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A novel *RHCE*ce 48C, 733G* allele with nucleotide 941C in exon 7, encodes an altered red cell e antigen

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Abstract

Background—Several *RHCE*ce* alleles have in common a 733C>G (Leu245Val) change. Some encode an altered expression of e on RBCs and individuals with such RBCs can make e-like alloantibodies. The identification of an apparent anti-hr^B in the serum of an E–e+ African American patient prompted us to analyze her DNA, which revealed a novel RHCE*ce allele. We also screened blood samples from African Americans to determine the frequency of the novel allele.

Methods—Hemagglutination tests and molecular analyses were performed by standard procedures.

Results—Analysis of the proband's DNA revealed *RHCE*ce* 48C/C, 733G/G, 941T/C, and 1006G/T. Of 272 samples from African Americans, 257 were *RHCE*941T/T* (wild type), and 15 (6%) were *RHCE*941T/C*. Of these 15, 14 were *RHCE*ce/ce*; ten with 733C/G, and four with 733G/G; and one was *RHCE*ce/cE*, 733C/G. Cloning experiments confirmed the nucleotide 941 change and showed that 48C, 733G, and 941C were carried on the same allele. RBCs from the 15 samples carrying the RHCE*941C variant typed V/VS+ and hr^{B+W}.

Conclusion—This study identifies a novel allele, *RHCE*ce* 48C, 733G, 941C, which is predicted to encode 16Cys, 245Val, and 314Ala and was shown to encode c, V/VS, and an altered expression of e and hr^B antigens. The clinical significance of the antibody found in the proband is not established because E+e– RBC components were transfused to the patient. The novel *RHCE*ce* 48C, 733G, 941C allele was present in 5.5% of samples from African Americans and thus, in this small cohort, it had a frequency of 0.028.

Keywords

Blood groups; Rh blood group system; blood transfusion; partial antigen

Introduction

The RH locus is the most polymorphic of those encoding the antigens of the 30 blood group systems and antigens in the Rh blood group system are highly immunogenic. Two

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homologous genes, *RHD* and *RHCE*, each spanning 10 exons encode, respectively, the RhD and RhCE proteins.1 Nearly 50 different antigens, in addition to the five major Rh antigens (D, C, E, c, e), are encoded by these genes. The Rh blood group system is further complicated by the existence of partial antigens. Alterations in *RHD* and *RHCE* can arise from nucleotide changes or gene rearrangements and may encode a variant protein with qualitatively or quantitatively altered expression of a blood group antigen. Some variant proteins express a low-prevalence antigen that serves as a useful marker for the variant. Individuals whose RBCs express a so-called partial blood group antigen can produce an alloantibody to the portion(s) of the antigen lacking on their RBCs. Although part of the antigen is lacking, in the hybrid proteins carrying such partial antigens, the amino acids normally present are replaced with, typically, those encoded by the other RH gene; i.e., RhD amino acids are often replaced by amino acids from RhCE and vice versa. Numerous partial e antigens have been described.1 Several are encoded by RHCE*ce alleles that have in common a 733C>G change (Leu245Val). Individuals, predominantly African American, with such alleles have an altered expression of the e antigen on their RBCs and can make alloanti-e or anti-hr^B, which are not necessarily mutually compatible.2 Some people with partial e antigens have a broader immune response and make antibody to a high prevalence Rh antigen that is lacking only their RBCs and from those of other people with the same variant or from Rh_{null} or D- - RBcs. For example, *RHCE*ceS(340)* does not encode the high prevalence antigen CEST (Rh57),3 and *RHCE*ceCF* does not encode the high prevalence antigen CELO (Rh58).4 The respective anti-CEST and anti-CELO, both produced as a result of the presence of altered e antigens, are not mutually compatible

The identification of an apparent alloanti-hr^B in the serum of an E–e+ African American patient prompted us to analyze her DNA, to determine whether the e-like antibody was an alloantibody or an autoantibody. This analysis revealed a novel RHCE*733G allele. We then studied the frequency of the novel allele in a cohort of African Americans and found the altered RHCE allele in 15 of 257 samples.

Material and Methods

Samples

Blood samples from an African American patient with E–e+ RBCs and an apparent anti-hr^B in her serum, and from 272 African Americans [217 donors and 55 patients with sickle cell disease (SCD)] were collected according to approved Institutional Review Board protocols.

