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The low prevalence Rh antigen STEM (RH49) is encoded by two different RHCE*ce818T alleles that are often *in cis* to RHD*DOL

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Abstract

Background—STEM (RH49) is a low prevalence antigen in the Rh blood group system. A scarcity of anti-STEM has precluded extensive study of this antigen. We report that two alleles with a *RHCE*ce818C>T* change encode a partial e, and a hr^S–, hr^B+, STEM+ phenotype and that both alleles are frequently *in cis* to *RHD*DOL1* or *RHD*DOL2*.

Materials and methods—Blood samples were from donors and patients in our collections. Hemagglutination, DNA and RNA testing was performed by standard techniques.

Results—Fourteen STEM+ samples were heterozygous *RHCE*ce818C/T*: six had *RHCE*ceBI* and eight had a novel allele, *RHCE*ceSM*. Eleven were heterozygous for *RHD*DOL1* or *RHD*DOL2*. Eleven samples, previously typed STEM–, had *RHCE*ce818C/C* (consensus nucleotide). RBCs from informative STEM+ samples were e+/- hr^S– hr^B+. One person who was heterozygous *RHCE*ceBI* and *RHCE*ceE* had an anti-e-like antibody in her plasma, and one person, who was hemizygous for *RHD*DOL2* had anti-D in her plasma.

Conclusions—We show that two alleles with a *RHCE*ce818C>T* change (*RHCE*ceBI* and *RHCE*ceSM*) encode a hr^S– hr^B+ STEM+ phenotype. In addition, both alleles are frequently *in cis* to *RHD*DOL1* or *RHD*DOL2* and *RHCE*ceBI* encodes a partial e antigen. In the small cohort of samples tested, *RHD*DOL* invariably traveled with *RHCE*ce818T*. Our study also confirmed the presumption that *RHD*DOL2*, like *RHD*DOL1*, encodes a partial D antigen and the low prevalence antigen DAK.

Keywords

Rh blood group system; RHCE variant; partial D phenotype; low prevalence Rh antigen; STEM

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Introduction

STEM (RH49) is a serologically defined low prevalence antigen that was shown, by family studies, to be in the Rh blood group system.¹ A scarcity of monospecific anti-STEM has precluded extensive study of this antigen. An article by Marais and coworkers published in 1993¹ is the sole original report in the literature regarding STEM and anti-STEM. These authors reported that STEM has a variable expression, which is an inherited characteristic, and is associated with an altered e antigen. They also found that RBCs from approximately 65% of hr^S- and 30% of hr^B- South African donors typed STEM+ and that anti-STEM induced mild hemolytic disease of the fetus and newborn (HDFN).¹

RHD and *RHCE* are well studied and numerous variants, and the phenotypes they encode, have been described.²⁻⁴ The high degree of diversity is a consequence of the homology between *RHD* and *RHCE*, their opposite orientation and close proximity on chromosome 1p, which favor the exchange of nucleotides. Some of the variant alleles encode so-called partial antigens, which are identified when an antigen-positive person makes the corresponding alloantibody. Several variant *RHD* and *RHCE* alleles have been shown, especially in African Americans, to be inherited *en bloc*. Examples, represented by the transfusion medicine-friendly allele names that have been proposed by the ISBT Working Party on Red Cell Immunogenetics and Blood Group Terminology (see [ISBT-web.org](http://www.isbt.org)), are *RHD*DAU-RHCE*ceMO*,⁵ *RHD*DIIIa-RHCE*ceS*, and *RHD*DAU-RHCE*ceMO*.⁵ Variant phenotypes can express more than one low prevalence antigen, e.g., *RHD*DIIIa-RHCE*ceS* encodes V, VS, and DAK. Similarly, a low prevalence antigen can be encoded by more than one allele, e.g., DAK is expressed on RBCs with the DIIIa, R^N, or DOL phenotypes that are encoded by *RHD*DIIIa*, *RHCE*CeRN*, *RHD*DOL1*, and *RHD*DOL2*, respectively.⁶

