Mutation of *ABO* Gene in Thai Blood Donors with A_3 Phenotype

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The ABO system is the most important of all blood group systems in transfusion practice. The subgroup gives a weak reaction when treated with anti-A or anti-B. The most common subgroup found in Thai blood donors is subgroup A_3, which is characterized by mixed-field agglutination when reacted with anti-A and anti-A,B, was caused by mutation in the ABO gene, especially in the exon 7. In the present study mutation in A_3 were charactered in exon 7 of the ABO gene in 10 A_3 phenotype Thai blood donors from the National Blood Centre, Thai Red Cross Society by PCR amplification and DNA sequencing.

Mutations in exon 7 were identified in the A allele of six cases. In four cases, mutations were detected at positions 646T>A, 681G>A, 771C>T and 829G>A. One case showed a double mutations at positions 467C>T and 745C>T and one case showed a mutation at position 467C>T. Four cases showed wild type exon 7 as A101 allele. These mutations were previously reported in BGMUT database and no novel mutation was identified. These data suggest genetic heterogeneity in A_3 phenotype in Thai blood donors.

Keywords: *A_3 phenotype, Blood group, ABO gene, Genetic mutation*

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ABO, the firsat human blood group system, was discovered by Landsteiner in 1900. The ABO system is the most important of all blood groups in transfusion practice. The blood group ABO system constitutes of A and B antigens on red cells[1,2]. Expression of A and B antigens are controlled by the *ABO* gene[3]. The *ABO* gene encodes glycosyltransferases that produce the A and B antigens[4]. The A and B antigens are carbohydrate structure, presenting on red cell membrane. The substrate for A and B transferases is a structure called H antigen. It contains the monosaccharide fucose at the terminal residue. The A transferase, product of A allele, transfers the monosaccharide *N*-acetylgalactosamine from the donor substrate uridine diphosphate (UDP)-*N*-acetylgalctosamine to the fucosylated galactosyl residue of the H antigen, to produce an active structure. The B transferase, product of B allele, transfers galactose from UDP-galactose to the fucosylated galactose of H, to produce a B-active structure. The O allele produces no active enzyme, therefore on the group O red cells, the H antigens remain unconverted. *N*-acetylgalactosamine and galactose are the immunodominant sugars of A and B antigens respectively[5].

The *ABO* gene is located on the long arm of chromosome 9 (9q34.1-q34.2)[6]. It consists of seven exons spanning about 18 to 20 kilobase pairs (kb). The exon size ranges from 28 to 691 base pairs (bp), with most of the coding sequence lying in exon 6 and 7[7]. Three major alleles (A, B, O) were first cloned and sequenced by Yamamoto et al[8]. The B allele is distinguished from A allele at 7 nucleotide positions 297, 526, 657, 703, 796, 803 and 930. The sequence of O allele is identical to A allele except for a single-base deletion at position 261[6]. In addition to common ABO phenotype, the weak phenotypes or subgroups which are characterized by the reduced expression of antigen on erythrocytes are found[9]. The red cells of these variants are weakly agglutinated with anti-A or anti-B. Subgroup may have clinical relevance. If a subgroup in the donor sample is interpreted as group O, the anti-A,

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anti-B in group O recipient might cause the destruction of the transfused red cells[10]. Previous studies have shown that a subgroup is a result of genetic mutation in the *ABO* gene, particularly in the exon 7 of the *ABO* gene[11-14]. In 2000, Barjas-Castro et al analyzed four A_3 donors and members of three in four families. The 467C>T, 646T>A, 829G>A mutations and 1060C deletion were found[15]. The study of Sun CF et al in China, 838C>T missence mutation have also been found in one case of A_3 phenotype[16]. In Taiwan, Lei Li et al studied 9 cases of subgroup A. Three of them were classified as A_3 phenotype. In this group, 820G>A mutation was found in 2 cases and 745C>T mutation was found in one case[17]. Because there is no data in the Thai population and the most frequently found subgroup A in Thai blood donors is subgroup A_3 therefore it should be done. Mixed-field agglutination when treated with anti-A or anti-A,B is serological characteristic of the subgroup A_3. The present study aimed to detect the mutation in exon 7 of the *ABO* gene and to characterize the pattern of mutation in A_3 phenotype in Thai blood donors.

