Rh-Matched Transfusion through Molecular Typing for β -Thalassemia Patients Is Required and Feasible in Chinese

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Keywords

β -Thalassemia · *RHCE* genotyping · Rh-matched transfusion

Summary

Background: Molecular typing for *RHCE* blood group alleles has been established in many countries for patients and blood donors. In the Chinese literature nearly 80% of transfused patients with alloimmunization have antibodies specific for antigens of the Rh blood group system. We investigated if it is feasible to match packed red blood cells (RBCs) for Chinese β -thalassemia patients by *RHCE* genotyping. **Methods:** In this study, 481 patients with β -thalassemia were enrolled. They were genotyped for *RHCE* alleles by a simple PCR method with sequence-specific primers (PCR-SSP). Among these patients, 203 continuously received RBCs of the identical Rh subgroups according to the genotyping results for at least 3 months. Subsequently, their phenotypes were tested through a micro-column gel card method. For validation purposes, 400 donors were serologically typed with the same technology, of which 164 were genotyped too. Finally, the C, c, E, and e frequencies and the feasibility of the simple genotyping method were analyzed. **Results:** All patients showed mixed-field agglutination in the Rh subgroup gel cards before the same Rh subgroups in blood donors were selected for blood transfusion. The results, however, lacked mixed-field agglutination in all

203 cases after transfusion with RBC concentrates selected for the patient's C, c, E, and e antigens for at least 3 months. The genotyping results of 164 donors were all consistent with the serological results. Whole coding regions of *RHCE* were sequenced in 7 individuals with weak c, E, or e antigens. In only one sample we observed a 1059G>A nucleotide mutation coding for a truncated RhCE polypeptide (GenBank KT957625), in the other 6 samples no sequence variant was found. Both patients and donors were predominantly CcEe and CCee, with a prevalence of 55.3% and 24.9% for patients or 49.3% and 31.3% for donors, respectively. It revealed that about 80% of Chinese could receive Rh-matched RBCs easily. **Conclusion:** A simple *RHCE* genotyping technique is safe enough for Rh-matched transfusion of β -thalassemia patients in Chinese Han.

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Introduction

Many patients with β -thalassemia chose regular blood transfusion rather than bone marrow transplantation. Transfusion with effective iron chelation therapy has dramatically improved life expectancy of β -thalassemia patients in China in recent years. Many of them can live normally. However, 'red cell alloimmunization'

became a new challenge for them [1]. The incidence of alloantibodies varies in different ethnic groups, and so is the antibody specificity [2–4]. About 15–40% of β -thalassemia or other chronically transfused patients are alloimmunized according to the literature [5–7]. In China, investigations from Taiwan and Hong Kong independently reported that 10–23% of transfused β -thalassemia patients have alloantibodies [8, 9]. According to the Chinese literature, including studies with large sample numbers, about 50–80% of antibodies are specific for the Rh subgroup antigens C, c, E, and e [10–13]. Therefore, Rh-matched transfusion is clinically important for Chinese patients, especially for those individuals who receive blood transfusions regularly [14].

In China, β -thalassemia patients are mainly located in the southern part, including Guangdong, Guangxi, Fujian, Hong Kong (near Guangdong province), and Taiwan (near Fujian province). Most of them choose therapy of regular blood transfusion in local hospitals. Unfortunately, very few studies investigated the relevance of Rh-matched transfusion in China, and according to the transfusion guidelines, red blood cell (RBC) concentrates have to be matched for ABO and RhD only. Outside China, there are many reports of Rh-matched blood transfusions in chronically transfused patients, some of them demonstrate that *RHD* and *RHCE* genotyping methods are feasible and helpful for the determination of the patient's correct Rh blood group [15, 16]. However, some reports describe that a few patients could still develop anti-Rh antibodies, even following Rh-matched transfusions. For example, Chou et al. [17, 18] published in 2013 that 45% of African-American patients, who received Rh-matched RBCs according to PCR-SSP results, developed anti-Rh antibodies at the Children's Hospital of Philadelphia. The authors concluded that genetic variations caused false-positive results in the PCR-SSP tests. In this study, we predicted the RhCcEe phenotypes with a simple *RHCE* genotyping technique in β -thalassemia patients in our hospital and performed transfusion with RBCs from donors with the same Rh subgroup, if available. Thus, we evaluated the feasibility of Rh-matched transfusion in Chinese clinics.

