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Molecular genetics and clinical applications for *RH*

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Abstract

Rhesus is the clinically most important protein-based blood group system. It represents the largest number of antigens and the most complex genetics of the 30 known blood group systems. The *RHD* and *RHCE* genes are strongly homologous. Some genetic complexity is explained by their close chromosomal proximity and unusual orientation, with their tail ends facing each other. The antigens are expressed by the RhD and the RhCE proteins. Rhesus exemplifies the correlation of genotype and phenotype, facilitating the understanding of general genetic mechanisms. For clinical purposes, genetic diagnostics of Rhesus antigens will improve the cost-effective development of transfusion medicine.

Keywords

Rhesus; blood group; molecular genetics; molecular diagnostic; transfusion

1. Introduction

Molecular biology has been applied extensively in characterizing the genetic basis of blood group systems and developing clinical diagnostic tools for immunohematology and transfusion medicine[1–4]. There are now 51 antigens within the Rh system and more than 200 alleles for the *RHD* gene alone. *RHD* zygosity has been resolved, epitopes have been mapped, and many D variants with altered antigens have been identified. The relationship among the *RH* family members in various species contributes to our understanding of their biological importance [5].

Based on the homology of Rh polypeptides to the ammonia transporter AmpB, computational analyses have modeled the 3D structure of the RhD polypeptide to learn about additional potential functions of Rh polypeptides [6]. The reason for this interest is that *RHAG*, a gene located on chromosome 6 (6p11–p21), shares an identical exon structure and major regions of sequence identity with *RHD*/*RHCE*. Moreover, RhAG is essential for the expression of the Rh polypeptides and was identified in 2008 as the latest blood group system, no. 30, in its own right [7].

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To date, the function of RhD and RhCE appears associated with membrane integrity, and possibly transport of gases like carbon dioxide. On the other hand, the Rh-associated glycoprotein (RhAG) can transport ammonia [8], but whether it does so in red blood cells (RBCs) is debated. Also, RhAG may contribute to gas exchange across the plasma membrane, and its mutations are associated with hereditary stomatocytosis 9. Thus, expression of Rh polypeptides and associated proteins is complex, and molecular discoveries have broadened our understanding of this important blood group system. This review summarizes the progress that molecular analyses have made in furthering clinical applications for Rh.

Three clinically useful discoveries have been made since the cloning of *RHCE* and *RHD*: 1) the molecular basis underlying the common Rh-negative haplotype and the nucleotide polymorphisms associated with the common Rh antigens have been applied to predict risk for hemolytic disease of the fetus and newborn (HDFN); 2) the molecular distinction of partial D and weak D alleles, *DEL*, *RHD*-pseudogenes, and the *RHD*-deleted genome; and 3) the molecular basis of D antigen epitope expression on the RhCE polypeptide.

Recently efforts by a few independent research laboratories have begun to more fully characterize the molecular basis of RhCE variants and the allelic variation of *RHCE*. The results are applied to improve transfusion support for sickle cell disease (SCD, see a separate review in this issue) and to identify the deficiencies of monoclonal antisera in assigning antigen status accurately. It is now apparent that molecular analyses are the most accurate way to define the complex *RH* and other blood group systems. In a steadily increasing number of clinical settings, these molecular approaches facilitate preventing blood group incompatibilities, avoiding alloimmunizations and hemolytic transfusion reactions, and contributing to optimal RBC survival in transfusion-dependent immune disorders.

2. Molecular Basis of *RH*

RH is a bigenic locus comprising *RHD* and *RHCE* positioned in a tail-to-tail orientation toward the end of the short arm of chromosome 1 (p34–36). Another gene, *SMP1*, is interspersed between both *RH* genes in close proximity to the 3' end of *RHCE* (Figure 1) [10]; this minute technical feature was instrumental in resolving the physical structure of the *RH* gene locus [11]. Identification of the single murine equivalent in the mouse genome project provided evidence that *RHCE* evolved from the ancestral *RH* on the basis of the position and orientation of murine genes in the region (Figure 1). Therefore, *RHD* arose from a duplication event that predates modern humans [12]. During the duplication event, and possibly associated with its cause, two approximately 9,000 base-pair-long homologous repeat sequences, termed *Rhesus boxes*, were likely introduced that flank the *RHD* gene in the genomes of modern humans. *RHD* was lost from the genome through unequal crossing over involving the *upstream Rhesus box* and *downstream Rhesus box* (Figure 2), an event that may have occurred more than once. *RHD* and *RHCE* share sufficient sequence homology that RBCs function normally when no *RHD* gene is inherited. Why the *RHD*-deleted genomes have persisted to this day and become more prevalent is the topic of much worthwhile debate and some esoteric speculation [13,14].

More than 200 *RHD* alleles have been reported and may be grouped according to serological and molecular features (Table 1). Most of the alleles harbor either single nucleotide polymorphisms (SNPs) or present as *RHD*/*RHCE* hybrid alleles. The tail-to-tail orientation (Figure 3) may facilitate the large number of alleles; the identification of corresponding nucleotides in both genes suggests that most hybrid alleles arise through gene conversion events. A clinical benefit of the molecular characterization of the *RH* locus is that *RHD* zygosity can be assigned with near certainty. In the past, Rh haplotype tables based on

serological analyses were used to predict the 'most probable genotype' and thus *RHD* zygosity for Caucasians and African Americans.