Material and Method

From 324,172 donors donated blood at National Blood Centre between January 2007- December 2007, there were 90 A_3 phenotype donors. Ten A_3 phenotype donors were recruited in the present study with informed consent. Venous blood samples were collected from subjects into 2 tubes including clotted blood and EDTA blood. The present study was performed at the World Health Organization Co-operation Section National Blood Centre, Thai Red Cross Society. The present study was approved by Siriraj ethics committee.

Blood group serology

A_3 subgroup was confirmed by serological method (test tube technique)[4]. Clotted blood was tested for ABO grouping. Cell grouping was performed with anti-A, anti-A1, anti-B, anti-A,B monoclonal antibody reagent (National Blood Centre, Thai Red Cross Society) and serum grouping was performed with A cell, B cell (National Blood Centre, Thai Red Cross Society).

Extraction of genomic DNA

Genomic DNA were extracted from 5 ml EDTA blood by QIAamp DNA blood mini kit (QIAGEN, Germany) following manufacturer's instructions.

Amplification and sequencing strategies

The exon 7 of the coding sequence of the *ABO* gene was amplified and sequenced. The PCR product size was 744 base pairs. Oligonucleotide primers for amplification and direct sequencing are listed in Table 1. All primers sequences were picked from previous study[15].

PCR conditions

The PCR reaction was carried out in a 0.2 ml of PCR tube by adding the following solutions: *(i)* 10 µl of 1X GC rich buffer, *(ii)* 2 µl of 10 µM dNTP mix, *(iii)* 5 µl of 0.2 µM primer (forward and reverse), *(iv)* 1.5 µl of 3% Dimethylsulfoxide, *(v)* 0.5 µl of 0.5 mM $MgCl_2$, *(vi)* 0.5 µg DNA template, *(vii)* 0.25 µl of 0.02 U/µl DNA polymerase (Finnzymes, Finland). The total volume of the PCR mixture was 50 µl adjusted by adding DNase,RNase free water. The polymerase chain reaction was performed in a thermal cycler (GeneAmp 9600, MJ Research,USA). After an initial denaturation

Table 1. Primer used in this study

Name	Sequence	Location
Primer 1F[(a)]	5' CCGTCCGCCTGCCTTGCAG 3'	Intron 6
Primer 1R[(a)]	5' TGCCGGCAGCCCTCCCAGAG 3'	3' UTR
Primer 2F[(b)]	5' CCGTCCGCCTGCCTTGCAG 3'	Intron 6
Primer 2R[(b)]	5' CACACCAGGTAATCCACCTC 3'	Exon 7
Primer 3F[(b)]	5' CGCATGGAGATGATCAGTGACTTC 3'	Exon 7
Primer 3R[(b)]	5' TTGGCCTGGTCGACCATCATG 3'	Exon 7
)Primer 4F[(b)]	5' TGCAAGAGGTGCAGCGGCTC 3'	Exon 7
Primer 4R[(b)]	5' TGCCGGCAGCCCTCCCAGAG 3'	3' UTR

[(a)] Detection primer
[(b)] Sequencing primer

step at 95°C for 10 minutes, the PCR was conditioned for 10 cycles at 94°C for 1 minute then 67°C for 1 minute and 72°C for 30 seconds. After that, the reaction was performed at 94°C for 1 minute, at 65°C for 1 minute and at 72°C for 30 seconds for 30 cycles. An additional 1 minute at 72°C was done to the last cycle to complete the amplification.

After amplification, the PCR product was analyzed by electrophoresis at 50 Volts for 90 minutes on 2% agarose gel stained with ethidium bromide (10 mg/ml). The PCR product was visualized on UV transilluminator. The DNA fragment of PCR product (744 bp) was purified by a gel extraction kit (Qiagen, Germany) following the manufacturer's instructions.

