SEROLOGICAL PROFILING AND MOLECULAR DETECTION OF VARIANT D IN RHESUS NEGATIVE BLOOD DONORS IN PERAK

BY

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ABBREVIATIONS

Anti	Antibody
AHG	Anti Human-Globulin
cDNA	complementary deoxyribonucleic acid
DNA	Deoxyribonucleic Acid
DEL	D-elute
D^U	Weak D test
EDTA	ethylenediamine tetra-acetic acid
g	gram
HDFN	hemolytic disease of fetal and newborn
HUSM	Hospital Universiti Sains Malaysia
HRPB	Hospital Raja Permaisuri Bainun
Hb	Hemoglobin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
mls	mililiter
NBC	National Blood Centre
PCR-SSP	Polymerase chain reaction – sequence specific primer
Rh	RhD

RhIg	Rho(D) immune globulin
RHD	D gene
RHCE	C and E gene
RHAG	RhD associated glycoprotein
RBC	red blood cell
RNA	Ribonucleic Acid
ul	microliter
ug	microgram
μM	micromolar
mM	millimolar

ABSTRAK (MALAY LANGUAGE)

PROFIL SEROLOGI DAN UJIAN MOLEKULAR UNTUK VARIAN D DI KALANGAN PENDERMA DARAH RESUS NEGATIF DI PERAK

Individu dengan varian D mempunyai potensi untuk menghasilkan anti-D antibodi yang boleh menyebabkan tindak balas transfusi hemolitik dan penyakit hemolitik pada janin dan bayi baru lahir. Walaubagaimanapun, varian D biasanya tidak dapat dikesan dengan baik dan dilabel sebagai Rhesus D (RhD) negatif kerana kelemahan ujian serologi yang sedia ada. Oleh itu, adalah penting untuk menyelesaikan masalah percanggahan keputusan ujian serologi akibat varian D ini dan seterusnya ujian molekular diperlukan untuk membuat pengkelasan varian D secara tepat. Sehubungan itu, tujuan kajian ini adalah untuk mengenal pasti kekerapan varian D di kalangan penderma darah RhD negatif dengan menggunakan ujian serologi dan molekul.

Seramai 175 orang penderma darah RhD negatif dari pusat pungutan darah bergerak dan tabung darah Hospital Raja Permaisuri Bainun telah dikumpul dari bulan September 2015 hingga Mac 2016. Melalui ujian serologi, ujian kumpulan darah ABO dan pengelasan RhD telah dilakukan dengan menggunakan antibodi monoklonal IgM (TH-28), Diaclone (Diamed) menggunakan plat microtitre dan IgM/IgG,EpicloneTM-2 (CSL) menggunakan kaedah jubin dan tiub. Pengelasan Rh yang lain pula dilakukan dengan anti-c /C/E/e IgM/IgG, EpicloneTM-2 (CSL). Ujian D lemah atau D^u telah dilakukan dengan menggunakan kaedah tiub dengan gabungan IgM/IgG oleh EpicloneTM-2 (CSL). Ujian molekular menggunakan Polymerase Chain Reaction – Sequence Specific Primer (PCR-SSP) untuk mengesan DEL varian (mutasi RHD 1277A atau K

(409) K) telah dijalankan ke atas semua sampel, manakala kit BAGene RH TYPE dan kit partial D TYPE (kit komersial) telah dijalankan ke atas sampel yang positif ujian D^u dan positif RHD 1277A/K(409) K oleh Polymerase Chain Reaction – Sequence Specific Primer (PCR-SSP).

Kumpulan darah RhD negatif yang paling kerap adalah O (46.9%) diikuti oleh B, A dan AB. Fenotip ccddee (81.7%) adalah yang paling kerap di kalangan penderma darah RhD negatif dan yang paling kerap dalam semua kaum. Fenotip Ccddee pula kerap di kalangan orang Cina (43.8%). Ujian D^u menunjukkan, 6 orang (3.4%) merupakan varian D dengan fenotip CcDdee (4), CcDDEe (1), and CCDDee (1). Ujian molekular pula mendapati sebanyak 10 orang merupakan varian D, iaitu 3 adalah RHD 1277A/K (409) K (semuanya orang Cina), 5 adalah D Separa (tak berkelas), 1 adalah DVI Separa jenis III dan 1 adalah RHD-CE(2–9)-RHD.

Kesimpulannya, prevalens varian D di kalangan penderma darah RhD negatif adalah rendah (3.4%). Ujian serologi dapat mengesan kebanyakan varian D separa tetapi tidak dapat mengesan varian DEL. Hanya ujian molekular mampu mengesan varian DEL (RHD 1277A/K(409)K). Kaum Cina yang didapati adalah RhD negatif dengan fenotip Ccddee secara serologi disarankan untuk menjalani ujian molekular kerana mempunyai kekerapan yang tinggi untuk DEL RHD1277A/K (409)K.

ABSTRACT (IN ENGLISH)

SEROLOGICAL PROFILING AND MOLECULAR DETECTION OF VARIANT D IN RHESUS NEGATIVE BLOOD DONORS IN PERAK

D variant individual has the potential to cause anti-D production resulting in hemolytic transfusion reactions and hemolytic disease of the fetus and newborn. However, D variants are usually under detected and mistyped as Rhesus D (RhD) negative due to limitations of the available serological tests. Hence it is important to solve serological discrepant cases and further accurately classify D variants by molecular testing. In view of this, the aim of this study is to identify the frequency of D variant among the RhD negative blood donors in this population using serological and molecular tests.

