Insights into *RHCE* Molecular Analysis in Samples with Partial *D* Variants: the Experience of Western France

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Summary

Background: Although systematic blood group genotyping of patients/donors is virtually possible, serological studies remain the gold standard to identify samples of clinical interest that may be further genotyped. In this context, we sought to identify variant *D* alleles that are likely to be clinically relevant in terms of other Rh antigens in a subset of population genotyped in Western France. **Methods:** Samples presenting with the *RHD**weak *D* type 4.2.2 allele (*n* = 47) were selected for the study. *RHCE* exons 1–7 were directly sequenced, and expression of Rh antigens was predicted on the basis of the molecular data. **Results:** Of the 47 samples tested, 19 (40.4%) were predicted to be of potential clinical interest. Moreover, we could show that selecting the samples to be genotyped by the nature of their variant *D* allele (i.e., *RHD**weak *D* type 4.2.2 allele) rather than by their Duffy-null status appears to increase significantly the likelihood of identifying clinically relevant individuals for Rh status. **Conclusion:** On the basis of our findings we suggest that all individuals genotyped as weak *D* type 4.2.2 should be systematically screened for *RHCE* variants by molecular analysis on a routine basis.

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Introduction

Systematic genotyping of blood donor samples is currently technically feasible by high-throughput platforms [1–7] or next-generation sequencing in a near future [8, 9], although it remains to definitely confirm whether the clinical benefit significantly overpasses the economic cost. Serological analyses, which remain the gold standard, are known to be potent and robust to investigate blood group status and are sufficient to achieve this aim most of the time, unless specific antisera are not available. Therefore, an extensive, systematic genotyping of major blood group genes (for example *RHD* and *RHCE* in Rh; *DARC* in Duffy; *SLC14A1* in Kidd; *KEL* in Kell; *GYPB* and *GYPB* in MNS) in all donors may not be required from a strict transfusion point of view. This statement is however challenging regarding multi-transfused patients suffering from hemoglobinopathies, such as sickle cell disease (SCD). These patients are treated by transfusions on a regular basis with red blood cell (RBC) units from different donors that are practically not ethnically matched. Because this population of African descent is prone to express partial antigens (e.g., partial e) while others commonly found in Caucasians are not expressed (e.g., Fy), transfused RBC units may induce allo-immunization, thus complicating subsequent cross-matching.

In that context, extended genotyping in selected subsets of samples may be particularly worthwhile. The Rh system is extremely complex, not only in terms of molecular genetics but also because of the 54 antigens reported so far, some of them being of major clinical significance [10]. For years it has been demonstrated that some *RHD* variants, particularly those of the three African clusters [11], segregate with *RHCE* variants, which result in the expression of partial/rare antigens [12–19]. But it is currently unknown to

	Allele name	Allele details	Predicted phenotype*
ce	Reference	Reference
ce.01	ce(c.48C)	Weak e
N/A	ce(c.48C, c.105T)	(Weak e)?
ce.04	ce(c.48C, c.712A, c.733G, c.787G, c.800A, c.916G)	Partial c, partial e, hr ^s -, Hr-, V+ ^w , VS-
N/A	ce(c.712A, c.733G, c.787G, c.800A, c.916G)	(Partial c, partial e, hr ^s -, Hr-, V+ ^w , VS-)?
ce.05	ce(c.48C, c.712A, c.787G, c.800A)	Partial c, partial e, hr ^s -, Hr-
ce.06	ce(c.254G)	Partial e, hr ^s -, CEAG-
ce.20.01	ce(c.733G)	Partial c, partial e, hr ^s -, Hr ^B -, V+, VS+
ce.20.02	ce(c.48C, c.733G)	Partial c, partial e, hr ^s -, Hr ^B -, V+, VS+

Fig. 1. *RHCE* alleles and associated phenotype found in *RHD**weak *D* type 4.2.2 samples. The left panel illustrates the *RHCE* gene, including the 10 exons (each represented by a white square; left-to-right: exons 1 to 10) and the respective position of variants in each allele (black, vertical bar); note that exons 8 to 10 have not been sequenced. N/A: not applicable; *Phenotype different from the reference *RHCE**ce allele.