Sequencing of PCR product

Sequencing was done by dye terminator cycle sequencing. The sequencing reaction was performed in a 20 microlitre sample by adding the following solution *(i)* 9.2 µl of H_2O, *(ii)* 4 µl of 5X Big Dye sequencing buffer (Applied Biosystems, USA), *(iii)* 0.8 µl of Big Dye Terminators 3.1 (Applied Biosystems, USA), *(iv)* 3 µl of 1 µM sequencing primer, *(v)* 3 µl DNA (PCR product). The PCR cycle was performed at 96°C for 10 seconds, at 55°C for 5 seconds and at 60°C for 1.30 minutes with the total of 25 cycles. After that, the sequencing product was purified by ethanol/sodium acetate precipitation method. After purification, sample was loaded into ABI 310 genetic analyzer (Applied Biosystems, USA). Data from genetic analyzer was analyzed by comparing the obtained sequence with the consensus sequence (A101; GenBank accession number AF134412).

Results

Blood group serology

The ABO blood group of all donors were rechecked by test tube method using clotted blood. The serologic results of ABO blood group of ten donors are shown in Table 2. All cases showed 1+ to 2+ mixed-field agglutination with anti-A and anti-A, B. The sera of all donors contained strongly positive anti-B.

PCR and direct sequencing result

The extracted genomic DNA sample were amplified for exon 7. PCR product was shown in Fig. 1. The DNA extracted from agarose gel were sequenced using ABI prism 310 genetic analyzer. The exon 7 was located at nucleotide position 375-1,065 of coding sequencing of *ABO* gene. In four cases, the nucleotide sequences of exon 7 were identical to the consensus sequence (A101; GenBank accession number AF134412). The nucleotide sequences of the other six cases are different from the consensus sequence. Mutation in the *ABO* gene of six A_3 donors were shown in Table 3. As shown in Fig. 2, the heterozygous state for C and T at position 467 was demonstrated in one case (Sample no. 1, shown as Y). As shown in Fig. 3, the heterozygous states for C and T at position 467 and 745 were demonstrated in one case (Sample no. 4, shown as Y). Four cases showed the same results of 4 mutations together (Sample no. 5, 6, 8, 10). As shown

Table 2. The serologic results of A_3 phenotype

Sample	Reaction of RBCs with				Reaction of serum with	
	Anti-A	Anti-B	Anti-A,B	Anti-A_1	A cell	B cell
1	$1+^{mf}$	-	$1+^{mf}$	-	-	4+
2	$2+^{mf}$	-	$2+^{mf}$	$1+^{mf}$	-	4+
3	$2+^{mf}$	-	$2+^{mf}$	2+	-	4+
4	$1+^{mf}$	-	$2+^{mf}$	$1+^{mf}$	-	4+
5	$1+^{mf}$	-	$2+^{mf}$	W	-	4+
6	$1+^{mf}$	-	$1+^{mf}$	$1+^{mf}$	-	4+
7	$2+^{mf}$	-	$2+^{mf}$	W	-	4+
8	$2+^{mf}$	-	$2+^{mf}$	$1+^{mf}$	-	4+
9	$2+^{mf}$	-	$2+^{mf}$	1+	-	4+
10	$1+^{mf}$	-	$2+^{mf}$	W	-	4+

$1+^{mf}$ = 1+ mixed-field agglutination (few small agglutinates in a pool of free red cells)
$2+^{mf}$ = 2+ mixed-field agglutination (few small agglutinates in a pool of free red cells)
W = weak

