Mutations in the ribosomal protein genes in Japanese patients with Diamond-Blackfan anemia

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ABSTRACT

Background

Diamond-Blackfan anemia is a rare, clinically heterogeneous, congenital red cell aplasia: 40% of patients have congenital abnormalities. Recent studies have shown that in western countries, the disease is associated with heterozygous mutations in the ribosomal protein (RP) genes in about 50% of patients. There have been no studies to determine the incidence of these mutations in Asian patients with Diamond-Blackfan anemia.

Design and Methods

We screened 49 Japanese patients with Diamond-Blackfan anemia (45 probands) for mutations in the six known genes associated with Diamond-Blackfan anemia: *RPS19*, *RPS24*, *RPS17*, *RPL5*, *RPL11*, and *RPL35A*. *RPS14* was also examined due to its implied involvement in 5q- syndrome.

Results

Mutations in *RPS19*, *RPL5*, *RPL11* and *RPS17* were identified in five, four, two and one of the probands, respectively. In total, 12 (27%) of the Japanese Diamond-Blackfan anemia patients had mutations in ribosomal protein genes. No mutations were detected in *RPS14*, *RPS24* or *RPL35A*. All patients with *RPS19* and *RPL5* mutations had physical abnormalities. Remarkably, cleft palate was seen in two patients with *RPL5* mutations, and thumb anomalies were seen in six patients with an *RPS19* or *RPL5* mutation. In contrast, a small-for-date phenotype was seen in five patients without an *RPL5* mutation.

Conclusions

We observed a slightly lower frequency of mutations in the ribosomal protein genes in patients with Diamond-Blackfan anemia compared to the frequency reported in western countries. Genotype-phenotype data suggest an association between anomalies and *RPS19* mutations, and a negative association between small-for-date phenotype and *RPL5* mutations.

Key words: protein genes, Diamond-Blackfan anemia, *RPL5* mutation.

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Introduction

Diamond-Blackfan anemia (DBA, MIM#105650) is a congenital, inherited bone marrow failure syndrome, characterized by normochromic macrocytic anemia, reticulocytopenia, and absence or insufficiency of erythroid precursors in normocellular bone marrow.[1] DBA was first reported by Josephs in 1936 and defined as a distinct clinical entity 2 years later by Diamond and Blackfan. Recent investigations have shown that the cellular defect in DBA fibroblasts is primarily caused by reduced proliferation and a prolonged cell cycle corresponding to the bone marrow characteristics of DBA.[2] DBA is a rare disease with a frequency of two to seven cases per million live births and has no ethnic or gender predilection.[1]

Approximately 90% of affected patients typically present in infancy or early childhood, although patients with a 'non-classical', mild phenotype are diagnosed later in life.[3,4] Macrocytic anemia is a prominent feature of DBA, but the disease is also characterized by growth retardation and congenital anomalies, including craniofacial, upper limb/hand, cardiac, and genitourinary malformations that are present in approximately half of the patients.[3-5] In addition, DBA patients have a predisposition to malignancies including acute myeloid leukemia, myelodysplastic syndrome, and osteogenic sarcoma.[3] The diagnosis of DBA is often difficult because incomplete phenotypes and wide variability of clinical expression are present.[4-6] The central hematopoietic defect is enhanced sensitivity of hematopoietic progenitors to apoptosis along with evidence of stress erythropoiesis, including elevations in fetal hemoglobin and mean red cell volume.[2] The majority of patients have an increase in erythrocyte adenosine deaminase activity.[7]

Proteins are universally synthesized in ribosomes. This macromolecular ribonucleoprotein machinery consists of two subunits: one small and one large. The mammalian ribosome comprises four RNA and 80 ribosomal proteins.[8] The first genetic anomaly identified in DBA involves the *RPS19* gene, which is mutated in approximately 25% of DBA patients. This gene is located at chromosome 19q13.2 and encodes a protein belonging to the small subunit of the ribosome.[9,10] Haploinsufficiency of the *RPS19* gene product has been demonstrated in a subset of cases[11] and appears to be sufficient to cause DBA. The RPS19 protein plays an important role in 18S rRNA maturation and small ribosomal subunit synthesis in human cells.[12,13] Deficiency of RPS19 leads to increased apoptosis in hematopoietic cell lines and bone marrow cells. Suppression of *RPS19* inhibits cell proliferation and early erythroid differentiation but not late erythroid maturation in RPS19-deficient DBA cell lines.[14]