A total of 175 RhD negative donors were recruited through mobile drives and collection centres in Hospital Raja Permaisuri Bainun from September 2015 till March 2016. Serologically, ABO blood grouping and RhD typing was done with monoclonal antibody, IgM (TH-28), Diaclone (Diamed) using the microtitre plate and IgM/IgG, EpicloneTM-2 (CSL) using the tile and tube method. Other Rh typing was done with the anti-c/C/E/e IgM/IgG, EpicloneTM-2 (CSL). Weak D or D^u test was done using tube method with IgM/IgG blend, EpicloneTM-2 (CSL). Molecular testing using Polymerase Chain Reaction – Sequence Specific Primer (PCR-SSP) to detect DEL variant (RHD 1277A or K (409) K mutation) were done on all samples while BAGene RH TYPE kit and Partial D TYPE kit (commercialized kits) were performed on D^u positive samples

and positive RHD 1277A/ K(409)K samples by Polymerase Chain Reaction – Sequence Specific Primer (PCR-SSP).

The most common RhD negative blood group was O (46.9%) followed by B, A and AB. The ccddee (81.7%) is the most common phenotype among RhD negative donors and most common phenotype in all races. The Ccddee phenotype was common among the Chinese (43.8%). Serologically by D^u test, 6 (3.4%) were found to be D variant with phenotypes CDe/cde (4), CDE/cDe (1), and CDe/CDe (1). Molecular test revealed a total of 10 donors with D variants in which 3 with RHD 1277A/K (409) K (all these belonged to Chinese), 5 with Partial D (unclassified), 1 with Partial DVI type III and 1 with RHD-CE(2–9)-RHD.

In conclusion, the prevalence of variant D among RhD negative donors was found to be low (3.4%). Serological testing was able to detect most partial D variants but not DEL variants. Only molecular testing is able to detect DEL variant (RHD 1277A / K (409) K). Molecular test is advised on RhD negative Chinese's with Ccddee phenotype, since the frequency of RHD DEL 1277 K (409) K is high among them.

Chapter 1

Introduction

1.0 INTRODUCTION

1.1 GENERAL INTRODUCTION

RhD antigen (RhD) was originally discovered in 1939, and was the first clinically important blood group to be discovered subsequent to the finding of ABO antigen 39 years earlier. (Landsteiner, 2010). Most individuals are designated to be either RhD positive or RhD negative; however there is an increased recognition towards the presence of D variants, frequently categorized as either weak D, partial D or D-elute (DEL). RhD variants which are inadequately defined are still a cause of confusion across blood banks and with transfusionist prompting numerous studies in this field. (Denomme *et al.*, 2005)

Among the Rh antigens, D antigen is the most immunogenic followed by c, E, C and e antigen (in that order). It is well known to cause immune transfusion reactions. All the antibodies toward Rh blood group system are clinically significant as it causes acute and delayed hemolytic transfusion reactions and hemolytic disease of the fetus and newborn.

Low or abnormal antigenic expression in D variants such as weak D, DEL and partial D variants may result in the failure of detection with certain anti-D reagents, causing these donors to be mistyped as RhD negative. The opposite also occurs in some variants (eg: Partial D IIIa) which has higher antigenic strength than normal D antigen, causing these donors to be typed as RhD positive patients. Subsequently, when these units are given to inappropriate recipients, alloimmunization towards antigen D antigen can occur. However most weak D variants do not

spur anti-D formation and can be safely classified as RhD positive. Therefore, correct classification of RhD blood group can greatly spare precious RhD negative units and prevent unwanted clinical consequences. (Anstee, 2009)

In view of this, RhD status is routinely determined in blood donors, transfusion recipients, and in mothers-to-be. Abundant serological techniques and reagents each with different sensitivities have greatly complicated RhD typing. Hence, molecular testing to resolve these discrepancies have been introduced in blood banks some parts of the world, especially in populations than have higher than average incidence of RhD antigen alloimmunization.(Rizzo *et al.*, 2012)

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(Jones *et al.*, 1995). Molecular techniques not only can overcome 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 RhD from RhD negative and DEL variants such as RHD 1227A/K(409)K (Denomme, 2011)

RHD 1227A/K(409)K is a DEL variant most commonly found in the Asian population, predominantly in Japanese and Chinese population. The DEL variant appears as RhD negative phenotype and is routinely identified as RhD negative with routine serological tests and either further elution and adsorption testing or molecular testing is needed to identify this variant. (Pornlada Nuchnoi, 2014)

This study will help profile the Rh phenotype in the RhD negative donor population in Perak and further screen for RHD 1227A/K409K gene and other RhD variants in this population. This will help proper categorization and correct supply of blood to recipients. An added benefit will be the donors who become recipients will have their RhD antigen screened for possible RHD mutations, ensuring they receive blood that doesn't prompt anti-D formation.

Chapter 2

Literature Review

2.0 LITERATURE REVIEW

2.1 RhD (Rh) Blood Group System

Blood group antigens were first discovered by Karl Landsteiner in 1900 and 1901 at the University of Vienna. These antigens are found to be made up of either sugars or proteins, attached to various components on the red blood cell (RBC) membrane that determine an individual's blood group. The basic molecule is the H antigen, where its modification results in various blood groups. Over 30 blood group systems have been defined, but the two main blood group systems are called ABO and RhD (Rh) systems. The Rh blood group consists of about 45 antigens and is the most clinically significant in transfusion medicine next to ABO blood system (Avent and Reid, 2000).

