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RHD-specific microRNA for regulation of the DEL blood group: integration of computational and experimental approaches

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ABSTRACT

Objective: The discovery of specific microRNAs (miRNA) mediates a better understanding of molecular mechanisms, diagnosis and prognosis of complex phenotypes. Synthesis of the RhD blood group involves multiple factors causing variation in the expression of RHD antigens. The mechanism underlying the extremely weak expression of RHD antigen associated with the RHD variant called DEL (D-elute) is incompletely understood. Down-regulation of gene expression through miRNA is a guide to the potential involvement of miRNAs in the DEL blood group. In order to determine the association of miRNAs and Rh-DEL blood donors with DEL variant, we investigated the expression level *RHD*-specific miRNA.

Methods: Blood samples were serologically tested for RhD blood group determination. DNA was analysed using SSP-PCR for the Asian-type DEL allele (*RHD* 1227 G>A). Bioinformatics analyses were applied for prediction of candidate *RHD*-specific miRNA. The *RHD*-specific miRNA expression level was quantitated using a real-time-qPCR approach. The miRNA expression levels of various RhD blood groups were compared and statistically analysed.

Results: The bioinformatics tools (n = 3) for prediction of miRNA targeting on *RHD* identified miR-98 as the miRNA potentially specific for the 3′ UTR of *RHD*. The relative expression levels of miR-98 among D-positive (n = 50), D-negative (n = 49) and DEL (n = 63) subjects showed no statistically significant differences (P-values = 0.58).

Conclusion: This is the first attempt to determine whether miR-98 is involved in RHD expression using computational and experimental approaches. Further investigations are necessary to fully characterize the miRNA genetics in DEL blood group regulation.

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Introduction

The Rh Blood Group System, coded by *RHD* and *RHCE*, is highly polymorphic. The deletion of *RHD* and *RHD-CE-D* hybrid as well as inactive pseudo-D gene (*RHD**Ψ) mediated D-negative phenotype, which is explained by no *RHD* expression. The complex combination of human genetic recombination and single nucleotide polymorphisms (SNPs) account for RhD variants. In addition, the specific pattern of genetic variation and distribution of *RHD* allele illustrate genetic differences among distinct ethnic populations [1–3]. Protein structural changes and variability of *RHD* expression levels mediate the heterogeneity of RhD variants such as partial D, weak D and DEL [4]. DEL (D-elute) is the variant with the least RhD antigen expression and generally serotypes as D-negative. Transfusion of DEL blood to D-negative recipients leads to primary and secondary anti-D immunization [5–8]. In patients who need multiple blood transfusion, this would increase the difficulty of blood transfusion service (allo-antibody production) and might lead to the haematological transfusion reaction (HTR). These evidences highlight the urgent need of additional laboratory testing for discriminating DEL and true D-negative samples. The prevalence of the DEL variant is 22–30% among D-negative populations in East Asia, such as Japanese and Chinese, and is recognized as the most frequent RhD variant in the region [9]. The number of D antigens per red blood cell (RBC) is extremely low (~22–36) in DEL compared to 30,000 sites in normal D cells and 1500–7000 sites in cells with the weak D variant [10–12]. Numerous *RHD* alleles have been detected among RBCs with the DEL phenotype. *RHD*(K409K: 1227G>A), *RHD*(IVS3+1G>A), *RHD*(IVS5-38del4), *RHD*(M295I) and *RHD*(X418L) are the DEL-associated alleles reported in Asians and Europeans [2,13]. *RHD*(K409K; 1227G>A) is

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the most common DEL-associated allele identified in Asians and is designated as the 'Asian type' DEL allele [8,12]. The *RHD*1227G>A codon is located at the splice site of exon 9. The relationship between *RHD*1227A and splicing out of exon 9 mRNA in DEL continues to be debated [1,12]. Furthermore, the different DEL-associated alleles exert different effects on the level of D antigen per red blood cell. The *RHD*(M295I) allele demonstrates the highest number of D sites per RBC [12]. This underlines the important role of genetic variation in the regulation of quantitative differences in D antigen density associated with DEL-associated alleles. Interestingly, a high prevalence of Ccee, ccEe and CCee phenotypes has been detected on DEL variant cells [14,15]. The Ce haplotype is strongly linked to *RHD*1227G>A in Chinese with the DEL variant [16]. In addition, Rh membrane molecules (RhAG: Rh50, CD241) exhibit a post-transcriptional role on RhD expression. The evidence mentioned above indicates that multiple genetic mechanisms regulate DEL phenotype expression.

