RHCE*E and RHCE*e genotype incompatibility in a southern Thai Muslim population

Poonyapa Tanwarawutthikul, Kamphon Intharanut, Supattra Mitundee, Oytip Nathalang

Abstract:
CONTEXT: The formation of red cell alloantibodies resulting from both transfusion and pregnancy can cause adverse effects from allogeneic blood transfusions. Alloanti-E is commonly detected among Thai and Asian populations.

AIMS: This study aimed to determine RHCE*E and RHCE*e genotype incompatibility in a southern Thai Muslim population and to compare it with those previously reported for other populations.

SUBJECTS AND METHODS: Nine hundred and twenty-seven DNA samples obtained from 427 unrelated healthy blood donors from southern Thai Muslims and 500 samples from Central Thais were included. Samples were genotyped for RHCE*E and RHCE*e using an in-house polymerase chain reaction with the sequence-specific primer technique.

RESULTS: Significant differences were found when we compared the allele frequencies of the RHCE*E and RHCE*e between southern Thai Muslims and Central Thais: RHCE*E 0.162 versus 0.197 and RHCE*e 0.838 versus 0.803 and also found in Chinese, American native, Japanese, Korean, Alaskan native, Hawaiian, South Asian, Brazilian Japanese-descendant, and Malay Malaysian populations (P < 0.05). In addition, the E/e incompatibilities among southern Thai Muslims and Central Thais were 24.23% and 26.71%, respectively.

CONCLUSIONS: This study was the first to determine the RHCE*E and RHCE*e genotype incompatibility among southern Thai Muslims, enabling the estimation of their potential alloimmunization risk. These data could be useful to provide safe blood transfusions across ethnic populations.

Keywords: E/e incompatibility, RHCE*E and RHCE*e genotype, southern Thai Muslims

Introduction

Red cell antibodies of a certain specificity are produced following the immunization of a corresponding antigen-negative individual through pregnancy or transfusion. Blood group antigen frequencies vary in different populations.\(^1\)\(^,\)\(^2\) Factors influencing red cell alloimmunization depend not only on the dose and immunogenicity of the antigen but also on different antigen distribution-related variables. In Thailand, anti-MNS7 (Mi\(^a\)) and anti-E are usually found among multitransfused patients.\(^3\)\(^-\)\(^5\) In particular, anti-E is detected in a single antibody of those patients at about 13.19%, while found in combinations, such as anti-E plus -Mi\(^a\), -c or -Jk\(^a\), at about 30.24%.\(^3\) Hence, the antigen typing of Rh (C, c, E, and e) and MNS7 is an essential requirement before the first transfusion among patients with thalassemia.\(^6\) Moreover, the implementation of antigen typing to identify phenotype-matched donors could reduce alloimmunization or hemolytic transfusion reactions.\(^7\)\(^,\)\(^8\)

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At present, red cell genotyping is more feasible than standard serological phenotyping for large-scale population screening determinations. The RHCE gene is located on chromosome 1p36.11 and encompasses 10 exons spanning over 58 kb of genomic DNA. The E and e antigens are associated with a biallelic polymorphism caused by a single-nucleotide variation, c.676C>G (rs609320) in exon 5 predicting Pro226 (E) and Ala226 (e) of the RhCE protein. A comparative study of RHCE*E/*e alleles between Central and northern Thais revealed no significant difference. In contrast, other alleles, for example, GYPB*S/*s, DI*A/*B, and JK*A/*B, among southern Thai Muslims significantly differed from Central and northern Thais. Therefore, those data have helped achieve decreased risk for transfusions in three Thai populations. However, data regarding RHCE*E/*e alleles among southern Thai Muslims remain limited. This study thus aimed to determine RHCE*E and RHCE*e genotype incompatibility in a southern Thai Muslim population and compare it with those previously reported for other populations.