3. Modeling of Rh Polypeptides

The Rh proteins belong to the ammonium transport (*E. coli* AmtB)/methylammonium permease superfamily. Initially, molecular modeling of both Rh proteins and the RhAG protein on the crystal structure of *E. coli* AmpB suggested that the Rh proteins and RhAG form trimers in the red cell membrane, and may therefore function as ammonium transport proteins [15]. A few independent investigators have shown that RhAG facilitates ammonium transport in yeast and *Xenopus* oocytes without co-transfection of RhD and RhCE polypeptides [8]. Similar ammonium transport activity was shown directly in erythrocytes [16]. However, RhD and RhCE do not appear to transport ammonium or carbon dioxide; key amino acid substitutions in the transmembrane channel do not appear to meet the requirements for facilitating transport [17].

The model of red cell Rh proteins is consistent with the 6 exofacial loops that were first proposed on the basis of computational hydropathy plots [18]. A critical component of the structure of RhD and RhCE is the amino acids that reside in the extracellular Rhesus vestibule of the channel formed by the transmembrane regions (Figure 4) [19]. A few studies have shown that amino acid changes in the vestibule alter the molecular structure sufficiently to make persons susceptible to the formation of anti-D, although their red cells phenotype as Rh-positive [19,20]. Thus, molecular studies have convincingly confirmed the hypothesis of Tippett and coworkers that some D variants lack the expression of some D antigen epitopes and can permit an immune response to the missing parts [21].

4. Molecular Classification of Rh Phenotypes

RHD and *RHCE* share regions of identity, with the translated RhD polypeptide differing at up to 36 amino acid positions depending on which RhCE polypeptide it is compared with. Both Rh polypeptides comprise 12 transmembraneous protein segments and 6 extracellular / intracellular loops (Figure 4). Historically, serologic studies classified the D antigen into six major categories (DII through DVII, with DI being obsolete). Three epitope models were proposed comprising 9-epitopes or 37-epitopes or the combination of both based on the serological reaction pattern of > 80 monoclonal anti-D antibodies [22,23]. Many variants express altered D antigen, but no absolute correlation exists between phenotypic expression and clinical relevance of *RHD* alleles. *RHD* alleles have been classified on the basis of their phenotypic relationship to the molecular variation: partial D, weak D types, DEL, and nonfunctional alleles [24,25].

partial D

The classification of partial D variants is based on the premise that certain amino acid substitutions on an extracellular loop affect linear D epitopes or, more often, the 3-dimensional conformation of that loop. Many partial Ds are identified using monoclonal antibodies that target specific domains or loops on the surface of the erythrocyte [26]. The D categories (DII to DVII) represent a subset of all partial Ds. DII and DVII are caused by single extracellular amino acid substitutions, while DIII, DIV, DV, and DVI are caused by *RHD-CE-D* hybrid alleles and comprise several subtypes each. The classification as partial D is of clinical relevance because carriers often produce anti-D upon exposure to the normal D antigen R [27].

The inclusion of D categories among partial D makes intuitive sense, because they share the common feature of exofacial amino acid substitutions in different spatial arrangements.

However, for many partial D, anti-D immunization events are apparently rare, and for several partial D there has been no observation of any patient with anti-D so far. These facts are compatible with the conclusion that carriers of several distinct partial D may be at a very low or no risk of anti-D immunization.

weak D

A weak D type is a variant of the RhD protein that comprises an amino acid substitution located in the transmembraneous or intracellular segments and expresses a reduced amount of D antigen (generally less than 5,000 D antigens per RBC) [28]. A group of 16 distinct weak D types were described originally, but the total number of weak D types including their subtypes now exceeds 80. The substitutions are thought to cause folding problems during integration of the protein into the RBC membrane, which can impede protein integration, affecting palmitylation or anchoring of the polypeptide to the RBC cytoskeleton 29. Hence, the amount of D antigen expressed on the RBC surface is quantitatively reduced, but the D antigen itself remains, by-and-large, qualitatively unchanged. Therefore, the normal D antigen is not usually immunogenic 28,30–[36].

Like the mentioned exception for several partial D that cannot be immunized, there is an exception for some weak D types. Anti-D immunization in weak D carriers is rare, but there are exceptions: examples include weak D type 15, weak D type 4.2, also known as DAR, and weak D type 7 [37–39]. The weak D types 1, 2, 3, and 4.0/4.1, which are the most prevalent in any European and Caucasian population, represent more than 95% of all weak D types. To date, more than 10 years after their molecular description, the literature has not documented any carrier of weak D types 1 through 4.1 being alloimmunized and producing allo-anti-D. Those observed produce low titer antibodies of autoantibody nature. The observation that the common weak D types fail to make allo-anti-D is particularly relevant in the prevention and management of anti-D alloimmunization in pregnancy, which we will address in detail below.

DEL

A very weakly expressed D antigen is called DEL (formerly D_{el}) because it was originally detected only if anti-D adsorbed and then eluted from RBCs. Thus, the D antigen detection is by elution only (DEL). Typically, RBCs with DEL express 200 or fewer copies of the D antigen per RBC [40].

The most common DEL is caused by the *RHD*(K409K) allele harboring the C1225A nucleotide substitution in exon 9 41. Because it is very prevalent in D negative Asians, it has been dubbed the “Asian type” DEL 36. This substitution is a silent single nucleotide polymorphism (SNP), i.e. the amino acid lysine (K) at position 409 remains unchanged. However, the substitution causes missplicing mRNA such that the complete full messenger mRNA has never been documented and at most represents a very minor form of transcript for translation 42.