MicroRNAs (miRNA) are small non-coding RNAs involved in post-transcriptional gene regulation. miRNAs mediate negative control of human gene expression by nucleotide paring to the 3′ untranslated region (3′ UTR) of target mRNA. The role of miRNAs has been shown in various cellular processes, especially erythropoiesis [17,18] and in erythrocytic disorders [19,20]. In addition, miRNA demonstrated the important role in various types of cancer such as pancreatic ductal adenocarcinoma [21], osteosarcoma [22] and gastric cancer [23]. The molecular interaction between miRNA and *RHD* mRNA is hypothesized in the DEL variant. The aim of the present study was to investigate the relationship between *RHD*-specific miRNA expression and the DEL variant. We, integrated computational and experimental approaches to identify miRNAs interacting with the DEL variant. Ultimately, we intended to further elucidate the molecular mechanism underlying DEL blood group and advance the knowledge of red blood cell genetics.

Materials and methods

Based on the prevalence of DEL (20% in Thai with D-negative) and D-negative (0.3% in Thai population), a total of 162 blood samples were randomly collected from donors at the National Blood Center, Thai Red Cross Society during 2013–2015. The samples were categorized as one of three groups: (1) D-positive (n = 50); (2) D-negative (n = 49); (3) DEL (n = 63). All subjects gave written informed consent using a National Blood Center registry donation form. This study was approved by the Ethical Committee of National Blood Center, Thai Red Cross Society (NBC8/2012).

Blood samples were tested for RhD phenotype using anti-D IgM (National Blood Center, Thai Red Cross Society) and automate analyzer (PK7200® Automated Microplate System, Beckman Coulter Inc., CA, USA). RhD-negative results were confirmed using a tube technique according to National Blood Center guidelines. RhCE phenotypes were investigated using monoclonal IgM antibodies: anti-c, anti-E (both from the National Blood Center, Thai Red Cross Society) and anti-C, anti-e (both from DiaClon, DiaMed GmbH, Bio-Rad Laboratories, USA). D-negative sample with C(+) was further analysed by absorption-elution test and *RHD1227A* SSP-PCR for DEL phenotype.

Samples which were D-negative [using an indirect anti-globulin test (IAT)] and C-positive were subsequently tested for DEL phenotype using an adsorption-elution test. Two hundred microlitres of packed RBCs were washed with normal saline solution (NSS) and mixed with an equal volume of human polyclonal IgG anti-D (DiaClon, DiaMed GmbH, Bio-Rad Laboratories). The mixture was incubated at 37 °C for 1 h and the RBCs were then washed thoroughly with NSS. The supernatant and eluate from the last wash were kept for testing with Rh-positive and Rh-negative control cells.

Genomic DNA was extracted from peripheral blood samples using QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). The SSP-PCR protocol was carried out using a set of previously published primers [9]. PCR reactions were carried out at a total volume of 10 µL, each containing 1 µL of genomic DNA, 0.5 U of DNA polymerase (Genet Bio, Korea), 200 µL of dNTPs, primers (10 ng of each primer) and a 2.5-mmol/L concentration of magnesium chloride in a buffer provided by the manufacturer. Forty cycles were programmed on a thermocycler (GeneAmp® PCR System 9700, Applied System, Singapore) as follows: denaturation at 94 °C for 5 min, then 35 cycles of 30 s at 94 °C and 40 s at 68 °C. PCR products were visualized in a 2% agarose gel with ethidium bromide staining [9].