**Subjects and Methods**

**Subjects and controls**

Peripheral venous blood was collected in EDTA-anticoagulated blood from 927 unrelated healthy Thai blood donors. Five hundred samples included 300 samples from our previous study and an additional 200 samples from the National Blood Centre, Thai Red Cross Society, Bangkok. In addition, 427 samples were acquired from the Regional Blood Centre 12th Songkhla, Thai Red Cross Society, Songkhla, Thailand. Informed consent was obtained from each subject. This study was approved by the Committee on Human Rights Related to Research Involving Human Subjects, Thammasat University, Pathum Thani, Thailand (COE No. 018/2562), and the Committee for Research In Human Subjects, National Blood Centre, Thai Red Cross Society (COA No. NBC 18/2019). Genomic DNA was extracted from peripheral blood samples using the Genomic DNA Extraction Kit (REAL Genomics, RBC Bioscience, Taipei, Taiwan) and then stored at −20°C until used for genotyping. Three identified samples of DNA consisting of E + e−, 1 E + e+, and 1 E − e + phenotypes, confirmed by DNA sequencing, were used as controls.

**RHCE*E and RHCE*e genotyping by polymerase chain reaction-sequence-specific primer**

RHCE*E and RHCE*e alleles were detected using standard polymerase chain reaction-sequence-specific primer (PCR-SSP). The sequences of the primer combinations used in this study, the product sizes, and final concentrations (μM/L) are shown in Table 1. All primers were designed by NCBI software. In brief, 1 μL of genomic DNA (50 ng/μL) was amplified in 10 μL of total volume (1 μL of 5 μM RH-E forward primer and 1 μL of 5 μM RH-CE reverse primer) to detect the RHCE*E. To detect the RHCE*e allele, 1 μL of 5 μM rh-e forward primer and 1 μL of 5 μM RH-CE reverse primer were used. The human growth hormone (HGH) gene was co-amplified with 1 μL of 6 μM HGH forward primer and 1 μL of 6 μM HGH reverse primer and used as an internal control. A standard PCR technique was used with a reaction mixture of 5 μL of 2X PCR (OnePCR Plus, GeneDirex, Taiwan) using a T100 Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

The PCR technique consisted of one cycle of 95°C for 5 min, followed by 30 cycles at 95°C for 30 s, 62°C for 40 s, and 72°C for 30 s. The final step was a 5-min extension at 72°C, followed by storage at 4°C. After being amplified, the newly created products were electrophoresed at 100 volts with a 1.5% agarose gel using a 1X Tris-borate-EDTA buffer containing a 10,000X fluorescent DNA gel stain (SYBR Safe DNA gel stain, Invitrogen, Paisley, UK) and visualized using blue-light illumination. The product size of the PCR samples for both RHCE*E and RHCE*e alleles was 202 bp, whereas that of the HGH gene internal control was 434 bp.

**DNA sequencing**

The results of the PCR-SSP were confirmed by sequencing the genomic DNA of 30 genotyped donors (10 RHCE*E/RHCE*E, 10 RHCE*E/RHCE*e, and 10 RHCE*e/RHCE*e). A 560 bp fragment that contained single nucleotide polymorphisms (SNPs) (c. 676C/G) was obtained from PCR amplification. The PCR conditions of the DNA sequencing were similar to those for the RHCE*E and RHCE*e genotyping. The sequences of primer pairs of the gene target are shown in Table 1. For each PCR reaction, 2 μl of genomic DNA (50 ng/μL) was amplified in a total volume of 50 μL, using 3 μl of 10 μM SE-RHCE forward primers, and 3 μl of 10 μM SE-RHCE reverse primer for each reaction. The PCR was performed with 25 μL of a 2X PCR reaction mixture (Phusion High-Fidelity PCR Master Mix; New England BioLabs, Ipswich, MA, USA) and 17 μL of sterile distilled water in a T100 Thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Subsequently, the PCR products were purified using a gel extraction kit (GeneJET gel extraction kit, Thermo Fisher Scientific, MA, USA), and eluted fragments were then sequenced (First Base Laboratories Sdn Bhd, Selangor, Malaysia) using those PCR primers.