Other *DEL* alleles have underlying molecular changes that cause more pronounced effects than in weak D and strongly impede but do not completely abrogate membrane integration [41,43–45]. Even combined, all DEL phenotypes are rare in Europeans. Up to 30 % of seemingly D-negative East Asian people carry the DEL *RHD*(K409K), but other *DEL* alleles are also more frequent in Asia than in Europe.

DEL is of interest worldwide because of its potential to cause anti-D alloimmunization when DEL-positive blood donors are inadvertently labeled as D negative [46]. In addition, *DEL* alleles can cause genotype-phenotype discrepancies and should be taken into consideration when fetal blood group genotyping methods depend on the ethnicity of the parents

31,33,47,[48]. The fetal inheritance of DEL would not be considered a risk of hemolytic disease of the fetus and newborn (HDFN).

D negative

The most common D negative haplotype in all populations is caused by the deletion of the whole *RHD* gene with the concomitant presence of the *hybrid Rhesus box* (Figure 2). However, other D negative haplotypes exist [49,50]. Some individuals who are D negative can harbor a nonfunctional *RHD* allele. One of the first nonfunctional *RHD* alleles was termed *RHD* pseudogene (*RHDψ*). Since that time, several *RHD-CE-D* hybrid alleles have been reported, including *Cde*^{s} with its characteristic hybrid *RHD/CE* exon 3 (Table 1). Both nonfunctional alleles occur rather frequently in African populations. Less common D negative alleles are caused by a host of different hybrid *RHD-CE-D* alleles or nonsense and frame shift mutations 41,[51]. It is important to note that the distinction between apparent D negative and DEL phenotypes by serology may be somewhat arbitrary. But the clinical significance is not: DEL blood transfused to D negative transfusion recipients is immunogenic, and the common "Asian type" DEL is not prone to making anti-D after its carrier is transfused with normal D positive RBC units. Therefore, in Asian populations, in whom D negative blood is rare, identifying DEL transfusion recipients (approximately 1/3 of all serological D negative) could significantly reduce the demand for Rh-negative blood [42,52,53].

Rh_{null}

The lack of both RhD and RhCE proteins may be caused by the inheritance of two nonfunctional *RHCE* alleles in the background of an *RHD* deleted haplotype. This constellation gives rise to the amorph type Rh_{null} phenotype (lack of any Rh protein), in which neither D nor CE antigens are expressed [54,55]. Alternatively, because the expression of either Rh protein requires the presence of RhAG for appropriate assembly on the RBC membrane, defects in *RHAG* alleles cause the lack of both RhD and RhCE proteins. This biological background explains why defects in *RHAG* alleles cause the regulator type Rh_{null} phenotype (lack of expression of Rh protein), in which D and CE antigens may be undetectable but are in principle expressed [56].

Rh_{null} alloimmunization in pregnancy can be extremely difficult to manage in the setting of HDFN, largely due to the lack of compatible allogenic blood. Maternal blood has been used as a source of blood for the fetus and neonate [57].

RhCE variants

Partial antigens have been reported for the common RhCE antigens; C, c, E, and e, although several *RHCE* alleles have been characterized and many other alleles may exist [58–68]. Moreover, as with partial D, carriers of partial CE antigens can make antibodies to epitopes that are missing on the variant RhCE protein. Unlike *RHD*, *RHCE* is not often deleted. Therefore partial CE antigens are less obvious from serology, because they are covered by the regular RhCE protein from the second chromosome. For example, erythrocytes expressing an e variant may be assumed to be E homozygous. Few people carry these variants, which is one reason that alloimmunization is uncommon. The clinical relevance of RhCE variants may be more appreciated once molecular analysis allows deeper insight into their associated immunization events, like, for instance, in sickle cell disease (SCD) patients, in pregnancies, and in chronically transfused patients.

5. *RHD* Phylogeny

The phylogenic study of *RHD* alleles delineated 4 clusters: the Eurasian D cluster with the consensus *RHD* (Genbank mRNA accession NM_016124.3), the most common allele expressed in humans, and three African clusters designated DIVa, DAU, and weak D type 4. Clusters are defined by an allele that differs from the consensus *RHD* allele and comprise many alleles that gained additional amino acid substitutions [69]. As the genetic characteristics are unraveled and we gain more information about *RHCE* alleles (Genbank mRNA accession NM_020485.3) and their linkages to specific *RHD* alleles in haplotypes, the actual phylogenic tree will become more complete and well defined [19].

For example, it is expected that a few distinct *RHD* alleles are associated with *RHCE* alleles lacking hrS or hrB expression, and it may be possible to identify specific non-sister chromatid exchanges between haplotypes through such studies. On the other hand, there are examples of similar alleles that arose from independent gene conversion events within the Eurasian D cluster. Some *DV* alleles appear to have been caused by random or independent molecular events, and for these reasons, the DV group of partial D is not defined as a cluster [70].

6. Population Studies

It is apparent from systematic studies in African populations as well as sporadic observations in alloimmunized patients, as in those with SCD, that the allelic variation among Africans is much larger and more variable than in any other population. The reason for the presence of so many *RH* alleles in Africans remains unknown. Identifying a potential selective pressure or advantage may shed light on the function of Rhesus. The prevalence of distinct alleles in South Africans and West Africans also differs, and has not been fully evaluated, while studies from East Africa are largely lacking.

Europeans and East Asians share a small and overlapping subset of the African alleles called the Eurasian D cluster. The primordial alleles of this cluster are of African origin and are still fairly prevalent in African populations. The Eurasian D cluster may have more known alleles than the other 3 clusters combined but this almost certainly represents an observation bias and hints to the host of yet-to-be discerned alleles in all populations. Arab and Indian peoples represent the largest populations for which *Rhesus* gene polymorphisms have hardly been explored.

