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Original article

RHD positive among C/E+ and D-negative blood donors in Tunisia

Dépistage du gène RHD chez des donneurs de sang de phénotype D-négatif et C/E+

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Abstract

Purpose of the study. – The aim of this study was to investigate *RHD* alleles among Tunisian blood donors with D-negative phenotype and positive for C and/or E antigen.
Patients and methods. – A total of 100 D-negative and C/E+ samples were analyzed by *RHD* genotyping using an initial test for *RHD* exon 10. In case of a positive reaction, further molecular investigations including real time quantitative PCR, allele specific PCR and nucleotide sequencing were done to elucidate the *RHD* involved mechanisms.
Results. – Seventy-five percent of the studied samples lacked the *RHD* gene. Twenty-three percent carried the hybrid *RHD-CE-D* alleles (16 *RHD-CE(3-7)-D*, 5 *RHD-CE(4-7)-D*, 1 *RHD-CE(4-8)-D*, 1 *RHD-CE(3-8)-D*) and 2% were weak D (1 weak D type 1 and 1 weak D type 5).
Conclusion. – Our study proved the high frequency of *RHD* gene among serologically D-negative samples, positive for C and/or E antigen. Thus achieving systematically RHCE phenotyping in all transfusion centers on the Tunisian territory and considering blood donated from D-negative C/E+ persons as D-positive will be recommended to reduce anti-D allo-immunization.

Keywords: C/E+ D-negative phenotype; *RHD* gene; *RHD* genotyping; *RHD* alleles; Tunisians

Résumé

But de l'étude. – Le but de cette étude est de dépister les allèles *RHD* chez des donneurs de sang tunisiens de phénotype D-négatif ayant l'antigène C et/ou E positif.
Patients et méthodes. – Un total de 100 échantillons de phénotype D-négatif et C/E+ ont été analysés par génotypage *RHD* en utilisant un test initial ciblant l'exon 10 du gène *RHD*. En cas de réaction positive, d'autres investigations moléculaires incluant une PCR quantitative en temps réel, une PCR allèle spécifique et un séquençage ont été réalisées afin d'élucider le mécanisme moléculaire impliqué.
Résultats. – Soixante-quinze pour cent des échantillons étudiés avaient une délétion totale du gène *RHD*. Vingt-trois pour cent des cas avaient des allèles hybrides (16 *RHD-CE (3-7)-D*, 5 *RHD-CE (4-7)-D*, 1 *RHD-CE (4-8)-D*, 1 *RHD-CE (3-8)-D*) et 2 % présentaient des D faible : 1 D faible type 1 et 1 D faible type 5.
Conclusion. – Notre étude a confirmé la fréquence élevée du gène *RHD* chez les sujets sérologiquement D négatif et positif pour l'antigène C et/ou E. Ainsi, il serait recommandé de systématiser la réalisation du phénotypage RHCE dans tous les centres de transfusion sanguine en Tunisie et de considérer les dons de sang des personnes ayant le phénotype D-négatif C/E+ comme étant D positif afin de réduire le risque d'allo-immunisation transfusionnelle.

Mots clés : Phénotype D négatif C/E+ ; Gène *RHD* ; Génotypage RHD ; Les allèles RHD ; Tunisiens

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1. Introduction

The antigen D is of critical importance for the blood transfusion strategy and the most important blood group antigen, as anti-D immunizations can occur readily in D-negative recipients [1]. The frequency of D-negative varies widely among ethnic populations; 15% to 17% in Caucasians [2], 3% to 7% in Africans [3]. For Tunisians, 9% are D-negative [4], the main reason for D negativity was found to be linked to the deletion of the entire *RHD* gene [5]. Nevertheless, some unexpressed *RHD* (2 *RHDψ*, 4 *RHD-CE-D*) and allelic *RHD* variants (DBT-partial D, weak D type 4.0, weak D type 29, 2 weak D type 11) have been reported in 448 Tunisian blood donors, most of which were associated to a positivity for antigen C or antigen E or both according to an independent study [5].

The preferential occurrence of antigen D-negative *RHD* gene positive allele in *Cde* and *cdE* haplotypes was also observed in Caucasians, Japanese and other ethnic groups.

Wagner and colleagues [6] reported the molecular background in 754 D-negative Caucasian individuals with positivity for at least C or E. Five individuals with four different *RHD-CE-D* hybrid alleles and 15 individuals with three different *Del* alleles [6].