Mutations in two other genes, *RPS24* and *RPS17*, encoding proteins of the small ribosomal subunits have been found in approximately 2% of patients.[15,16] Furthermore, mutations in genes encoding large ribosomal subunit-associated proteins, *RPL5*, *RPL11* and *RPL35A*, have been reported in 9% to 21.4%, 6.5% to 7.1%, and 3.3% of patients, respectively.[17-19] To date, approximately 50% of DBA patients in western countries have been found to have a single heterozygous mutation in a gene encoding a ribosomal protein.[1,3] These findings imply that DBA is a disorder of ribosome biogenesis and/or function. However, there have been no studies of the incidences of these mutations in Asian DBA patients.

In this study, we screened 49 Japanese DBA patients (45 probands) for mutations of the six known DBA genes and *RPS14*, which has been implicated in the 5q- syndrome, a subtype of myelodysplastic syndrome characterized by a defect in erythroid differentiation.[20]

Design and Methods

Patients

Forty-nine patients were studied in order to define the frequency and type of mutations of ribosomal protein genes associated with DBA in Japan. Eight patients were from families with more than one affected member, whereas 41 were from families with only one affected patient. The diagnosis of DBA was based on the criteria of normochromic, often macrocytic anemia; reticulocytopenia; a low number or lack of erythroid precursors in bone marrow; and, in some patients, congenital malformations, without known causes of single cytopenia including acquired or congenital infection, transient erythroblastopenia of childhood, metabolic disorders, malignancies, or autoimmune diseases. All clinical samples were obtained with informed consent from 28 pediatric and/or hematology departments throughout Japan. Additional information was obtained by a standardized questionnaire including information on birth history, age of onset or diagnosis, family history, physical examination (especially regarding malformations), hematologic data, response to therapeutic procedures, and prognosis. This study was approved by the Ethics Committee of Hirosaki University Graduate School of Medicine.

Ribosomal protein gene analysis

DNA was extracted from peripheral blood using a standard proteinase K, phenol and chloroform protocol.[21] A polymerase chain reaction (PCR) was used to amplify fragments from genomic DNA using primer sets designed to amplify the coding exons and exon/intron boundaries of the *RPS19*, *RPS17*, *RPS24*, *RPS14*, *RPL5*, *RPL11* and *RPL35A*. PCR products were directly sequenced in the forward and/or reverse direction using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Tokyo, Japan) on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). *RPS19* was analyzed by determining the genomic DNA sequence of the non-coding first exon, with flanking regions, and the 450-base pair (bp) sequence upstream of the first exon (5'UTR) for each DNA sample, as previously described.[5]

To clarify the sequence of heterozygous insertion/deletion sequence variations, the respective PCR products were cloned into a TA pCR 2.1 vector (Invitrogen, Carlsbad, CA, USA) and their sequences were confirmed.

Genotype-phenotype correlations and statistical analysis

Physical abnormalities in the Japanese DBA patients were evaluated from a viewpoint of correlations with genotype. Although growth retardation can be modified by several factors such as steroid therapy, chronic anemia, and iron overload, the retardation was considered pathognomonic for DBA if it was marked, being below -3 standard deviations (SD). Response to treatment is usually seen within 1 month of treatment in DBA, but a prediction of response has not been reported previously.[1,3] We, therefore, also examined the correlation between genotype and response to the first round of steroid therapy. Associations between two groups of variables were assessed with Fisher's exact test. All tests were two-sided and P levels less than 0.05 were considered statistically significant. Data were analyzed with SPSS 11.0J software (SPSS Inc., Chicago, IL, USA).

Results

Patients' characteristics

Overall, 49 patients (45 probands) were available for analysis. The male to female ratio was 1:1.2. Forty-one index cases were classified as sporadic without unexplained anemia in first-degree relatives, while the remaining eight patients were from four families. All patients were Japanese except two cases: case 10 was Chinese and case 23 was a Brazilian of Japanese extraction. Case 15 had a Filipina mother and a Japanese father.

