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Original article

Molecular characterization of rare *D--/D--* variants in individuals of Indian origin

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Background - Rh antigens are critical in haemolytic disease of the foetus and newborn (HDFN). The D-- phenotype is a rare blood group characterised by the lack of expression of C, c, E and e antigens at the surface of red blood cells because of mutations in both RHCE alleles inactivating the expression of a "normal" protein. The aim of the study was to determine the molecular basis of D-- individuals of Indian origin.

Materials and methods - Ten Rh D-positive postnatal women who had produced antibodies against all Rh antigens, except D, leading to HDFN and foetal loss, were investigated. Extensive serological and molecular (polymerase chain reaction [PCR] using sequence-specific primers), quantitative multiplex PCR of short fluorescent fragments (QMPSF), and Sanger sequencing analyses were carried out.

Results - Serological testing with anti-C, anti-c, anti-E, and anti-e reagents showed absence of the four antigens in all ten index cases, as well as in three siblings. Flow cytometry indicated absence of these antigens with a typical exalted expression of the D antigen, thus confirming the rare D-- phenotype. Molecular analysis by QMPSF suggested homozygous CE-D hybrid alleles causing the D-- phenotype: *RHCE-D(3-9)-CE* (n = 11), *RHCE-D(3-8)-CE* (n=1), and *RHCE-D(2-6)-CE* (n=1).

Discussion - For the first time, we report the molecular basis of the D-- phenotype in the Indian population. Identification and characterisation of *RHCE*-null variants by molecular methods can help resolve transfusion-related problems in these individuals. Family studies of index cases helped to identify rare blood donors and offer counselling to females of child-bearing age on the complications involved in such pregnancies.

Keywords: *rare blood group, RHD variants, D--, rearrangement, RHD-RHCE hybrid.*

INTRODUCTION

The Rh is the only known protein-based blood group system with more than 50 antigens[1]. The two *RH* genes, namely *RHD* and *RHCE*, hold >90% sequence similarity and are separated by 30 kilobases (kb) with an interspaced *TMEM*50A gene[2,3]. The *RHD* gene encodes only for D antigen. Conversely, the polymorphic *RHCE* gene encodes the antithetical C and c antigens on the one hand, and the antithetical E and e antigens on

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the other[4]. Alloantibodies produced against these five Rh antigens (D, C, c, E and e) are known to be involved in haemolytic disease of the foetus and newborn (HDFN) and haemolytic transfusion reaction (HTR)[5-9].

Beyond the eight standard Rh haplotypes (i.e., *DCe, Dce, dce*, etc.) found at various frequencies in different populations[10], rare Rh phenotypes, such as Rh_{null} and D--, encoded by unusual variant haplotypes, are rarely encountered in routine blood bank practice[11]. Rh_{null} phenotype is characterised by a complete absence of the D, C, c, E, and e antigens[12]. In the D-- phenotype, Rh antigens encoded by the RhCE protein, including C, c, E, and e, are all absent with a typical exalted D antigen expression to the extent that IgG anti-D reagents can agglutinate the red blood cells (RBCs) in the saline phase[10].

In developing countries, such as in India, blood banks do not usually type for C, c, E and e antigens on a routine basis[13]. Thus D-- phenotype is commonly detected during antibody screening/identification or crossmatching in individuals who have developed alloantibodies against RhCE antigens or in a woman with a problematic obstetric history[14,15]. If sensitised with missing Rh antigens, these individuals form anti-Rh17 (or anti-Hr_0) alloantibody[16]. Therefore, given the scarcity of compatible donors, an individual with this rare phenotype poses a challenge to transfusion medicine. Conversely, blood units from donors with such rare phenotypes are the most valuable for transfusion purposes. Since the first report of D-- in 1951[17], several cases have been reported involving various mutational mechanisms in *RHCE*, including single nucleotide variations (SNVs), such as substitutions, insertions/deletions, and splice site mutations, and structural variants (hybrid genes)[10]. Here, we report for the first time the molecular bases of D-- phenotype in the Indian population.

