopsy samples that were taken at the initial presentation was performed in two patients, S1 and S5. We found variable degrees of nuclear staining in the patient's bone marrow biopsies, with S5 showing a stronger nuclear staining than S1; normal bone marrow, which was used as a control, showed no significant nuclear staining (Figure 3).

Genotype–phenotype correlation

We compared the clinical features of patients with *RPS19* mutations in our cohort with those of previously reported DBA patients who shared identical or associated genotypic aberrations (Supplementary Table S1). Mutations affecting the first translation initiation codon were identified in seven patients. Two of these patients had associated malformations,
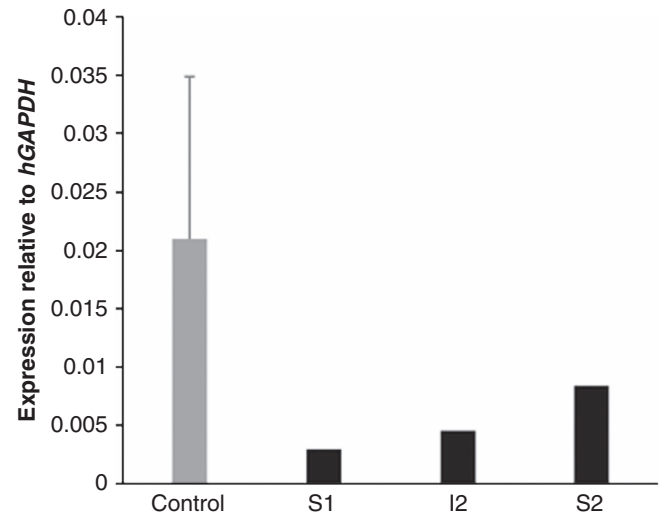

Figure 2 The relative expression of *RPS19* mRNA relative to the controls in three Korean DBA patients with identified *RPS19* mutations. *GAPDH* was used to normalize the data.

whereas the others did not. Four of these patients did not respond to steroids, while two did respond. *RPS19* mutations involving the valine residue at codon 9 and the c.328delC mutation were found in two patients, respectively, and mutations resulting in exon 5 skipping were found in five patients. However, there was no apparent correlation between the *RPS19* genotypes and the clinical phenotypes.

DISCUSSION

In this study, we investigated the molecular aberrations of known DBA genes in Korean DBA patients. Seven distinct mutations, including three novel mutations, were identified in three RP genes, *RPS19* (4/7), *RPS26* (2/7) and *RPS17* (1/7), of seven probands. The frequency of *RPS19* mutations in our cohort was higher than the reported frequency (25%) in western countries and in a Japanese DBA cohort.[8] In the Japanese cohort, 12 (27%) of the 45 DBA patients had mutations in the RP genes, and mutations in the *RPS19* gene were detected in 5 (11%) of the 45 DBA patients.[8] Therefore, the higher detection rate of *DBA* gene mutations in our cohort is due to the higher incidence of *RPS19*
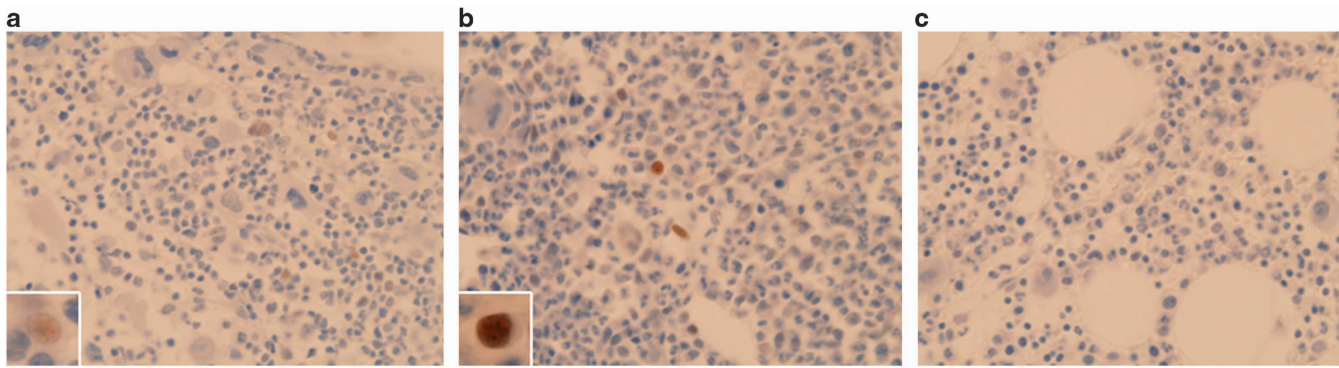
Figure 3 Immunohistochemical demonstration of p53 expression in bone marrow biopsies. Panels **a**–**c** show the p53 immunohistochemical staining results from the DBA patients (**a**) S1, (**b**) S5 and (**c**) a normal control ($\times$ 400); the insets in the bottom left corner represent a higher magnification, $\times$ 1000.

mutations. However, due to the small number of patients included in the present cohort, the significance of these findings needs to be further confirmed using a larger number of DBA patients.