Genetics

RPS19

Five different mutations were detected in five probands out of 45 families (11%) (Table 1). The median age at presentation of the index cases with *RPS19* mutations was 1 month (range, 0 to 2 months). There appears to be a lower percentage of *RPS19* mutations in Japanese DBA patients than in patients from western countries. All mutations were in the coding region of the gene. Missense mutations resulting in amino acid substitutions were noted in four index cases. The three mutations, p.R62Q in case 30, p.R62W in case 44 and p.0 in case 43, have been reported in seven, ten and two families, respectively,[6,10,11,22-26] whereas one mutation, p.G95V in case 25, was novel, and could not be found in the Single Polymorphism Database (dbSNP at *www.ncbi.nlm.nih.gov/SNP*). Furthermore, the mutation was not observed in DNA from 50 normal individuals. An insertion of one nucleotide was found in one case (case 28), resulting in a novel frameshift mutation.

RPL5 and RPL11

The human *RPL5* gene consists of eight exons and is located on chromosome 1. Four novel mutations were found among the 45 probands (9%) (Table 1). The median age at presentation of the index cases with *RPL5* mutations was 10 months. A deletion of two nucleotides was found in case 10, and an insertion of one nucleotide was found in case 65, each affecting the reading frame. Two cases (cases 41 and 55) had point mutations that resulted in a loss of the translation initiation codon.

The human *RPL11* gene, which consists of six exons, is also located on chromosome 1. All exons and exon/intron boundaries were PCR-amplified and sequenced in DBA patients who were negative for mutations in *RPS19* and *RPL5*. Two mutations (4%) were found, and they were diagnosed at 18 and 20 months old, respectively (Table 1). A deletion of two nucleotides was found in case 9, and a deletion of one nucleotide was found in case 23, in each patient leading to a shift in the reading frame and the introduction of a premature stop codon.

RPS17

The *RPS17* gene is located on chromosome 15, and consists of five exons. *RPS17* mutations are rare and have been reported in only two patients with DBA. A novel one-nucleotide deletion in *RPS17* was identified in one patient (2%), resulting in the introduction of a premature stop codon (Table 1). The patient with the *RPS17* mutation (case 56) was born to healthy non-consanguineous parents and diagnosed as having DBA at the age of 1 month. He responded to the initial steroid treatment, and had a course of steroid-dependent therapy. No physical anomalies were seen in this patient.

RPL35A, RPS24 and RPS14

Mutations in *RPS24* and *RPL35A* are rare and have been reported in only eight and six patients with DBA, respectively. DBA patients were screened for *RPS24* and *RPL35A*, in addition to *RPS14*, which is implicated in the 5q- syndrome. No mutations were detected in *RPS24*, *RPL35A* or *RPS14* in Japanese DBA patients.

In total, sequence changes were found in four out of seven screened ribosomal protein genes (Table 2). Mutations in *RPS19*, *RPS17*, *RPL5*, and *RPL11* were detected in 11%, 2%, 9%, and 4% of the probands, respectively. The frequency of ribosomal protein gene mutations in Japanese DBA patients was 27%.

Genotype-phenotype correlations: congenital anomalies

The patients' characteristics are summarized in Table 3.

Table 1. Mutations identified in *RPS19*, *RPL5*, *RPL11*, and *RPS17* in Japanese DBA patients.

Patients (gender)	Inheritance	Age at diagnosis	Mutation	Predicted amino acid change
Mutations in the *RPS19* gene				
43 proband (F)	Sporadic	0 D	Exon2:g.3G>A	p.0
28 proband (M)	Sporadic	6 D	Exon3:g.130_131insA	E44fsX50
44 proband (F)	Sporadic	1 M	Exon4:g.184C>T	R62W
30 proband (F)	Familial	1 M	Exon4:g.185G>A	R62Q
30 father (M)	Familial	0 M	Exon4:g.185G>A	R62Q
25 proband (M)	Sporadic	2 M	Exon4:g.284G>T	G95V
Mutations in the *RPL5* gene				
10 proband (F)	Sporadic	0 M	Exon5:g.473_474delAA	K158fsX183
41 proband (F)	Sporadic	1 Y	Exon1:g.3G>T	p.0
55 proband (F)	Sporadic	3 Y	Exon1:g.3G>A	p.0
65 proband (F)	Sporadic	4 M	Exon2:g.37_38insT	F13fsX14
Mutations in the *RPL11* gene				
9 proband (F)	Sporadic	1 Y 10 M	Exon2:g.58_59delCT	L20fsX53
23 proband (M)	sporadic	1 Y 6 M	Exon5:g.460delA	R154fsX189
Mutations in the *RPS17* gene				
56 proband (F)	Sporadic	1 M	Exon2:g.26delT	V9fsX17

