Vox Sanguinis (2003) **85**, 216–220
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ORIGINAL PAPER

Molecular genetic analysis of the B_{el} phenotype

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Vox Sanguinis

Background and Objectives In addition to the common ABO phenotypes, numerous phenotypes with a weak expression of the A or B antigens on the red blood cells have been found. This study describes the molecular genetic analysis of the B_{el} phenotype in Taiwanese individuals.

Materials and Methods The exon 6–7 region of the *ABO* gene of an individual with the B_{el} phenotype was amplified by the polymerase chain reaction (PCR), cloned, and the sequences of the exons and their adjacent splice sites were analysed. A PCR-based restriction fragment length polymorphism (RFLP) analysis was designed to detect the 502C>T nucleotide change identified in the B^{el} allele. Six unrelated individuals with the B_{el} phenotype were analysed, and samples from 40 randomly selected individuals with the common B phenotype were also assessed.

Results All six unrelated Taiwanese individuals with the B_{el} phenotype were shown to possess a *B* gene with the 502C>T mutation. The mutation was not detected in the general group B population. The 502C>T nucleotide change predicts an amino acid alteration of Arg168→Trp in the encoded B transferase.

Conclusions The results suggest a new molecular basis, a 502C>T missense mutation in the *B* allele, for the B_{el} phenotype and an association of the B^{el}*502C>T* allele with the B_{el} phenotype in the Taiwanese population.

Received: 15 May 2003, revised 20 June 2003, accepted 23 June 2003

Key words: ABO, B_{el}, blood group, subgroup.

Introduction

The A and B antigenic determinants of the human histo-blood group ABO system are distinguished by a difference of a single carbohydrate residue – Gal*N*Ac in the A antigen and Gal in the B antigen – which are synthesized by the respective glycosyltransferase products of the *A* and *B* alleles at the *ABO* locus [1,2]. The *A* allele encodes the α-1,3-*N*-acetylgalactosaminyltransferase (A transferase), which catalyses the transfer of Gal*N*Ac to the H-precursor structure (Fucα1–2Galβ1-R) to give the A determinant (Gal*N*Acα1–3[Fucα1–2]Galβ1-R), and the α-1,3-galactosyltransferase (B transferase), encoded by the *B* allele, is responsible for the formation of the B determinant (Galα1–3[Fucα1–2]Galβ1-R) by the transfer of Gal to the same H precursor. The molecular genetic basis of the human ABO blood group system was elucidated by Yamamoto *et al.* in 1990, who first characterized the respective nucleotide sequences of the three major alleles (A^1, *B* and *O*) of the *ABO* locus [3,4]. A^1 and *B* allelic cDNAs have seven nucleotide dissimilarities and this results in differences at four amino acids (residues 176, 235, 266 and 268), which are believed to be responsible for the difference in specificity between the A and B transferases [5–8]. Most examples of the *O* cDNA possess a deletion at nucleotide 261, which leads to a reading frameshift, and thus this allele fails to encode a complete transferase product. The *ABO* gene was found to consist of seven exons [9,10], which span ≈ 19·5 kb of genomic DNA. Recently it was found that, in addition to the known complete transcript structure, the *ABO* locus expresses another variant form, which contains a different starting exon 1 region through utilization of an alternative promoter [11,12].

The ABO blood group is, without doubt, the most important blood group system in transfusion medicine. In addition

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to the common ABO phenotypes (A_1, A_2, B, A_1B, A_2B, and O), numerous phenotypes with a weak expression of the A or B antigens on the red blood cells (RBCs) have been found. Each of these subgroups, such as A_3, A_x, A_{el}, *cis*-AB, B_3, B_x, B_{el} and B(A), has defined serological characteristics [2,13]. Many *A* and *B* suballeles, responsible for the formation of subgroups, have been identified [14,15]. Most of these minor alleles have mutation(s) in the coding sequence of the *ABO* gene, and most of the mutations are single-nucleotide substitutions, leading to an amino acid alteration. Splice-site mutations in the *A* and *B* alleles have also been correlated with subgroups. Thirteen out of 14 Taiwanese subjects with the B_3 phenotype have been shown to have a *B* allele with a G>A mutation at the +5 nucleotide of intron 3 [intervening sequence (IVS)3+5G→A] [16]. The IVS3+5G→A splice donor-site mutation leads to the skipping of the exon 3 of the *B* transcript during mRNA processing. The *A* allele of individuals with the A_{finn} phenotype was found to possess an A>G mutation at the +4 nucleotide of intron 6 [17], while the *A* allele with the IVS6+5G→A mutation has been shown to be associated with the A_{el} phenotype in Taiwanese individuals [18].