**Statistical analysis**

Gene and allele frequencies were simply estimated by counting the number of times each gene and allele was observed in samples from southern Thai Muslims and Central Thais. The Chi-square (χ²) test
was used to test for the Hardy–Weinberg equilibrium for the RHCE (E/e) gene. A Pearson’s Chi-squared test was conducted between the independent variables of RHCE*E and RHCE*e allele frequencies among southern Thai Muslims and the independent variables of previously reported populations,\[9,13‑16\] using the allele frequencies in a 2 × 2 contingency table to determine whether the allele frequencies of southern Thai Muslims differed significantly from those of other populations. All statistical analyses were conducted using SPSS, Version 16.0 (SPSS Inc., Chicago, IL, USA). *P<0.05 was established as statistically significant.

In addition, the percentage of predicted E/e incompatibilities was calculated by *\(\frac{\text{E}/\text{E} \times \text{E}/\text{E} + \text{E}/\text{E} \times \text{E}/\text{e} + \text{E}/\text{e} \times \text{E}/\text{e} + \text{E}/\text{e} \times \text{e}/\text{e}}{\text{E}/\text{E} \times \text{E}/\text{e} + \text{E}/\text{e} \times \text{E}/\text{e} + \text{E}/\text{e} \times \text{e}/\text{e}}\)\* and \(\frac{\text{E}/\text{E} \times \text{E}/\text{e} + \text{E}/\text{e} \times \text{E}/\text{e} + \text{E}/\text{e} \times \text{e}/\text{e}}{\text{E}/\text{E} \times \text{E}/\text{e} + \text{E}/\text{e} \times \text{E}/\text{e} + \text{E}/\text{e} \times \text{e}/\text{e}}\)\* the percentage of genotype frequencies in each population. The estimated risk of E/e alloimmunization was obtained by multiplying the probability of being predicted by the E/e-negative phenotype frequency by the probability of having a predicted E/e phenotype frequency.\[10,17\] i.e., anti-E or anti-e by considering the E/e pair of antigens in four conditions:

1. E/e donor to e/e patient (risk of anti-E production): E/e x e/e
2. E/E donor to e/e patient (risk of anti-E production): E/E x e/e
3. e/e donor to E/E patient (risk of anti-e production): E/e x E/E
4. E/e donor to E/E patient (risk of anti-e production): E/e x E/E.

Overall, the global risk of alloimmunization related to RhE/e incompatibilities is the sum of those calculations in four conditions.

**Results**

The results of a PCR-SSP were used to differentiate between the RHCE*E and RHCE*e alleles. The first and second mixes could identify the RHCE*E and RHCE*e alleles with an amplified product size of 202 bp, as shown in Figure 1. The validated genotyping results of 3 DNA controls were consistent with each other, and 30 DNA samples tested by PCR-SSP showed a 100% concordance with the DNA sequencing results [Figure 2].

A total of 427 DNA samples from southern Thai Muslims and 500 samples from Central Thais were examined for the RHCE*E and RHCE*e alleles using the standard PCR-SSP technique. The RHCE*E and RHCE*e genotype and allele frequencies in southern Thai Muslims and Central Thais are shown in Table 2. The genotypes of the 427 southern Thai Muslims and 500 Central Thais were consistent with each other according to the Hardy–Weinberg equilibrium \(\chi^2 = 4.751, \text{DF} = 2, P = 0.093\) and \(\chi^2 = 0.056, \text{DF} = 2, P = 0.962\), respectively. Moreover, \(\text{RHCE}^\text{e}/\text{RHCE}^\text{e}\) was the most common genotype (71.89% and 64.60%), followed by \(\text{RHCE}^\text{E}/\text{RHCE}^\text{e}\) (23.89% and 31.40%) and \(\text{RHCE}^\text{E}/\text{RHCE}^\text{E}\) (4.22% and 4.00%).