MATERIALS AND METHODS

Patients and serological analysis

Ten RhD positive postnatal women with recurrent foetal loss were referred to the Department of Transfusion Medicine (ICMR-NIIH, Mumbai, India) for detailed laboratory evaluation from 2016 to 2019. Informed consent was obtained from all the subjects. ABO blood group was determined with both forward and reverse grouping. Samples were typed for D, C, c, E, and e antigens with antisera from four different manufacturers (Bio-Rad Laboratories, Irvine, CA, USA; Immucor Inc., Norcross, GA, USA; Tulip Diagnostics, Goa, India; Alba Bioscience Ltd., Penicuik, Scotland, UK). Absence of RhCE antigens was further confirmed by ether-elution technique. Direct antiglobulin test (DAT), and antibody screening and identification were performed according to the manufacturers' instructions. The family members of the index cases available for extended investigation during the study period were also screened for the D-- phenotype.

Flow cytometric studies

Flow cytometric analysis for the expression of Rh antigens was carried out on a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA, USA) with the use of blood grouping monoclonal IgM anti-D, anti-C, anti-c, anti-E, anti-e reagents (BioRad, Diamed GmbH, Cressier, Switzerland) and in-house human polyclonal IgG anti-D. Anti-human globulin produced in goat (FITC-labelled polyvalent anti-human immunoglobulin [G, A, M], Sigma-Aldrich GmbH, Schnelldorf, Germany) was used as a secondary antibody. Appropriate control RBCs (R_1R_2, R_1r, R_2r, R_1R_1, R_2R_2, rr) were selected according to the Rh antigen being analysed. The test was carried out according to the procedure previously described[18].

Molecular typing

DNA was extracted using the commercially available FlexiGene® DNA Extraction Kit (QIAGEN®, Hilden, Germany). Polymerase chain reaction using sequence-specific primers (SSP-PCR) was initially performed to confirm the absence of C, c, E and e antigens at the molecular level[19]. Copy number variations (CNVs) within the *RHD* and *RHCE* genes were further investigated by the quantitative multiplex PCR of short fluorescent fragments (QMPSF) according to the method described previously[20]. Briefly, all the exons were co-amplified by multiplex PCR in two separate tubes (i.e., one per gene) using FAM-labelled universal primers. Two control primer sets targeting the *HFE* and *F9* genes were used along with exon-specific primers. The amplified products were run by capillary electrophoresis (Genetic Analyzer 3730xl, Applied Biosystems, Waltham, MA, USA) using the GeneScan 500 LIZ dye (Thermo Fisher Scientific, Waltham, MA,USA) as the size standard. Output data were analysed with GeneMapper software (Thermo Fisher Scientific). Presence of any sequence variations within the *RHCE* gene was assessed by the conventional Sanger sequencing method as previously described[21].

RESULTS

Ten cases with a history of consecutive pregnancy loss and anaemia were referred to our institute for antibody identification. The first delivery of all except one woman was normal. However, during the second pregnancy, every foetus developed severe anaemia and jaundice. In one case, the first child died of HDN as the woman had been transfused with an ABO-compatible, Rh D-positive RBC unit in her childhood.

All referred samples displayed normal forward grouping, but reverse grouping showed the presence of an alloantibody. When tested with different clones of anti-D, -C, -c, -E and -e reagents, all samples only showed the presence of D antigen, suggesting that all CcEe antigens were absent. While performing RhD typing with IgG antisera, agglutination was observed in the saline phase depicting the exalted expression of D antigen. To confirm whether any Rh CcEe antigens were present in a weaker form, all samples along with known RBC controls (R_1R_1, R_1r, R_2r and rr) were adsorbed separately with commercial anti-C, -c, -E, and -e antibodies. After adsorption, ether-elution was performed to remove any bound antibody. In all cases, adsorption/elution test on patients' RBCs confirmed the total absence of Rh CcEe antigens, while controls showed positive reaction (*data not shown*). Antibody study showed panagglutination reaction, suggesting the presence of alloantibodies. In all cases, DAT was negative. The antibody titre in D-- cases ranged from 1: 4 to 1: 64 in IgM phase and 1: 64 to 1: 512 in IgG phase. Family history revealed that, in four families at least, the parents had consanguineous marriages. Out of 41 family members of seven probands, three siblings showed absence of Rh CcEe antigens. Detailed serological studies indicated that all ten women and their three siblings belong to the very rare D-- blood group.