The first Rh gene, RHCE gene was discovered in 1990 and the second RHD gene two years subsequently(Flegel, 2007). Both these genes, comprise 10 exons each, are highly homologous are located in the short arm of chromosome 1 at (1p34.1-1p36). (Wagner and Flegel, 2000). As one of the most immunogenic and clinically significant RBC antigens, Rh antigen has been extensively studied. The ability to clone complementary DNA (cDNA) and sequence genes encoding the Rh proteins has directed to an understanding of the molecular bases connected with some of the Rh antigens (Avent and Reid 2000). RhD and RhCE proteins differ by 32-35 out of 416 amino acids and comprise of 10 exons each. They encode the RhD and Rh C, C, c, E and e protein that in turn make the Rh antigens: C, c, D, E and e (Flegel, 2007). Rh antigens are expressed only in the membranes of red blood cells

and their immediate precursors, unlike other antigens of the blood group system (Avent, 2000). The function of the Rh complex remains unclear and they may play a role in ammonium transportation (Neil D. Avent, 2000).

The difference between C and c antigens is based on the difference of four amino acid sequence. They contrast by the amino acid change in Cys16Trp (cysteine at residue 16 replaced by tryptophan) encoded by exon 1, and Ile60Leu, Ser68Asn, and Ser103Pro encoded in exon 2. While the difference between E and e antigens is only one amino acid sequence, Pro226Ala, situated on the fourth extracellular loop of the protein in exon 5. RhD positive individuals have both RHD and RHCE gene while RhD negative individuals most often have a complete absence of the RHD gene but the RHCE gene is intact (Figure 2.1).

The expression of Rh antigen depends on the RHAG to direct these antigens to the RBC membrane. None of the Rh antigens are expressed if RHAG is absent. RHAG share about 35% of their primary sequence with Rh proteins, and is a similar trans-membrane protein. It is not polymorphic and does not carry the Rh antigens (Figure 2.2) (Neil D. Avent, 2000).

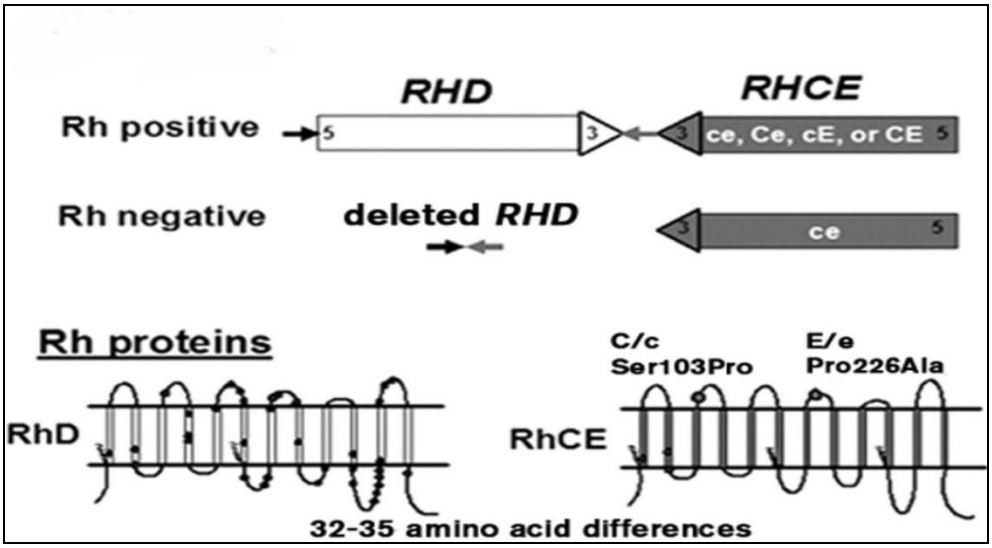

Figure 2.1: Diagram of the *RHD* and *RHCE* Locus and Rh Proteins in RBC Membrane. (Adapted from Westhoff Connie M., 2007)

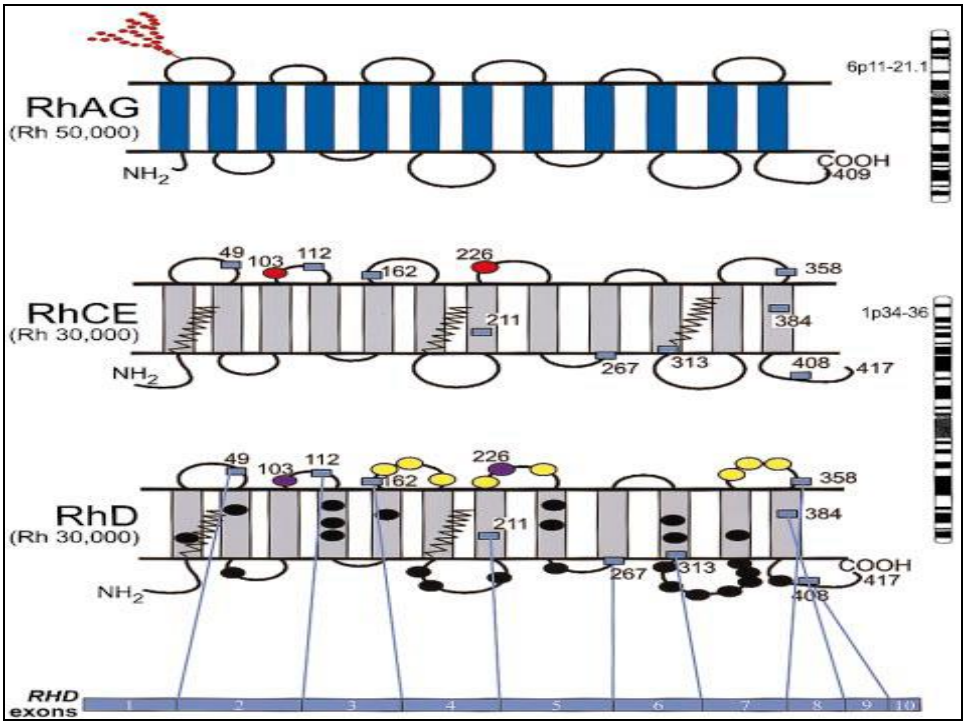

Figure 2.2: Model of topology for RhAG, RhCE, and RhD antigen (Adapted from Westhoff Connie M., 2007)