As for the genotype–phenotype correlation, clinical data from European and American DBA registries showed that the frequency of malformations was 31% in patients with *RPS19* mutations, which was not significantly different from the entire DBA population.[17] However, in the Japanese DBA patients, all six patients with *RPS19* mutations had physical anomalies, and there was a statistically significant difference in the frequency of thumb abnormalities between the *RPS19* mutated and *RPS19* nonmutated groups.[8] In this study of Korean DBA patients, anomalies associated with DBA were found in five patients (56%), and of the four patients with an *RPS19* mutation, only two (50%) patients had physical anomalies. Therefore, the high frequency of anomalies found in the *RPS19* mutated Japanese DBA patients was not found in our cohort of Korean DBA patients, and the frequency of the physical malformations in *RPS19* mutated patients was similar to the entire DBA population.

In addition, patients with *RPS19* mutations have been shown to display a significantly lower sensitivity to steroids in the European and American DBA registries,[17] whereas the Japanese cohort exhibited no statistically significant difference in the response rates of the *RPS19* mutated DBA patients.[8] In this study of Korean DBA patients, six patients (67%) responded to steroid therapy and three of the four patients with *RPS19* mutations were steroid responders. Therefore, the response rate of *RPS19* mutated Korean DBA patients was not different from the entire DBA population, a finding that was similar to the Japanese DBA cohort.

Among the DBA patients harboring *RPS19* mutations, *RPS19* alterations have not been correlated with any specific phenotype.[5] When we compared the clinical features of the *RPS19*-mutated DBA patients in our cohort with those of the previously reported DBA patients who share identical or associated genotypic aberrations, no apparent correlation was found between the *RPS19* genotype and the clinical phenotype, which is similar to previous reports.

DBA is a disease that is genetically linked to an autosomal heterozygous RP mutation. According to the literature, mutations in the known genes encoding RPs of both the small (RPS) and large (RPL) ribosomal subunits can be found in ~50% of DBA patients. However, the molecular mechanisms that underlie the causal effect between RP mutations and anemia have not been clearly elucidated. Nevertheless, a generally recognized pathogenetic hypothesis is that the heterozygous RP mutations cause haploinsufficiency, which in turn interferes with ribosome biogenesis, due to aberrant rRNA maturation at different steps, depending on which RP is affected.[21]

In the present study, quantification of the *RPS19* mRNA level, normalized to GAPDH expression, in three patients with *RPS19* mutations demonstrated a two- to fourfold decrease in the levels of *RPS19* mRNA compared with the normal controls. These patients had an initiation codon mutation (S1), a deletion mutation resulting in a premature stop codon (I2) and a splice-site mutation (S2). This finding is in agreement with a previous study that showed both the protein and RNA were reduced in DBA patients that contained either a missense or frameshift mutation.[22] The reduced RP mRNA level identified in our Korean DBA patients signifies that haploinsufficiency is the underlying pathogenetic mechanism of DBA in the *RPS19* mutation-positive Korean DBA patients. This result demonstrates that this reduced mRNA level is not confined to patients with disruption or deletion of the entire *RPS19* allele,[23] but it is also found in nondeletion mutations.

How the haploinsufficiency of RP genes causes the hematological and other clinical phenotypes of DBA remains elusive. Increasing evidence has supported the role of p53 activation in animal models of ribosome dysfunction, as well as in human cells. *p53* is a tumor suppressor gene that becomes activated to induce cell cycle arrest, senescence or apoptosis in response to cellular stress, including ribosomal

stress.[24] In a zebrafish model, *RPS19* deficiency led to an upregulation of p53 and defective erythropoiesis resembling DBA, which was reversed upon suppression of p53.[25] Similarly, in human hematopoietic progenitor cells, partial knockdown of *RPS14* and *RPS19* led to p53 accumulation, resulting in lineage-specific p53 target gene expression, cell cycle arrest and apoptosis.[26] The most recently identified DBA gene, *RPL26*, is also a positive regulator of p53 activity, as are *RPL5*, *RPL11* and *RPS7*, which are also mutated in DBA.[4]

In the present study, immunohistochemical analysis of p53 expression in bone marrow biopsy samples taken at the time of initial presentation in two patients, one with an *RPS19* initiation codon mutation (S1) and one with no mutations in the eight known DBA genes (S5), showed strong nuclear p53 staining when compared with the normal control. Previous reports on the immunohistochemical analysis of p53 in bone marrow biopsies from eight DBA patients also demonstrated strong nuclear staining in two cases and weak nuclear staining in others.[26] This increased p53 expression is both a potential diagnostic marker in bone marrow failure states, such as DBA and 5q − syndrome, as well as a target for pharmacological intervention, as has been shown with pifithrin-α, a compound that blocks the transcriptional transactivation activity of p53.[26]

In conclusion, heterozygous mutations in the known DBA genes *RPS19*, *RPS26* and *RPS17* were detected in seven out of nine Korean DBA patients. Among these mutations, *RPS19* was the most frequently mutated gene, and mutations in this gene were present in four out of the nine Korean DBA patients. No mutations were detected in the *RPL35A*, *RPS10*, *RPL5*, *RPL11* or *RPS24* genes, and the array-CGH results showed no genomic rearrangements or deletions in the known DBA gene regions. In addition, consistent with other reports, we found no genotype–phenotype correlation in the Korean DBA patients with *RPS19* mutations. Furthermore, the decreased *RPS19* mRNA expression and p53 overexpression in the Korean DBA patients support the hypothesis that haploinsufficiency and p53 hyperactivation underlie the pathogenesis of DBA.

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Supplementary Information accompanies the paper on Experimental & Molecular Medicine website (http://www.nature.com/emm)