Flow cytometry analysis also showed absence of the CcEe antigens on proband RBCs, while control RBCs with $r_yr/r'r''$ phenotype were positive (**Figure 1**). In addition, exalted expression of D antigen in a homozygous D-- individual was observed when compared with R1R1 or heterozygous D-- controls (R_2/D-- and R_1/D--). Overall, flow cytometry analysis confirmed the D-- phenotype.

While molecular screening by SSP-PCR specific for polymorphic sites encoding C/c and E/e antigens was negative, the presence of CNVs in *RHD* and *RHCE* potentially defining hybrid genes was investigated by QMPSF as described previously. In all index cases (n=10), gene conversion events between *RHCE* and *RHD* were shown, defining the *RHCE-D(3-9)-CE* (n=8), *RHCE-D(3-8)-CE* (n=1), and *RHCE-D(2-6)-CE* (n=1) hybrid genes resulting in the rare D-- phenotype when present at the homozygous state (**Figure 2**). When a total of 41 family members of the seven probands were screened, three more individuals with the D-- phenotype were identified; all presented with the *RHCE-D(3-9)-CE* rearrangement at the homozygous state in accordance with the variant allele identified in their respective families (**Table I**).

A detailed family study of two probands was further performed. CNV analysis of their parents revealed the

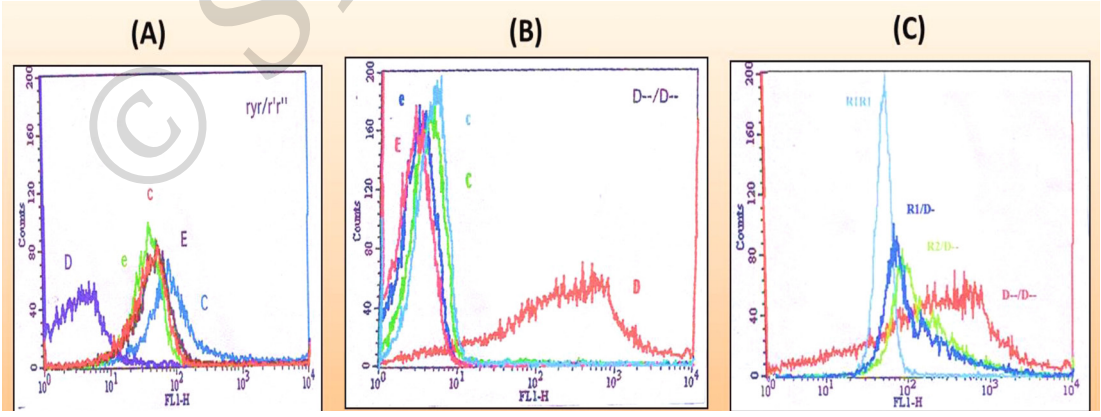

Figure 1 - Flow cytometric analysis
(A) Positive control ($r_yr/r'r''$) for C, c, E, and e antigen expression. (B) Individual with a D-- phenotype showing the absence of the C, c, E, and e antigen expression. (C) Homozygous D-- individual showing exalted D antigen expression when compared with R1R1 individual and heterozygous D-- individuals.

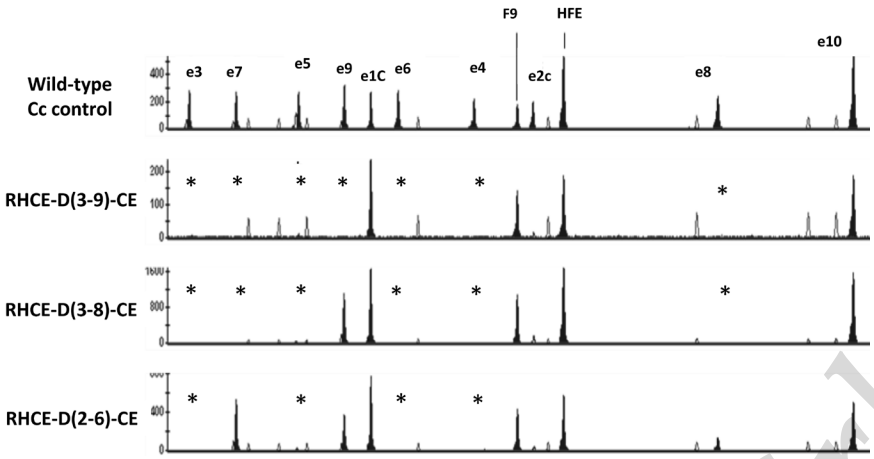

Figure 2 - RHCE QPMSF analysis in Indians with a D—phenotype
*Undetected RHCE exons.

