Multiplex ligation-dependent probe amplification enhances molecular diagnosis of Diamond-Blackfan anemia due to RPS19 deficiency

Diamond-Blackfan anemia (DBA,#MIM105650) is a rare congenital pure red cell aplasia characterized by normochromic macrocytic anemia, reticulocytopenia, and normocellular bone marrow with a selective deficiency of erythroid precursors. Defects in the *RPS19* gene on chromosome 19q13.2 are the main known cause of DBA and account for 20-25% of DBA patients.[1-3] This gene comprises six exons that span ~11 kb of genomic DNA, and encodes the ribosomal protein S19. Mutations in the *RPS24* and *RPS17* genes have been reported, though they are only mutated in a minority of patients.[4,5] The 8p23.3-8p22 region has also been implicated in DBA, but the gene has not yet been identified.[6] Eighty-three unique RPS19 mutations scattered throughout the entire gene and all found in heterozygosity with the wild type sequence have so far been reported in 127 families.[7] Since only 8 patients have been found to carry a complete or partial RPS19 deletion, it may be supposed this category of mutations is present in around 6% of *RPS19*-mutated patients.[7] Standard PCR-based methods used for conventional mutation detection fail to identify heterozygous deletions because the normal allele masks the deleted segment. In sequence analysis, apparent loss of heterozygosity of one or more intragenic single nucleotide polymorphisms (SNPs) may be the only sign of a deletion in family studies. Strategies that detect copy number variations, such as Southern blotting, real-time PCR, and fiber FISH, are thus necessary to integrate sequencing. Development of the MLPA (Multiplex Ligation-dependent Probe Amplification) technique to detect complete or partial gene deletions or duplications has greatly improved mutation screening.[8] This technique is an easy and sensitive method based on the simultaneous hybridization and ligation of several probes that matched to single exons in a single reaction tube, followed by PCR and analysis by capillary electrophoresis. If a deletion is present, even on only one exon of a single allele, the corresponding peak is reduced (Figure 1); enhanced peaks suggest duplication.

Here we describe MLPA first application to the *RPS19* gene in the molecular diagnosis of DBA.

Routine sequence analysis of the *RPS19* gene in 123 DBA patients identified 31 mutated subjects, whereas 92 seemed normal.[9] Informed consent was obtained from all patients included in the study. Our PCR primers also allow interrogation of six SNPs distributed along the gene

