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Elucidation of Weak D Phenotype among Malaysian Blood Donors using Molecular Basis

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Abstract: The Rh blood group system is the most complex, polymorphic and immunogenic protein-based blood group system in humans. Some individuals produce a weak expression of D antigen on RBCs surface as a result of missense RHD mutations and amino acid substitutions that lead to distinct serologic phenotypes and anti-D immunization. This study aimed to elucidate the molecular genetic basis of weak D phenotypes in Malaysian blood donors with multiracial population. A total of 183 Rh-negative blood samples were phenotyped for D, C, c, E and e antigens. Weak D samples that identified by Indirect Antiglobulin Test (IAT) were amplified by polymerase chain reaction-sequence specific primers for weak D type determination. A total of 183 multiracial Rh-negative donors were reviewed, consisting of 88 (48.1%) Indians, 56 (30.6%) Malays, 23 (12.6%) Chinese and 16 (8.7%) other minority ethnics. Four samples were found to be positive for weak D phenotype using IAT. Of these four samples, two samples were reported with weak D type 15. Both samples were from Chinese blood donor with D^{weak}Ccee and D^{weak}ccEe phenotype, respectively. This study provide the first database of a molecular basis of weak D in Malaysian blood donors which improved the understanding of molecular mechanisms underlying D antigen expression in Malaysian population. Thus, increase the transfusion safety in highly racial mixed population.

Key words: Rh negative, weak D phenotype, Malaysian blood donors, Malaysian population, polymerase chain, transfusion safety

INTRODUCTION

The Rh blood group system is the most complex, polymorphic and immunogenic protein-based blood group system in humans (Zacarias *et al*., 2016). It represents the largest number of antigens out of the 36 known blood group systems which comprised of 54 antigens numbered RH1 to RH61 and more than 200 alleles for RHD gene alone (Flegel, 2011; ISBT., 2016). The Rh antigen are encoded by two highly homologous genes (RHD and RHCE) in Rh locus that localized in chromosome 1p34.4-p36.1. These genes are placed in opposite orientations and share more than 90% homology of all introns and coding region (Chen *et al*., 2016).

The D blood group antigen is a clinically significant protein of Rh system due to its association in haemolytic disease of newborn, haemolytic transfusion reactions and autoimmune haemolytic anaemia (Cruz *et al*., 2012).

The RHD gene encoded the RhD protein that expressing the D antigen while the RHCE gene encoded for RhCcEe protein that expressing Ce, CE, ce or cE antigens. In transfusion medicine, each individual is clinically classified as Rh-positive or Rh-negative depending on the presence or absence of the D antigen on the Red Blood Cell (RBC) membrane. Rh-positive was determined by expression of D antigen that encoded by normal and functional RHD gene intact with RHCE gene on a single locus. Meanwhile, Rh-negative indicates total absence of D antigen that commonly caused by complete absence of the RhD protein which clarifying the high immunogenicity of D. Genetic background of Rh-negative explained the difference among the majors human population. The prevalence of Rh-negative phenotype was estimated <3% in Asians, 15-17% in Caucasians and 5% in Black Africans (Cruz *et al*., 2012). The RHD gene polymorphism contributes to phenotypic polymorphism

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D variants including weak D, Del and partial D (Wafi *et al*., 2016). A serologic weak D phenotype is defined by reduced RhD expression on RBCs membrane compared to its expression in the vast majority of Rh-positive individual (Chen *et al*., 2012). The reactivity of weak D RBCs with anti-D giving no or >2+ reaction (<2+) in initial testing and it can only react with IgG anti-D by Indirect Antiglobulin Test (IAT) (Sandler and Flegel, 2015). Weak expression of D antigen is caused by missense RHD mutations and amino acid substitutions of weak D types are positioned in intracellular and transmembraneous protein segments and clustered in four regions of the Rh protein (Chen *et al*., 2012). The prevalence of weak D antigen ranges from 0.2-1% and these incidence occurred varies in different ethnic groups, populations and geographical locales (Chen *et al*., 2012). It was demonstrated that persons with weak D type 1, 2, 3, 4.0 or 4.1 have never been reported to cause formation of alloanti-D. However, it was documented that certain other types of weak D including weak D type 4.2, DAR, type 11, 15, 21 and 57 have been reported to be associated with alloimmunization (Sandler *et al*., 2015).

In fact, some weak D are not detected by serologic techniques. This become a worldwide concern in blood transfusion due to its potential to cause anti-D alloimmunisation when donors with certain weak D types being transfuse to Rh-negative patient. Considering the risk of forming alloanti-D following blood transfusion and the diverse ethnicity of Malaysian, this study aims to elucidate the molecular genetic basis of weak D phenotypes in Malaysian blood donor, a representative sample of a genetically diverse population due to its high degree of miscegenation.

MATERIALS AND METHODS

Blood specimen collection: This study was carried out in accordance with the standards recommended by Medical Research and Ethics Committee (MREC), Ministry of Health Malaysia (NMMR-15-1622-25347). A total of 183 Rh-negative unrelated blood donor's samples were collected in Ethylenediaminetetraacetate (EDTA) tubes from National Blood Centre (NBC) between Mar. to Jun 2016.

