Implication of novel *RH* alleles in blood transfusion therapy

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Abstract: The Rh blood group system is one of the most complex and clinically significant blood group systems, with numerous alleles that can affect compatibility between donors and recipients. Over the years, with advancements in molecular techniques an increasing number of novel *RH* alleles arising from genetic variations in the *RHD* and *RHCE* genes have been discovered. These novel alleles can lead to discrepancies between serological and genotypic typing methods and may result in altered Rh protein structure or expression levels, potentially affecting antigen presentation and immune recognition. In general, new alleles have emerged with advances in molecular techniques with very little information about the antigens they encode or the formation of antibodies. Understanding these functional implications is crucial for predicting compatibility and managing the risk of alloimmunization and transfusion reactions effectively. The risk of alloimmunization is often associated with the type of variant Rh antigen that the new allele encodes, so further serological and clinical studies would be valuable to determine its clinical significance. This review aims to explore the implications of novel *RH* alleles in transfusion therapy and to discuss future directions, including the incorporation of 3D analysis and computational tools to predict the functional consequences of novel *RH* alleles. These tools can aid in the interpretation of molecular data and provide insights into the potential clinical phenotype associated with specific variants.

Keywords: Rh blood group system; *RH* variants; novel *RH* alleles; alloimmunization; transfusion safety

Introduction

Background

The Rh blood group system is among the most immunogenic and polymorphic blood group and consists of 56 antigens encoded by two homologous genes located on chromosome 1, *RHD* and *RHCE*. Most antigens are encoded by the *RHCE* gene and a small number are encoded by *RHD* or hybrid *RHCE* with *RHD*. The most common antigens are D (RH1), C (RH2), c (RH4), E (RH3) and e (RH5) detected by commercial reagent antibodies. There are over 400 *RHD* and over 150 *RHCE* alleles recognized by International Society of Blood Transfusion (ISBT) (1), but many more alleles can be found in other database inventories as the RhesusBase (2), RHeference database (3), Erythrogene (4). Those alleles are result of single nucleotide variants, short/large insertions/deletions, gene rearrangements and gene deletion (5). This level of complexity can result in altered expression of D and CE antigens on red blood cells (RBCs) with critical transfusion outcomes and high rates of alloimmunization. Clinical or biological evidence of these effects are not available for all *RH* variant alleles as not all Rh antigens have been associated with antibody formation and not all alloantibodies produced are clinically significant (6,7).

Rationale and knowledge gap

There are 2 types of Rh variants, the weak Rh antigens that conceptually do not lack immunogenic epitopes and have weak expression and the partial Rh antigens that conceptually lack immunogenic epitopes and have weak or normal expression (8,9). Historically, weak antigens do not induce alloimmunization and partial antigens induce

alloimmunization and are considered clinically significant, as the carrier of partial antigens exposed to a complete antigen can produce antibodies against the missing epitopes and against high-frequency antigens such as RH31 (hrB) and RH19 (hrS) (10-12). However, the limit of these two concepts is not simple as demonstrated by Daniels *et al.* (13), since it is not possible to predict that all patients with weak antigens will not produce antibodies and that all patients with partial antigens are at risk of antibody formation.

Although the clinical significance of Rh variants is not easy to establish, RH genotyping has been recommended to resolve serologic weak phenotypes or discrepancies found in the routine and to differentiate weak and partial Rh antigens (8,10-16). However in recent years, an increasing number of novel *RH* alleles have been discovered but their serological descriptions, antibody formation risk and implication in blood transfusion have still been difficult to demonstrate as many of these alleles have only been observed once in a donor or in a non-alloimmunized patient. In addition, the variant type, allelic forms, and trimeric conformation of Rh proteins influence the clinical significance of antibodies. In many cases, functional analysis and intraprotein interactions have been used to characterize the effect of the variations at the molecular level (17-20).

Objective

A better understanding of alloimmunization risk and clinical significance of the antibodies produced by these novel *RH* alleles are necessary to improve transfusion safety.