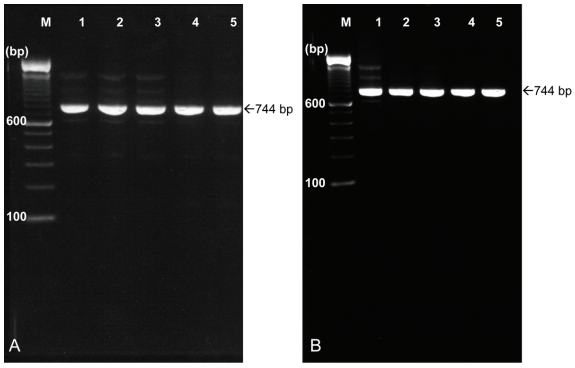

Fig. 1 The PCR product of exon 7 in *ABO* gene of A_3 sample. A) The 744 bp PCR products of the A_3 samples (Sample no.1-5) were shown in lane 1-5. Lane M shows the 100 bp DNA ladder. B) The 744 bp PCR products of the A_3 samples (Sample no.6-10) were shown in lane 1-5. Lane M shows the 100 bp DNA ladder

in Fig. 4, each sample possessed heterozygous state of A and T at nucleotide position 646 (shown as W), of A and G at position 681 (shown as R), of C and T at position 771 (shown as Y), as well as of A and G at position 829 (shown as R).

Discussion

The molecular basis of the ABO blood group was first elucidated by Yamamoto et al in 1990[8]. Their studies characterized nucleotide sequences of the three major ABO alleles (A, B, O). After the elucidation of the three major ABO alleles, the variants of ABO alleles have been reported and submitted to Blood Group Antigen Gene Mutation (BGMUT) database. However, there were a few studies about molecular biology of the blood groups in Thai blood donors and the present study is the first recent on molecular study about the subgroups in Thai blood donors. The most frequently subgroup of ABO in Thai is A_3 phenotype. The ABO subgroup was defined by serological characteristic.

According to the BGMUT database, the mutations of the A_3 phenotype were found only in exon 7[18]. The direct sequencing results showed the

Table 3. Mutations in the *ABO* gene of six A_3 donors

Sample	Nucleotide position					
	467	646	681	745	771	829
A101 allele	C	T	G	C	C	G
Sample No. 1	T					
Sample No. 4	T			T		
Sample No. 5		A	A		T	A
Sample No. 6		A	A		T	A
Sample No. 8		A	A		T	A
Sample No. 10		A	A		T	A

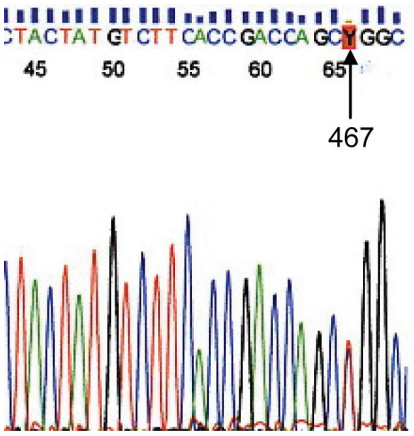

Fig. 2 Part of DNA sequencings of the exon 7 of the *ABO* gene in A_3 phenotype of sample no. 1. The arrow showed the heterozygous state of C and T (Y) at position 467 of exon 7 in the *ABO* gene. This heterozygous state can be defined as the mutation in A allele with A/O genotype and the A allele possessed 467C>T mutation

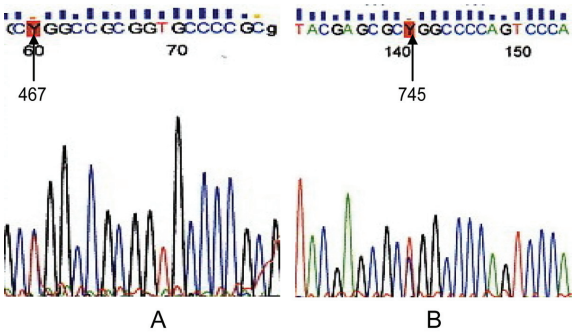

Fig. 3 Part of DNA sequencings of the exon 7 of the *ABO* gene in A_3 phenotype of sample no. 4. A) showed the heterozygous state of C and T (Y) at position 467. B) showed the heterozygous state of C and T (Y) at position 745. These heterozygous states can be defined as the mutation in A allele with A/O genotype. The A allele at position 467 and 745 possessed 467C>T and 745C>T mutations, respectively