The RHCE*E and RHCE*e genotype and allele frequencies in southern Thai Muslims and other populations\[9,13‑16\] are shown in Table 3. Significant differences were found when we compared the RHCE*E and RHCE*e allele frequencies among southern Thai Muslims and Central Thais: RHCE*E 0.162 versus 0.197 and RHCE*e 0.838 versus 0.803. Furthermore, the allele frequencies among southern Thai Muslims also displayed significant differences with Chinese, American native, Japanese, Korean, Alaskan native/Aleut, Hawaiian/Pacific Islander, South Asian, Brazilian Japanese-descendant, and Malay Malaysian populations (\(P<0.05\)).
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For the next step, E/e incompatibilities were predicted in different populations [Table 3]. The E/e incompatibilities among southern Thai Muslims and Central Thais were 24.23% and 26.71%, respectively. To sort from low to high, the South Asian population had the lowest percentage of prediction of E/e incompatibilities, followed by the Italian (Naples), Filipino, northern Thai, southern Thai Muslim, Southeast Asian, Central Thai, Hawaiian/Pacific Islander, Chinese, Brazilian Japanese-descendant, American native, Japanese, Alaskan native/Aleut, Malay Malaysian, and Korean populations. In addition, the risk of E and e alloimmunization among populations was analyzed, as shown in Table 3. An important finding of our study is that, according to the genotype data, the proportion of estimated risk of E alloimmunization was more than 1.24-fold higher in the Korean population (0.2498) than in southern Thai Muslims (0.2021).

Discussion

In general, the Rh blood group antigens are highly immunogenic; hence, the prevalence of anti-E is often detected in multiparous women and in patients with repeated red cell transfusions among Asian populations. They may require antigen-negative donor’s red cells for transfusions. The accessibility of appropriate transfusions depends on the compatibility of a patient’s ancestral genetic background and the ancestry of the majority of blood donors in the population. [18] Southern Thai Muslims usually share strong ethnic, linguistic, religious, and cultural bonds with the people across the Thai-Malaysian border, resulting in higher genetic similarities with Malaysians than with people in other regions of Thailand. [19] For RHCE*E and RHCE*e allele frequencies in northern and Central Thais, no significant difference was observed. [9]

In this study, we developed the PCR-SSP to determine those two alleles, and the validated PCR-SSP genotyping results agreed with the DNA sequencing results; hence, the genotyping results were accurate and reliable. The RHCE*E and RHCE*e allele frequencies among southern Thai Muslims were described and compared with other populations. For three Thai populations, the RHCE*E allele among southern Thai Muslims was significantly lower than that of Central Thais, but no significant difference was found compared with northern Thais. [9] In addition, we observed marked differences across other Asian populations. [13,16] The differences and similarities of the two alleles could lead to predicting E/e incompatibilities and the risk of E and e alloimmunization. Concerning the South Asian population, our data displayed the lowest estimated E/e incompatibilities and risk of E alloimmunization. Those estimated results, however, were not perfectly correlated with the actual alloanti-E that can be frequently observed.

Table 2: RHCE*E and RHCE*e genotype and allele frequencies among southern Thai Muslims and Central Thais

| Allele    | Allele frequencies | Genotype         | Observed (%) | Expected (HWE) | $\chi^2$ | P (DF=2) |
|-----------|--------------------|------------------|--------------|----------------|----------|----------|
| RHCE*E    | 138 (0.162)        | RHCE*E/RHCE*E    | 18 (4.22)    | 11             | 4.751    | 0.093    |
| RHCE*e    | 716 (0.838)        | RHCE*E/RHCE*e    | 102 (23.89)  | 116            |          |          |
|           |                    | RHCE*e/RHCE*e    | 307 (71.89)  | 300            |          |          |
| Central Thais (n=500) |                         |                    |              |                |          |          |
| RHCE*E    | 197 (0.197)        | RHCE*E/RHCE*E    | 20 (4.00)    | 19             | 0.056    | 0.962    |
| RHCE*e    | 803 (0.803)        | RHCE*E/RHCE*e    | 157 (31.40)  | 158            |          |          |
|           |                    | RHCE*e/RHCE*e    | 323 (64.60)  | 323            |          |          |

