Distribution of Rhesus blood group antigens and weak *D* alleles in the population of Albania

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Background. Determination of Rhesus (Rh) status is of critical importance in the field of both transfusion and obstetric medicine. As the distribution of Rh phenotypes was unknown in the Albanian population, we investigated the donor population in Albania to estimate the prevalence of each phenotype, as well as to identify and characterise the variants at the molecular level.

Materials and methods. A total of 38,836 blood donors were phenotyped for Rh D, C, c, E and e antigens by routine serological methods, and samples with reduced D antigen expression underwent molecular characterisation by a Tm-shift genotyping method and direct sequencing.

Results. Among all donors 89.00% and 10.86% were D-positive and D-negative, respectively, while 0.14% (n=55) of the donors were found to be weak D-positive. Overall 45/55 samples (81.8%) were resolved by Tm-shift screening, showing that approximately 67% of the variant *D* alleles were weak *D* type 1, while weak *D* type 3 (9.1%) and weak *D* type 2 (3.6%) were less common. A novel c.932A>G (p.Y311C) variant was also found in the heterozygous state by direct sequencing.

Discussion. This extensive study reveals the distribution of Rh phenotypes in the Albanian population, the low prevalence of individuals with a weak D phenotype, and the specific pattern of distribution of the three most common variant alleles in this Caucasian population.

Keywords: blood group, genotype, phenotype, Rh, weak D types.

Introduction

The Rhesus (Rh) blood group is associated with the expression of the highly homologous *RHD* and *RHCE* genes located on human chromosome 1, which respectively encode the RhD and the RhCE polypeptides[1-6]. The D antigen is of major clinical relevance in the fields of transfusion medicine and obstetrics. Given its marked immunogenicity, D antigen can induce the production of alloantibodies resulting in a haemolytic transfusion reaction in alloimmunised patients or haemolytic disease of the foetus and the newborn in alloimmunised, D-negative pregnant women[7]. So far, dozens of variant alleles have been reported in the *RHD* gene, most of which are associated with either a weak or partial D phenotype (www.uni-ulm.de/~fwagner/RH/RB2/). It is well known that *RHD* gene variations differ between populations with specificities in all ethnic groups[8-13]. In Caucasians it has been reported that 0.2-1.0% of individuals exhibit a weak D phenotype caused by a single-point variant or a combination of them resulting in amino acid substitution(s) in intracellular and/or transmembrane domains of the RhD protein, while qualitatively altered partial D phenotypes, caused by amino acid substitutions in extracellular domains or *RHD-RHCE* gene rearrangements, are rare[9].

Albania is a small country in southern Europe with a population of more than three million inhabitants. From a genetics point of view, studies have clearly shown that the Albanian population is a typical Indo-European population[14]. A peculiarity of this country is that it was isolated from the rest of Europe during the second half of the 20th century for political reasons. Because no Rh data have been reported so far in this population, we investigated the distribution of Rh phenotypes and sought to identify and characterise samples with weak D reactivity at the molecular level by a combination of previously published approaches[15,16].

Materials and methods

Samples

From January 2010 to April 2013, blood samples from 38,836 donors originating from all districts (n=26) of Albania and, therefore, fully representative of the whole country, were collected and included for routine blood group typing at the immunohaematology laboratory of the National Blood Transfusion Centre

(NBTC, Tirana, Albania), which centralises blood testing in Albania.

Serological Rhesus typing

Rh typing for D, C, c, E and e antigens was performed for all donors. All samples were investigated using DiaClon monoclonal antibodies that detect the presence of the DVI variant (DiaMed, Cressier, Switzerland). The RhCcEe phenotype was determined by ID-card human antibodies (DiaMed). Individuals who tested negative for D antigen were further subjected to weak D testing using anti-D blend (clones TH-28/MS-26) (CE-Immundiagnostika, Eschelbronn, Germany). After the final wash, the saline was decanted and one to two drops of anti-human globulin serum (Biotec, purchased from Lab21 Healthcare, Dorset, UK) was added. Samples showing agglutination after incubation were considered to be weak D-positive.