Material and Methods

Subjects

Among regular transfused β -thalassemia patients in the First Affiliated Hospital of Shenzhen University School of Medicine, 481 patients were enrolled from October 2013 to September 2017 after informed consent. We applied *RHCE* genotyping in these patients and selected RBCs with the same Rh subgroup type, if possible. We excluded RhD-negative (less than 0.3% in Chinese Han population and only 1 β -thalassemia patient in our hospital), autoantibody-positive patients (about 4% who apply for washed RBCs), and patients with unidentified adverse transfusion reactions in the medical history. In addition, we included 12 patients with anti-CcEe (2 of anti-C, 1 anti-e, 8 anti-E, and 6 anti-c), but excluded those who had alloantibodies different from anti-C, anti-c, anti-E, or anti-e (more than 1% in our hospital). All enrolled patients are Han Chinese. The majority was from Guangdong, Guangxi, and Fujian provinces, others were from Sichuan, Hubei, and Hunan. There were 270 males and 211 females (1:0.78). The age distribution of the patients was as follows: 141 patients were 0–5 years old, 181 between 6 and 10 years, 71 were 11–15 years, 59 were 16–20 years, 25 were 21–30 years, 3 were 31–40 years, and 1 patient was 42

years old. Most of the patients received the first blood transfusion at the age of 7–8 months, others at 3 months or after 2 years. All patients were diagnosed with β -thalassemia by genetic tests in our hospital or in their local hospitals. Most patients came for blood transfusion 1 to 2 times per month.

Controls

400 samples of suspended leukocyte-reduced RBCs were collected, and the Rh subgroup phenotype was determined. Additionally, in 164 donations collected at the Shenzhen Blood Center, whole blood samples were available for DNA isolation and *RHCE* genotyping, which include all weak C, c, E, or e phenotype samples stored also for detailed sequencing analysis.

Blood Components

All patients were transfused with leukocyte-reduced RBCs [19], which have been filtered to remove leukocytes and centrifuged to remove plasma. RBCs contained mannitol, citric acid, sodium citrate, glucose, sodium dihydrogen phosphate, adenine, and sodium chloride (MAP storage solution) for the preservation of RBCs. All RBCs were prepared by the Shenzhen Blood Center. In most cases, patients received components from donors with the same Rh subgroups within about 10 days. If a patient was transfused in another city during this study, he or she received RBCs prepared according to the same standard operation procedure (SOP) [19].

Blood Group Serology

Serologic typing for the Rh subgroups C, c, E, and e was conducted by a micro-column gel card method [20] using RhDCcEe gel cards (Changchun Boxun Biotechnology Co., Ltd., Changchun, China) or RhCeEe gel cards (Diagnostic Grifols, Parets del Vallès, Spain). The detailed SOP followed the instructions of the manufacturer. Supplied centrifuges were used.

Molecular Analysis of *RPHCE*

DNA was extracted from whole blood or buffy coat. *RHCE* genotyping was carried by Tianjin Xiupeng Biotechnology Development Co., Ltd. (Tianjin, China) using its own products (20153400740 through a sequence-specific primer PCR technique (PCR-SSP). The *RHCE**C primers target to the 109-bp insert in intron 2 of the *RHCE**C allele. *RHCE**c primers target to exon 2 of *RHCE**c. Finally, *RHCE**E and *RHCE**e were genotyped by testing for the allele-specific nucleotide polymorphism in exon 5. 10 μ l of DNA and dNTP buffer were added into PCR tubes with primers and amplified with 96 °C for 120 s, 5 cycles of 96 °C for 20 s and 68 °C for 60 s, 25 cycles of 96 °C for 20 s, 65 °C for 50 s and 72 °C for 45 s, and 72 °C for 120 s at last. In several samples the full-length coding region of *RHCE* was sequenced as previously described [21].