Table 1. Examples of *RHCE* exon dosage by QMPFSF#

Predicted genotype	Panel ^a	HFE	F9	1C ^b	2c ^b	3	4	5	6	7	8	9	10
*ce.04/*ce.04	A	2	2	2	2	2	2	0	0	2	0	2	2
*ce.04/*ce(c.712A, c.733G, c.787G, c.800A, c.916G)	B	2	1	1	2	2	2	0	0	2	0	2	2
*ce/*ce.04	C	2	1	1	2	2	2	1	1	2	1	2	2
*ce/*ce.05	D	2	2	1	2	2	2	1	2	2	1	2	2
*ce.20.01/*ce.20.02	E	2	2	1	2	2	2	2	2	2	0	2	2
*Ce/*ce (calibrator)	F	2	1	1	1	2	2	2	2	2	2	2	2

#Numbering of markers discordant with the wild-type alleles is underlined. 1 to 10: *RHCE* exons.
^aPanel in fig. 2.
^bMarkers 1C and 2c target positions c.48C and c.307C in exons 1 and 2, respectively [22].

what extent expression of various antigens carried by the *CE* allele is linked to the expression of variant *D* alleles. On the basis of that statement, we sought to identify variant *D* carriers that are likely to be clinically relevant in terms of the other Rh antigens in the population of blood donors and patients in Western France. To this aim the *RHCE* gene was genotyped in a subset of 47 samples carrying the *RHD**weak *D* type 4.2.2 allele. Phenotypes were predicted on the basis of the sequencing data. Here we report the results of that study.

Material and Methods

DNA Samples

Among the 2,000 DNA samples from donors and patients collected and addressed to our laboratory by the local sites of the French Blood Center (Etablissement Français du Sang, France) in Western France from 2003 to 2013, DNA samples characterized locally by routine molecular methods [20–22] as *RHD**weak *D* type 4.2.2 were selected for the study, for a total of 47 unrelated DNA samples. For simplicity, samples presenting with heterozygous sequencing profiles in the *RHD* gene were excluded from the analysis, implying that only hemizygous and homozygous *D* variants were further studied.

Molecular Analyses and Phenotype Prediction

Exons 1 up to 7 of the *RHCE* gene (NM_020485.4) were specifically PCR-amplified and directly sequenced by following previously published conditions [23]. *RHCE* exon 6 was alternatively PCR-amplified by using forward primer rb25 [24] prior to direct sequencing. To simplify the molecular testings and because a limited number of *RHCE* alleles including variants within exons 8, 9, or 10 have been reported so far (ISBT Blood Group Allele Terminology; www.isbtweb.org).

www.isbtweb.org/fileadmin/user_upload/WP_on_Red_Cell_Immunogenetics_and/004_RHCE_alleles_v2.0_110914.pdf), the three last exons of the gene were not investigated.

RHD zygosity and gene rearrangement within *RHD* and *RHCE* were tested by quantitative multiplex of PCR short fluorescent fragments (QMPFSF) [22]. This assay is a quantitative method that calculates the copy number of each exon in the respective genes.

The c.-67 nucleotide position within the 5'-untranslated region (UTR) of the *DARC* gene (NM_002036.3) was also assessed by PCR amplification and direct sequencing in all DNA samples with primers 5'-TGTAAGTCCCAAC-CAGCCAAATC-3' and 5'-GGCTAGCATAGGAAGGAGAGAA-3' in similar conditions. Output sequencing data were analyzed with Sequencher™ 5.2 (Gene Codes Corporation, Ann Arbor, MI, USA). Because no serological data was available, and neither familial study nor RNA sequencing could be carried out, the most likely *RHCE* alleles were deduced from the literature and reference databases [13] (ISBT Blood Group Allele Terminology; RhesusBase v2.0: www.uni-ulm.de/~fwagner/RH/RB2/; New York Blood Center *RHCE* database: nybloodcenter.org/rhce/). Samples are considered of clinical interest when two alleles producing a partial antigen or resulting in the silent expression of another antigen are combined together, or when an alternative antigen is predicted to be produced by either allele.

Statistical Analysis

Statistical analysis was performed using the StatCalc calculator module of Epi Info™ software (v6.04 – CDC, Atlanta, GA, USA). A *p* value less than 5% was considered as significant.

Results

Variants were found by direct sequencing in *RHCE* exons 1, 2, 5, 6, and 7, but not in exons 3 and 4, for a total of nine different

Fig. 2. Typical *RHCE*-QMPFSF profiles obtained in samples with genotypes **A** *RHCE***ce.04*/*RHCE***ce.04*, **B** *RHCE***ce.04*/*RHCE***ce*(*c.712A*, *c.733G*, *c.787G*, *c.800A*, *c.916G*), **C** *RHCE***ce*/*RHCE***ce.04*, **D** *RHCE***ce*/*RHCE***ce.05*, **E** *RHCE***ce.20.01*/*RHCE***ce.20.02*, and **F** *RHCE***ce*/*RHCE***ce* (calibrator). Respective peak IDs are indicated below the figure; e1C to e10: *RHCE* exons 1 to 10. *Exon numbering different from what expected for a homozygous *RHCE***ce* sample [22].