RHD molecular genotyping

Genomic DNA from all weak D-positive samples was extracted from a 2-mL EDTA blood sample by the QIAmp DNA Blood Mini kit (Qiagen, purchased from Mediline d.o.o., Kamnik, Slovenia) according to the manufacturer's instructions. *RHD* gene variant screening was performed at the Blood Group Molecular Genetics Laboratory (French Blood Institute - Bretagne, Brest, France) using previously published methods. Briefly, samples were first screened for weak *D* type 1, 2 and 3 alleles by a Tm-shift assay[15]. The ten *RHD* exons in inconclusive samples were then amplified by polymerase chain reaction (PCR) and sequenced directly[15]. *RHCE* exon 6 was amplified with gene-specific primers (forward: 5'- AGTAGTGAGCTGGCCCACCG-3'; reverse: 5'-TGAAGCCAATAAGAGAATGCACCA-3'), and sequenced (forward sequencing primer rb25[17]; reverse sequencing primer: 5'- AGAGAATGCACCAACACCTGCCTA-3') in conditions described before[15]. Sequencing data were analysed with Sequencher® v5.1 sequence analysis software (Genes Codes Corporation, Ann Harbor, MI, USA). Finally, exon gene dosage was determined by a quantitative, multiplex PCR of short fluorescent fragments (QMPSF) on the remaining samples[16]. This quantitative method is particularly potent for studying gene rearrangements by calculating individual exon copy numbers both in the *RHD* and *RHCE* genes.

Results

Rhesus phenotypes

Over a period of 28 months and in a total of 38,836 D-phenotyped blood donors, 34,564 (89.00%) were typed D-positive in our conditions (Table I). Of the remaining 4,272 samples tested for weak D reactivity in a second round of investigations, 58 were found to be positive and submitted to subsequent molecular analysis for variant investigation, while 4,214 were typed D-negative.

In the whole Albanian population the Ccee phenotype is the most prevalent (Table I) followed respectively by CCee, CcEe, ccee, ccEe, and ccEE. The CCEe and CcEE phenotypes were very rare and not a single case of CCEE was found.

Variant *RHD* alleles

The Tm-shift assay[15] designed to screen for the three most common weak *D* variants in the Caucasian population (i.e., weak *D* type 1; weak *D* type 2; and weak *D* type 3) resolved the genotype of 45/58 (77.6%) samples, including one compound heterozygous sample carrying both weak *D* type 1 and weak *D* type 3 alleles (Table II). Of the remaining 13 samples, the profiles obtained in the Tm-shift assay suggested that in three cases the DNA was homozygous for the deletion of

Table I - Rh status in 38,836 Albanian donors.

RhCcEe phenotype	D+		Weak D		D-		Total (population)	
	n[a]	%	n[a]	%	n[a]	%	n[a]	%
CCee	9,989	25.72	21	0.05	3	<0.01	10,013	25.78
Ccee	12,340	31.78	30	0.08	225	0.58	12,595	32.43
Ccee	508	1.31	1	<0.01	3,988	10.27	4,498	11.58
CCEe	34	0.09	0	0.00	0	0.00	34	0.09
CcEe	6,222	16.02	1	<0.01	0	0.00	6,223	16.02
ccEe	4,390	11.30	2	<0.01	1	<0.01	4,393	11.31
CCEE	0	0.00	0	0.00	0	0.00	0	0.00
CcEE	10	0.03	0	0.00	0	0.00	10	0.03
ccEE	1,071	2.76	0	0.00	0	0.00	1,071	2.76
Total (status)	34,564	89.00	55	0.14	4,217	10.86	38,836	100.00

Three weak D-positive samples genotyped as *RHD*-negative were excluded from the calculations; [a]n: occurrence.

Table II - Occurrence, phenotype and allele frequency of the weak *D* types 1, 2, and 3 alleles in the Albanian population.

Weak *D* allele	n[a]	Phenotype (occurrence)	Allele frequency	CI[b] (95%)
Type 1	38	Ccee (25); CCee (13c)	1:2,044	1:2,890 -1:1,490
Type 2	2	ccEe (2)	1:38,836	1:320,512 - 1:10,751
Type 3	6	Ccee (5); CCee (1[c])	1:12,945	1:35,336 - 1:5,948

[a]n: number of samples observed in 38,836 Albanian donors, including one heterozygous (weak *D* type 1 + weak *D* type 3) sample; [b]CI: confidence interval (Poisson distribution); [c]Including the heterozygous (weak *D* type 1 + weak *D* type 3) sample.

the whole *RHD* gene (data not shown)[15], implying that these patients do not express the D antigen. The ten other samples (10/55, 18.2%) could not be resolved by the Tm-shift assay and were further studied by direct sequencing.