RH alleles and blood transfusion therapy

RHD alleles and risk of anti-D

It is important to be aware that not all carriers of variant *RHD* alleles will develop anti-D. The distinction between *RHD* alleles encoding weak D and partial D antigens can be of clinical importance to predict the risk of alloimmunization and their implication in blood transfusion therapy. There are over 700 *RHD* alleles registered in the RHeference database but only 30% of them have been associated with anti-D formation (3). In a narrative review of *RH* alleles and antibodies, Floch (21) reported the most common *RHD* alleles associated with production of anti-D according to the literature. Most are *RHD* alleles encoding partial D but weak D types 11, 15, 21, 41, 42 and 45 have also been associated

with anti-D formation. Due to the lack of evidence on alloimmunization in carriers of the 5 most prevalent weak D types 1, 2, 3, 4.0, 4.1, it has been recommended that they should be treated as D-positive to conserve the limited supply of D-negative blood while the less common types including weak D type 4.2 that is commonly found in Africans should be treated as D-negative (22). This is one of the reasons that RHD genotyping is being recommended to differentiate the weak D types and partial D antigens in transfused patients (8,16,23-25). In a recent study, Miranda *et al.* (11) performing systematic RHD genotyping in transfused patients serologically typed as weak D receiving D-positive RBC units showed that 63.2% of them had *RHD* alleles encoding partial D and therefore should be treated as D-negative. The prevalence of novel alleles can vary significantly among different populations and there are still rare reports on alloanti-D immunization of these sporadic *RHD* variant alleles. Therefore, in the absence of clinical information about anti-D formation patients carrying these rare *RHD* alleles have been treated as D-negative (21). Testing RBCs with different anti-D clones can provide potential insight into D epitope profile as the absence of epitopes suggests that these alleles encode partial D phenotypes with potential risk of D formation (26,27). Bioinformatics studies have also revealed the effects of amino acid substitutions on interactions within the RhD molecule and can be helpful in assessing new variants (20,28). The type of nucleotide variation also provides information, for example, changes that occur at an exon splicing site are predicted to result in a reduced level of D transcripts responsible for weak D typing, but not an amino acid change, and therefore these alleles would not be predicted to be associated with alloimmunization (29). Prospective studies on the anti-D formation risk of novel *RHD* alleles, especially those reported in a large number of carriers are necessary to understand their clinical significance. D epitope expression of the novel *RHD* alleles determined by serological tests and expression studies *in vitro* would also be helpful. Without these studies it seems premature to exclude their implication in blood transfusion. In addition, there are some alleles encoding antigens with a very low antigen density which tend to type as D-negative and therefore the carriers are less likely to receive D-positive RBC units (30). There are also some intriguing situations regarding the production of antibodies by *RHD* alleles encoding variant antigens as for example the individuals with a compound heterozygous variant *RHD* alleles. Even in the presence of an allele encoding a partial

antigen, depending on the other allele, the association with *RHCE* allelic forms and the trimeric conformation of Rh proteins, the risk of anti-D formation may vary (10,12,23). Identifying compound heterozygous individuals can be clinically relevant because patients with weak D types 1, 2, 3, 4.0, or 4.1 will not need to be transfused with D– RBCs (22). Novel *RHD* alleles often produce discrepant or inconclusive serological and molecular results but the causes for such discrepancies should be identified for the benefit of patient care and the advancement of allele databases. The bioinformatics analysis of RhD protein can help us understand the impact of *RHD* variants on antigen differences and guide blood transfusion strategies. The impact of novel *RHD* molecular variations located in splicing regions has also been investigated using a functional test, namely minigene splicing assay that could be another tool for interpretating the functional effect and/or related phenotype of novel *RHD* alleles to provide more precision transfusion guidance (17,31,32). The decision to treat a patient as D+ or D– should consider, in addition to the specific allele, the patient age, gender, history of antibody production, transfusion needs and the blood supply.