We report here an expansion of our initial findings⁷⁻⁹ that two alleles with a *RHCE*ce818C>T* change encode the e+/- hr^S- STEM+ phenotype. These alleles are (i) the previously reported *RHCE*ceBI*¹⁰ (IBST provisional name *RHCE*01.08*, or *RHCE*ce.08*), which was previously not known to encode a novel low prevalence antigen, and (ii) the novel allele, *RHCE*ceSM*, (ISBT provisional name *RHCE*01.09*, or *RHCE*ce.09*). Both alleles are frequently *in cis* to *RHD*DOL1* (ISBT provisional name *RHD*12.01*) or *RHD*DOL2* (ISBT provisional name *RHD*12.02*). Furthermore, we confirm that *RHCE*ceBI* encodes a partial e antigen, and that, like *RHD*DOL1*, *RHD*DOL2* encodes a partial D antigen and the DAK antigen.

Material and Methods

Samples and hemagglutination testing

Blood samples were freshly collected samples or were recovered from storage in liquid nitrogen. They were either referred for investigation, found in DNA screening tests, or received from numerous colleagues over many years. Reagent RBCs and antibodies were from our collections. Hemagglutination was performed by methods that were optimal for the antibody being used. STEM typings were determined in our laboratories.

Analyses of DNA and RNA

RNA was isolated from the reticulocytes by standard methods using TriZol and PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA). Reverse transcription was carried out with gene-specific *RHD* and *RHCE* primers and Superscript III according to manufacturer's instructions (Superscript III first-strand synthesis SuperMix, Invitrogen), and polymerase chain reaction (PCR) amplification was carried out for 35 cycles with primers to amplify exons 1 to 4 and exons 5 to 10 in *RHD* and *RHCE* as described previously.¹¹ RT-PCR

products were checked for appropriate size and purity on agarose gels, purified using PCR product clean-up reagent according to manufacturer's instructions (ExoSAP-IT, USB Corporation, Cleveland, OH), and directly sequenced by GENEWIZ, Inc. (South Plainfield, NJ). Sequences were aligned, and protein sequence comparisons were performed with Sequencher v4.9 (GeneCodes, Ann Arbor, MI).

For those blood samples where RNA extraction failed, and for confirmatory testing, genomic DNA was isolated using the QIAamp DNA Blood Mini Kit (QIAGEN, Inc. Valenica, CA). Samples were tested for *RHCE*ceBI* and *RHCE*ceSM* by analyzing *RHCE*ce818C/T* and *RHCE*ce1132C/G* on PCR amplicons using *RHCE* exon-specific primers as previously described,¹² followed by sequencing by GENEWIZ, Inc. To screen for *RHD*DOL1*, and *RHD*DOL2*, genomic DNA was amplified using specific primers flanking *RHD* exon 4 and exon 8 as previously described,¹² and products were submitted for sequencing to GENEWIZ, Inc to determine the presence of *RHD*DOL1* (nt 509T>C in exon 4) and *RHD*DOL2* (nt 509T>C in exon 4 and nt 1132C>G in exon 8). The *RHD*410T>C* nucleotide change in exon 3 that would discriminate *RHD*DOL3*, was not analyzed.

RHD zygosity analyses were performed to detect the presence or absence of the hybrid box as previously reported.¹³

Results

Two alleles encode STEM and are linked to *RHD*DOL*

RHD and *RHCE* cDNA, prepared from samples with a historic STEM+ or STEM- type, were sequenced. We found that two *RHCE* alleles (*RHCE*ceBI* or *RHCE*ceSM*) and two *RHD* alleles (*RHD*DOL1* or *RHD*DOL2*) were present in the STEM+ samples. We then tested samples from people of African origin for the presence of these alleles using a combination of PCR-based assays (see Material and methods section).

Taken together, a total of fourteen STEM+ samples were tested: all were heterozygous for *RHCE*ce818C/T*: six (including the original index case, Stemper) were *RHCE*ceBI* [ce48C (16Cys), 712G (238Val), 818T (273Val), 1132G (378Val)]¹⁰ and eight had a new allele, which we name *RHCE*ceSM* [ce 48C (16Cys), 712G (238Val), 818T (273Val); so named from the first and last letters of 'STEM']. Although *RHCE*ceBI* has been reported¹⁰, it previously had not been shown to encode the STEM antigen. Of the six samples with *RHCE*ceBI*, three were heterozygous for *RHD*DOL1* [509C (170Thr), 667G (223Val)] and three were heterozygous for *RHD*DOL2* [509C (170Thr), 667G (223Val), 1132G (378Val)]. Of the eight samples with *RHCE*ceSM*, five were heterozygous for *RHD*DOL1*, and three did not have either a *RHD*DOL1* or a *RHD*DOL2* allele (see Table 1).