2.2 Rh Nomenclature and Rh Phenotype

Generally 8 Rh haplotypes are commonly found: R°, R1, R2, Rz, r, r', r'', ry and are classified with the Fischer- Race or Weiner nomenclature (Table 2.1). Each haplotype is inherited from each parent and expressed in various combinations. This system of Rh depiction eases transfusion medicine personnel and laboratory staff in communicating the Rh phenotype of a patient or donor (CM, 2004). In the Malaysian population, Rh blood group system has shown a diverse distribution among ethnic groups (Table 2.2). The RhD positive individuals were almost 97.5% of the population with the distribution of 99.5% in Malays, 98.5% in Chinese, and 91.7% in Indians. The RhD negative phenotype comprises of roughly 2% of the population, mostly found in the people Indian ethnicity (7.4%) followed by the Malays (5.2%), Chinese (2.3%) and others (2.3%) (Table 2.2) (Musa, 2014)

Table 2.1: The Phenotypes and Genotypes of the Rh Blood Group System

Rh Phenotype	Fisher-Race	Weiner	Antigens
	Dce	D,c,e	R°
RhD positive	DCe	D,C,e	R^1
	DcE	D,c,E	R^2
	DCE	D,C,E	R^z
	dce	c,e	r
RhD negative	dCe	C,e	r’
	dcE	c,E	r’’
	dCE	C,E	r^y

Note: An uppercase R is used to describe haplotypes that produce D antigen and a lowercase r is used when D is absent. The C or c and E or e Rh antigens carried with D are represented by 1 for Ce (R1), 2 for cE (R2), o for ce (Ro), and z for CE(Rz). The symbols prime (’) and double prime (”) are used with r to designate the CcEe antigens; for example, prime is used for Ce (r’), doubleprime for cE (r”), and “y” for CE (ry). The R versus r terminology allows one to convey the common Rh antigens present on one chromosome in a single term (a phenotype). (Adapted from Helena Day 2015 ; www.wikipedia.org/wiki/Rh_blood_group_system)

Table 2.2: Frequencies of Rh Phenotypes among Blood Donors in NBC, Malaysia

	Malay (%)	Chinese (%)	Indians (%)
CDe/CDe (R1R1)	61.5	53.6	50.0
cDE/cDE (R2R2)	1.0	9.1	0.8
CDe/cDE (R1R2)	15.0	24.8	12.5
CDe/cde (R1r)	15.0	6.7	23.4
cDE/cde (R2r)	2.5	2.9	5.0
CDe/CDE (R1Rz)	3.5	2.2	0.8
cDE/CDE (R2Rz)	1.0	0	0
cde/cde (rr)	0.5	0.7	7.5

(Adapted from:, Rozi Hanisa Musa, 2012)

2.3 Variation of RhD antigen expression

2.3.1 RhD positive

Almost 95% of the world population is Rh positive. Rh positive phenotypes have an intact RHD gene and complete expression of the D antigen on the surface of the RBC membrane denotes RhD positivity (Figure 2.3). All 10 exons of the D antigen are expressed and are

detected without ambiguity with anti-D. The Rh C, c, e, E antigen is also almost always expressed in various combinations(Avent and Reid, 2000)

2.3.2 RhD negative

There are wide racial differences not only in the frequency of Rh phenotypes but also at its molecular level. The distribution of the RhD negative phenotype is varied across different ethnicities. The RhD negative phenotype is most commonly found in Caucasians (15%), less common in Blacks (8%), and rare in Asians (1%). The Rh CE protein is almost always present in RhD negative phenotypes. Inactive or silent RHD gene rather than the entire gene deletions are the reason behind RhD negative phenotypes in the African and Asian populations as compared to the Caucasians (Wagner, 1995; Daniels, 2013). There are many mutated RHD genes and hybrid genes responsible for RhD negative phenotypes and the most common Rh negative genotypes are a result of homozygosity or compound heterozygosity for an RHD deletion, RHD pseudogene (RHDΨ) (a D gene inactivated by a 37 bp duplication in exon 4) and RHD–CE–D^S gene which is a hybrid of CE and D antigen, with an abnormal C antigen and no D antigen produced (Figure 2.3)(Daniels, 2013). In Asians, 70% of RhD negative genotypes are due to RHD gene deletion and the rest have an intact but poor expression of the RHD gene such as DEL variants(Okuda *et al.*, 1997).

2.3.3 D Variant

There are largely 2 classes of D Variant. The first is a quantitative variant where the entire RHD gene is intact but has reduced expression levels. This heterogeneity in expression is termed weak D and weaker expressions are classified as DEL (Pornlada Nuchnoi 2014). The second is a qualitative variant where structural changes to the D protein or antigen results in absence of some or many epitopes resulting in partial D and RHD-CE-D hybrid genes (Daniels, 2013). Molecular mechanism underlying variant D's are generally a result of single nucleotide polymorphisms (SNP's), point mutations or genetic recombination's resulting in a RHD-CE-D hybrid gene (Daniels, 2013; Kappler-Gratias *et al.*, 2014).