PCR, PCR-RFLP, and RHD zygosity

Genomic DNA was prepared from 200 μL of the buffy coat layer of peripheral blood using a DNA extraction kit (QIAamp DNA Blood Mini Kit, Qiagen, Valenica, CA, USA). The region of *RHCE* that includes and flanks exon 7 was amplified using *RH* specific oligonucleotide primers, CEInt6F (5′-ccattgatgtgagtacacattc-3′) and CEInt7R (5′-ctggacataatttctgaataaatc-3′) (Invitrogen, Carlsbad, CA). Five microliters of DNA per reaction were amplified by 5U *Taq* DNA polymerase (HotStarTaq Master Mix Kit; Qiagen) in a 50 μL final reaction mixture containing 3.0 mM $MgCl_2$, 1X PCR buffer, 0.2 mM dNTPs, and 100 ng of forward and reverse primer. Amplification was achieved over 35 cycles with an annealing temperature of 58°C and a final extension time of 10min. The 456 bp amplicon was then treated using ExoSAP-IT (USB, Cleveland, OH) prior to direct sequencing of the amplicon and cloning of the amplicon (GENEWIZ, Inc., South Plainfield, NJ).

RHCE was further analyzed by amplification of exon 1, followed by *Hph*I-RFLP for 48G>C detection; and amplification of exon 5, followed direct sequencing to detect the 733C>G

polymorphism. Allele-specific PCR was performed to determine the 1006G>T polymorphism. *RHD* zygosity was determined by assaying for the presence of the hybrid Rhesus box and was confirmed by *Pst*I-RFLP.5,6

RNA extraction and Rh cDNA cloning and sequencing

RNA was isolated from reticulocytes using standard techniques (TriZol and PureLink Micro-to-Midi Total RNA Purfication System, Invitrogen, Carlsbad, CA). Reverse transcription was carried out with gene-specific *RHD* and *RHCE* primers as published previously7 and Superscript III, according to manufacturer's instructions (Supercript III First Strand Synthesis SuperMix, Invitrogen, Carlsbad, CA). PCR amplification was carried out as described previously with primers cRHx1F and cRHx5R to amplify exons 1–4; and cRHx4F and cRHx10R to amplify exons 5–10 in *RHD* and *RHCE* cDNA7 using HotStarTaq Master Mix Kit (Qiagen). PCR products were checked for purity on agarose gels, cleaned using ExoSAP-IT (USB Corporation) according to manufacturer's instructions and directly sequenced (GENEWIZ Inc).

An additional PCR product was generated from one sample for cloning purposes. Primers InternalRHx1F (5′-ctctaagtacccgcggtctg-3′) and InternalRHx6R (5′-ggagaagggatcaggtgaca-3′) were used on *RHCE* cDNA to amplify exons 1–6 using HotStarTaq Master Mix Kit as above. This product served to determine linkage of the 48C and 733G variants. The overlapping product encompassing exons 5–10 in this sample were generated as described above for cloning to determine linkage of 733G and 941C. PCR products were cloned, and sequenced with either InternalRHx6R or cRHx4F, respectively, by GENEWIZ, Inc. Sequences were aligned and analyzed using Sequencher v4.8 (GeneCodes, Ann Arbor, MI).

Hemagglutination tests

Hemagglutination tests were performed by standard procedures using tubes or gel cards (MTS, Ortho Clinical Services) by the technique that was most suitable for the reagent being used. Monoclonal antibodies were kindly provided by Malcolm Rhodes and Mikoto Uchikawa. Commercial reagents were from various manufacturers and polyclonal antibodies were from our collection of rare reagents.

Results

The proband's serum contained an anti-e-like antibody reactive ($2+^{W}$) in the indirect antiglobulin test with enhancement media (albumin, LISS, PEG) and with papain pre-treated panel RBC samples. As her serum was non-reactive with e+, hr^{B}– RBC samples (n = 7), it was concluded that the reactivity was anti-hr^{B}. The proband had been recently transfused and her RBCs were positive ($2+^{W}$) in direct antiglobulin test, which prevented meaningful typing for e, hr^{B}, and hr^{S} to attempt to determine whether this was an alloantibody or an autoantibody and thus DNA-based testing was performed.

PCR-RFLP and AS-PCR analyses of DNA from the proband showed *RHCE*ce* 48C/C, 733G/G, 941T/C, and 1006G/T [only differences from the reference *RHCE*ce* (Accession number NM_020485) are given]. The 941T/C change in exon 7 was found by sequencing the PCR amplicon from gDNA *RHCE* exon 7. Cloning of the gDNA from the proband demonstrated 6 clones having *RHCE*941T* and 4 clones having *RHCE*941C*. The *RHCE*941C* variant ablates the restriction enzyme site, *Set*I. However, repeated attempts to digest the PCR amplicon using this enzyme led to degradation of the PCR product.

To determine the frequency of the *RHCE*941C* variant allele, we reviewed sequence data from cDNA samples from 272 black African Americans (217 donors; 55 patients with SCD)

that had been collected previously for other purposes. Of these samples, 257 (94.5%) were *RHCE*941T/T* (wild type) and 15 (5.5%) were *RHCE*941T/C*, representing an allele frequency of 0.028 The interpretation of the most likely RHCE allele combinations for these samples are given in Table 1.