Implication of RHCE variant alleles in transfusion

The genetic diversity of the RH locus in populations of African origin contributes to the high prevalence of altered *RHCE* alleles and with high rate of alloimmunization in sickle cell disease (SCD) patients (10,33). In addition to variant *RHCE* alleles predicting partial antigens there are many alleles that also predict the loss of high prevalence antigens such as hrB (RH31) and hrS (RH19) that should be taken into account in the selection of RBC units for transfusion (34). Anti-e is the most frequent alloantibody observed in SCD patients with variant *RHCE* alleles, although not all are implicated in hemolytic transfusion reactions (HTRs) suggesting that the alloimmunization risk and the clinical significance of Rh antibodies produced may vary according to the specific variant inherited (21). In a recent study, we observed that most of patients with the *RHCE*01.20.01* (*RHCE*ce733G*) allele who developed anti-e had a good survival of the transfused RBCs when exposed to conventional e antigen while patients with the *RHCE*01.04.01* (*RHCE*ceAR*) allele produced clinically significant anti-e and anti-hrS (34). Indeed, there is evidence that some *RHCE* alleles such as *RHCE*01.01* (*RHCE*ce48C*), *RHCE*01.20.01* (*RHCE*ce733G*) and *RHCE*01.20.02* (*RHCE*ce 48C,733G*) have a low risk for antibody formation

or implication in severe hemolytic episodes (6,10,34). In addition, it has been suggested that the presence of one conventional *RH* allele is not protective against alloimmunization (10,35,36). *RHCE* alleles previously associated with antibody formation to the corresponding antigens were reported with RH10 (V) and RH20 (VS) phenotypes (10,21) and no antibody formation was associated with rare *RHCE* alleles, such as, *RHCE*02.02*, *RHCE*02.03* and *RHCE*02.11* with no RH10 or RH20 phenotypes (37-39). Although extremely important, the clinical significance of *RHCE* variant alleles is not easy to establish in polytransfused patients, especially whether the antibody formed is an alloantibody or autoantibody as auto-adsorptions are not recommended for recently transfused patients (40). Moreover, autoantibodies can cause hemolysis and therefore the antibody implication in an HTR may be difficult to ascertain. The difficulty in finding donors with well-characterized *RHCE* alleles to select units based on patient's *RH* alleles, associated with little knowledge of the risk of alloimmunization and the clinical significance of the antibody, makes the transfusion decision difficult (41). In order to understand the implication of the novel *RHCE* alleles in transfusion therapy, it is important to know their frequency in the population studied and demonstrate that the allele predicts an antigen at risk for antibody formation and a loss of the high prevalence antigens hrB (RH31) and hrS (RH19) (6,21,36). It is important to emphasize that the selection of compatible blood for a patient with a rare allele also depends on the availability of genotyped donors for RH variant alleles as this request is filled with RH genotype-selected units.

Novel RH alleles, alloimmunization and hemolytic risks

High resolution genotyping has revealed a plethora of novel *RH* alleles in different populations as a result of serological discrepancies or discordances between serologic typing and genotype-predicted phenotype (36,42-44). However, many alleles have been reported in abstract form, have no prevalence associated, and few of the novel alleles reported to-date were associated with production of antibody to the expressed antigen. Most of *RH* novel alleles have been described in donors or in non-alloimmunized patients and in many of these studies, even when an antibody is produced, the serology is not well detailed, mainly in the discrimination of auto and alloantibodies. The risk of alloimmunization is often associated with the type of variant Rh antigen that the new

allele encodes, that is, a weak antigen or a partial antigen, however, more serological, and clinical studies would be valuable to determine the risk for antibody formation. It is difficult to measure the risk of alloimmunization of novel alleles, since most patients carrying partial Rh antigens are not routinely recognized unless they form unexpected antibodies. Furthermore, the Rh antibodies formation may be influenced by personal characteristics, since it is known that some individuals are less likely to produce alloantibodies and are "low responders" despite multiple transfusions (45). There are some *RH* alleles widely accepted for producing partial antigens and are associated with the formation of alloantibodies if exposed to a conventional antigen as for example the *(C)ceS* haplotype but even so it varies in different populations (10,12,34,35). Therefore, it is very difficult to establish a strategy to reduce the risk of alloimmunization if we do not properly know the antigen characteristic. In general, new alleles have emerged with advances in molecular techniques with very little information about the antigens they encode or the formation of antibodies. Furthermore, a study observed that altered epitopes on donor red cells can also induce alloimmunization and this is very difficult to control (46). In the discovery of novel *RH* alleles, it is very important to provide serological data, even if incomplete, alloimmunization information and clinical data so that we can assess the implication of that allele in blood transfusion therapy and propose a strategy to prevent alloimmunization and HTRs (47). In a study performed by Coleman *et al.* (35) on alloimmunization and implication of antibodies produced with delayed hemolytic transfusion reactions (DHTRs) they observed that 47% of clinically significant Rh antibodies occurred in patients with at least one allele encoding any partial Rh antigen. Although phenotype and clinical data are the standard determinants for assessing the effect of genetic variations, the use of bioinformatics prediction and modeling tools have evolved and currently studies of 3D intraprotein interactions combined with epitope mapping and antigen density have been used in assessing the risk of alloimmunization in carriers of RH variants (19,20). An investigation of intraprotein interactions of the product of 25 *RHD* variant alleles on the risk of anti-D alloimmunization performed by Floch *et al.* (19) showed that extracellular changes and the alteration of intraprotein interactions were correlated with the risk of anti-D formation in 76% of variants with anti-D. Currently, functional and clinical interpretation of variations in blood group genes by 3D-protein structure investigation