2.3.3(a) RHD-CE-D hybrid gene

RHD-CE-D hybrid gene is a result of rearrangements and exchanges between RHD and RHCE gene (Kappler-Gratias et al., 2014). Since the two Rh genes are closely situated with an inverted orientation, this enhances opportunity for genetic exchange. Exchanges between these two genes encode hybrid proteins that may have RhCE and RHCE residues in RhD protein. These generate new antigens and alter or weaken expressions of the conventional RhD antigens. (Figure 2.3) (Avent and Reid, 2000)

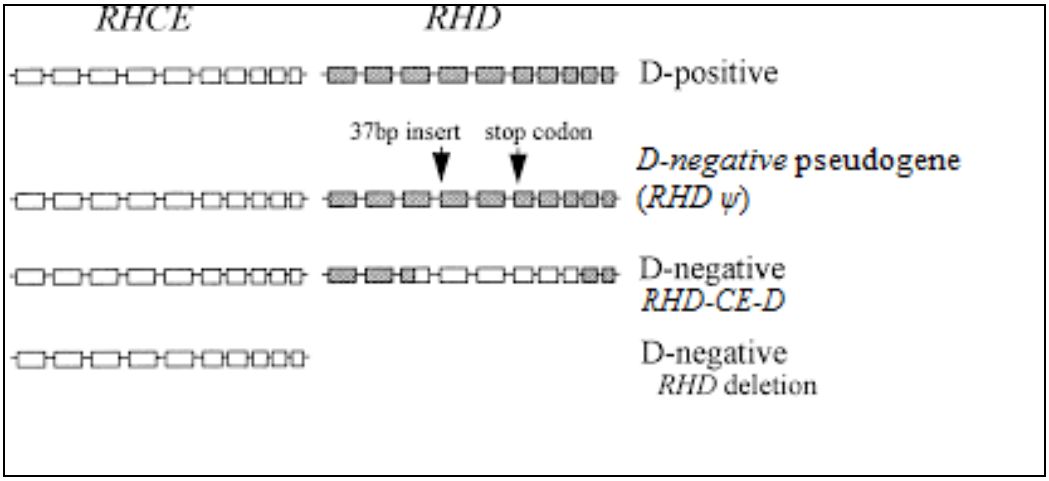

Figure 2.3: Representation of the genomic organization of the RhD positive haplotype and 3 RhD negative haplotypes. (Adapted from Belinda K, 2000)

2.3.3(b) Partial D variant

Partial D variant is a qualitative variant, where either some or many epitopes are lacking on the D antigen. Individuals who lack certain parts of the D antigen may be immunized with normal D RBC's and produce antibodies to the missing parts. These antibodies behave as anti-D in routine serological testing. Analysis of epitopes of the D antigen on partial D red cells by monoclonal antibodies and sequencing of genes encoding the D antigen led to classification of partial D antigens into categories. These categories were initially numbered with Roman numerals from DII (DI was made obsolete) to DVII, and further molecular analysis discovered other partial D variants such as DBT, DFR, and DHAR The molecular basis of partial D is shown in (Figure 2.4). The strength of reactivity of partial D antigens with the anti-D that react with them varies. Some reactions may be weaker than that of normal RhD positive cells (e.g. DVI), similar to normal RhD positive cells (e.g. DIII), or can even seem to have raised D expression (e.g. DIVa) (Daniels, 2013). Almost all Partial D can produce anti-D with partial DVI to be most commonly reported(Zabern *et al.*, 2013).