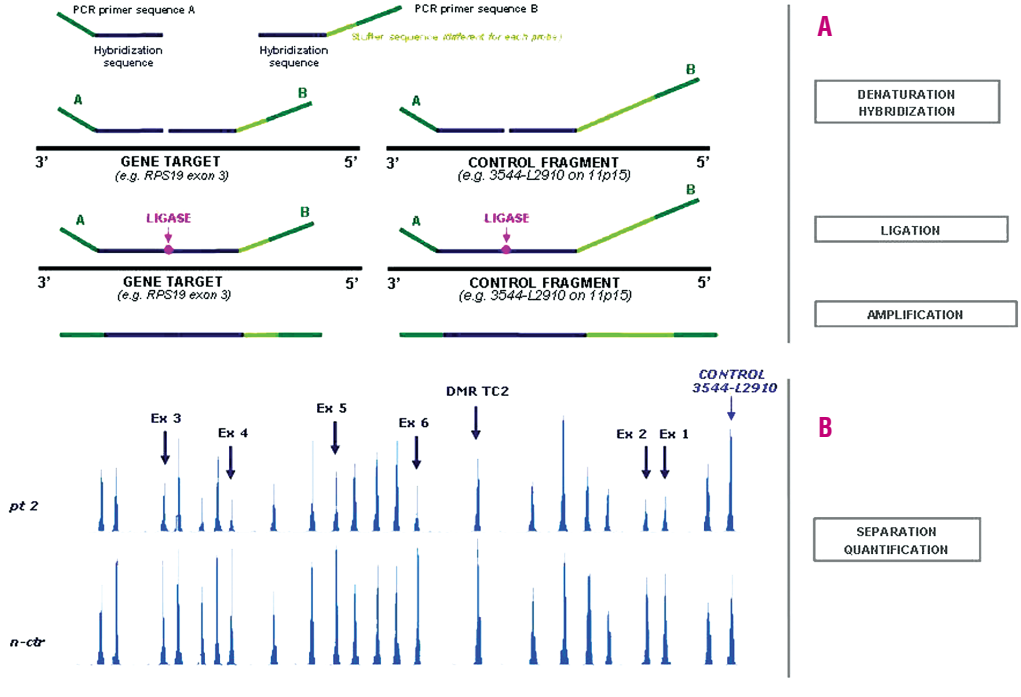

Figure 1. MLPA technique. (A) Denatured genomic DNA is hybridized with a mixture of 23 probes. Each MLPA probe consists of two oligonucleotides. The two parts of each probe hybridize to adjacent target sequences and are ligated by a thermostable ligase. A universal primer pair is used to amplify all ligated probes. The amplification product of each probe has a unique length ranging from 130bp to 400bp. Fragments are separated on an ABI Prism 3100-Avant automatic sequencer by using POP4 polymer and GS-ROX-500 molecular marker (Applera, Foster City, CA, USA), and analyzed with the Genescan software ver.3.1. Relative amounts of probe amplification products, as compared to control DNA sample, reflect the relative copy number of target sequences. (B) MLPA chromatogram of patient 2 carrying a whole gene deletion and a control subject (n-ctr). RPS19 peaks are labeled with their exon numbers. Unlabeled peaks represent genes in the 8p23 region and control genes (only the control probe 3544-L2910 is indicated). The values of peak sizes and area from patients and controls were used and normalized as follows: 1) the area of each peak (As) was divided by the sum of all 23 peak areas ($\sum$As) of that sample; 2) for each peak this ratio (*n*As=As/$\sum$As) was divided by the relative peak area of the corresponding probe calculated as the average from (two or three) control DNA samples (*n*Ac=Ac/$\sum$Ac). A ratio (*n*As/*n*Ac) ranging from 0.8 to 1.2 was considered as a normal exon dosage; a deletion was suspected for ratio <0.7; a duplication was suspected for ratio >1.3.

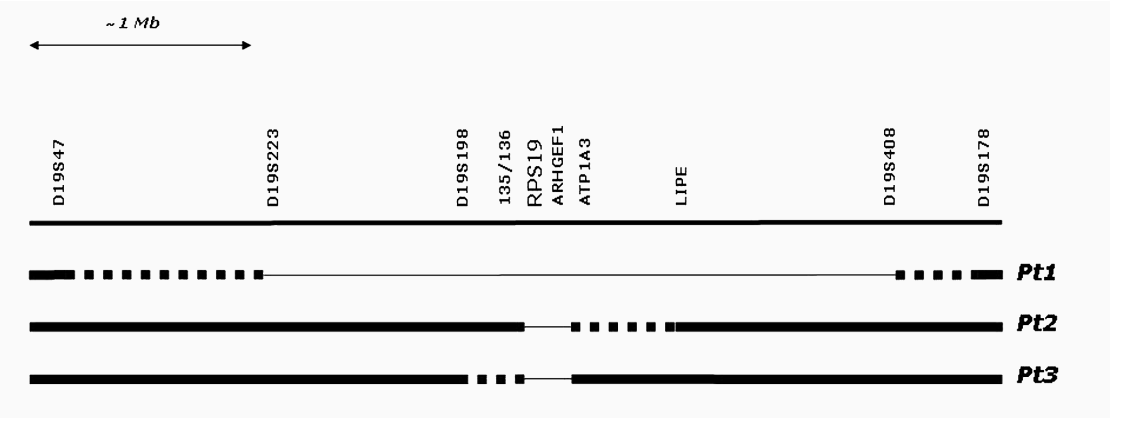

Figure 2. Extension of the 3 deletions in the region 19q13. Horizontal bars represent the chromosome regions present in the 3 patients. The deleted regions are indicated by an intervening line. The undefined regions are represented by dotted bars.

in not coding regions and usually transmitted *en bloc*.[2,10] These SNPs were used to divide the 92 non-mutated patients into two groups according to their haplotypes. Heterozygotes were 38/92 (41%). None displayed a loss of heterozygosity for one or more SNPs, suggesting the absence of intragenic deletions. Homozygotes were 54/92 (59%). It was assumed that some of them could carry a complete or partial deletion. MLPA was also used to look for duplication in both groups.

MLPA was performed on genomic DNA with the MLPA DBA test kit (MLPA KIT P212 DBA, MRC-Holland, Amsterdam, The Netherlands) according to the manufacturer's instructions (*http://www.mrc-holland.com*). The probemix was composed of 23 probes: six probes mapped the *RPS19* gene (one for each exon), one probe a region close to *RPS19* (8.8 Kb upstream from the exon 1 probe), and ten the 8p23 region formerly suggested as a candidate for an unknown *DBA* gene. Six were control fragments. In each experiment, MLPA efficiency was tested by using a patient carrier of a known complete gene deletion of the *RPS19* gene due to an unbalanced translocation (1;19) (p32; q13).[1]

MLPA revealed a significant reduction of the peak heights of all *RPS19* exons in 3/54 homozygotes, suggesting the presence of heterozygous deletions of the entire *RPS19* gene. A probe located 8.8 Kb centromeric to the *RPS19* gene (*DMRTC2*) was also deleted in these 3 patients. All DNA samples showing evidence of a deletion were confirmed in a second MLPA experiment. Normal results in the other 89 patients showed the absence of intragenic deletions and duplications. No genomic rearrangement was discovered in the 8p23.3-8p22 region.

MLPA was also performed on the parents of each deleted patient; the absence of abnormalities in both parents proved that the three deletions were *de novo*.