Four additional samples, that could not be tested with anti-STEM (because the RBCs did not recover from cryopreservation and fresh samples were not available), were heterozygous for *RHCE*ceBI* and heterozygous for *RHD*DOL1* (Table 1). Thus, of the 18 samples with a *RHCE*ce818C>T* change, 12 had *RHD*DOL1*, three (including the original index case) had *RHD*DOL2*, and three did not have a *RHD*DOL* allele (Tables 1 and 2).

Two samples from two South African donors (code names, Roman and Alson) were historically typed as STEM+^w. However, these samples did not have *RHCE*ce818T* or *RHD*509C*, 667G. The reason for this apparent discrepancy could not be determined because our concerted efforts to obtain fresh samples for extensive testing have been unsuccessful and other samples with this unique genotype were not available. Both RBC

samples were non reactive by the direct antiglobulin test. As the STEM typing of both samples was considerably weaker than that of samples with *RHCE*ce818T*, it is highly probable that the agglutination was caused by an additional, but unidentified, antibody in the test plasma and was not due to the anti-STEM. A paucity of plasma containing anti-STEM precluded us from being able to perform absorption and elution studies.

As few samples tested for STEM, either positive or negative, are available, we have included our testing of eleven STEM- samples, which included hr^S- and hr^B- samples; all had the consensus nucleotide, i.e., *RHCE*ce818C/C*. The RHD and RHCE alleles associated with these STEM- samples are given in Table 1. Attempts to perform *RHD* zygosity testing confirmed that discrepant results are frequently obtained in the presence of an RHD*DOL allele and, thus the results are not given.¹⁴⁻¹⁶

STEM+ and variant e antigen

Hemagglutination results given in Table 1 are a compilation of those obtained from historic records and those performed during this study. Accessibility of RBCs, the condition of the RBC sample, or lack of anti-STEM prevented us from being able to test all samples.

RBCs from eight informative STEM+ samples [with a hr^S- haplotype (*RHCE*cE*, *RHCE*ceJAL*, *RHCE*ceAR*, *RHCE*ceEK*, or *RHCE*ceMO*) in trans to the *RHCE*ceBI* or *RHCE*ceSM*] were tested by hemagglutination and found to be hr^S-. In contrast, RBCs from the only two informative samples with a hr^B- haplotype (*RHCE*cE*) in trans to *RHCE*ceBI* were hr^B+. RBCs from the original index case (Stemper) and from another *RHCE*ceBI/RHCE*cE* sample (MA72-09) carried a single dose of the variant e antigen, they were agglutinated by four commercial anti-e and five single clone monoclonal anti-e (MS16, MS21, MS69, HIRO41, HIRO43), but were not agglutinated by three other monoclonal anti-e (MS19, MS62, MS63), indicating that *RHCE*ce818T* encodes an altered e antigen. One person (MA72-09) with *RHCE*ceBI/RHCE*cE* had anti-e-like antibodies in his plasma, providing the first evidence that *RHCE*ceBI* encodes a partial e antigen.

RHD*DOL encodes a partial D

One person (MA170-09) with *RHD*DOL2* (presumed to be hemizygous) had anti-D in her plasma. Given the similarity in D epitope expression between DOL1 and DOL2, the presumption can be made that DOL2 is also a partial D but the presence of anti-D in this patient provides the first actual evidence that this allele encodes a partial D antigen. One person (MG303-00) with *RHD*DOL1* had anti-D in her plasma, confirming that this allele encodes a partial D antigen.