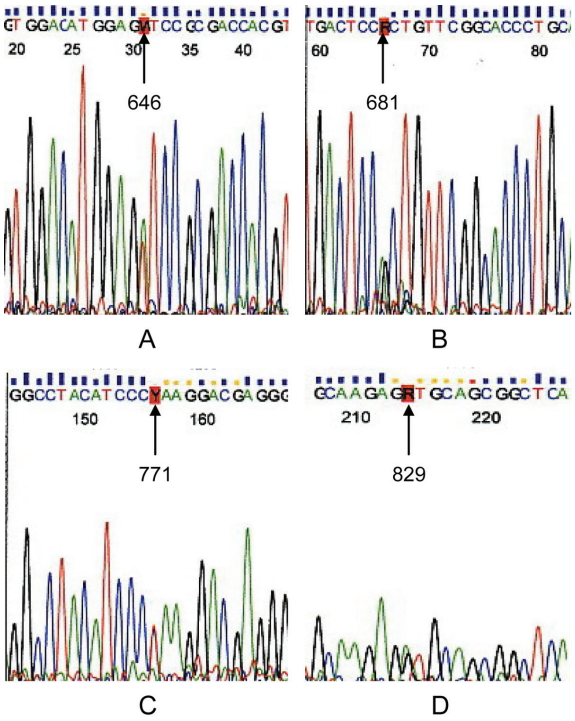

Fig. 4 Part of DNA sequencings of the exon 7 of the *ABO* gene in A_3 phenotype of sample no. 5, 6, 8,10. A) showed the heterozygous state of A and T (W) at position 646. B) showed the heterozygous state of A and G (R) at position 646. C) showed the heterozygous state of C and T (Y) at position 771. D) showed the heterozygous state of A and G (R) at position 829. These heterozygous states can be defined as the mutation in A allele with A/O genotype.The A allele possessed 646T>A, 681G>A, 771C>T and 829G>A

heterozygous state of C and T nucleotide at position 467 in one A_3 sample. It is very likely that the mutation is located on the A allele. This heterozygous state can be defined as the mutation in A allele with A/O genotype and the A allele possessed 467C>T mutation. The study of Barjas-Castro et.al also found 467C>T mutation in one A_3 donor[15]. The 467C>T substitution is resulting in an amino acid alteration of Pro 156Leu. However, this mutation was also identified in other subgroups A according to BGMUT. Therefore, it can be concluded that 467C>T substitution is unlikely to be specific characteristic of A_3 phenotype but it can be found in other subgroups A.

The heterozygous state of C and T nucleotide at positions 467 together with 745 are found in one sample. These heterozygous states can be defined as the mutation in A allele with A/O genotype. The A allele at position 467 and 745 possessed 467C>T and 745C>T mutations, respectively. Amino acid alterations derived from 467C>T mutation was Pro 156Leu and from 745C>T mutation was Arg 249Trp. According to BGMUT, these nucleotide substitutions can be classified as A307 allele. It was first elucidated by Lei Li et al in 2007. He also suggested that these nucleotide substitutions could reduce the activity of the A transferase[17]. Thus, it can be concluded that the 467C>T mutation together with 745C>T mutation are specific characteristic of A_3 phenotype.

Four samples demonstrated four nucleotide substitutions in heterozygosity. The A allele possessed 646T>A, 681G>A, 771C>T and 829G>A. Two of four positions can caused amino acid alterations (Phe 216Ile, Val 277Met). These mutations are not a specific characteristic of A_3 phenotype because they can be found in other subgroup alleles according to BGMUT. Nevertheless, the study of Barjas-Castro also described these mutations in exon 7 in one homozygous A_3 individual[15]. None of seven A_3 alleles listed in the database also possess these four mutations but one A_3 allele (A302) possess the 829G>A mutation. However, the A302 allele has another mutation, 1061delC. These data can be suggested that 646T>A, 681G>A, 771C>T and 829G>A mutations are can be found in A_3 phenotype in Thai blood donors.