Only one variant could be identified: c.932A>G in exon 6 (Figure 1). Because c.932G is found in *RHCE* and to investigate whether the sequencing profile could result from co-amplification of a potential variant allele in *RHCE* exon 6, we specifically sequenced this exon in our sample of interest and paid attention to six positions (i.e., c.916, c.932 and c.939+21_24) defining gene-specific haplotypes (Figure 1). A wild-type sequence was found in *RHCE* exon 6 (Figure 1), suggesting that no *RHCE* variant allele is carried by the donor in this exon, excluding the potential risk of co-amplification with *RHD*-specific primers. The c.932G nucleotide is, therefore, likely to be carried by a *RHD* allele. No other genetic alteration was found in this sample, which was then included in the final step of the screening for exon dosage by QMPSF[16].

The QMPSF analysis actually confirmed the homozygous whole deletion of the *RHD* gene in three samples, suggesting that they were incorrectly typed. These samples were then considered as D-negative. On the basis of these findings, 10.86% of Albanian individuals are D-negative, while 0.14% have weak D

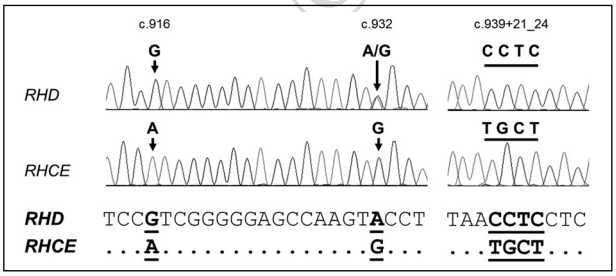

Figure 1 - Direct sequencing of *RHD* and *RHCE* exon 6 in a heterozygous *RHD* c.932A>G sample (electropherograms).
Positions of interest are indicated by an arrow (i.e., c.916 and c.932) and a black line (c.939+21_24). *RHD* (NM_016124.4) and *RHCE* (NM_020485.4) reference sequences with bold, underlined gene-specific nucleotides are mentioned below the electropherograms. Dots in the *RHCE* sequence indicate homology with the *RHD* sequence.

(Table I). No other abnormality (i.e., gene deletion or rearrangement) was found either in the heterozygous c.932A>G sample or in the remaining nine samples. On the basis of the sequencing data and considering that no gene rearrangement was identified, we suggest that the *RHD* c.932A>G is a novel variant *RHD* allele. This variant, which was observed in a single sample carrying two *RHD* copies (i.e., heterozygous), is assumed to result in the p.Y311C amino acid substitution, and affects a residue predicted to be located within the tenth transmembrane domain of the mature polypeptide[18].

Discussion

Appropriate assignment of Rh status, RhD most importantly, is of critical importance in transfusion and obstetric medicine. We investigated the Rh status of blood donors in the Albanian population, which has remained completely unexplored so far.

In our cohort of 38,836 blood donors we found that 10.86% are D-negative and 0.14% of individuals exhibit a weakly agglutinating D phenotype (Table I). These rates are both significantly lower than those commonly observed in Caucasian populations, in whom the proportion of D-negative people is 15-17%[19], while weak expression of the D antigen is observed in 0.2-1.0% of such populations[9]. These results highlight the specific pattern of RhD status in the Albanian population.

In terms of CcEe antigens, the global distribution fits well with that previously reported for the UK population[19] although there is a significant difference in the ccee phenotype (17.17% *vs* 11.58% in our study). This difference may be attributed, at least in part, to the lower rate of D-negative individuals in Albania than in the UK (~16.80%[19] *vs* 10.86% in our study), which contributes to reducing the prevalence of the *cis*-associated *RHce* haplotype, but also to the specific structure of the Albanian population.