Rhesus phenotyping: The Rhesus phenotype for C, c, D, E and e of all donor's RBCs samples were determined by standard serologic protocols. The commercial antibody reagents used for Rhesus typing were from CSL (Australia), Millipore (United Kingdom) and Bio-Rad (Switzerland). Samples that showed weak agglutination with anti-D sera (<2+) were determined for weak D phenotype using an IAT.

DNA extraction and quantification: Genomic DNA was isolated from peripheral blood cells using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA concentration and purity were determined using biophotometer (Eppendorf, USA) and purity index in between 1.7-2.0.

PCR-SSP genotyping for weak D types: For molecular analysis, BAGene PCR-SSP kit (BAG Health Care, Lich, Germany) was used to identify specific weak D allele according to the manufacture's instruction. BAGene PCR-SSP for weak D kit was designed to identify weak D type 1, 1.1, 2, 3, 4.0 or 4.1, 4.2, 5, 11, 15, 17 and 20. PCR strip for weak D genotyping consisted of 8 reaction mixture. Reaction 1 consists of multiplex-PCR reaction targeting weak D allele type 1 (150 bp) and 1.1 (150 and 250 bp). Reaction 2 consists of multiplex-PCR reaction targeting weak D allele type 2 (126 bp). Reaction 3 consists of multiplex-PCR reaction targeting weak D allele type 3 (165 bp). Reaction 4 consists of multiplex-PCR reactions targeting weak D allele type 4.0 or 4.1 and weak D allele type 4.2 (101 bp). Reaction 5 consists of multiplex-PCR reaction targeting weak D allele type 4.2 (130 bp) and weak D allele type 17 (83 bp). Reaction 6 consists of multiplex-PCR reaction targeting weak D allele type 5 (112 bp). Reaction 7 consists of multiplex-PCR reaction targeting weak D allele type 11 (198 bp) and weak D allele type 17 (83 bp). Reaction 8 consists of multiplex-PCR reaction targeting weak D allele type 15 (153 bp). The internal control product will appeared in all lanes at 434 bp. PCR products were visualized in 2% agarose gel.

RESULTS AND DISCUSSION

Serologic test results: The prevalence of Rh-negative blood group differs considerably among ethnics groups and populations. The frequency of Rh-negative was estimated >3% in Asians, 15-17% in Caucasians and 5% in Black Africans (Cruz *et al*., 2012). In this study, the distribution of Rh-negative phenotype was carried out among Malaysian blood donor which represent highly racial mixed population. Malaysia, a multiracial population comprising of three major races (Malays, Chinese and Indian) and other ethnic groups. Thus, this study aimed to investigate the distribution of Rh-negative phenotypes among the ethnic groups in the Malaysian population.

Between the periods of Mar to June 2016, a total of 183 Rh-negative blood donors were collected at NBC (Table 1). Of these blood donors, 88 (48.1%) Indians, 56 (30.6%) Malays, 23 (12.6%) Chinese and 16 (8.7%) other

Table 1: The distribution of different Rh phenotypes among 183 blood donors of various races at NBC, Kuala Lumpur, Malaysia

		Races			
Rh phenotype	Frequency (n = 183)	I	M	C	O
dccee	162 (88.52%)	85	44	17	16
dCCee	12 (6.56%)	2	8	2	0
dCcee	4 (2.18%)	0	2	2	0
dCcEe	1 (0.55%)	0	1	0	0
D^{weak}Ccee	2 (1.09%)	1	0	1	0
D^{weak}ccEe	1 (0.55%)	0	0	1	0
D^{weak}ccee	1 (0.55%)	0	1	0	0
TOTAL*	183 (100%)	88	56	23	16

I: Indian, M: Malay, C: Chinese, O: Others

minor ethnic groups. The commonest phenotype recorded was found to be dccee, 162 (88.52%). This was observed in 85 (96.6%) Indian, 44 (78.6%) Malays and 17 (73.9%) in Chinese. The remaining 16 (100%) consisted of donor from minor ethnic groups. The prevalence of dccee phenotype in Rh-negative blood donor in Malaysia reported by Kyaw *et al*. (2012) was found to be 750 out of 911 Rh-negative blood donor between the period of January 2003 and September 2008, showed nearly similar with present findings. Out of 750 dccee phenotype, 91.5% Indian, 74.6% Malays, 55.5% Chinese and 92.5% others (minor ethnic groups). Meanwhile, Rh-negative blood donors with C and or E antigens including CCee, Ccee and CcEe were reported to be 6.56% (12/183), 2.18% (4/183) and 0.55% (1/183), respectively. No dccEe phenotype was recorded in this study. Malays showed the most prevalence Rh-negative with C and or E antigens 8 compared to Chinese 4 and Indian 2 (Table 1).