Anomalies associated with DBA were found in 27 patients (55%). Sixteen had two or more malformations (33%). All six patients with an *RPS19* mutation had physical anomalies, and three of them had multiple anomalies. In contrast, clinical data from European and American DBA patients showed that the frequency of malformations was 31% in patients with *RPS19* mutations, which is not significantly different from that of the entire DBA population.[26] *RPS19* mutations are characterized by a wide variability of phenotypic expression.[26] A mutation is frequently associated with various degrees of anemia, different responses to treatment, and dissimilar malformations. Even various family members having the same mutation in *RPS19* present with different clinical expressions. Cases 30, 44 and 43 harbored the same *RPS19* mutations reported in multicase families (p.R62Q, p.R62W, p.0).[6,10,11,22-27] Comparable to previous observations, no consistent clinical features were found in patients from different families displaying mutations in *RPS19*. For example, the father of case 30 harboring the same mutation had no finger anomalies, although case 30 had syndactyly and thumb polydactyly.

Consistent with reports that patients with *RPL5* and *RPL11* mutations are at high risk of developing malformations,[17,18] all four patients with *RPL5* mutations had physical anomalies. Furthermore, three of them had multiple physical anomalies, particularly case 41, who had very severe congenital heart disease (Table 3). One of two patients with *RPL11* mutations had physical anomalies. In contrast, of the 36 patients with no mutations, physical anomalies were seen in 16 (44%).

Nine patients had craniofacial anomalies. Of these, two had *RPL5* mutations, while the remaining patients had no mutations. Gazda *et al.* suggested an association between *RPL5/RPL11* mutation and cleft lip and/or palate.[17] Data in the Diamond-Blackfan Anemia Registry (DBAR) of North America also suggest that the DBA phenotype associated with cleft lip/palate is caused by non-*RPS19* mutations.[4] In our cohort, the frequency of cleft palate was significantly different between *RPL5*-mutated and *RPL5* non-mutated groups ($P<0.05$): cleft palate was seen in three patients, two of whom had *RPL5* mutations while the other patient belonged to the *RPL5* non-mutated group.

Thumb anomalies were seen in six patients, four of whom had *RPS19* mutations while two had *RPL5* mutations. There was a statistically significant difference in the frequency of thumb anomalies between *RPS19*-mutated and *RPS19* non-mutated groups ($P<0.05$). Flat thenar was seen in one patient with an *RPL5* mutation. In contrast to previous reports on patients with *RPL11* mutations, thumb anomalies were not found in our patients with these mutations.

A small-for-date phenotype was seen in seven patients (14%): one had an *RPS19* mutation, one had an *RPL11* mutation, and the four others had no mutations. None of the patients with *RPL5* mutations was born small-for-date.

Genotype-phenotype correlations: therapeutic response

Corticosteroids and transfusions are the mainstays of DBA treatment.[1,3] Of 45 patients evaluable for first treatment response, 73% responded to steroid therapy, 8% did not respond and 16% were never treated with steroids. The proportions of patients who responded to the first steroid treatment were 5/5 (*RPS19*), 2/3 (*RPL5*), 1/2 (*RPL11*), 1/1 (*RPS17*), and 22/27 (no mutation). There were no significant differences in the response rates among these patients.

Sixty-nine percent of patients received red blood cell transfusions. Of 48 patients available for therapy in follow-up, 8 patients (17%) were transfusion-dependent, 18 patients (37%) were steroid-dependent, and 18 patients (37%) were transfusion-independent with no other treatment. Three patients received bone marrow transplants and were alive and well (Table 3). A malignancy was detected in one case (case 50, proband), who developed a myelodysplastic syndrome 1 year after the diagnosis of DBA.

Table 2. Summary of sequence changes in seven ribosomal protein genes identified in Japanese DBA patients.