Table I - *Summary of serology, molecular and family studies of index D-- cases*

Case n.	ABO blood group and Rh phenotype	Molecular background	N. of family members screened	Family member with D--/D--phenotype
1	B D--/D--	*RHCE-D(3-8)-CE*	0	0
2	A D--/D--	*RHCE-D(2-6)-CE*	5	0
3	O D--/D--	*RHCE-D(3-9)-CE*	9	0
4	B D--/D--	*RHCE-D(3-9)-CE*	8	1 (AB D--/D--)
5	B D--/D--	*RHCE-D(3-9)-CE*	4	0
6	A D--/D--	*RHCE-D(3-9)-CE*	3	1 (B D--/D--)
7	B D--/D--	*RHCE-D(3-9)-CE*	9	1 (B D--/D--)
8	A D--/D--	*RHCE-D(3-9)-CE*	0	0
9	B D--/D--	*RHCE-D(3-9)-CE*	0	0
10	A D--/D--	*RHCE-D(3-9)-CE*	3	0
Total			41	3

presence of the *RHD* gene at the homozygous state with a single functional copy of the *RHCE* gene. As in the parents, some of the siblings were found to carry the D-- haplotype at the heterozygous state (**Figure 3**).

DISCUSSION

The proximity and tail-to-tail orientation of the highly homologous *RHD* and *RHCE* genes promote gene rearrangement during crossover[3]. Beside numerous SNVs in both genes, the genetic mechanisms involved in the expression of Rh variants include deletion or rearrangement of some or entire coding regions between these two genes. Some of the Rh-deficient phenotypes, including Rh_{null} and D--, are the outcome of this phenomenon. Most potent forms of Rh deficiency, which may cause RBC disorders (stomatocytosis, spherocytosis, etc.), are Rh_{null} (complete absence of the RhD and RhCE proteins at the RBC surface) and Rh_{mod} (severe decrease in the expression of both proteins)[22]. Other haplotypes causing Rh deficiency lacking E/e antigens include Dc-, DC^{w}-, D--, D··, and DIV(C)-[10].

The D-- phenotype is characterised by the lack of expression of C, c, E and e antigens at the surface of the RBCs. This phenotype is strongly linked to consanguinity and has been found in diverse populations with an estimated frequency of 0.0005, 0.0047, and 0.0032, in Sweden,

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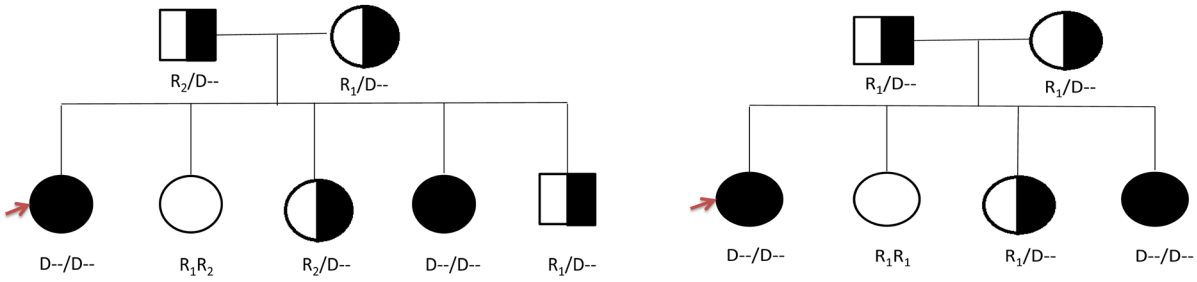

Figure 3 - Pedigree analysis of two families with two D-- cases showing the presence of heterozygous D-- allele in parents
Arrows: index cases.