Weak D phenotype is defined by weak expression of RhD antigen on RBCs membrane which result in reduced reactivity of anti-D, giving no or >2+ reaction (<2+). This type of phenotypic polymorphism D variants can only react with IgG anti-D by Indirect Antiglobulin Test (IAT) giving moderate to strong agglutination (Chen *et al*., 2012; Sandler *et al*., 2015). Out of 183 Rh-negative blood samples, 4 samples were found to be positive for weak D phenotype. The two samples that positive for weak D phenotype were present with Ccee and ccEe phenotypes. The incidence of weak D worldwide range from 0.2-1% as reported by Gupta *et al*. (2016) and majority are associated with weak D type 1-3. While the molecular genetic basis of weak D are well investigated in Caucasian and other population to the best of our knowledge, there is no data on weak D distribution among Asian population.

Molecular analysis of weak D type by PCR-SSP: Genomic DNA of the four weak D phenotypes samples were subjected to commercially available PCR-SSP for detection of weak D types. Of these four samples, two samples were reported with weak D type 15. Both samples were from

Table 2: Weak D phenotype and associated types in Malaysian population positive with IAT (n = 4)

Sample	Weak D phenotype	Race	Weak type by PCR-SSP
1	D^{weak}Ccee	C	Type 15
2	D^{weak}ccee	I	No type detected
3	D^{weak}ccEe	C	Type 15
4	D^{weak}Ccee	M	No type detected

I:Indian, M:Malay, C: Chinese

Chinese blood donor with D^{weak}Ccee and D^{weak}ccEe phenotype, respectively. Wagner *et al*. (1999), Virk and Sandler (2015) have reported that weak D type 15 has associated with cDE haplotype. In addition, Shao *et al*. (2012) also have similarly reported that weak D type 15 was observed with cEe phenotype. While present study reported that weak D type 15 was observed with the present of Ccee and ccEe phenotype. In such case, weak D type 15 was assumed has independently related with C and or E antigen and molecular genotyping for weak D was optional for weak D phenotype present with either C and or E antigen (Table 2).

Figure 1 shows analysis of weak D type present in individual with weak D phenotype using PCR-SSP. The PCR products were analyzed by agarose gel electrophoresis. Weak D type 15 was observed in lane 9 with product size of 153 bp. In all lanes (lane 2-9), a 434 bp fragments was amplified representing the internal control. M, 100 bp ladder marker.

Distribution of weak D among countries showed difference of occurrences. Based on that reason, previous study recommended that determination of molecular basis of weak D should be applied in own country. Studies on molecular basis of weak D in Europe have underlined three major occurrence weak D types which are weak D type 1-3. Even that the occurrences of the three major weak D types showed difference in frequency among countries in Europe. In Portugal, weak D type 2 showed the most common among the three major weak D types. Weak D type 1 was the most common in Northern and Southwestern Germany while weak D type 3 was the common Tyrol, Austria. In African, weak D type 4 was detected. Findings of type weak D in Europe and African were differed from the finding of present study which recall back that distribution of types of weak D were showed difference among countries.

Similar finding of weak D type 15 also can be observed in Chinese population of China. Shao *et al*. (2012) firstly observed weak D type 15 in Chinese individual. Moreover, Yan *et al*. (2007) found that weak D type 15 was highly observed in Chinese individuals. Currently, weak D type 15 found to be predominant type of weak D in Chinese individuals specifically in Zhejiang Hans population (He *et al*., 2015). Therefore, weak D type 15 known to be the type of weak D that commonly can be

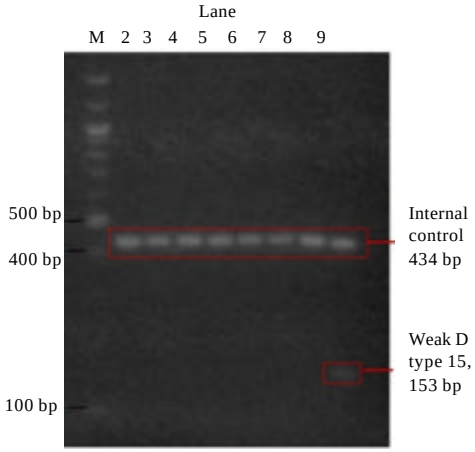

Fig. 1: Analysis of weak D type

observed in Chinese individuals. Besides, weak D type 15 also was identified from Korean blood donors (Luettringhaus *et al*., 2006). Other than that similar finding also was observed in Taiwanese (Lin *et al*., 2003). Based on previous studies in Asia and also present study, weak D type 15 was assumed to represent the common type of weak D that can be observed in Malaysian population.

CONCLUSION

We investigated the molecular mechanism of weak D phenotype among Rh-negative in Malaysian population. Out of 183 Rh-negative blood donors, 4 samples were positive for weak D phenotype by IAT. However, among these 4, 2 samples were reported with weak D type 15 based on amplification using PCR-SSP. Both samples were from Chinese blood donor with D^{weak}Ccee and D^{weak}ccEe phenotype, respectively. This study added to the understanding of molecular mechanisms underlying D antigen expression in Malaysian population and provides useful information for the medical representative to adopt suitable genotyping approaches in routine test. It is recommended to discover more on weak D type in Malaysia in other to effectively utilize the negative blood and to avoid unnecessary Anti D prophylaxis.

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