Although they tested D-negative in the first round of experiments, 58 (0.15%) samples exhibited weak D reactivity with a second set of reagents. Subsequent molecular analysis of these samples was carried out and identified three samples homozygous for the deletion of the whole *RHD* gene (3/58; 5.2%). This result suggests that these samples are likely false-positive for weak D

testing. A typical Caucasian pattern, as expected, was also highlighted with 81.8% (45/55) of the weak *D* alleles being either type 1, 2 or 3 (Table II). The study further indicated that the weak *D* type 1 allele is by far the most prevalent weak *D* allele, followed respectively by weak *D* type 3 and weak *D* type 2 (Table II). When comparing our findings with data from other European countries, such a high prevalence of the weak *D* type 1 allele is also observed in Germany and Denmark, but major differences are reported for the two other common alleles (Table III). Interestingly, data from Croatia, which is geographically close to Albania, tend to fit with those of Austria rather than those of Albania (Table III). Although these latter observations should be viewed with caution considering that different sets of reagents, which may result in different serological cut-offs, were used by the respective laboratories, these data nevertheless highlight the distinct pattern of the weak *D* variants in the Albanian population. Albania is considered as a country with insufficient blood donation and the low frequency of D-negative individuals in the overall population (i.e., 10.86%) makes D-negative units a critical problem in blood bank facilities. The molecular analysis that confirmed the prevalence of the three most common weak *D* variants in most variant *D* carriers may contribute to managing them as D-positive[20], and thus rationalising the use of D-negative stock units.

The c.932A>G (p.Y311C) variant in exon 6 was found in the heterozygous state in a single individual. This variant has already been reported in several complex haplotypes involving gene rearrangements with the paralogous *RHCE* gene (www.uni- ulm.de/~fwagner/RH/RB2/). In our case, no *cis*-linkage with any other variant could be detected by the genotyping approach, suggesting that the sporadic *RHD* c.932A>G substitution is a novel variant allele. Additional serological tests with a fresh blood sample from the donor carrying this allele will help to define the underlying phenotype more precisely. As in *trans* of this latter allele, no variant was identified in nine DNA samples accounting for a significant total of 16.4% (9/55) samples with weak D reactivity, as found by serological analysis, which are still unexplained. We and others have already reported this situation in various populations, such as the French[15,21], Brazilians[22] and Argentineans[23]. At least three independent scenarios or a combination of them may be hypothesised: (i) these samples, which were initially typed as D-negative, are actually false-negative and should be tested again in the future to define their phenotype precisely; (ii) the low antigen reactivity is due to the suppressive effect of C[24], which is however absent in one out of our nine weak D-typed, "wild-type" *RHD* samples; or (iii) a founder variant, which is specific to the population of interest, is located within an unexplored region of the *RHD* gene and quantitatively alters its expression. Resequencing the whole locus by next-generation sequencing in these samples may help to identify a putative variant in this last hypothesis.

In conclusion, this is the first description of the distribution of Rh phenotypes and the prevalence of weak *D* variants in the Albanian population.

Acknowledgements

The authors are grateful to Déborah Jamet (EFS-Bretagne, Brest, France) for useful discussions on serological typing and Laurence Bryckaert and Christine Guerry (EFS-Bretagne, Brest, France) for their technical assistance in the genotyping procedure.

Funding

This study was supported by the French Embassy, Tirana, Albania, the French Blood Institute (EFS-Bretagne) and the National Institute of Health and Medical Research (Inserm), France.

Table III - Reported prevalence (%) of the three most common weak D types in Caucasian populations[a].

Country (Region)	n[b]	Frequency (%)					Reference
		Type 1	Type 2	Type 3	Types 1 + 3	Others	
Albania	55	67.3	3.6	9.1	1.8	18.2	This study
Germany (south)	159	59.7	27.0	4.4	0.0	8.9	[9]
Germany (north)	260	65.0	16.9	17.3	0.0	0.8	[25]
Austria (Tyrol)	130	33.1	7.7	50.0	0.0	9.2	[25]
Portugal	99	16.1	63.6	14.1	0.0	6.2	[26]
France (west)	230	40.4	27.4	4.8	0.0	27.4	[27]
Denmark	91	65.9	12.1	9.9	0.0	12.1	[28]
Croatia	167	37.7	3.6	46.1	0.0	12.6	[29]

[a]Because of different serological cut-offs among these studies, the reported frequencies cannot be compared directly; [b]n: number of weak D-positive samples in the respective studies.

The Authors declare no conflict of interest.

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Arrived: 03 September 2013 - Revision accepted: 30 December 2013
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