have been considered an additional tool for assessing the risk of antibody formation in patients carrying novel variant *RH* and may become valuable for guiding the implication of novel alleles in hemolytic risks, especially when no serological or clinical data are available. *In silico* processed bioinformatics analysis may also improve our understanding of the influence of genetic variants on antigen expression and its implications for blood transfusion therapy (48-50). *Figure 1* illustrates a case of a patient with SCD typed as D-positive with anti-D in his serum on how 3D analysis could be used when new *RH* variants are detected (46). In this particular sample, the authors attempted to take advantage of combining the molecular data and available silico tools for the functional interpretation of the new variant, in order to get insights into the clinical phenotype that may be predicted a priori from genotyping.

Conclusions

It's worth noting that medical advancements and improved understanding of the RH system have led to more accurate testing and screening procedures. High-resolution RH typing is beginning to be performed using advanced sequencing technologies with increased average read length that allows identification of structural variants, sequencing repetitive regions, phasing of distant nucleotide changes, and distinguishing highly homologous genomic regions, thereby improving the characterization of *RHD* and *RHCE* genes, and increasing the discovery of variants relevant to blood transfusion. Immunization potential of novel *RHD* and *RHCE* alleles is difficult to predict. For now, the recommendation is that patients be treated as antigen negative in transfusion, until evidence emerges that can prove the risk or not of antibody formation. In the pregnancy management, without knowledge of the potential risk of alloimmunization, all women with a novel *RHD* allele will be considered D– and will receive comprehensive monitoring, including additional antibody detection test, *RHD* fetal genotyping and RhIG injection. Hopefully, more serological, epidemiological, and clinical data with the incidence and severity of DHTRs associated with antibody formation, particularly if documented for larger cohorts, will be reported with the novel alleles being discovered so that the transfusion recommendation and pregnant management will be more accurate. A better understanding of the involvement of novel *RH* alleles with the production of antibodies and with a potential risk of causing HTRs may refine RH molecular matching and increase transfusion