A variety of barely or completely unexpressed *RHD* alleles were also reported among D-negative black individuals with positivity for at least C antigen. The identified alleles included frequently *RHD-CE-D* hybrids with large parts of the *RHCE* gene associated to the d(C)ce^{s} haplotype [7,8]. *RHD* gene positive D antigen negative persons have been evenly observed in East Asia including frequently *DEL* phenotypes [9–11].

Taking into account the above-mentioned *RHD* allele ethnic specificities, we aimed in the present study to investigate *RHD* alleles in a target group of RHD negative blood donors with positivity for either at least C or E antigen.

2. Materials and methods

Ethylenediaminetetracetate anticoagulated blood samples were colleted from 100 Tunisians blood donors, at the Centre Régional de Transfusion Sanguine of Sousse. The samples were from donors who had previously been categorized as D-negative but C+ and/or E+ based on serologic tests. Serologic typing for D, C/c, and E/e antigens was performed for all samples using the anti-D antibody Diagast reagent (France) (clones P3x61 + P3x21223B10 + P3x290 + P3x35) and Bio-rad reagents (France) for RHCE phenotyping, anti-C (clone MS24), anti-c (clone MS33), anti-E (clone MS260) and anti-e (clones MS16, MS21, MS63) antibodies were used.

All tests were performed in accordance with the manufacturer's instructions. In addition, an indirect antiglobulin test was performed for all samples to detect some weak D phenotype.

DNA was isolated from 5 to 10 mL of blood by a modified salting out procedure as described by Miller [12].

The study was approved by the committee of ethics and research for the university hospital Farhat Hached of Sousse.

3. RHD molecular analysis

In a first step, all samples were screened for *RHD* exon 10 by polymerase chain reaction (PCR) performed as described elsewhere [5]. Samples from which a specific product was obtained, were then screened for *RHD* exon 5 and 7 by a real time multiplex PCR with TaqMan chemistry using ABI PRISM 7900 Sequence detection system (Life technologies) to identify the involved *RHD* alleles. Primers/probes and TaqMan amplifications reactions were applied as described previously [5].

Thus two different patterns were obtained: samples positive for exon 5 and 7 were further characterized by nucleotide sequencing of the 10 *RHD* exons until they could be assigned to a distinct RHD allele, using primer sets described by Lemaréchal [13], with a sequencing kit (Big Dye Terminator v1.1, Applied Biosystems, Forsty city, CA) and automated fluorescence-based genetic analyser (ABI PRISM 3130, Life technologies) however samples negative for *RHD* exon 5 and 7 were further investigated for the presence of the remaining *RHD* exons by PCR-simplex, using the same primers used for sequencing, to identify properly the several involved *RHD-CE-D* hybrids.

Amplifications were carried out in a final volume of 50 μL containing 50 ng of genomic DNA, 2.5 U of *Taq* polymerase, 200 μM dNTPs, 0.4 μM of each primer and 1.5 mM Mgcl2.

The cycling conditions used were as follows: denaturation at 95 °C for 10 min and then 35 cycles of 45 s at 95 °C, 30 s at 55 °C and 1 min 30 at 72 °C, all PCRs were terminated after a 10-min extension at 72 °C. PCR products were visualised using the Agilent 2100 bio-analyzer (Agilent technologies) according to the manufacturer's protocol.

4. Results

We analysed 100 blood donors typed D-negative by serology but C+ and/or E+ of which 86 were ddCcee, 13 ddccEe and one had a ddCcEe phenotype. DNA samples were screened with an initial test for *RHD* exon 10. In case of positive reaction, the genotype was further analysed by real time PCR specific for *RHD* exons 5 and 7 and an *RHD* exon scanning PCR specific for *RHD* exons 1,2,3,4,8 and 10 and in some cases nucleotide sequencing of the *RHD* gene.

Molecular analysis showed that 75% of the studied samples were negative for *RHD* exon 10. The 25 remaining samples were found to be positive for *RHD* exon 10, further *RHD* genotyping revealed two weak D (1 weak D type 1 and 1 weak D type 5). Twenty-three were hybrid *RHD-CE-D* alleles, among these, *RHD-CE(3-7)-D* was the most frequent allele, found in 16 samples, the remaining cases were 5 *RHD-CE(4-7)-D*, 1 *RHD-CE(4-8)-D* and 1 *RHD-CE(3-8)-D*.

Summarized results are shown in Table 1.

5. Discussion

We studied the molecular basis for the C/E+ D-negative phenotype in Tunisians to investigate *RHD* alleles among individuals with the above-mentioned phenotype. Thus, the results of our study confirm that *RHD* gene deletion was considerably