Rh-Matched Transfusion

Every Wednesday and Saturday are Rh-matched transfusion days for β -thalassemia patients in our hospital. Patients should make an appointment at least one day ahead. According to the name list and their *RHCE* genotyping results, the immunohematology laboratory searched the same Rh subgroup type from all available RBCs for every patient by using micro-column gel cards and then labeled the component packages with the patient's name. For some Rh phenotypes, we had to provide RBCs with a different Rh subgroup because RBCs with the required type were not available. Furthermore, in line with the national transfusion guidelines an antibody screening was performed before each RBC dispatch, and a wet crossmatch was performed too. During or after transfusion, the ward personnel recorded any adverse transfusion reaction in the medical record and provided the information through an adverse event reporting system.

Results

Rh Subgroup Phenotyping in Patients

We tried to phenotype and record samples from 50 β -thalassemia patients before starting RBC transfusion with the same

Fig. 1. RhCcEe typing before and after Rh-matched transfusions. No. 1–5 showed mixed field agglutination in the gel column in different antigen detections or in all CcEe antigen tests before Rh-matched transfusion performed for those patients. According to the genotyping results, No. 1–3 performed identical RhCcEe phenotype transfusions at least for 3 months. Those patients then had clear CcEe phenotyping results in the gel cards. In No. 4 and No. 5, C+ c+ E+ e+ donor RBCs were transfused.

Rh subgroup. As expected, there was no patient with an unequivocal Rh subgroup phenotyping result using a micro-column gel card method. Most samples had mixed-field agglutination results in 2 or 3 gel columns or even all 4 Rh-subgroup testing columns (left column of fig. 1), which means ‘4+’ positive agglutination RBCs at the top of the micro-columns and ‘0’ negative cells at the bottom of the same micro-column, simultaneously. Totally, more than 90% (47/50) samples showed mixed-field agglutination with anti-c and anti-E, whereas, less than 50% were found positive for anti-C and anti-e. These results suggested that c- and E-matched transfusion is most important for Chinese patients.

Rh-Matched Transfusion

During the study period, 481 β -thalassemia patients were genotyped for *RHCE* by a PCR-SSP method (fig. 2). The immunohematology laboratory screened blood components according to the patient’s genotyping results and issued an RBC with the same Rh subgroups through a transfusion management information system whenever possible. No feedbacks of adverse transfusion reaction were received during or after the transfusions in all patients.

Starting from June 2015, we used the transfusion management information system to search for patients who received always RBCs with the identical RhCE type for at least 3 consecutive

Fig. 2. *RHCE* genotyping by PCR-SSP. C, c, E, and e indicated RhCE antigens. No. 1 was a control sample of CcEe phenotype, No. 2–6 were patients of CcEe, ccEe, CCee, Ccee, and ccEE types.

months. Thus 203 patients identified, of whom 115 patients were found with CCee, 78 with CcEe, and 10 with Ccee. In all these patients, we tested RhCcEe phenotypes with micro-column gel cards again. All samples showed clearly readable results. Unexpectedly, no mixed-field agglutination was observed, and no weak positive results were found. Finally, all 203 phenotyping results were concordant with the genotyping results (1, 2 and 3 of fig. 1), the PCR-SSP did not reveal false-positive or false-negative results. It seems that a simple DNA genotyping technology is reliable for Rh-matched RBC transfusion in clinics for Chinese.