In order to identify candidate miRNA involving the DEL blood group, a bioinformatics-based prediction approach was applied. The 3′ untranslated region (3′ UTR) of the *RHD* gene (NM_016124) was retrieved from a genome database (http://genome.ucsc.edu). We used a combination bioinformatics tools, RegRNA [24]. Targetscan [4] and miRanda software [25], which were based on different principles of target prediction in order to enhance the sensitivity and specificity of *RHD*-specific miRNA prediction [26,27]. The thermodynamic stability of miRNA–mRNA duplexes, sequence conservation and base-paring patterns are the principles of putative miRNA prediction used in this study. miRNA derived from at least two of the three softwares were accepted as *RHD*-specific miRNA. The profile of miRNAs preferentially expressed in human erythrocytes was also used as criteria for erythrocyte-specific miRNA selection [20]. The interaction of candidate miRNA–*RHD* mRNA was further

analysed for target structure based on a hybridization model.

The thermodynamic chemistry of *RHD*-miRNA's secondary structure was systematically computed using the Sfold RNA package (http://sfold.wadsworth.org/cgi-bin/index.pl). The effect of *RHD* secondary structure is crucial for target accessibility. The energy cost for opening the local secondary structure was evaluated as a reflection of the probability of target accessibility.

In order to prevent miRNA loss, the miRNA isolation step was systematically processed within 2 h of blood donation. The EDTA blood samples were washed three times using NSS. Subsequently, the buffy coat was carefully removed. The isolations of miRNA from fresh packed RBCs were performed using an miRNA isolation kit (Favorgen Biotech Corporation, Taiwan). Total miRNA was subjected to two-step reverse transcription PCR (RT-PCR) for cDNA synthesis using Taqman® MicroRNA Reverse Transcription Kit (Applied Biosystem, Foster City, CA, USA). The miRNA quantitative real-time PCR was carried out using Taqman miRNA assay according to the manufacturer's instructions. The hsa-mir-98 (candidate miRNA) and RNU6B (endogenous control) expression levels were measured using StepOnePlus™ Real-Time PCR System (Applied Biosystem, Foster City, CA, USA). The threshold cycle (C_T) was defined as the number of PCR cycles in which fluorescence reporter dye crossed the threshold in the real-time PCR. The relative expression of miR-98 was calculated using the C_T method. ΔC_T was calculated by subtracting the C_T value of RNU6B from the C_T of hsa-miR-98. $\Delta\Delta C_T$ was then calculated by subtracting the mean of ΔC_T in control samples from the ΔC_T of the tested sample. The relative quantification was calculated using the $2^{-\Delta\Delta C}{}_T$ method and expressed as fold changed relative to the reference sample [28,29]. The miRNA expression quantification of each sample was experimentally run in triplicate.

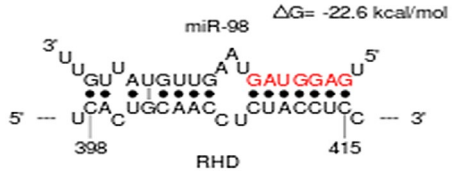
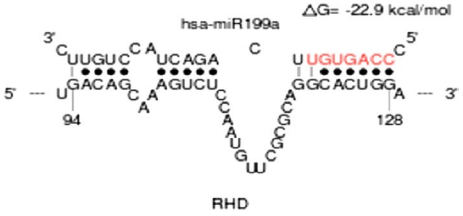
Normality to assess the normal distribution of the data was determined using Kolmogorov–Smirnov and Shapiro–Wilk tests. The Mann–Whitney U Test was applied to evaluate the difference of miRNA relative expression levels between each two groups. *P*-values < 0.05 were considered significant. Analyses were computed using SPSS software (SPSS Inc., Chicago, IL).

Results

We analysed the miRNA binding sites in 3′ UTR of the human *RHD* gene using RegRNA, Targetscan and miRanda software. Five miRNAs were predicted by all three software as candidate *RHD*-specific miRNAs. Among the five predicted miRNAs, miR-98 and miR-199a have been demonstrated to be expressed preferentially in human erythrocytes [20]. We found that the nucleotide binding sites of miR-98 and miR-199a localized in evolutionarily conserved regions of human, chimpanzee and rhesus genomes. This indicates that the predicted regions are functionally important. Considering the minimal free energy, the miR-98 demonstrated higher stability of miRNA–*RHD* mRNA hybridization compared to those of miR-199a. The pattern of seed sequence matching between the 5′-ends of miR-98 and miR-199a, and the 3′-end of *RHD* show perfect Watson–Crick complementarity of seven consecutive bases (Table 1). As shown in Table 1, the entire nucleotide paring pattern between miR-98 and *RHD* is much more favourable for target recognition than that between miR-199a and *RHD*.