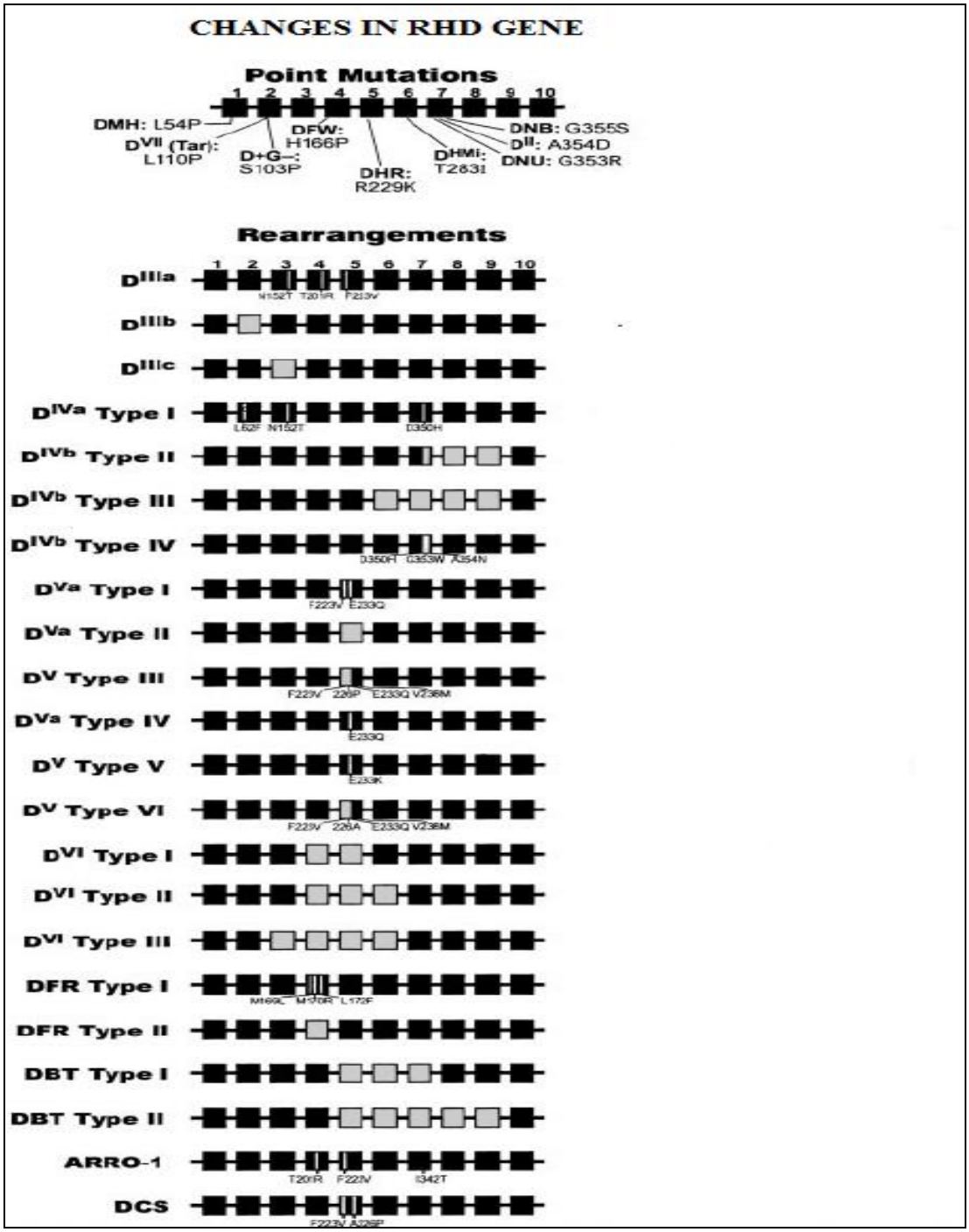

Figure 2.4 : Molecular basis of Partial D phenotypes(Avent and Reid, 2000).

2.3.3(c) Weak D variant.

Weak D variant is a result of normal RHD sequence but with severely reduced expressions. Genetic analysis of the weak D phenotype, demonstrated a standard RHD sequence but with a reduced expression indicating a defect at transcription or pre-mRNA processing levels. Recent research on weak D RBCs found missense mutations within the transmembrane or cytoplasmic domains of RhD antigen(Garratty, 2005). Molecular basis of weak D variants is shown in (Figure 2.5).The normal number of D antigen sites in normal red blood cell (RBC) is 10,000-30,000 antigen sites and 1500 to 7000 sites for weak D and in DEL RBC's is estimated to be less than 40 (Nagl, 2014; Pornlada Nuchnoi, 2014). Individuals with weak D type 4.2-11 and weak D type 15 can be immunized to make anti-D and should be treated as RhD negative. These weak D types should be transfused with RhD negative red blood cells. Individuals with weak D types 1, 2, 3, 4.0, 4.1 and 5 can be treated as Rh positive and be transfused by Rh positive red blood cells (Rizzo *et al.*, 2012).

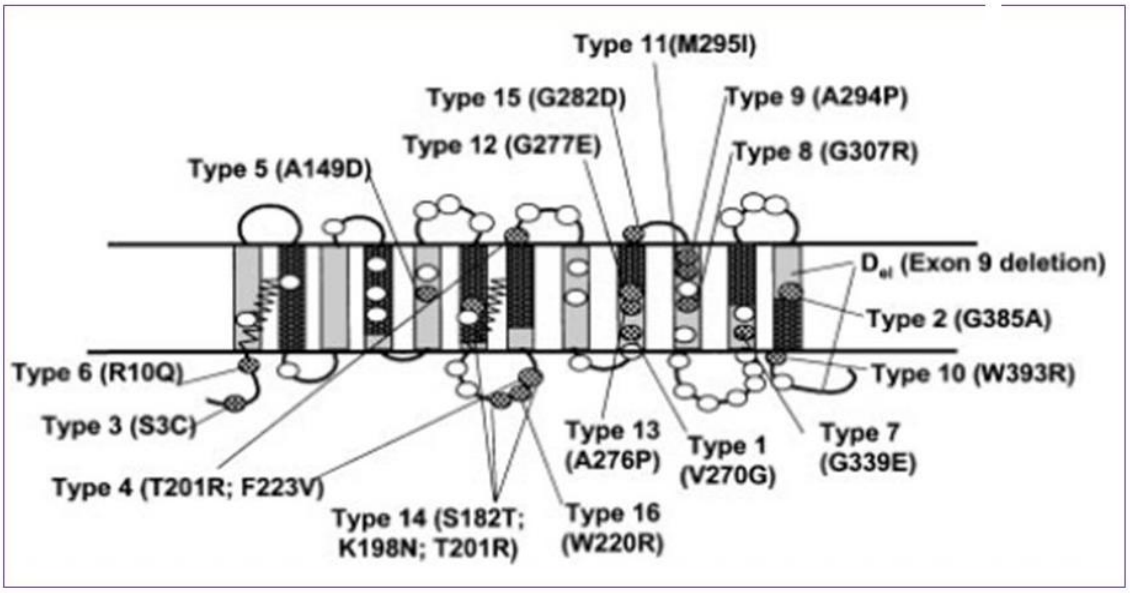

Figure 2.5: Molecular basis of weak D phenotypes.
Note: This figure depicts missense mutations in the RHD gene associated with weak D phenotypes. The locations of these mutations on the predicted topology of the RhD protein are depicted as checkered ovals; the D specific amino acids are shown as open ovals. Most of the missense mutations are located within nonconserved membrane spans (gray) and cytoplasmic regions. Regions of conserved Rh protein family sequence are indicated as black rectangles. (Adapated from: Neil D. Avent and Marion E. Reid, 2000)

2.3.3(d) DEL variant

Some RhD antigen are so weakly expressed in blood donors and is below the detection limit of standard serological screening platforms at Blood Banks, have been termed 'DEL' or D-elute. The tern D-elute or 'DEL' is from anti–D adsorption-elution test performed to identify this D variant.