alleles on a *RHCE***ce* background (fig. 1), including the recently reported *RHCE***ce*(*c.48C*, *c.105T*) allele [25], which is reminiscent to the *RHCE***ce.01* allele with an additional silent *c.105C>T* variant, and a yet unreported *RHCE***ce*(*c.712A*, *c.733G*, *c.787G*, *c.800A*, *c.916G*) allele, which only differs from the *RHCE***ce.04* allele by the *c.48G>C* transversion that is not found here. QMPFSF analysis of the 47 samples genotyped as weak *D* type 4.2.2 exhibited aberrant results in five DNAs (data not shown), which is a typical feature of degraded genomic DNA [22]. Then *RHD* zygosity could not be investigated in these samples. Of the 42 remaining samples, four were found to carry two copies of the *RHD**weak *D* type 4.2.2 allele, while the 38 others carry one variant *D* copy (data not shown). In terms of *RHCE* exon numbering, five different profiles affecting some or all exons 1, 5, 6, and 8 were found (table 1, fig. 2). Patterns with altered numbering of exons 1, 5, and 6, for a total of 36 samples, are linked to the respective base substitutions carried by the *RHCE***ce.04* allele that directly impair QMPFSF primer annealing. Only two samples display alternative profiles (table 1): in the *RHCE***ce*/*RHCE***ce.05* sample, one copy of markers 1C and 5 are found, while the *RHCE***ce.20.01*/*RHCE***ce.20.02* sample exhibits

one copy of marker 1C. As observed before, exon 8 marker, which targets a genomic sequence upstream exon 8, may vary between 0 and 4 in both genes depending on its origin [22].

Initially discrepant results were found between QMPFSF and direct sequencing in eight samples in *RHCE* exon 6. Indeed, while QMPFSF data indicated one copy of this marker with a forward primer targeting the *c.916A*, *RHCE*-specific position, direct sequencing suggested a homozygous G nucleotide at that position. *RHCE* exon 6 was then PCR-amplified by using an alternative forward primer (i.e., *rb25*, see 'Material and Methods') and then sequenced, resulting in a heterozygous A/G pattern at position *c.916*, as expected. Overall the QMPFSF profiles are in full accordance with the sequencing data.

Finally a total of nine genotypes could be predicted (table 2). As reported in the literature, *RHCE***ce.04* (also known as *ceAR*; 45/47 individuals, 95.7 %) and *RHCE***ce.05* (also known as *ceEK*; 1/47, 2.1 %) were found to be linked to the *RHD**weak *D* type 4.2.2 allele [15, 26–29], while only one DNA does not carry either allele. Further serological investigation will be required to characterize more precisely antigen expression resulting from these allele combina-

Table 2. *RHCE* allele, phenotype prediction and Fy(a–b–) status in 47 weak *D* type 4.2.2 samples

<i>RHCE</i> genotype prediction	Occurrence	Potential clinical interest	Fy(a–b–)	Fy(a–b–) + potential clinical interest
*ce.04/*ce	22 (46.8%)	no	8 (17.0%)	N/A
*ce.04/*ce.20.01	8 (17.0%)	yes	8 (17.0%)	8 (17.0%)
*ce.04/*ce.06	6 (12.8%)	yes	4 (8.5%)	4 (8.5%)
*ce.04/*ce.04 ^a	3 (6.4%)	yes	3 (6.4%)	3 (6.4%)
*ce.04/*ce.01	3 (6.4%)	no	3 (6.4%)	N/A
*ce.04/*ce(c.48C, c.105T) ^b	2 (4.3%)	no	2 (4.3%)	N/A
*ce.04/*ce(c.712A, c.733G, c.787G, c.800A, c.916G) ^a	1 (2.1%)	yes ^c	1 (2.1%)	1 (2.1%)
*ce.05/*ce	1 (2.1%)	no	1 (2.1%)	N/A
*ce.20.01/*ce.20.02	1 (2.1%)	yes	1 (2.1%)	1 (2.1%)
Total	47 (100.0%)	19 (40.4%)	31 (66.0%)	17 (36.2%)

N/A = Not applicable.

^aHomozygous *RHD**weak *D* type 4.2.2 samples.

^bThe c.105C>T substitution is a silent variant.

^cTo be confirmed by further serological analyses.

tions, especially regarding partial c and partial e. But on the basis of the molecular data, 19 samples are predicted to be potentially of clinical interest (table 1).

