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 B50–60% 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. Experimental & Molecular Medicine (2014) 46, e88; doi:10.1038/emm.2013.159; published online 28 March 2014

Keywords: array-CGH; Diamond-Blackfan anemia; ribosomal protein; sequencing

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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Received 13 August 2013; revised 23 October 2013; accepted 5 November 2013
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.\(^7\) 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:\(^1\) 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. KC11EIS0836).

#### Mutation analysis of eight known DBA genes

Genomic DNA was isolated from the peripheral blood leukocytes using the QIAamp 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,\(^14\) 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 Sequencer 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.\(^15\)

#### 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 NimbleGen 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 (Fromega, 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 NimbleGen).

### 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)\(^6\)\(^,\)\(^7\)\(^,\)\(^8\) and one splice-site acceptor mutation. The deletion mutation, c.328delC in case I2, and one missense mutation, c.3G\(^4\)A in case S1, have been previously reported.\(^6\)\(^,\)\(^7\)\(^,\)\(^8\) The splice-site acceptor mutation in intron 4, c.357-2A\(^4\)G in case S2, and the other missense mutation, c.26T\(^4\)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\(^4\)A resulting in Val9Glu, \textit{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.\(^9\)

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\(\rightarrow\)C, was identified in intron 1.\(^13\) In case S4, a novel nonsense mutation, c.259C\(\rightarrow\)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\(\rightarrow\)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/Reticulocyte (%) | Associated anomalies | Growth retardation | Steroid responsive | Reference |
|--------------|----------|-----------------|--------------|---------------|--------------------------|------------------------|----------------------|---------------------|-------------------|---------------------|
| Mutations in the RPS19 gene | | | | | | | | |
| S1 (F) | Sporadic | c.3G\(\rightarrow\)A | Ex 2 | p.Met1? | At birth | 5.0/0.36 | Hydronephrosis | Yes | No | 16 |
| I1 (M) | Sporadic | c.26T\(\rightarrow\)A\(^a\) | Ex 2 | p.Val9Glu | 5 | 2.8/2.45 | Polydactyly | No | Yes | 7 |
| I2 (F) | Sporadic | c.328delC | Ex 4 | p.Leu110\(^*\) | 3 | 6.1/0.25 | None | No | Yes | |
| S2 (F) | De novo (f,m,b normal sequence) | c.357-2A\(\rightarrow\)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\(\rightarrow\)C | Int 1 | — | 1 | 2.3/NA | Strabismus, SNHL | No | Yes | 13 |
| S4 (M) | Sporadic | c.259C\(\rightarrow\)T\(^a\) | Ex 3 | p.Arg87\(^*\) | 2 | 4.0/0.92 | None | No | No | |
| Mutations in the RPS17 gene | | | | | | | | |
| O1 (M) | Sporadic | c.1A\(\rightarrow\)G | Ex 1 | p.Met1Val | 2 | 1.9/NA | Infantile spasms | Yes | Yes | 20 |
| Patients without mutation of eight RP genes | | | | | | | | |
| S5 (F) | Sporadic | | | | | | | |
| S6 (F) | Familial | | | | | | | |

Abbreviations: ASD, atrial septal defect; b, brother; F, female; f, father; M, male; m, mother; SNHL, sensorineural hearing loss.

\(^a\)Represents novel mutations.
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. 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, 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. 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. Therefore, the higher detection rate of DBA gene mutations in our cohort is due to the higher incidence of RPS19.
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. 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. 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, whereas the Japanese cohort exhibited no statistically significant difference in the frequency of thumb abnormalities between the RPS19 mutated and RPS19 nonmutated groups. 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.

Among the DBA patients harboring RPS19 mutations, RPS19 alterations have not been correlated with any specific phenotype. 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.

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. 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, 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...

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 (×400); the insets in the bottom left corner represent a higher magnification, ×1000.
stress. In a zebrafish model, RPS19 deficiency led to an upregulation of p53 and defective erythropoiesis resembling DBA, which was reversed upon suppression of p53. 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. 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.

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. This increased p53 expression is demonstrated strong nuclear staining in two cases and weak nuclear staining in others. 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.

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 five 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.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

ACKNOWLEDGEMENTS
We thank the patients and their parents, as well as the Catholic Genetic Laboratory Center, for assisting us in performing this study and compiling this report. This study was supported by a grant from the Korea Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (A120175).

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