Iceland, and Japan, respectively[10]. While incidence of the D-- phenotype in Indians has not been studied so far, not a single case of Rh-deficient phenotype has been discovered in our institute (i.e., ICMR-NIIH, Mumbai, India) after typing more than 30,000 individuals for Rh antigens, which emphasises the rarity of these blood groups in India. Ethnic diversity was observed in individuals with D-- phenotype. Five D-- cases belonged to Muslims and the remaining five individuals were from the Hindu community. Distribution according to geographical region showed that the majority of the cases (n=5) were reported from the western part of India, followed by north (n=2), south (n=2) and east (n=1) part of the country. Although the molecular basis of D-- phenotype expression remained unidentified in a few reports[23,24], different mutational mechanisms that alter the RHCE locus resulting in this rare phenotype have been reported (**Table II**). These mutations include: non-functional hybrid genes (as identified here)[24-30], large deletions[31,32], and SNVs[30,33]. As hybrid genes have been commonly reported, QMPSF analysis was carried out preferentially for the molecular characterisation of D-- individuals.

Table II - *Molecular mechanisms of individuals with D-- phenotype in this study and in various populations*

***RHCE* allele**	**Origin/ethnicity**	**Reference**
RHCE-D(3-9)-CE	Indian	This study
RHCE-D(3-8)-CE	Indian	This study
RHCE-D(2-6)-CE	Indian	This study
Apparent wild-type *RHCE*	Caucasian (France)	Chérif-Zahar B *et al.*, [23]
Apparent wild-type *RHCE*	Native Americans (Iroquois)	Kemp TJ *et al.*, [24]
RHCE-D(3-9)-CE	Caucasian (Italy, Iceland, UK)	Kemp TJ *et al.*,[24]
RHCE-D(3-8)-CE	Caucasian (Italy, UK)	Kemp TJ *et al.*,[24]
RHD(1-9)-CE	Caucasian (Italy)	Chérif-Zahar B *et al.*,[25]
RHCE-D(3-7)-CE	Caucasian	Cheng GJ *et al.*,[26]
RHCE-D(3-7)-CE	East-Asian (Japan)	Okuda H et al.,[27]
RHCE-D(3-8)-CE	Asia (Malaysia)	Flatt JF *et al.*,[29]
RHCE-D(4-9)-CE	Asia (Malaysia)	Flatt JF et al.,[29]
RHCE*Ce(c.87insT)	Caucasian	Ochoa-Garay G *et al.*,[30]
RHCE*Ce(c.148+5G>A)	Caucasian (Turkey)	Ochoa-Garay G *et al.*,[30]
RHCE*cE(c.221G>A)	Caucasian (Cajun)	Ochoa-Garay G *et al.*,[30]
RHCE-D(3-8)-CE	East-Asian (South-Korea)	Ochoa-Garay G *et al.*,[30]
RHCE-D(3-9)-CE	Hispanic	Ochoa-Garay G *et al.*,[30]
RHCE*cE(c.907delC)	Hispanic	Westhoff CM *et al.*,[33]

Three large rearrangements that have already been reported previously were observed: *RHCE-D(3-9)-CE*, *RHCE-D(3-8)-CE*, and *RHCE-D(2-6)-CE*. In our study, the RHCE-D(3-9)-CE hybrid allele, also found in Caucasians[24,30], was the most common(n=11) cause of the D-- phenotype.

The *RHCE-D(3-8)-CE* hybrid allele has been reported in Europe and in individuals of Malaysian D-- origin[24,29]. Finally, we observed the *RHCE-D(2-6)-CE* rearrangement in one index D-- case. A similar rearrangement had previously been observed in an individual with the D·· (Evans) phenotype[26]. Additional testing will be carried out to conclusively confirm the phenotype in this latter individual.

Interestingly, family screening of index cases helped to identify three more D-- members. These members can be registered as future blood donors as they have not developed any alloantibody. Unfortunately, family members of three D-- index cases could not be contacted.

CONCLUSIONS

Molecular mechanisms involving gene rearrangement between *RHD* and *RHCE* is responsible for D-- phenotype in Indians. We found QMPSF to be an invaluable tool in detecting Rh variants formed by hybridisation. Family studies should be performed to detect similar variants in other family members who could be blood donors for an individual with this rare phenotype. Identification of rare phenotypes and their characterisation by molecular methods can help resolve transfusion-related problems in such individuals. This warrants the need to develop a national rare donor registry for better transfusion management of transfusion-dependent patients.

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AUTHORSHIP CONTRIBUTIONS

SK and YF designed the research study. SK, DG, AKB, SPS, AC, and RS contributed to sample management. GM, HG, DP and YF carried out the experiments. SK,GM and YF wrote the manuscript. MM and CF revised and approved the manuscript.

The Authors declare no conflicts of interest.

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