Recently, Kappler-Gratias et al. [30] reported a study in which systematic *RH* genotyping was carried out in 316 blood donors of African origin selected on the basis of the Fy(a–b–) phenotype that is typical of sub-Saharan populations [31]. Of the 31 donors carrying one or two variant *D* alleles of various molecular structures resulting in a partial *D* phenotype, two samples (i.e., 1 *RHD**weak *D* type 4.2.2 and 1 *RHD***DIIIa*; 6.5%) were reported with a predicted, rare Rh phenotype. Because we did not have access to the Fy phenotype in our cohort, we genotyped the c.-67 nucleotide position of the *DARC* gene by direct sequencing, which is characterized by a T>C substitution at the homozygous state in Fy(a–b–) samples [32]. 31 out of 47 samples (66.0%) were predicted to be Fy(a–b–) (table 2), which is in the range of what reported before (63.0%; $\chi^2 = 0.12$; $p = 0.7276$) [33] and confirms the African origin of our cohort. Of this subset, 17 were potentially of clinical interest (table 2), which is significantly more than what was observed by Kappler-Gratias et al. [30] (6.5% vs. 54.8% in this study; $\chi^2 = 17.07$; $p = 0.000036$). This latter result suggests that selecting samples to be genotyped within the *RHCE* gene on the basis of their variant *D* allele rather than their African origin may contribute to optimize the probability to identify rare, clinically relevant Rh phenotypes.

Discussion

Systematic molecular genotyping of blood group genes has been made technically feasible by the tremendous improvements of high-throughput technology over the past 10 years. Although the clinical benefit of mass-scale genotyping has been reported [34, 35], the economic issue remains a major hurdle to routinely implement such a strategy. To gain insights into the accurate identification of samples of potential clinical interest that may deserve fur-

ther genotyping, we focused our study on a subset of samples selected on the basis of preliminary molecular criteria and took advantage of our local DNA bank. Our analysis stressed on the *RH* genes, which are known to express more than 50 antigens [10]. Because many variant *RHCE* alleles have been reported in people of African origin, we sought to evaluate the relevance of genotyping that gene in 47 samples addressed for routine *RHD* molecular analysis and presenting with a *RHD**weak *D* type 4.2.2 variant at either the hemizygous or the homozygous state. The respective Rh phenotype was subsequently deduced from molecular data. It is worth mentioning that i) segregation of the variant *D* allele with the *RHCE* allele could not be investigated and ii) this prediction is exclusively based on the genotyping results in line with the data found in the literature, suggesting that further serological investigations are mandatory to confirm our predictions. From another point of view it is also important to note that in the context of systematic molecular mass-scale screening of all donors, i) haplotyping would not be carried out and ii) assignment of blood group status would be preliminary based on these molecular data, then suggesting that our prospective study falls into that latter area.

Nine different allele combinations could be deduced in the 47 samples, among which 19 (40.4%) (table 2) were assumed to be clinically relevant in terms of Rh status. While the clinical significance of anti-V and anti-VS has remained mild if any so far [10], expression of partial c and partial e as well as lack of expression of the high-frequency antigens Hr and Hr^B together with hr^S and hr^B, respectively, is a critical issue [15, 28, 29, 36–40], particularly in a transfusion context in patients that are prone to be transfused on a regular basis. Of note, it must be mentioned that anti-c has been reported in individuals carrying *RHCE**ce.04 [28, 29], this latter allele being importantly found in 45/47 samples.

As expected, it is interesting to underline that the QMPFS assay [22] easily provided clues about the nature of the *RHCE**ce.04 allele by displaying a typical pattern. Indeed, the hybrid *CE-D* exon 5 is not amplified by the QMPFS primers and the c.916A>G transition

found in this allele impairs the specific primer annealing in exon 6, resulting in 1 or 0 copy calculated for both exons in compound heterozygous or homozygous *RHCE***ce.04* samples. It is also worthwhile to mention that, if this latter test had not been performed, the c.916 position in *RHCE* exon 6 would have been incorrectly genotyped as homozygous G/G instead of heterozygous A/G in 8/47 samples. Once again this example highlights the complexity of the molecular genetics of the *RH* genes, because of the high degree of similarity and the multiple gene rearrangements occurring in both coding and non-coding regions, and more specifically the critical challenge to genotype accurately the *RHCE* gene.

A recent paper reported an important study of systematic *RH* genotyping in a cohort of French donors of African origin [30]. The authors interestingly concluded that specific mutations defining *RHD* and/or *RHCE* variant alleles, as well as *D-CE* haplotype inheritance, should be taken into consideration for extended molecular analysis. Our results are definitely in favor of that statement. Furthermore, although most of the *RHCE* variant alleles are carried by people of African ancestry [39], we showed that selecting samples of interest on the basis of their variant *D* allele for subsequent *RHCE* genotyping would be more appropriate than by their origin (i.e., Fy(a-b-) phenotype) (table 2).

In conclusion we have provided molecular data suggesting that both donors and patients genotyped as weak *D* type 4.2.2 should be systematically screened for *RHCE* variants by molecular analysis on a routine basis. This strategy may help to prevent Rh alloimmunization in a transfusion context by reinforcing data to favor genetic matching, especially by considering that these individuals are particularly prone to be involved in SCD management. It will be mandatory to confirm and complete these preliminary results by serological studies and/or clinical data to define general guidelines for blood group typing as well as to carry out such studies with variants *D* samples of other clusters.

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Disclosure Statement

The authors declare that they have no conflict of interest.

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