DAK typing

All of 14 STEM+ samples tested with anti-DAK (Akom, Riz, Jep), were positive. Twelve gave strong reactions: nine had *RHD*DOL1*, two had *RHD*DOL2*, one had *RHD/RHD*DIIIA*. Two gave weak reactions: both had the *RHD*weak D type 4.2.2/RHD*D186T* haplotype¹⁷ (Table 1). RBCs with DOL or DIIIA phenotypes have been previously shown to express DAK.⁶ The DAK+ result on RBCs from two samples with *RHD*DOL2* (Stemper and RFA-NYBC221) showed that this allele, like *RHD*DOL1*, encodes DAK. The unexpected reactivity of anti-DAK, albeit weak, with RBCs from the two samples with *RHD*weak D type 4.2.2/RHD*186T* is likely due to the 186G>T change that is predicted to encode Phe instead of Leu at amino acid residue 62, because this change is present on RBCs with the DIIIA phenotype. However, more samples need to be tested to confirm this hypothesis. The RHD*D186T allele is novel and we are reporting it elsewhere.¹⁷

Discussion

We have identified two RHCE alleles, both with a change of nucleotide 818C>T in *RHCE*ce*, that encode the STEM antigen. One allele (*RHCE*ceBI*) has been previously reported but was not known to be associated with STEM,¹⁰ and the other is a novel allele (*RHCE*ceSM*). Of the 18 samples with *RHCE*ce818C/T*, 12 had *RHD*DOL1* and three had *RHD*DOL2* (hereafter collectively called *RHD*DOL*). Thus, in this small cohort of 18 samples, *RHD*DOL* travelled with one of the two *RHCE*ce818T* variants in 15 cases. Three samples with *RHCE*ce818C/T* but not *RHD*DOL* were STEM+ and provided evidence that STEM is encoded by the *RHCE*ce818T* variant alleles and not by *RHD*DOL*. Two of these samples had both *RHD*weak D type 4.2.2* and the novel allele, *RHD*D186T*.¹⁷ The third sample had *RHD/RHD*DIIIa*.

In a separate, previously reported, study, we tested 705 samples that were not serologically typed for STEM and found four had *RHCE*818C/T*.⁹ Of these 705 samples, 70 samples were from patients with sickle cell disease and 220 samples were from blood donors who self-identified as being of African descent. Two of the 220 donors were heterozygous for *RHCE*818C/T* and 1132G (*RHCE*ceBI*); both samples had *RHD/RHD*DOL*. In these 290 samples, no other *RHD*DOL* were found. This shows the alleles encoding STEM are not as rare as previously thought, an impression due in part, no doubt, to the dearth of anti-STEM. Predicting the STEM type of samples by DNA analyses provides a means to have appropriate RBCs available to aid in the identification of anti-STEM, to provide matched blood products to immunized patients, and to overcome the problem of the scarcity of anti-STEM. Furthermore, testing plasma from patients who have been transfused with blood predicted to be STEM+ could reveal a source of anti-STEM and/or anti-DAK.

Our serologic tests indicate that *RHCE*818T* does not produce hr^S and the plasma of one STEM+ person contained an anti-e-like antibody. The exact specificity of this antibody remains to be determined. Other hr^S- RBCs exist, namely those with ceAR, ceMO, and ceEK phenotypes, but there is no evidence that these RBCs are STEM+. The anti-hr^S made by hr^S- STEM- people may not be mutually compatible and are likely to be incompatible with hr^S- STEM+ RBCs, and vice versa. Indeed, Peyrard, *et al.* briefly reported that the antibody made by a *RHCE*ceMO/RHCE*ceMO* person reacted strongly (4+) with RBCs from a *RHCE*ceBI/RHCE*ceBI* person.¹⁸ Neither ceBI nor ceSM express hr^S but, based on two informative samples, do express hr^B. Thus, it is clear why the original study¹ found that approximately 65% of hr^S- samples were STEM+ but it is unclear why it found that approximately 30% of hr^B- samples were STEM+.