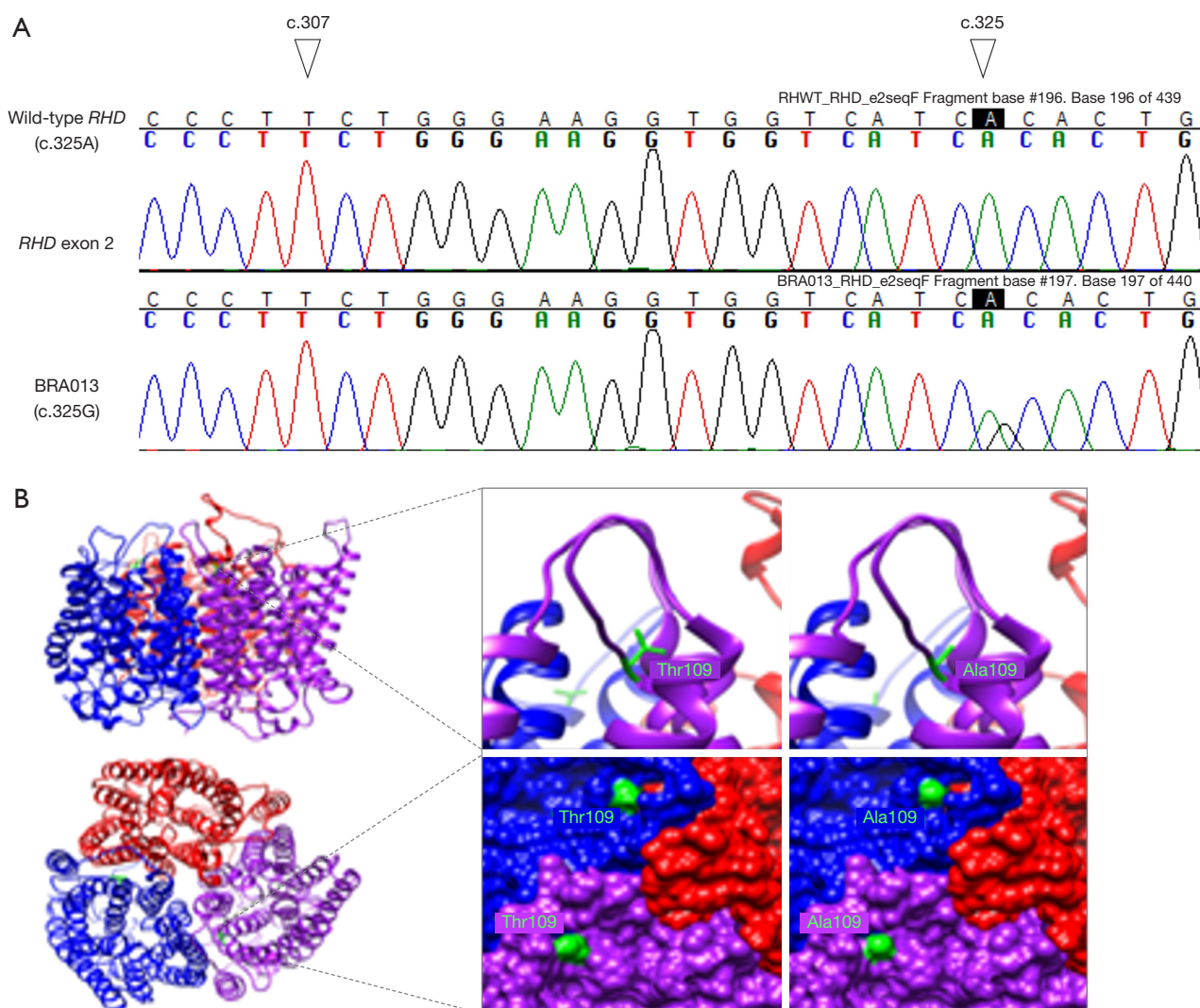
Figure 1 Example of a 3D analysis used for the functional interpretation of a new *RHD* variant. (A) The single c.325A>G variation was found in exon 2 leading to a threonine-to-alanine substitution at amino acid position 109 (p.Thr109Ala). (B) A 3D structural model of the RhD-RhD-RhAG heterotrimer was used to visualize the position of variations and predict their putative functional/clinical effect. Amino acid 109 is located in RhD protein extracellular loop 2, and is thus thought to alter D antigen structure, resulting in a partial D phenotype. This hypothesis is in accordance with the anti-D found in the patient's serum. 3D, three-dimensional.

safety. The bioinformatics of these alleles can extend our knowledge of Rh variants and help assess their implications in blood transfusion therapy. For the past years, due to the advent of next-generation sequencing and the subsequent identification of numerous rare variants, bioinformatics prediction and modelling tools have evolved and currently help physicians in diagnostics, clinical management and genetic counselling. Although phenotype and clinical symptoms remain definitely the standard determinants to assess the effect of genetic variations, use of those

approaches may soon become valuable for guiding subsequent investigations in immunohematology.

Overall, the discovery and understanding of novel *RH* alleles contribute to the improvement of blood transfusion practices. They help enhance donor-recipient compatibility, refine screening and testing procedures, and ensure the safety of transfused blood products. Regular updates and advancements in blood bank protocols are crucial to accommodate the evolving knowledge of *RH* alleles and reduce the risk of alloimmunization. Accurate determination

of RhD status, identification of RhCE variants, and monitoring for alloimmunization risk are essential in providing safe and effective transfusion therapy to patients. Molecular techniques play a crucial role in identifying novel *RH* alleles and improving transfusion practices.

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Footnote

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