Sequencing of cloned cDNA products from one *RHCE*ce, 48G/C, 733C/G, 941T/C* donor sample (RFA011) confirmed the nucleotide 941T>C change and showed that this nucleotide change was carried on the same allele as nucleotides 48C and 733G. PCR products spanning exons 1–6 contained the 48bp position as well as the 733bp and showed linkage of 48C and 733G on seven clones, and 48G and 733C (wild-type) linked on three clones. Overlapping PCR products from the same sample, but spanning exons 5–10, were also cloned and showed linkage of 733G and 941C on six clones and 733C and 941T on four clones. We conclude that the variant allele carries three nucleotide changes from the reference sequence, i.e., 48C, 733G, and 941C. The novel allele is named *RHCE*ce 48C, 733G, 941C*.

Analysis for *RHD* in the 15 samples (30 alleles) with *RHCE*941C*, showed *RHD* (n = 21 alleles), *RHD*DAU0* (n = 5 alleles), deleted RHD (n = 2 alleles); and 2 alleles that are still under analysis.

RBCs from the 15 *RHCE*941T/C* samples were tested by hemagglutination with anti-e reagents [in date commercial reagents (n = 4), monoclonal reagents (n = 9)], anti-V/VS, anti-hr^S, and anti-hr^B. The four commercial reagents and eight of the monoclonal anti-e (MS16, MS17, MS21, MS62, MS63, HIRO38, HIRO41, HIRO42) agglutinated RBCs from all samples. The ninth monoclonal anti-e, MS19, agglutinated RBCs from the nine samples but did not agglutinate four other RBC samples (see Table 1). The 15 RBCs samples were agglutinated by polyclonal anti-V/VS and anti-hr^S. All samples were agglutinated by a polyclonal anti-hr^B (NYBC 07-007) (Table 1). In tests using the proband's serum, of two ABO-compatible RBC samples, one from a donor with *RHCE*ce 733G in trans* to the *RHCE*941C* (RFA100) were weakly reactive, and one from a donor with *RHCE*cE in trans* to the *RHCE*941C* (RFA131) was non-reactive. These results provide further information that anti-hr^B are heterogeneous. Collectively, these results show the RHCE*ce 48C, 733G, 941C allele encodes a Rhce protein expressing c, V/VS, and hr^S antigens, an altered e, and a possible partial hr^B. Unfortunately, the proband was lost to follow-up, thus other rare Rhce variants could not be tested.

Conclusion

This study identified a novel allele, *RHCE*ce 48C, 733G, 941C* in a E– e+ patient who made an anti-hr^B. This allele is predicted to encode 16Cys, 245Val, and 314Ala in an Rhce protein expressing c, V and VS antigens, as well as altered expression of e and hr^B antigens. This is another example of an *RHCE*ce* with 48C and 733G changes that encodes an Rhce protein with altered e and hr^B antigens. This variant *RHCE*ce* differs from others previously described with 733G such as *RHCE*ceS* and *RHCE*ceCF* due to the presence of an *RHCE*941C in cis*.

Hemagglutination studies confirmed the predicted antigen typings; notably, monoclonal anti-e MS-19 showed that the e antigen was altered. The interpretation that the patient produced an alloanti-e-like antibody, or more precisely alloanti-hr^B, is consistent with the DNA test prediction. It is apparent that the anti-e-like antibodies made by individuals with different forms of the RHCE*ce 48C, 733G allele *in trans* to either the same allele (i.e., homozygote) or to *RHCE*cE* are not necessarily mutually compatible. Further investigations are required to clarify these complex antibody specificities.

The clinical significance of this antibody could not be established because serologically compatible, E+e– RBC components were transfused to this patient. The *RHCE*ce* 48C, 733G, 941C allele is not rare; it was present in 5.5% of samples screened in our African American cohort, indicating a frequency of 0.028.

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Table 1

RHCE alleles in the proband and 15 African American samples and reactivity of their RBCs with monoclonal anti-e MS19 and polyclonal anti-hr^{B}

Number of samples	RHCE* alleles	Reactivity with MS19	Reactivity with anti-hr^{B}
3	*ce 48C, 733G, 941C/ce*	1+ to 2+	1+ to 2+
5	*ce 48C, 733G, 941C/ce 48C*	1+ to 2+	1+ to 2+
4	*ce 48C, 733G, 941C/ce 733G*	0 (n = 3*)	1+ to 2+
2	*ce 48C, 733G, 941C/ce 254G*8	1+	1+
1	*ce 48C, 733G, 941C/cE*	0	1+
Proband	*ce 48C, 733G, 941C/ce 48C, 733G, 1006T*	Not tested	Not tested

* only 3 samples could be tested