RhD blood group variant DEL is most commonly reported in individuals of Japanese and Chinese ethnicity and generally in East Asians. (Pornlada Nuchnoi, 2014). The reported

prevalence is almost 30% in Asian RhD negative donors. Detailed examination and epitope mapping of the D antigen using the adsorption elution test and with different monoclonal antibodies have been done. Monoclonal antibodies detected that the RHD (IVS3+1g>a) allele, do not have a major proportion of normal D epitopes, expressing only epitopes 2 and 5. In contrast, RHD 1227A K409K, RHD (IVS5-38del4), RHD(X418L), and RHD (M295I) were found to possess all tested D epitopes suggesting a presence of a complete D antigen albeit with poor expressions. (Körmöczi *et al.*, 2005)

The molecular mechanism of DEL variant is unclear but can be mainly explained is through nucleotide modifications in the trans membrane and cytosolic domains of the RhD protein (Figure 2.6) (Pornlada Nuchnoi, 2014). So far, 17 RHD alleles (subtypes) that encode DEL phenotypes have been described (Nagl, 2014).

Due to the complexity of RHD gene it is challenging to detect the RHD architecture and associated modifier genes in DEL variant in different genetic backgrounds. One of the most common DEL alleles in the Caucasian population is IVS3+1 g>a (splice site mutation)(Daniels, 2013), whereas in East Asians the most frequent DEL allele RHD 1227A/K409K (Gu *et al.*, 2014). Various populations report different frequencies of DEL-associated alleles. (Table 2.3)(Pornlada Nuchnoi, 2014)

Table 2.3: Distribution of DEL-Associated Alleles among Different Ethnic Groups

DEL Allele	DEL-Carrying Individuals Per Ethnic Group, No.				
	Taiwanese (n = 94)	**Chinese (n = 26)**	**Chinese (n = 279)**	**Danish (n = 13)**	**German (n=47)**
K409K	**94**	**25**	**268**	**…**	**4**
IVS7+152A	…	1[a]	…	…	…
Hybrid allele[b]	…	…	3	…	1
3G>A	…	…	4	…	…
R10W	…	…	1	…	…
L18P	…	…	1	…	…
L84P	…	…	1	…	…
A137E	…	…	1	…	…
L153P	…	…	…	…	1
G212R	…	…	…	…	1
Y401X	…	…	…	…	1
X418L	…	…	…	…	2
IVS2–2A>G	…	…	…	2	…
IVS8+1G>A	…	…	…	1	…
659delG	…	…	…	2	…
885G>T	…	…	…	1	14
896T>C	…	…	…	1	…
IVS7–2A>C	…	…	…	1	…
IVS3+1g>a	**…**	**…**		**1**	**16**
93_94insT	…	…		1	2
147delA	…	…		…	4

a : Heterozygote of K409K and IVS7+152A b : RHD-CE-D hybrid. (Adapted from: Pornlada Nuchnoi 2014**)**

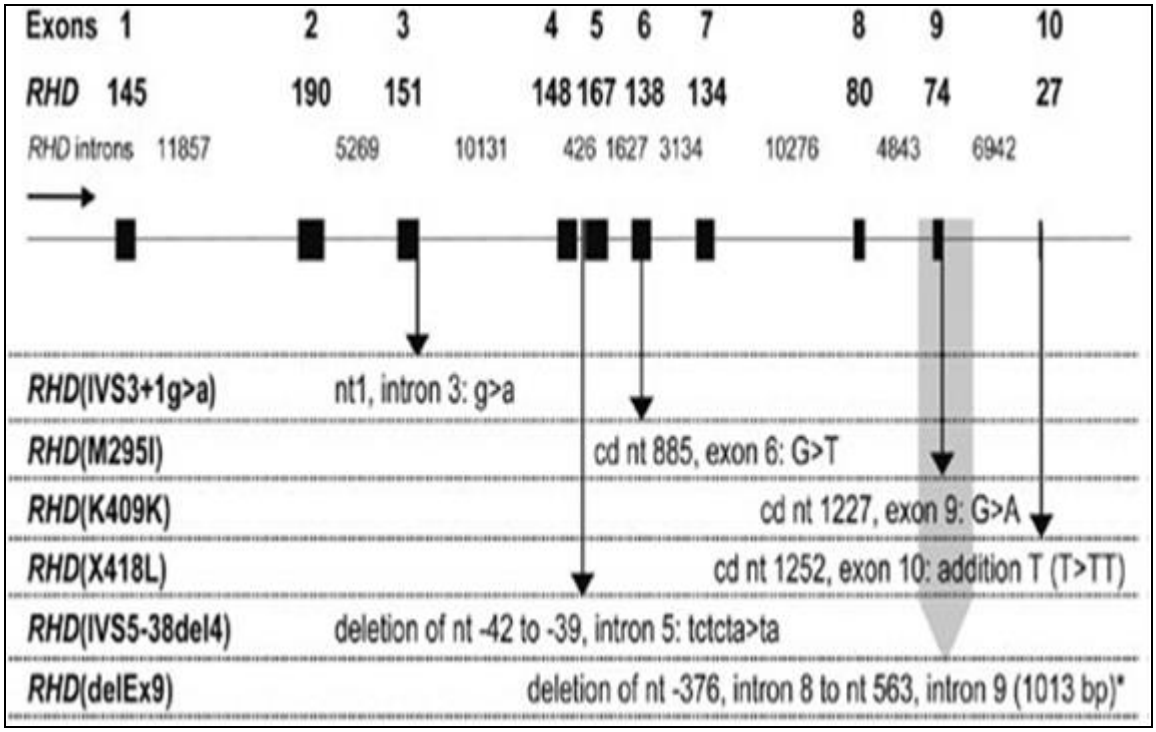

Figure 2.6: Molecular background of known DEL alleles. Note: An in-scale scheme of the RHD gene is depicted, with introns shown at 1/10th of their length. Exon count and exon and intron length in base pairs (bp) are given. (Pornlada Nuchnoi, 2014)