Depending on alleles carried on the *in trans* chromosome, patients with the *RHD*DOLRHCE*ce818T* haplotypes can make anti-D, anti-e-like/-hr^S, or anti-Rh18¹⁰ and their RBCs carry the low prevalence antigens STEM and DAK. The original JAL+ proband (J. Allen) has *RHD*D/RHD*DOL1* and *RHCE*ceJAL/RHCE*ceBI* and his RBCs express JAL, STEM, DAK, and extremely weak V and VS.¹⁹ In our relatively small study, two other samples (Delia and Keys) harbored the *RHCE*ceJAL* allele associated with expression of JAL. Our results highlight that many RBCs express multiple low prevalence antigens and this fact should be taken into account when identifying antibodies. From our results, most RBCs expressing STEM also express DAK and a high proportion express V and VS. It is possible that some antibodies have been misidentified and retesting them with a panel of RBC samples from people whose allele makeup has been determined will help to fine-tune the specificity(ies) within a given plasma. Having appropriate RBCs to test would resolve the conundrum of exactly which proteins express DAK.

In summary, our study revealed several new findings: *RHCE*ceBI* and *RHCE*ceSM* encode STEM; on limited testing, *RHCE*ceBI* encodes a slightly stronger expression of STEM than does *RHCE*ceSM*. This is consistent with the previously reported variable expression¹ and provides evidence that STEM is encoded by these variant *RHCE* alleles and not the *RHD*DOL*. *RHCE*ceBI* and *RHCE*ceSM*, which encode a partial e and lack of hr^S are often, although not invariably, *in cis* to *RHD*DOL1* or *RHD*DOL2*. In this study and our previously reported study⁹ in all samples with *RHD*DOL*, *RHCE*ceBI* or *RHCE*ceSM* were found *in cis*.

The variant RhD encoded by *RHD*DOL2*, is a partial D antigen. As anti-STEM is in short supply, typing for this antigen by hemagglutination is virtually impossible; however, DNA-based assays provide a useful tool to predict the presence of STEM. Knowledge of the molecular basis of various Rh antigens should help to not only establish which antigenic determinants are encoded by which alleles but also to accurately establish the specificity of an antibody, or antibodies, in a plasma. In table 1, when possible, the donor code name is used so that others who have these samples in their frozen library can use the knowledge of which alleles are present to aid selection of appropriate RBCs for inclusion on a panel used for antibody identification purposes.

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Table 1
Compiled hemagglutination results and allele assignments on STEM+ and STEM- samples

Name	D	C	E	c	e	hr ^S	hr ^B	V/V ^{S†}	STEM		DAK	RHD*	RHCE*
RHCE*ceBI and STEM+													
Stemper	+	0	+	+	+/0	0	+	0	+	+	+ ^S	DOL2 _D	ceBI ceE
MA 72-09	+	0	+	+	+/0	0	+	NT	NT	+	+ ^S	DOL1 DFV	ceBI ceE
RFA-221	+	0	0	+	+	+ ^w	+	0	NT	+	+ ^S	DOL2 DAU-0	ceBI ce 48C
MA 170-09	+/0	0	0	+	NT	NT	NT	NT	NT	+	NT	DOL2 Hemizygous presumed	ceBI ce 48C
J Allen	+	0	0	+	+	NT	NT	0	NT	+	+	DOL1 D	ceBI ceJAL
MA 372-09 RFLP only	+	NT	0	+	+	NT	NT	+	NT	+	+	DOL1 Hemizygous presumed	ceBI ceS
RH*CEceBI and STEM NT													
MG303-00 RFLP only	+/0	0	0	+	+	NT	NT	0	NT	NT	+	DOL1 Hemizygous	ceBI ce
RFA-075	+	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	DOL1 DIIa-CE(4-6)-D	ceBI ceS
PC73 RFLP only	+	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	DOL1 D	ce818C/T
LC donor RFLP only	+	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	DOL1 D	ce818C/T
RHCE*ceSM and STEM+													
Delia RFLP only	+	0	0	+	+	0	NT	0	+	+	+	DOL1 DAU-0	ceSM ceJAL
Keys	+	0	0	+	+	0	+	0	+	NT	+	DOL1 D	ceSM ceJAL
Hector	+	0	0	+	+	NT	NT	NT	+	NT	+	DOL1 DAU-0	ceSM ceMO
John	+ ^w	0	0	+	+	NT	NT	+	+	NT	+	DOL1 Weak D type 4.2.2	ceSM ceAR