RHD target accessibility is important for miRNA function. The region of *RHD* 3′ UTR at the nucleotide position 398–415 base pairs was predicted as the recognition site of miR-98. (Table 1). We therefore, analysed the *RHD* structural accessibility for the region containing this miR-98 recognition site (nucleotide position 386–431). The position of nucleotides with a probability of accessibility greater than 0.5 were indicated as an accessible region.

Table 1. Characteristics of RHD-specific miRNAs.

miRNA	MFE (kcal/mol^{-1})	Target score	Duplex Pattern	Nucleotide position
miR-98	–15.60	167		398-415 bp of 3′ UTR
miR-199a	–10.70	144		94-128 bp of 3′ UTR

Note: MFE: minimal free energy, bp: base pair.

The site for miR-98 binding was localized at nucleotide position 386–406. This result revealed that *RHD* region around the 386–406 nucleotide region has the possibility of being highly accessible to miR-98. We therefore, selected miR-98 as a potential *RHD*-specific miRNA candidate (Figure 1).

In order to address the role of miR-98 in the DEL blood group, the miR-98 expression level was measured in three types of RhD blood groups using quantitative real-time PCR. The miR-98 relative expression level was normalized to an RNU6B endogenous control. miR-98 was consistently detectable among members of all the RhD blood groups. In order to compare miR-98 expression level in each group, D-positive was selected as reference sample. The $2^{-\Delta\Delta C}{}_T$ method was used to determine the fold change of miR-98 normalized to the internal control and relative to the reference sample (D-positive). We found that miR-98 was expressed at a lower level in DEL and D-negative samples compared to that in the D-positive control. The miR-98 expression level in D-negative show slightly lower than those in DEL. There was no statistically significant difference in miR-98 expression among the three types of RhD blood group (*P*-values = 0.58 (Figure 2).

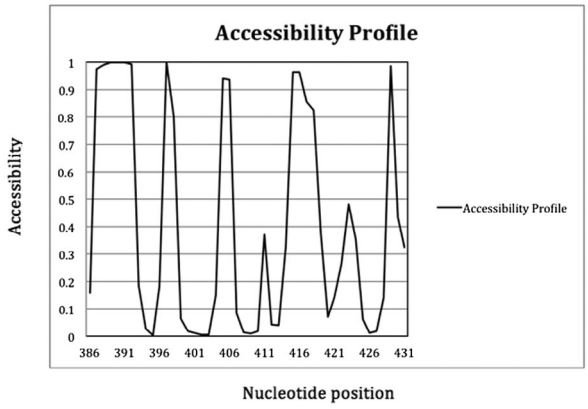


Figure 1. Accessibility profile of *RHD* mRNA. The region surrounding the miR-98 recognition site has been analysed for the probability of miR-98 accessibility.
Note: An accessibility score more than 0.8 indicated the area to be accessible.