2.4 RhD 1227A>G mutation

In ethnic Eastern Asians the RHD 1227A/K409K is the most common single nucleotide polyphormism mutation resulting in the DEL variant. RHD 1227A/K409K is related to a splice site in exon 9 of RHD gene, where the nucleotide guanine is replaced with alanine. This DEL variant is greatly associated with the C phenotype (CDe or cDE haplotypes)(Table 2.4) (Pornlada Nuchnoi, 2014)

In a study done in Malaysia 12 out of 17 donors of Chinese ancestry, belonging to the Ccee phenotype were found to be positive for the RHD 1227A/K409K or RhD DEL 1227A>G

mutation(Tay Za Kyaw, 2012). A precedent study that supports these findings is a study on the Molecular basis of DEL phenotype in the Chinese population conducted by Juan Gu et all centered in southeast China. Among 165 samples of RhD negative donors and 41(24.58%) of them were typed DEL phenotype by the Indirect AHG test and the adsorption-elution test. Further molecular test with sequence specific primers revealed 90.2% DEL samples were harboring the RHD1227A/K(409)K variant. The remaining samples noted to have RHD-CE-D hybrid alleles (Gu *et al.*, 2014) . Many other studies exploring the genetic basis of the DEL variant showed similar results. (Srijinda *et al.*, 2012; Sun *et al.*, 2008).

Table 2.4: RHCE Phenotypic distribution of DEL among distinct ethnic populations

	Population Frequency			
RHCE Phenotype	**Chinese**	**Taiwanese**	**Taiwanese**	**German**
Ccee	83.51%	83%	83.3%	82.97%
CCee	12.19%	15%	14.3%	10.65%
CCEe	0.36%	1%	0.8%	…
CcEe	3.23%	1%	1.6%	…
ccEe	0.72%	…	…	6.38%
ccee	…	…	2.13%	…

(Adapted from: Pornlada Nuchnoi 2014)

Table 2.5: D variants and Rh considerations for transfusions

RhD Phenotype	D antigen sites	Formation of anti-D	Reactivity with anti – D (monoclonal)	Genetic Basis	Detection	Rh consideration for transfusion
Rh Positive	10,000-30,000	No	Strong	Complete RHD gene present	Reacts at immediate spin	RHD positive
D variants						
Weak D	1,500-7,000	No, except D type 4.2,11 and 15	Weak	Point mutations in intracellular or cytoplasmic region of RHD	Reacts at AHG phase	Can be considered RhD positive for most cases
Partial D	10,000-30,000 (mutated)	Yes	Strong	Point mutations in the extracellular region of RHD	Forms anti-D following transfusion	RhD negative
DEL	22-30	Yes	Negative	RHD mutation and altered epitopes	Formation and elution of anti-D	RhD negative
RHD CE D hybrid gene	reduced	Yes	Strong : D^{HAR}, ce^{cf} antigen Weak : ce^{RT}, ce^{SL} antigen	Presence of D like amino acid sequence in Rhce protein reacting to some monoclonal anti-D	Negative with some and positive with some monoclonal antibodies	RhD negative
Rh negative	none	Yes	Negative	No RHD gene present		RhD negative

(Adapted from : Krishna Oza, 2011)

2.5 Clinical importance of D variants in patients and donors.

D variants are clinically important because there have been reported cases where RhD positive patients have produced anti-D with transfusion from RhD positive RBC's and RBC's from seemingly RhD negative donors have stimulated anti-D production in RhD negative patients. There are also cases where individuals show discrepant RhD typing when tested at different centres, for example RhD negative at Hospital A but RhD positive at Hospital B. Upon further investigation, these patients and donors were discovered to be D variant (Oza, 2015).

Individuals with RhD variant have been reported to experience acute and delayed hemolytic transfusion due to mislabeling of RhD type. In the field of obstetrics, women with D variant are known to produce anti- D with a potential for hemolytic disease of fetus and newborn (HDFN). It is important to appropriately classify D status so that women with D variants capable of making anti-D can be offered Rh IG prophylaxis (Dehua Wang, 2010).

Weak D types 1–3 are the most frequent of those variants with a 'weak D' designation, representing about 93% of those D variants in a Caucasian population (Wagner et al, 1999). Frequencies vary in different populations in Asia the most common variant D is DEL variant amounting to 30% of RhD negative individuals. As mentioned earlier, individuals with weak D type 4.2-11 and weak D type 15 are commonly found to produce anti-D. Almost all partial D variants can make anti-D, but the clinically important ones are DAR, DFR, and DAU and categories DIIIa, DVa, and DVI(Table 2.5 and Table 2.6) (Denomme *et al.*, 2005; Oza, 2015).

DEL variants found to produce anti-D include RHD 1227A K409K, RHD (IVS3 + 1G>A) allele, DEL-5, DEL-ex 8 del(Daniels, 2013; Nagl, 2014; Xu *et al.*, 2015)

Table 2.6: Some D variants associated with production of alloantibodies to RhD antigen.

Partial D		**Weak D**	**DEL**
DII	DFL	Weak D type 1a	**DEL-5**
DIII	**DFR**	Weak D type 2a	**DEL-ex 8 del**
DIVa	DFV	**Weak D type 4**	**RHD 1227A K409K,**
DIVb	DHAR	**Weak D type 11**	**RHD (IVS3 + 1G>A)**
DV	DHMI	**Weak D type 15**	
DVI	DMH	Weak D type 21	
DVII	DMI	Weak D type 57	
DAR	DNAK	Weak D type 2a	
DAU	DNB	Weak D type 4	
DBT	DOL	Weak D type 21	
DVII	DFV	Weak D type 57	
DAR	DHAR		

(Adapted from : Geoff Daniels,2013)