More clusters may be characterized, because two “orphan” alleles are known, which may represent the primordial alleles for two novel D clusters. Other topics for further research are the nucleotide sequence diversity beyond the coding region of *RH* alleles and the linkage of *RHCE* alleles to *RHD* alleles.

The prevalence of alleles in the Eurasian D cluster [69] differs widely between the European and East Asian populations. For example, weak D type 15 and 17 are common in East Asians and rare in Europeans, while the prevalent “European” weak D types 1 to 4.1 are rarely encountered in East Asians. A random survey has identified additional diversity within exon 5 of *RHD*, which seems to be the region with the largest allelic variation, but this may be another observation bias. Prudently devised population studies have proved to be instrumental and worthwhile for research in Rhesus and much of its current clinical application, but they are not often chosen for funding.

7. Clinical Applications

Evaluation of anti-D alloimmunization in pregnancies

It is important to recognize that, in spite of our efforts in the last 50 years, anti-D alloimmunization still occurs in 1:2000 D negative pregnancies, a number that seems to defy further reduction. Apart from the lack of appropriate prophylaxis, the reasons for anti-D alloimmunization include the inappropriate administration of prophylaxis, pre-alloimmunization due to maternal-to-fetal transmission of D positive blood, early transplacental passage of fetal cells in the pregnant woman, and administration of DEL positive RBC units. But the source of a potential immunizing event often is not explored and, hence, is unlikely to be recognized. Advances in preventing anti-D alloimmunizations can be realized by ongoing surveillance of anti-D immunization in D positive recipients and identification of the root cause [71].

Prenatal diagnostics

Fetal *RHD* genotyping is performed routinely as part of the management of HDFN. Most of these cases are still caused by anti-D in D-negative women, although it may occur in women with partial D as well [72,73]. The use of amniotic fluid-derived DNA to predict of fetal D and other blood group antigens is very reliable and has been the method of first choice for more than 10 years [74]. Obtaining fetal tissue by amniocentesis avoids the high-risk procedure of taking blood from fetal cord for blood group testing. However, amniocentesis will likely be abandoned in favor of an even safer procedure, testing of fetal DNA derived from mother's peripheral blood [75,76].

Cell-free fetal DNA in maternal plasma

The sensitivity of quantitative or real-time PCR in detecting cell-free fetal DNA in maternal peripheral blood was proposed in 1998 [77,78]. This fetal DNA represents rather small DNA fragments [79,80] found in maternal plasma, derived from fetal cells that are exposed to the maternal circulation. The advantage of cell-free fetal DNA is that this DNA is essentially cleared from maternal blood within hours after birth [81]. Any fetal cell with its cellular DNA that may remain in the mother's circulation for years is not tested at all. Several laboratories in various European countries have successfully implemented this technology to identify pregnancies at risk of HDFN, and algorithms have been devised to withhold RhIg prophylaxis for the *RHD* gene negative pregnancy [82–85].

Decision to administer RhIg in pregnancy [86]

Pregnant women with the prevalent weak D types 1 to 4.1 may be transfused with D positive blood, and there is no indication for RhIg prophylaxis. In fact, the risk of alloimmunization of these weak D types is so low that the potential risks of unknown infectious agents or other adverse effects of RhIg prophylaxis should be considered as equally risky; this limited exposure approach has been taken with neonatal transfusion and to a lesser extent in adults. In terms of cost, *RHD* genotyping performed early in the first pregnancy may spare woman several RhIg exposures and may be implemented at a cost-neutral endpoint [73,87]. Furthermore, utilizing *RHD* genotyping tests would identify women with rare weak D types who are prone to anti-D immunization and would benefit from RhIg prophylaxis. Transfusion medicine guidelines currently do not address this issue in any health care system, but a handy decision tree has been proposed to address this problem from a practical standpoint [88].

Monoclonal anti-D as therapeutics

Recombinant engineering has been used to design anti-D with the same variable region genes, i.e. identical epitope specificity, and Fc portions that do not elicit red cell sequestration, in effect to producing potential 'drugs' to prevention immune destruction of red cells *in utero* by the fetus [89]. Potentially, these or similar anti-D can be produced with recombinant technology and substitute for human blood-derived RhIg preparation [90]. It would be worthwhile to use such drugs in a clinical setting in the not-too-distant future. Further, the use of these molecular techniques is not limited to Rh antigens.

RHD zygosity in HDFN

The accurate determination of zygosity is important in the perinatal care of anti-D alloimmunized women and the study of Rhesus variants. Given today's world migration patterns and inter-racial marriage rates, it is more accurate to evaluate *RHD* zygosity than to rely on limited 'most probable genotype' tables or calculations. Zygosity can be determined on the basis of 1) detection of the *hybrid Rhesus box* harboring the chromosomal breakpoint for the *RHD* deletion 11‚74‚91,[92] and 2) quantitative PCR to determine *RHD* dosage. Both techniques are complementary and require proper controls. Altered *hybrid Rhesus boxes* can confound zygosity as can the presence of non-functional *RHD* alleles. Long-range high-fidelity nucleotide sequencing encompassing a long stretch across the *RHD* breakpoint [11] produces the least error, but it is technically challenging and hardly ever used in clinical applications.