Additionally, 5 patients with the more rare ccEe phenotype, who applied for Rh-matched transfusion, received identical Rh types or RBCs with the CcEe type because of the difficulty of finding donor blood with the identical Rh subgroups every time. The phenotyping results in the gel cards of those patients after 3 months transfusion showed mixed-field agglutination with anti-C, whereas there were exclusively clear results with anti-c, anti-E, and anti-e. This indicated obviously that the patients had previously received CcEe erythrocytes (4 of fig. 1). Moreover, 3 patients with ccEE received CcEe RBCs. Their gel card results then showed mixed-field agglutination with anti-C and anti-e, but clear results with anti-c and anti-E (5 of fig. 1). These observations further prove that the simple *RHCE* genotyping method predicts correct RhCE phenotypes and is feasible for Chinese.

RHCE Sequencing

The RhCE phenotypes of 400 donor samples were investigated and meanwhile 164 of them were genotyped (fig. 2). All genotyping results were in concordance with serology, confirming the micro-column gel card results mentioned above and further supporting the conclusion that *RHCE* molecular typing is a very reliable tool. Among 400 blood donors, 7 individuals with weak RhCE phenotypes were observed (table 1). The exons 1 to 10 of *RHCE* were amplified and sequenced in these 7 samples. One sample (No. 3, Ccee, table 1) revealed a G/A heterozygous result at nucleotide position 1059 (fig. 3), suggesting one base mutation (1059G>A) in exon 7 of

RHCE (Genbank KT957625) which results in a stop codon at amino acid position 353. But no nucleotide variation was observed in the other 6 samples.

Frequencies of RhCE Phenotypes

Both patients and donors were predominantly CcEe and CCee phenotypes, with a prevalence of 55.3% and 24.9% in patients and 49.3% and 31.3% in donors, respectively. This indicates that more than 80% of Chinese can easily receive Rh-matched RBC transfusions (table 2). The frequencies of C and e antigens were 72.8% and 78.3%, respectively, when the results from patients and donors were combined for calculation. Furthermore, the frequencies of c and E antigens were 27.2% and 21.7%, respectively.

Table 1. Serological and genotyping results of Rh weak expression samples

No.	Gel card types	Weak positive (≤2+)	PCR-SSP types
1	CCee	weak e	CCee
2	CcEe	weak c and weak E	CcEe
3	Ccee	weak c	Ccee
4	CcEe	weak E	CcEe
5	ccEe	weak e	ccEe
6	CcEe	weak e	CcEe
7	ccEe	weak e	ccEe

Table 2. Rates of RhCcEe phenotypes in this study and literature

Phenotypes	Patients	%	Donors	%	Chinese [34] [*] %	Caucasian [36] [§] %	African origin [25] [§] %
CCee	266	55.30	197	49.25	51.82	18.50	6.30
CcEe	120	24.95	125	31.25	32.45	13.37	0.90
Ccee	36	7.48	44	11.00	7.71	35.65	19.30
ccEE	25	5.20	16	4.00	3.98	2.33	1.20
ccEe	23	4.78	10	2.50	2.18	12.68	7.90
CCEe	7	1.46	5	1.25	1.24	0.20	0
ccee	1	0.21	1	0.025	0.39	17.17	64.20
CcEE	2	0.042	1	0.025	0.21	0.07	0
CCEE	1	0.21	1	0.025	0.01	<0.01	0
Total	481	100	400	100	100	100	100
*Only Rh(D)-positive Chinese Hans investigated.							
§Rh(D)-negative individuals included.							