Name	D	C	E	c	e	hr ^S	hr ^B	V/VS [†]	STEM		DAK	RHD*	RHCE*
									SA	NY			
Jack	+ ^w	0	0	+	+	0	+	+	+	+	+	DOL1 Weak D type 4.2.2	ceSM ceAR
KHV RFLP only	+	0	0	+	+	0	+	NT	+	NT	+	DIIla D	ceSM ceEK
Dennis	+	0	0	+	+	0	+	+/-0	+ ^w	+	+ ^w	Weak D type 4.2.2 DI186T (novel)	ceSM ceAR
Sybil	+	0	0	+	+	0	+	+/-0	+ ^w	+	+ ^w	Weak D type 4.2.2 DI186T (novel)	ceSM ceAR
STEM+ but not RHCE*ceBI or RHCE*ceSM													
Roman	+	0	0	+	+	+/-0	0	+V-	+ ^w	+ ^w	+ ^S	DIIla DIIla	ceS ce 48C, 733G
Alson	+	0	0	+	+	NT	NT	V-	+ ^w	NT	+	DIIla, 150C (novel) DIIla, 150C (novel)	ceS ceS
STEM- and not RHCE*ceBI or RHCE*ceSM													
MA127-08	+	+ ^w	0	0	0	+ ^w	0	0	NT	0	+	D D	CeRN CeRN
MA315-09	+	0	0	+	+	NT	0	NT	0	NT	+	DIIla DIIla	ceS ceS
MA156-08	+	0	0	+	+	+ ^w	0	+	0	NT	+	DIIla DIIla	ceS ceS
E. Dlamini	0	+	0	+	+	+	0	+	0 ^{AA}	0	0	DIIla-CE[4-7]-D DIIla-CE[4-7]-D	ceS ceS
M. Shaba	0	+	0	+	+	+	0	+	0	NT	0	DIIla-CE[4-7]-D DIIla-CE[4-7]-D	ceS ceS
MA314-09	0	+	0	+	+	+	0	+	0	0	0	DIIla-CE[4-7]-D DIIla-CE[4-7]-D	ceS ceS
Marcus	+ ^w	0	0	+	+	+	0	+	0	0	+ ^w	DIIlb (DIII type 7) DIIlb (DIII type 7)	ceS ceS
Norma	+ ^w	0	0	+	+	0	+	+	0 ^{AA}	NT	NT	Weak D type 4.2.2 Weak D type 4.2.2	ceAR ceAR
MA318-09 RFLP only	+	+	+	+	+	0	+	+	0	0	NT	Not analyzed	Not ceBI or ceSM
MA149-09	+	0	0	+	+	NT	+	+	NT	0	+ ^w	Weak partial D type 4.0	ce 48C, 733G Not ceBI or ceSM

Name	D	C	E	c	e	hr ^S	hr ^B	V/VS [†]	STEM		DAK	RHD*	RHCE*
									SA	NY			
CO 06-11	0	+	0	+	+	NT	NT	+	0 ^{^^}	NT	0	Not DOL1 or DOL2 Hybrid under investigation	Hybrid under investigation <i>ceS</i> <i>ce</i>

[†] = positive with a reagent containing anti-V and anti-VS
[^] = not available for repeat testing
^{^^} originally thought to be STEM+
RFLP = only PCR RFLP assays were performed. Full sequencing was done on the other samples.
NT = not tested

Table 2

Summary of STEM testing interpretations (no homozygous *RHCE*ce818T* or *RHD*509C, 667G* [*RHD*DOL*] were found) in a total of 31 samples

<i>RHCE*ceBI</i> (n = 10)
6 samples STEM+
6 had <i>RHD*DOL</i>
4 samples STEM not tested
4 had <i>RHD*DOL</i>
<i>RHCE*ceSM</i> (n = 8)
8 samples STEM+
5 had <i>RHD*DOL</i>
3 had other RHD alleles
<i>RHCE*ce818C</i> and <i>RHD*509T, 667T</i> (n = 13)
2 samples STEM+ ^W
2 had <i>RHD*DIIIa</i>
11 samples STEM–

See Table 1 for alleles