This study investigated, by molecular genetic analysis, blood samples from six unrelated Taiwanese subjects with the B_{el} phenotype. The B_{el} RBCs were not agglutinated by anti-B or anti-A,B, but the B antigen on the cells can be demonstrated after adsorption and elution. Mutations of 641T>G and 669G>T, which lead to the amino acid alterations Met214Arg and Glu223Asp, respectively, in the *B* allele, have been reported for the B_{el} phenotype in other ethnic groups [19]. In this study, however, a different molecular basis was identified in six Taiwanese B_{el} patients.

Materials and methods

Serology analysis

ABO grouping was performed by using the standard haemagglutination test. Adsorption and elution tests for testing the B_{el} red cells against monoclonal anti-B (Gamma Biologicals Inc., Houston, TX) were performed as described previously [20]. The salivary ABH substances were determined by using a salivary ABH inhibition test [20]. Peripheral blood samples were collected from 46 healthy blood donors (six unrelated individuals with the B_{el} phenotype and 40 randomly selected individuals with the common B phenotype). All of the assessed individuals belong to the Taiwanese population. Informed consent was obtained from all participants.

Sequence analysis of exons 6 and 7 of the *ABO* gene

The genomic DNAs of B_{el} individuals and group B individuals were prepared from their peripheral blood cells using the QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany). The polymerase chain reaction (PCR) and forward (GGGTGGTCAGAGGAGGCAGAAGCTGAGTGG, 91 bp upstream to exon 6) and reverse (GACGGGGCCTAGGCTTCAGTTACTCACAAC, antisense sequence, 99 bp downstream to the stop codon) primers for the *ABO* gene were used to amplify the DNA fragment encompassing the region from exon 6 through exon 7. One-hundred nanograms of genomic DNA from the propositus and 10 pmole of each primer were combined in 25 µl of PCR buffer containing 1·5 mM $MgCl_2$, 0·2 mM of each dNTP, and 0·5 U of Expand HiFi^{PLUS} DNA polymerase (Roche Diagnostics GmbH, Penzberg, Germany). The PCR conditions were as follows: 5 min at 94 °C, followed by 30 cycles of 0·5 min at 94 °C and 2·5 min at 72 °C. The PCR product, ≈ 2·1 kb in size, was cloned into the pCR4-TOPO vector using the TOPO TA Cloning Kit (Invitrogen, Groningen, the Netherlands). DNA sequencing was carried out using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

PCR-based restriction fragment length polymorphism (RFLP) analysis

Based on the nucleotide substitution of 502C>T identified in the *B* gene of the B_{el} propositus, a PCR-based RFLP analysis was developed to identify the presence of the mutation in the *ABO* genes of an individual. The sense (GTGGCTTTCCTGAAGCTGTTCCTGGAGACG) and antisense (TTGTAGGCGCSCACCTCCAGCACTGACAGTGGC) primers were designed to anneal to nucleotides 379–408 and 504–536 of the *ABO* gene, respectively (nucleotides numbered according to the *ABO* cDNA sequence, the codon for initiation methionine as nucleotides 1–3). In the antisense primer, the S (G or C) nucleotides, which correspond to nucleotide position 526 of the *ABO* cDNA, permitted the amplification of all the three *A*, *B* and *O* alleles, and two nucleotides (underlined) were changed from CT to TG, so that an *Msc*I recognition sequence (TGGCCA) would be produced when *ABO* alleles bearing a 502C>T mutation were amplified. One-hundred nanograms of genomic DNA and 10 pmole of each forward and reverse primer were combined in 25 µl of PCR buffer containing 2·0 mM $MgCl_2$, 0·2 mM each dNTP, and 0·5 U of *Taq* DNA polymerase (Promega, Madison, WI). The PCR conditions were as follows: 5 min at 94 °C; 30 cycles of 30 seconds at 94 °C and 30 seconds at 65 °C; and 1 min at 72 °C. The 158-bp PCR products were subjected to digestion by *Msc*I restriction endonuclease (New England BioLabs, Inc., Beverly, MA), and then analysed by electrophoresis on a 2·0% agarose gel.

Results

Serology

The individuals with the B_{el} phenotype were discovered as a result of discrepancies between forward and reverse typing

in routine ABO grouping. The red cells of individuals with the B_{el} phenotype were not agglutinated by polyclonal and monoclonal anti-B and anti-A,B reagents at room temperature. The sera of these individuals contained anti-A (4+) and weak anti-B (1+) activities. Adsorption and elution tests performed by testing B_{el} red cells against monoclonal anti-B produced eluate that reacted with the B cells. The saliva contained only H substance, as determined by a salivary ABH inhibition test.