The serological approach used the expression of the common Rh antigens to determine the 'most probable genotype' on haplotype tables published up to the 1970s. This approach should be abandoned, because exact empirical data for local populations are largely lacking and any molecular technique is more specific. Thus, this limitation of accurately assigning *RHD* zygosity was overcome by the genetic characterization of the *RHD*-deleted genome and the discovery of *Rhesus boxes* [11].

Methods that identify the *RHD* deletion, either directly or indirectly, can be used to determine the *RHD* heterozygous father. Therefore, a mother with an allo-anti-D can be assigned either a 50 % or 100% chance of conceiving a D-negative fetus [93]. This genetic information can be used to determine whether to apply either invasive or non-invasive tests to predict fetal inheritance of *RHD*.

Exalted D antigen expression

The lack of expression of the RhCE polypeptide [94] can cause an exalted expression of the D antigen. Expression of D epitopes by hybrid *RHCE-D-CE* alleles is another mechanism. Such RBC represent excellent reagents for anti-D antibody screening. Moreover, when exalted D antigen expression is found serendipitously, the nucleotide change leading to the *RHCE* null allele should be evaluated to gain a better understanding of the types of molecular changes leading to nonfunctional alleles or hybrid *RHCE-D-CE* alleles.

Molecular identification of partial D versus weak D types in patients

Problems with determining Rh status are most often associated with a restricted number of prevalent *RHD* alleles. D discrepancies observed in the transfusion service laboratory include carriers of *RHD* alleles who can be immunized by the normal D antigen [30], but also include alleles that are not known to make anti-D. Therefore, the distinction is clinically relevant. It is important to realize that monoclonal anti-D reagents have variable reactivity with both partial D and weak D types, so they cannot reliably distinguish partial D from weak D types. Generally, the reagents detect most D category and partial D RBC in the direct agglutination phase of testing. Molecular techniques not only prove limitations of

serology with polyclonal and monoclonal anti-D, they also meet the clinical need to distinguish partial D from weak D types and normal D from D negative.

Antibody investigations in the 1960s identified D category VI (DVI) as the most important allele at risk for D antigen alloimmunization in D positive patients. Later on a strategy was developed to deem DVI as D negative among Europeans 95. In the late 1980s, monoclonal anti-D reagents developed for use in direct hemagglutination tests allowed separation of DVI RBC from normal D positive RBC, a discovery that was not part of a purposeful design. By 1995, monoclonal anti-D reagents that do not detect DVI were widely accepted for use in routine D typing 95. Using this strategy, the DVI transfusion recipient and pregnant woman are typed “false negative” to avoid transfusion with D positive blood and anti-D immunization is prevented 96. Many other partial D variants, like DIV, could benefit from the same serological reagent design strategy 97. Moreover, some oligoclonal anti-D (mix of two or more monoclonal anti-D) reagents are very useful for donor typing, but they should be used judiciously in the transfusion laboratory and prenatal testing. We do not recommend using human polyclonal anti-D or polyclonal/monoclonal anti-D for any routine serological test.

Transfusion recipients

In contrast to the immunized carriers of partial D, anti-D alloimmunizations in weak D type 1 to 3 and 4.0/4.1 have not been observed. These alleles are the most common and together comprise more than 95 % of all weak D types in European or Caucasian populations. Transfusion recipients and pregnant women harboring these weak D types may be safely transfused with D positive blood. This may save up to 5% of D negative units, which are generally in short supply and should be reserved for patients who benefit from these D antigen matched transfusions [39,98,99]. Of note, in African populations approximately 50 % of the weak D types are weak D type 4 subtypes. One of them, the weak D type 4.2, also known as DAR, permits anti-D immunization and requires D negative transfusion in carriers and RhIg prophylaxis in pregnancy.

Transfusion recipients with DEL

The corollary to the transfusion of D positive RBC to weak D patients is the transfusion of normal D positive RBC to DEL patients. In this setting, the risk of alloimmunization is theoretically nil. In Asian populations, where D-negative blood is in short supply, it should be safe to use D positive blood in the DEL transfusion recipient. Adopting such a policy would lessen the demand for rare D negative blood, which is found in less than 1 % of Asian populations. Transitioning a third of all patients currently classified as D negative to the group of transfusion recipients who can safely receive D positive RBC is a significant step. Additional evidence needs to be gathered, but current results are very encouraging [42,52,53]. The “Asian type” DEL needs to be specifically detected as other DEL types are known or likely to be at risk of immunization.

Genetic diagnostics in specific diseases

Immunohematology investigations of transfused patients who have auto- and allo-hemolytic anemias are difficult to perform and often standard serology is not possible [74,100–102]. However, genetic typing can distinguish whether anti-D is alloor auto-immune in nature or whether an apparent null allele is present instead a case of antigen masking.

Blood donors

RHD genotyping in donors is beneficial to transfusion recipients, because it can exclude weak D and DEL donors among apparent D negative blood donors [41]. It is becoming

obvious that determining a donor's phenotype and genotype is a more powerful quality tool than two or more serological tests alone. Without such phenotype/genotype detection algorithms, transfusion recipients of weak D and DEL positive blood units have been anti-D immunized [31,45,48,103], an issue of significant practical relevance [47,98]. Another potentially serious risk is posed by serologically D negative donors who are D positive/D negative chimeras. These individuals carry few D positive red cells, albeit with normal expression, such that a single RBC unit transfusion contains as many RhD polypeptides as 10 mL of 'normal' D positive blood. Therefore, these transfusions are capable of causing an anti-D immunization [41] even though the D positive RBC are not detected through routine serological methods [47].