There is no mutation in exon 7 found in four donor samples as the sequences of these samples are identical to A101 allele in the last coding exon. For A_3 subgroup testing, the serological pattern of these four samples are not different from six samples which show the mutations. The study of Yamamoto et al also found that both A_3 individuals did not show a mutation in the last two coding exons[19]. Although the mutation was not found in the last coding exon it can not be concluded that there is no mutation in these four samples. Because the other exons and other regions of the *ABO* gene have not been investigated.

In conclusion, the mutation was found at six positions with three patterns in six donor samples. These results suggested the genetic heterogeneity in A_3 phenotype donors. No new mutation was found in these six donor samples. As the findings revealed only in exon 7 of the *ABO* gene, it cannot be concluded that other areas have no mutation. There should be an investigation of other exons of the *ABO* gene and genotype all subgroup A_3 donors for complete observation in molecular study. They may affect the activity of glycosyltransferase enzyme. However, ten

A_3 blood donors did not represent all Thai populations. A further study should be performed in more samples for complete investigation in a Thai population with A_3 phenotype.

The present study elucidated the nucleotide mutation and developed molecular protocol to detect mutation of A_3 phenotype in Thai blood donors. The details which were obtained from the present study served as a beginning of a database in Thai population and can be used for further study in other blood groups.

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Potential conflicts of interest

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การตรวจหาการกลายพันธุ์ของจีนเอบีโอ ในผู้บริจาคโลหิตคนไทยที่มีหมู่โลหิตเอทรี

อภิวันท์ พิพัฒน์วนิชกุล, ปาริชาติ เพิ่มพิกุล, ศศิจิต เวชแพศย์, วิมล ชินสว่างวัฒนกุล

หมู่โลหิตระบบเอบีโอเป็นหมู่โลหิตที่มีความสำคัญที่สุดในงานด้านเวชศาสตร์การธนาคารเลือด ซึ่งนอกเหนือจากหมู่โลหิตหลักแล้ว พบว่ามีหมู่โลหิตย่อยที่มีแอนติเจนบนผิวเม็ดเลือดแดงที่น้อยกว่าหมู่โลหิตหลัก ในคนไทยหมู่เลือด A_3 เป็นหมู่โลหิตย่อยที่พบได้บ่อยที่สุด เกิดจากการกลายพันธุ์ของจีนเอบีโอโดยเฉพาะใน exon 7 การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อตรวจหาการกลายพันธุ์ใน exon 7 ของจีนเอบีโอ ในผู้บริจาคโลหิตคนไทยที่มีหมู่โลหิต A_3 จำนวน 10 ราย โดยการสกัดดีเอนเอ จากตัวอย่างโลหิตจากนั้นนำมาทำการเพิ่มจำนวนสารพันธุกรรมด้วยวิธีพีซีอาร์ และทำการตรวจหาลำดับดีเอนเอ

ผลการศึกษาพบว่ามีการกลายพันธุ์ ใน exon 7 ของ allele A จำนวน 6 รายจาก 10 ราย ที่ตำแหน่ง 646T>A, 681G>A, 771C>T, 829G>A, 467C>T และทั้งตำแหน่ง 467C>T และ 745C>T และอีก 4 ราย ไม่พบ การกลายพันธุ์ที่พบในการศึกษานี้ได้มีรายงานมาก่อนหน้านี้แล้วในฐานข้อมูลของ BGMUT และมีลักษณะเดียวกันกับการศึกษาอื่น ๆ และไม่พบการเปลี่ยนแปลงลักษณะของจีนแบบใหม่ ผลการศึกษาครั้งนี้ สามารถสรุปได้ว่าคนไทยที่มีหมู่โลหิต A_3 เกิดจากพันธุกรรมที่หลากหลาย