To confirm these deletions, better define their extension and substantiate parental origin, we analyzed seventeen microsatellites spanning the 19q13 region from D19S47 to D19S178 (~2Mb centromeric and ~2Mb telomeric to the *RPS19* gene).[11,12] We also analyzed a microsatellite internal to *ARHGEF1* gene using a forward fluorescently-labeled primer 5'-TAGTTGTGGGGT-CAGGATGG-3' and a reverse primer 5'-GAAGTTCCTC-CCCGACTTCT-3'.

We found that the extension of the deletions varied from ~0.06 to ~3Mb; the deletion was of maternal origin in patients 1 and 2, and paternal origin in patient 3 (Figure 2). These patients (2 males, 1 female) displayed early-onset anemia (age at diagnosis 0-4 months) and malformations: cranio-facial dysmorphisms, strabismus and urogenital malformations (patient 1), growth retardation (patient 2), and cardiac malformations (patient 3). Patient 1 was also mentally retarded. Initial steroid response was reported in patients 1 and 3; patient 3 died of sepsis during the first steroid course. Patient 2 was steroid unresponsive and therefore transfusion dependent.

Like other authors, we found MLPA efficient and rapid for analysis of gene dosage alterations.[8] In this study, it identified three unsuspected heterozygous *RPS19* gene deletions missed by standard mutation screening PCR-based methods. Mutation screening of the *RPS19* gene with a combination of sequencing and MLPA reached an overall rate of 28% (34/123). This was equivalent to a 3% increase in the detection rate compared to sequencing alone. Furthermore, whereas the frequency of genomic deletions in DBA patients was previously at 6% of *RPS19* alterations,[7] this rises to 8.5% on the strength of our results. In our cohort, genomic deletions represent up to 12% of all mutations detected.

In conclusion, we stress that a gene-dosage technique, such as MLPA or FISH, should complement sequencing in a clinical environment since only a combined approach of this kind permits comprehensive detection of all mutations within the *RPS19* gene. We strongly suggest that it should be applied as a complement to *RPS19* sequencing to all subjects with a DBA phenotype.

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Immune-mediated pure red cell aplasia in renal transplant recipients

Pure red cell aplasia (PRCA) is defined by an isolated severe anemia, contrasting with a normal white blood cell and platelet counts, severe reticulocytopenia and a selective absence of erythroid progenitors in the bone marrow smears.[1] Although an immune origin is involved in the majority of cases,[1] the diagnosis of immune-mediated cytopenia is rarely considered in organ transplant recipients, given the immunocompromised status. Etiologies of post-transplant PRCA are rather dominated by chronic parvovirus B19 infections[2] and drug-induced bone marrow toxicity.[3] However, in most cases reported in the literature with drug-induced PRCA, cyclosporine A (CyA) was introduced to replace the incriminated drug. We, therefore, hypothesized that some post-transplant PRCA may be immune-mediated, especially in renal transplant recipients treated with a calcineurin inhibitor (CNI)-free regimen. This hypothesis is supported by an unexpectedly high frequency of LGL-like clonal disorders in organ transplant recipients,[4] and by a recent report of autoimmune cytopenia occurring in up to 5.6% of pancreas transplant recipients receiving a calcineurin inhibitor-free regimen.[5] We report here 3 renal transplant recipients admitted for PRCA, in whom thorough investigations provided compelling evidence of immune mechanisms. End stage renal failure was related to chronic primary glomerulonephritis in all of them, and they all received a first renal transplant in the early '80s. Between 1980 and 1990, 489 out of the 611 patients receiving a renal transplant in our center were given a calcineurin inhibitor (CNI)-free immunosuppressive regimen, including the 3 cases who later experienced immune-mediated PRCA.

The 3 patients were admitted for severe aregenerative anemia, while treated with prednisone and azathioprine for several years. Bone marrow smears confirmed the diagnosis of PRCA. None of them was given recombinant erythropoietin and the high erythropoietin levels found in our patients excluded the hypothesis of anti-erythropoietin antibodies (Table 1). Parvovirus B19 infection is a well-identified cause of PRCA in immunocompromized patients.[2] However, both serology and specific PCR ruled out this hypothesis in our 3 cases. A specific azathioprine-

Table 1. Laboratory examination of 3 patients with immune-mediated PRCA. Erythroid progenitors cultures were performed with 5×10^5 cells/mL. Values are means of duplicate culture of day 7 CFU-E-derived colonies per 5×10^5 plated cells.

	Normal Values	*Case 1*	*Case 2*	*Case 3*
		1st flare / 2nd flare		
Mean corpuscular volume (fL)	80-100	105	105	100
Erythropoietin (mU/mL)	4-14	1666 / 1920	ND	1220
Parvovirus B19 IgM		– / ND	–	–
IgG		+ / ND	+	+
PCR		– / –	–	–
T-cell clone		+ / +	+	+
LGL population		ND +	ND	ND
Coomb's reaction		– / –	–	–
ANA		–	–	–
CFU-E-derived colonies	100-1,000			
patient's serum		155 / ND	135	ND
control's serum		105 / ND	65	ND

ANA: anti-nuclear antibody; LGL: large granular lymphocyte; ND: not done; PCR: polychain reaction; (+), positive; (-), negative.