Discussion

Currently, it is not applicable in chronic transfusion patients to identify RhCE phenotypes for , unless serological blood group determination is performed before initiation of transfusion therapy. Therefore, molecular techniques are increasingly applied to predict RhCE antigens [22]. However, because different ethnic groups have different genetic background and especially numerous variant *RHCE* alleles, many DNA genotyping techniques were found not reliable. Tax et al. [23] mentioned that *RHCE**C genotyping based on 48C was not reliable because of a 48G>C mutation in the *RHCE**ce allele (false-positive prediction of C), and genotyping on intron 2 is unreliable because of r's (Cdes) alleles (false-negative prediction of C). Then, in France, Pham et al. [24] and Kappler-Gratias et al. [25] reported 572/806 individuals and 14.2% African blood donors, respectively, with RhCE variants or variant *RHCE* alleles. Both studies suggested to transfuse patients with sickle cell disease and RhCE variants not only with phenotypically matched but also with genetically matched RBC units. In Brazil, Prisco et al. [26] reported that 421 of 80,961 blood samples were tested with atypical D typing results; among them 94.9% presented variant *RHCE**ce. Overall, many authors proposed that Rh variants should be considered to perform Rh-matched transfusion through molecular methods [27, 28]. And it would be better to use a high-throughput DNA testing platform [29] or a next-generation sequencing (NGS) strategy [30] instead of a simple PCR-SSP as described in this study. In China, some Chinese papers [31–33] reported that the 48G>C mutation in the *RHCE**ce allele could be observed. Furthermore, Zhou et al. [31] reported in 2008 that 2 C-positive samples in 98 D-positive individuals lacked the 109 bp insertion in intron 2. Shi et al. [32] found 1 C-positive samples in 186 D-positive Chinese Hans from southern China with a 109 bp deletion in intron 2 of *RHCE*. However, Wang et al. [33] identified no individual with this deletion when studying 942 samples. According to our data from 203 patients and 164 healthy donors, there is no discrepancy between RhCE phenotyping and molecular genotyping results. The

varying results from different studies indicate that more data should be accumulated in Chinese. However, to our limited knowledge, a simple PCR-SSP technology may be practicable or at least preliminary feasible for Rh-matched transfusion in Chinese.

We also investigated, if the frequency of different RhCE phenotypes allows us to perform Rh-matched transfusion for β -thalassemia patients regularly. Both Chinese literature [34] and our work demonstrated that CcEe and CCee phenotypes are present in more than 80% of Chinese. This implies that a general hospital, if a large amount of RBCs is used, could provide RhCE-matched RBCs for most of the β -thalassemia patients independent from the blood center. Even the local blood centers do not provide Rh subgroup phenotype-labelled blood components currently. If the local blood center can provide RhCE-marked RBC units, then a hospital could serve more than 97% of the patients who apply for Rh-matched transfusions, which include the common phenotypes of CCee and CcEe as well as Ccee, ccEE, ccEe, and other rare phenotypes. According to our data from 2016 published in Chinese [35], 20 alloantibodies were detected in 14 of total 354 β -thalassemia patients. Among them, 80% (16/20) were Rh-specific alloantibodies and 88% (14/16) Rh-specific alloantibodies were anti-c and anti-E being in agreement with the results of some recent larger studies [10–13]. Therefore, Ce-matched transfusion may be important in some nationalities [36]. But it is more important to avoid mismatch for c (in CC individuals) and mismatch for E (in ee individuals) in Chinese. However, identical type transfusion of all CcEe factors is the safest way for patients in need for long-term regular transfusion. According to our last data collection [35], 2 β -thalassemia patients were newly detected with alloanti-

bodies, one with anti-S and the other one who formerly was anti-Jk^a positive with anti-Ce. However, both patients never performed Rh-matched transfusion. Overall, it seems that RhCE-matched transfusion is simple and practicable in clinics in China compared to other countries such as France, Brazil, the US, or Thailand [17, 24, 27, 37, 38]. Moreover we would like to suggest that β -thalassemia patients in China should receive Rh subgroup-compatible blood from the beginning of transfusion therapy. It does not have to be donor blood with the same Rh subgroup, but it should be compatible in order to prevent alloimmunization.

Finally, 7 cases of weak c, E, and e phenotypes were found in 400 donor samples, but only 1 weak c sample was associated with a genetic variation in exon 7 of *RHCE* as shown by sequencing analysis. This is in contrast to the results reported by Wang et al. [33]. They detected nucleotide variations in most of the 36 cases with weak CcEe phenotypes. Thus more data accumulation is needed for understanding the molecular background of *RHCE* in Chinese.

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Disclosure Statement

The authors declare no conflict of interest with regard to this paper.

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