Mass scale genotyping

No blood group system is as complex as the genetic basis of *RH*. The sheer number and complexity of alleles among various populations make it challenging to develop a comprehensive tool to identify all clinically relevant alleles. Mass scale genotyping may be an appropriate solution for widespread use in different clinical settings [47,98,104,105], and several such mass scale applications have been developed [106–109]. Modifications to the current static high-throughput technologies will address genotyping of large donor and patient cohorts and the 'dry matching' of genotyped units [110]. Furthermore, the computer systems to match donors and patients must be modified to present the appropriate allelic information; current commercial clinical database systems that house serological information are not particularly suitable.

8. Future Perspectives

Applications using genetic analysis of blood groups have become a reality in transfusion medicine [111–115]. The way the genetic results are used is not different than with serological testing, and no new legal or ethical issues have been raised [74]. Pregnant women expressing weak D type alleles or carrying D negative fetuses, which can be specifically detected by *RHD* genotyping, may be spared RhIg prophylaxis [52]. This policy could lower their overall health care bill while avoiding potential risks associated with RhIg and be implemented at a cost equal to current practice [75,99]. Such a strategy may involve some initial costs given that blood group genetic testing has yet to be implemented to a significant degree in most transfusion service laboratories. However, the genetic analysis of donor blood groups can avoid the transfusion of foreign antigens that can illicit red cell alloimmunization in transfusion recipients.

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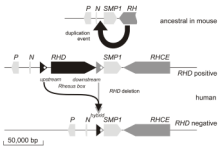

Figure 1. Duplication of the *RH* gene and loss of the *RHD* gene

The ancestral configuration is shown as represented by the *RH* gene locus in mouse. The single *RH* gene is in close proximity to the three genes *SMP1*, *P29-associated protein* (*P*), and *NPD014* (*N*). A duplication event introduced a second *RH* gene in reverse orientation between *N* and *SMP1*. At the two break points in front and behind the *RHD* gene, DNA segments of approximately 9,000 base pairs (bp) occur. Both DNA segments are flanking the *RHD* gene and dubbed "*upstream Rhesus box*" and "*downstream Rhesus box*". In the *RHD* positive haplotype, the *RHD* gene may have been lost by a recombination event (see Figure 3).