Gene symbol	N. of tested DNA samples from unrelated probands	N. of probands with mutations	N. of subjects with mutations	Mutation types
RPS19	45	5 (11%)	6	missense, loss of 1st methionine, small insertion
RPL5	45	4 (9%)	4	loss of 1st methionine, small deletion, small insertion
RPL11	34	2 (4%)	2	small deletion
RPS17	45	1 (2%)	1	small deletion

Discussion

This is the first report of an investigation of DBA patients in Japan. Twelve types of mutations were detected in four ribosomal protein genes. These mutations occurred in 27% of Japanese DBA patients. Mutations in *RPS19*, which have been found in 25% of patients in western countries,[26] were detected in only five of 45 probands (11%) in Japan, and two of these mutations were unique. Novel mutations in *RPL5* (four probands; 9%), *RPL11* (two probands; 4%) and *RPS17* (one proband; 2%) were identified. The frequencies of mutations in *RPL5*, *RPL11* and *RPS17* were very similar to those in western countries.[16-19] These results may suggest that a lower incidence of mutations in ribosomal protein genes in Japanese patients with DBA is due to a lower incidence of *RPS19* mutations, although we might have missed large deletions or re-arrangements in this study.

Physical abnormalities and growth retardation were detected in 55% of the Japanese DBA patients, consistent with previous reports from western countries.[4-6] Recent studies suggest that patients with *RPL5* mutation are more likely to have physical malformations including craniofacial, thumb, and heart anomalies.[17,18] Remarkably, patients with *RPL5* mutations tend to have cleft lip and/or palate or cleft soft palate, isolated or in combination with other physical abnormalities.[17,18] We found that three of four patients with *RPL5* mutations had multiple physical malformations, and two had cleft palate, whereas only one patient without an *RPL5* mutation had cleft palate. In the general population, 0.1% to 0.2% of children are born with cleft lip and/or palate.[28] Our data, and those from previous findings, suggest that *PRL5* mutations are associ-

Table 3. Characteristics of Japanese DBA patients.

Patient	Malformation status	Response to first steroid therapy	Present therapy
Patients with mutation of *RPS19*			
25 proband	Thumb polydactyly, growth retardation (-2.0SD), etc.	ND	ND
28 proband	Thumb polydactyly, CHD, etc.	Response	Steroid-dependent
30 proband	Thumb polydactyly, syndactyly, growth retardation (-3.4SD)	Response	Steroid-dependent
30 father	Growth retardation (-3.6SD)	NA	CR
43 proband	Thumb polydactyly	Response	Steroid-dependent
44 proband	SFD	Response	CR
Patients with mutation of *RPL5*			
10 proband	Flat thenar, cleft palate, CHD, etc.	Poor	Transfusion-dependent
41 proband	Craniofacial abnormalities, cleft palate, CHD, etc.	ND	Transfusion-dependent
55 proband	Thumb polydactyly	Response	Steroid-dependent
65 proband	Growth retardation (-3.0SD)	Response	Steroid
Patients with mutation of *RPL11*			
9 proband	CHD, SFD, etc.	Response	CR
23 proband	None	Poor	Steroid-dependent
Patient with mutation of *RPS17*			
56 proband	None	Response	Steroid-dependent
Patients without mutation of seven RP genes			
1 proband	Growth retardation (-4.0SD)	Response	CR
1 daughter	None	Response	CR
3 proband	Growth retardation (-3.6SD)	Response	Steroid-dependent
4 proband	Craniofacial abnormalities, SFD, short stature, webbed neck	Response	Steroid-dependent
5 proband	None	Response	CR
6 proband	Cleft palate, SFD, etc.	Poor	BMT
7 proband	Craniofacial abnormalities, SFD, growth retardation, etc.	Response	CR
8 proband	Growth retardation, webbed neck	Response	Steroid-depndent
13 proband	None	NA	CyA, BMT
14 proband	None	Response	CR
15 proband	None	Response	Transfusion-dependent
20 proband	Craniofacial abnormalities, CHD, etc.	Rsponse	Transfusion-dependent
21 proband	None	Response	Steroid-dependent
22 proband	None	Response	CR
24 proband	Growth retardation (-4.0SD)	Response	Steroid-dependent
26 proband	Growth retardation (-4.1SD), craniofacial abnormalities, etc.	Response	Transfusion-dependent
33 proband	None	Response	BMT
36 proband	Hypospadias, cryptorchidism	Response	Steroid-dependent
36 cousin	None	Response	Steroid-dependent
37 proband	Hypospadias, cryptorchidism	ND	CR
42 proband	None	Response	CR
45 proband	Craniofacial abnormalities, growth retardation, etc.	Poor	Transfusion-dependent
48 proband	Fetal hydrops	ND	CR
49 proband	None	Response	Steroid-dependent
50 proband	None	Response	Steroid-dependent, CBT (due to MDS)
50 sister	None	Response	Steroid-dependent
51proband	None	Poor	CR
54 proband	None	ND	Transfusion-dependent
59 proband	None	ND	Transfusion
60 proband	SFD	ND	Transfusion
61 proband	None	Response	Cyclosporine
62 proband	CHD, SFD, growth retardation (-3.1SD)	Response	Steroid-dependent
63 proband	Craniofacial abnormalities, growth retardation (-7.5SD)	Response	Steroid-dependet
64 proband	None	Response	Steroid-depndent
66 proband	None	NA	Transfusion-dependent
67 proband	None	NA	NA