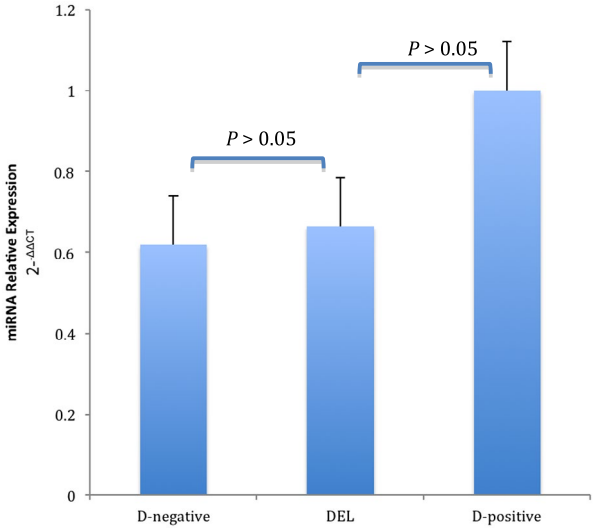


Figure 2. Expression level of miR-98 among Rh-negative (n = 49), Rh-positive (n = 50) and DEL (n = 63). The data were expressed as $2^{-\Delta\Delta C}T$. $\Delta C_T = (C_{T\ miR\text{-}98} - C_{T\ RNU6B})$, $\Delta\Delta C_T = (C_{T\ tested\ sample} - C_{T\ reference\ sample})$ and shown by mean value ± SD.
Note: The statistical significance was calculated by Mann–Whitney *U* Test.

Discussion

The present study began by addressing the role of miRNA in the regulation of the DEL blood group. Aberrant expression levels of miRNAs have been increasingly shown in haematologic and malignant diseases. Most published studies used microarray technology for investigating the miRNA expression profiles. In this study, we applied a bioinformatics approach for the prediction of potential *RHD* gene-specific miRNAs. Three bioinformatics software revealed miR-98 and miR-199a to be *RHD*-specific miRNAs. Considering the pattern of seed sequence paring, minimal free energy (MFE) and target score, miR-98 was selected as the candidate *RHD*-specific miRNA (Table 1).

We hypothesized that the lowest expression of D antigen, known as DEL, is controlled by miRNA. In order to investigate the role of miR-98 in DEL, we, therefore, examined the expression level of the miR-98 candidate in D-positive, D-negative and DEL. We applied the $2^{-\Delta\Delta C}{}_T$ method for determining the fold change of miR-98, normalized to RNU6B (internal control) and relative to D-positive (calibrator). We found no significant difference in miR-98 among three RhD blood groups (*P*-values = 0.58). Lower expression levels of miR-98 were detected in D-negative and DEL compared to D-positive samples. Our results indicated that the low expression of the RhD antigen in DEL is not associated with miR-98 expression.

The functional role of miR-98 has been addressed in various types of cancers. The up-regulation of miR-98 has been shown in cell lines from non-small cell lung cancer [resulting in the inhibition of tumour suppressor gene (*Fus1*) [30]] and in formalin-fixed paraffin-embedded (FFPE) tissue of breast cancer patients [31]. The aberrant expression of miR-98 in cancer indicates the major role of miR-98 as a controller of the tumour suppressor gene. The bioinformatics approach suggested that the miR-98 was a *RHD*-specific miRNA. However, human genes may be regulated by multiple miRNAs. In addition, the fine-tuning of *RHD* gene expression could be influenced by various mechanisms, including single nucleotide polymorphisms and hybrid genes. The limitation of the current study is that we selectively quantitated miRNA expression based on bioinformatics analysis. Furthermore, DEL group harbours *RHD1227A* (K409K) which is common in Thai population. Further study is therefore, necessary to fully characterize the functional role of miRNA in the DEL blood group of each ethnicity using microarray expression profile.

The discrimination between DEL and true D-negative is clinically important for clinical and laboratory practice. In order to apply molecular testing in clinical laboratory, the body of knowledge about genetic basis underlying DEL mechanism should be clearly emphasized [32]. The present study investigated miR-98 expression level in DEL, D-negative and D-positive. The relative expression levels of miR-98 showed no statistically significant differences (*P*-values > 0.05). Though negative finding, this study would enhance the advance biomedical knowledge to clinical laboratory scientist community. In the future, the application of molecular analysis and routine conventional test would be clinically benefit for blood transfusion safety and donor management [33]. This work represents an advance in biomedical science because it integrated bioinformatics tools and an experimental approach for elucidating the role of miR-98 in the DEL blood group.

Summary table

What is known about this subject:
- The mechanism of extremely weak expression of D antigen associated with the *RHD* variant DEL is not completely understood.
- Transfusion of DEL blood to D-negative recipients leads to primary and secondary anti-D immunization.
- The down regulation of human gene expression through miRNAs guides the potential involvement of miRNAs in the DEL blood group.

What this paper adds:
- The *RHD* gene may be regulated by microRNA.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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