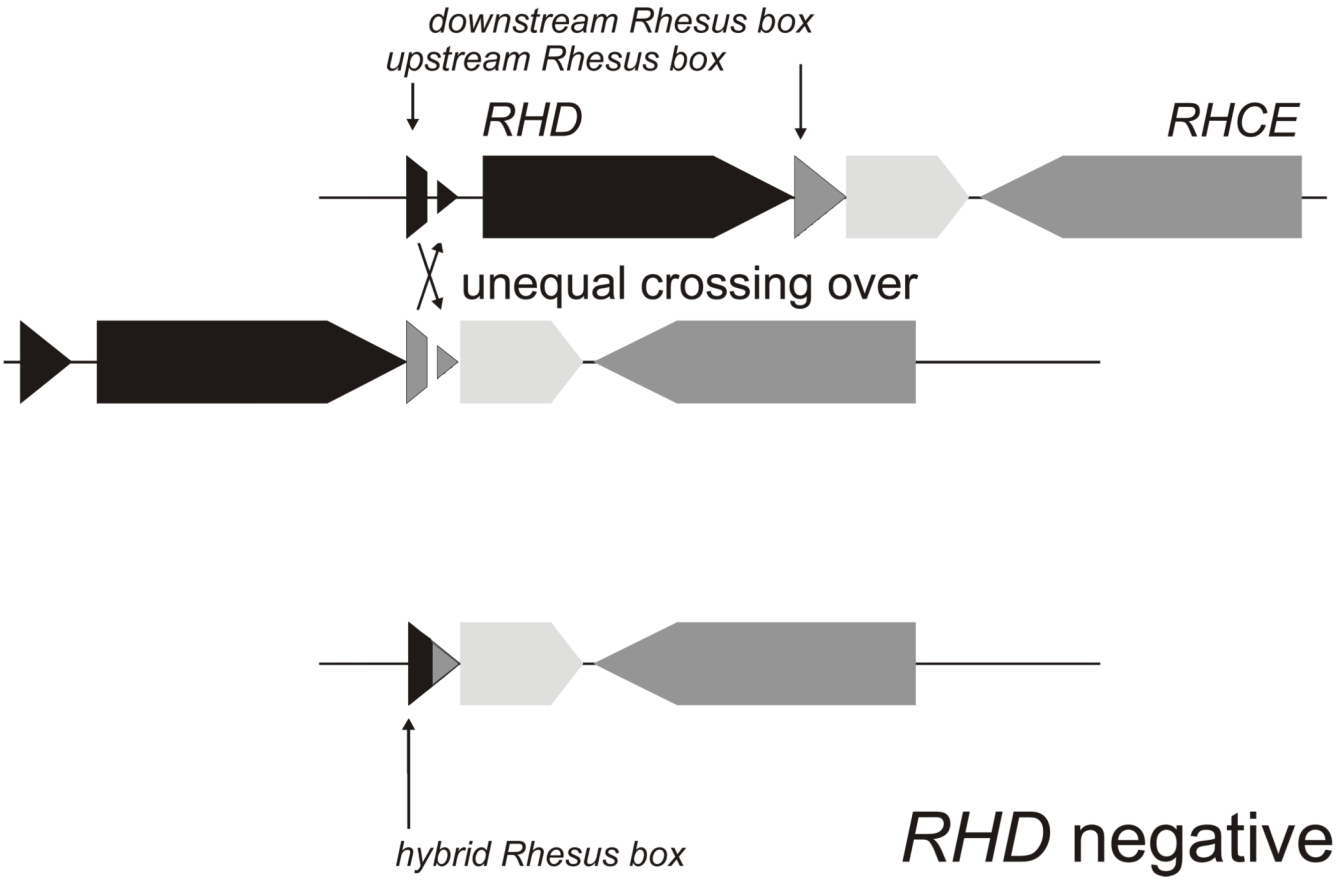

Figure 2. *RHD* deletion

An unequal crossing over event between an *upstream Rhesus box* and a *downstream Rhesus box* caused the *RHD* deletion. If one of the two crossed-over chromosomal threads are resolved, an *RH* gene locus results that lacks the *RHD* gene completely and harbors a hybrid *Rhesus box*.

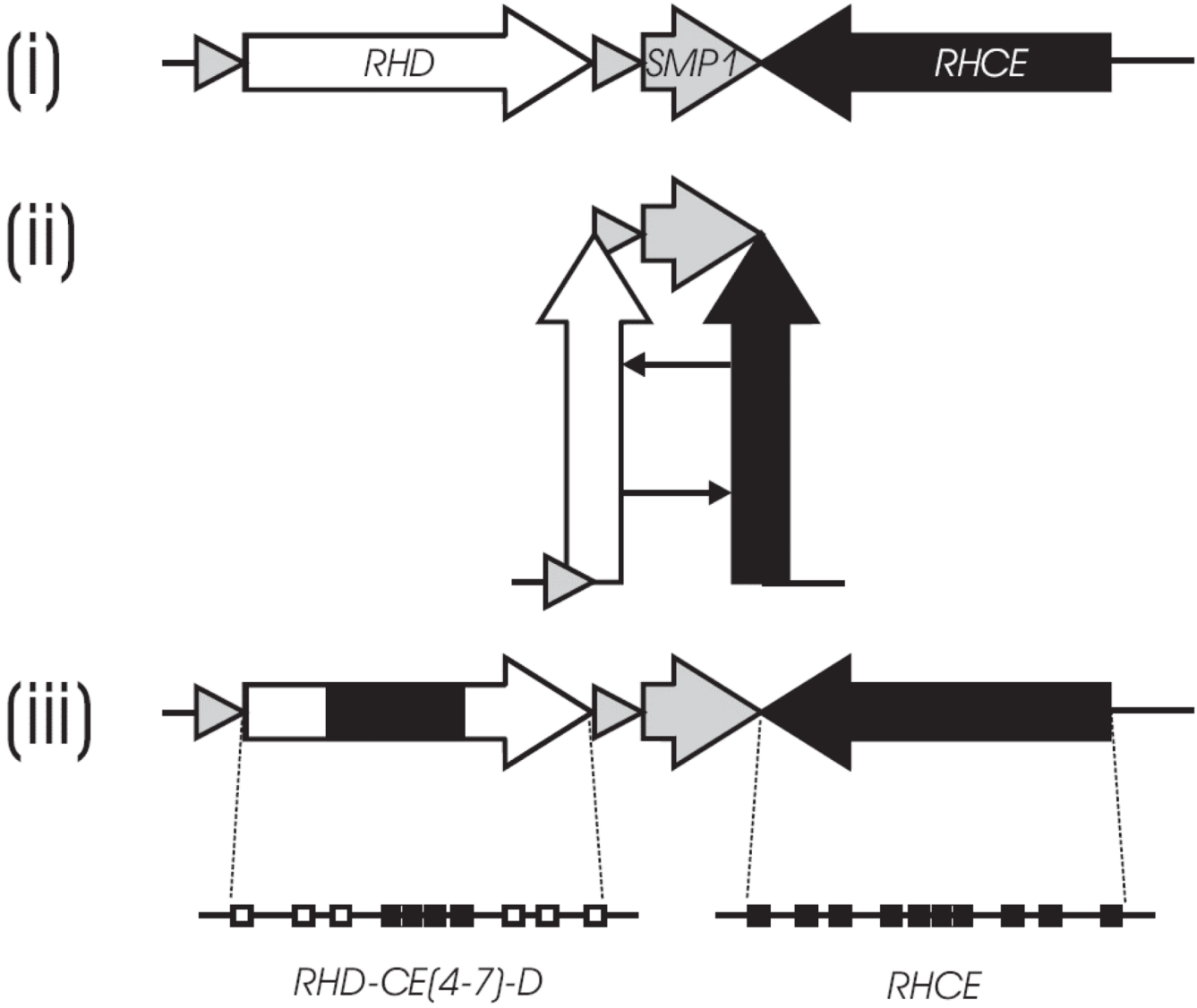

Figure 3. *RHD*/*RHCE* hairpin formation

The schematic diagram depicts the mechanism of gene conversion at the Rhesus gene locus on one chromosome. (i) The *RHD* and *RHCE* genes are inversely orientated, which is typical for clustered genes. (ii) A hairpin formation of the chromosome would generate the close proximity of homologous segments in identical orientation. This structural feature is generally instrumental in gene conversion events *in cis.* (iii) Resolving the hairpin yields an *RHD-CE-D* hybrid gene structure, many of which have been observed to date at the *RH gene* locus. The *RHD-CE(4–7)-D* hybrid exon structure shown here is an example. Modified from Wagner et al., licensee BioMed Central Ltd. Reprinted with permission.