*ND: not done; NA: not available; SFD: small-for-date; CHD: congenital heart disease; MDS: myelodysplastic syndrome; BMT: bone marrow transplantation; CBT: cord blood stem cell transplantation; CR: complete remission. * RPS19, RPS24, RPS17, RPS14, RPL5, RPL11, RPL35A.*

ated with multiple physical abnormalities, especially cleft lip and/or palate.

Cmejla *et al.* reported that 87.5% of *RPL5*-mutated patients were born small-for-date, whereas only 42.9% of *RPS19*-mutated patients were born small-for-date.[18] However, in our series, the small-for-date phenotype was seen in seven patients, and all of them were *RPL5*-non-mutated patients. Our data suggest that *RPL5* mutations in Japanese DBA patients have no relevance to the small-for-date phenotype, which may be a unique characteristic of Japanese DBA.

According to recent studies, the frequency of malformation, particularly thumb anomalies, in *RPS19*-mutated patients, was relatively low compared to that in *RPL5*- or *RPL11*-mutated patients.[22-24,29] In Italian DBA patients, the risk of malformation was 7-fold higher in *RPL5*-mutated patients than in *RPS19*-mutated patients.[29] In contrast, all of the Japanese DBA patients with *RPS19* mutations had one or more malformations. The frequency of thumb anomalies was significantly higher in patients with *RPS19* mutations, as well as in patients with *RPL5* mutations, compared to in the other groups of patients.

Although steroid therapy is one of the established treatments for DBA, the mechanism of action is unknown and reliable prediction of response to initial steroid therapy is not available.[1,3] *RPS19* mutation status has not been predictive of response in any series.[3] In our cohort, responsiveness to first steroid therapy in Japanese DBA patients was as good as that reported in western populations.[1,3] In this study, no significant differences in response to initial steroid therapy were found between *RPS19*-mutated and *RPS19*-non-mutated groups, or between the groups with *RPS19* mutations and other ribosomal protein gene mutations.

In summary, we found that heterozygous mutations in *RPS19*, *RPL5*, *RPL11* or *RPS17* were present in 27% of Japanese DBA patients. No mutations were detected in *RPS14*, *RPS24* or *RPL35A*. We observed a slightly lower frequency of mutations in ribosomal protein genes in our cohort of Japanese DBA patients than the frequencies reported previously from western countries, although the data from both populations are based on relatively low numbers of patients and values showing significant differences between populations are lacking. Our data suggest an association between *RPL5* mutation and malformations, especially cleft palate, and between *RPS19* mutation and malformations, particularly thumb anomalies. This study also suggests that no association exists between *RPL5* mutations and the small-for-date phenotype or between *RPS19* mutations and non-responsiveness to initial steroid therapy in Japanese DBA patients.

Authorship and Disclosures

EI was the principal investigator and takes primary responsibility for the paper. YK, TT, ST, GX, RNW, KT, and SO performed the laboratory work for this study. SO, TH, AH, SK, DH, YK, RY, KK, RK, TI, TH, MHP, and KS enrolled the patients. EI and YK wrote the paper.

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