DF=Degree of freedom, HWE=Hardy-Weinberg equilibrium

Figure 2: Electropherograms of the RHCE gene at the RHCE*E and RHCE*e polymorphism region, single-nucleotide variation c.676C>G (rs609320). (a) Homozygous state of the c.676C identified in the E+e−phenotype, (b) heterozygous state of the c.676C/G identified in the E+e+phenotype, and (c) homozygous state of the c.676G identified in the E−e+phenotype.
Table 3: Comparison of allele and genotype frequencies, prediction of E/e incompatibilities, and alloimmunization risk from different ethnic populations

| Population                          | n   | RHCE alleles (%) | RHCE genotypes (%) | E/e incompatibilities (%) | Alloimmunization risk |
|-------------------------------------|-----|------------------|--------------------|---------------------------|-----------------------|
|                                     |     | *E   | *e    | *E/*E | *E/e | *e/e | Anti-E | Anti-e |
| Southern Thai Muslims (current study) | 427  | 0.162 | 0.838 | 4.2   | 23.9 | 71.9 | 24.23  | 0.2021 0.0402 |
| Central Thais (current study)       | 500  | 0.197 | 0.803 | 4.0   | 31.4 | 64.6 | 26.71  | 0.2287 0.0384 |
| Northern Thais[9]                   | 300  | 0.163 | 0.837 | 2.0   | 28.7 | 69.3 | 23.24  | 0.2126 0.0198 |
| Chinese[10]                         | 1,715| 0.221 | 0.779 | 4.7   | 34.8 | 60.5 | 28.38  | 0.2390 0.0448 |
| Filipino[10]                        | 1,333| 0.140 | 0.860 | 2.2   | 23.5 | 74.3 | 21.25  | 0.1910 0.0215 |
| American Native[11]                 | 970  | 0.240 | 0.760 | 6.8   | 34.3 | 58.9 | 30.55  | 0.2421 0.0634 |
| Japanese[12]                        | 1,022| 0.260 | 0.740 | 6.1   | 39.8 | 54.1 | 30.56  | 0.2483 0.0573 |
| Korean[13]                          | 1,033| 0.311 | 0.689 | 10.7  | 40.7 | 48.6 | 34.54  | 0.2498 0.0956 |
| Alaska Native/Aleut[13]             | 621  | 0.305 | 0.695 | 9.0   | 43.0 | 48.0 | 33.15  | 0.2496 0.0819 |
| Hawaiian/Pacific Islander[13]       | 522  | 0.198 | 0.802 | 4.8   | 30.1 | 65.1 | 27.29  | 0.2269 0.0460 |
| South Asian[13]                     | 922  | 0.105 | 0.895 | 1.3   | 18.3 | 80.4 | 17.04  | 0.1576 0.0128 |
| Southeast Asian[13]                 | 942  | 0.171 | 0.829 | 3.4   | 27.4 | 69.2 | 24.60  | 0.2131 0.0329 |
| Italian (Naples)[14]                | 225  | 0.124 | 0.846 | 3.1   | 18.7 | 78.2 | 20.05  | 0.1704 0.0301 |
| Brazilian Japanese descendants[15]  | 209  | 0.254 | 0.746 | 4.3   | 42.1 | 53.6 | 28.99  | 0.2487 0.0412 |
| Malay-Malaysian[14]                 | 360  | 0.257 | 0.743 | 10.0  | 31.4 | 58.6 | 33.26  | 0.2426 0.0900 |

*P<0.05, allele frequencies differed from those among southern Thai Muslims. n=Number of subjects

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Conflicts of interest
There are no conflicts of interest.

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