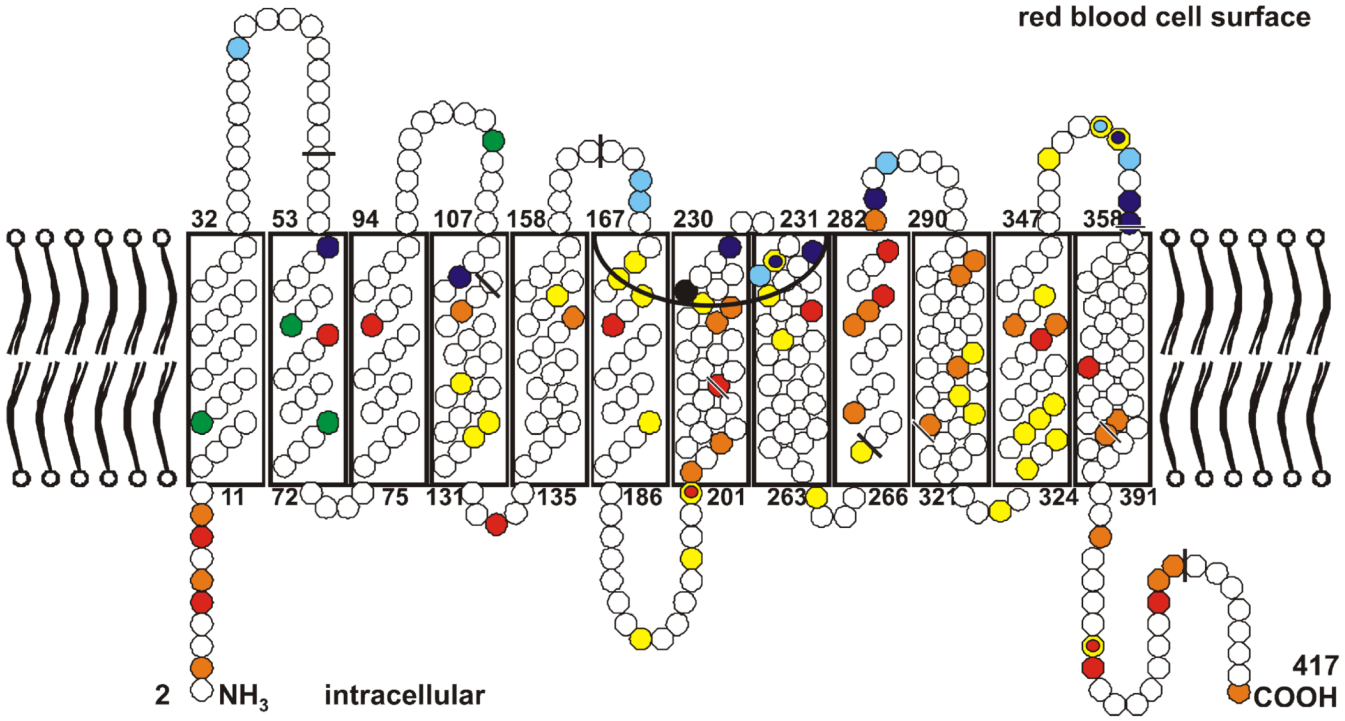

Figure 4. Model of Rhesus proteins in the red blood cell membrane

Both Rhesus proteins comprise 417 amino acids, shown here as circles. Mature proteins in the membrane lack the first amino acid. The amino acid substitutions that distinguish the RhCE from the RhD protein are marked in yellow, with the 4 amino acids that code for the C antigen in green and the one that codes for the E antigen in black. The single amino acids substitutions which code for partial D are in blue, and those that code for weak D are in red. The mutations that had been identified at the Ulm Institute since 1999 are in light blue and orange. The extracellular Rh vestibule is depicted by the inverted black arc and bordered in part by amino acids of loops 3 and 4. The nine exon boundaries in the *RHD* cDNA, as reflected in the amino acid sequence, are indicated by black bars.

Table 1

Representative molecular changes in *RHD* alleles expressing distinct phenotypes of the D antigen

classification of antigen variation	D antigen Phenotype	molecular basis		representative example		novel Rhesus antigen
		protein variation	Mechanisms	*RHD* allele	trivial name	
partial D	qualitative change	amino acid substitution on the RBC surface	missense mutation	*RHD*(G355S)	DNB	Unknown
		protein segment exchange on the RBC surface	gene conversion (hybrid protein)	*RHD-CE*(3–6)-*D*	DVI type 3	BARC
weak D	quantitative change	amino acid substitution in the membrane or intracellularly	missense mutation	*RHD*(V270G)	weak D type 1	Unknown
DEL	major quantitative	grossly reduced translation or	missense mutation	*RHD*(M295I) in *CDe*	not applicable	unknown
	change	protein expression	mutation at splice site	*RHD*(K409K)	not applicable	unknown
D negative	D negative	lack of protein expression	gene deletion	*RHD*-Deletion	D negative	unknown
			nonsense mutation	*RHD*(Y330X)	not applicable	unknown
			Frame shift mutation	*RHD*(488del4)	not applicable	unknown
			modifying gene	defect of *RHAG* gene	Rh_{null}	unknown
		protein segment exchange on the RBC surface	gene conversion (hybrid protein)	*RHD-CE(3–7)-D*	Cdes	Unknown
antithetical antigens of the RhCE protein	expression of antigen E or antigen e	amino acid substitution on the RBC surface	missense mutation at amino acid position 226 in *RHCE*	*RHCE* allele: Ala226 coding antigen e Pro226 coding antigen E	not applicable	E versus e