The B_{el} propositus possesses a *B* gene with a 502C>T missense mutation

The DNA region encompassing exon 6 through exon 7 of the *ABO* gene of an individual with the B_{el} phenotype, the propositus, was PCR-amplified and cloned, and the sequences of the exons and the adjacent splice acceptor and donor sites were analysed. Recombinant plasmids containing the PCR products could be separated into alleles of *O* and non-*O* origin, according to the presence of the 261G deletion in the *O* allele. The results demonstrated that the individual harboured an O^1 gene with consensus exon 6 and exon 7 sequences for this allele. The exon 6 and exon 7 regions of a number of non-*O* allele clones were sequenced, and all the sequences demonstrated that the *B* gene had a correct sequence, except for a nucleotide substitution of 502C>T (translation initiation codon of *ABO* cDNA as nucleotide 1–3). Direct sequencing of the PCR product demonstrated the heterozygous status with C and T nucleotides at nucleotide position 502. The 502C>T substitution in the *B* gene predicts an amino acid alteration of Arg to Trp at residue 168.

The 502C>T mutation is present in six unrelated individuals with the B_{el} phenotype, but is not detected in the general group B population

A PCR-based RFLP analysis system was developed and used to identify the 502C>T mutation in the other five unrelated B_{el} individuals and in 40 randomly selected individuals with the common group B phenotype. By changing two nucleotides in the PCR primer, an *Msc*I recognition sequence was produced when *ABO* alleles with a 502C>T mutation were amplified (detailed in the Materials and methods).

The 158-bp PCR product amplified from the mutant allele was cleaved into 126- and 32-bp fragments by *Msc*I digestion, while that from the consensus allele was resistant to digestion. As shown in Fig. 1, all of the other five B_{el} individuals (lanes 2–6) had one allele with the 502C>T mutation at their *ABO* loci, as did the B_{el} propositus (Fig. 1, lane 1). None of the *B* or *O* alleles of the 40 randomly selected group B individuals possessed the mutation (one such analysis is shown in lane B). Exons 6 and 7 of the *ABO* genes of these five B_{el} individuals were further analysed by direct sequencing after PCR amplification. The results demonstrated that, similarly to the B_{el} propositus, all of the five B_{el} individuals possessed the 502C>T mutation and were heterozygotes with one *O* allele.

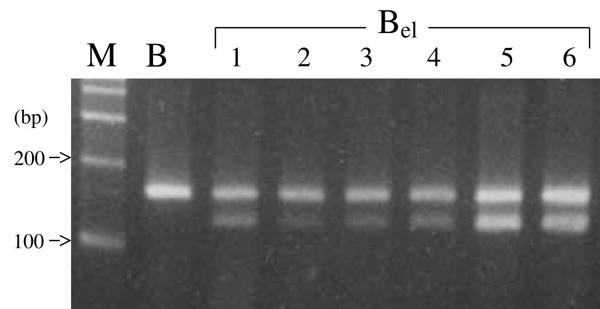

Fig. 1 Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis of the 502C>T mutation in the *ABO* gene. Genomic DNA samples from the B_{el} propositus (lane 1) and five other unrelated B_{el} individuals (lanes 2–6), together with samples from 40 randomly selected group B individuals, were subjected to the PCR–RFLP analysis. One of the results obtained from the group B individuals is shown in lane B. Lane M shows the molecular mass standard, a 100-bp ladder. The PCR primers were designed to permit the amplification of all three *A*, *B* and *O* alleles and to produce the *Msc*I recognition sequence when an *ABO* allele, bearing the 502C>T mutation, was amplified (see the Materials and methods). After digestion with the *Msc*I restriction endonuclease, the 158-bp PCR product amplified from the gene with the 502C>T nucleotide substitution yielded 126- and 32-bp fragments, while the 158-bp PCR product from a consensus allele was resistant to digestion. The *Msc*I-cleaved products were analysed by electrophoresis on a 2·0% agarose gel. The results indicate that all six B_{el} Taiwanese sibjects possessed one allele with a 502C>T mutation, while none of the 40 group B individuals had this mutation.

This result shows that all six B_{el} individuals carry a *B* gene with a 502C>T missense mutation, while the mutation is not detected in the general group B population.

Discussion

In this study, the molecular change – 502C>T in the *B* gene – was identified in Taiwanese subjects with the B_{el} phenotype, and when six unrelated B_{el} individuals were analysed, all were found to possess the 502C>T mutation. This suggests an association of the B^{el}*502C>T* allele with the B_{el} phenotype in the Taiwanese population. The PCR–RFLP analysis, designed to detect the 502C>T mutation in the *ABO* genes, as shown in Fig. 1, provides a rapid genotyping method for the presence of the B^{el}*502C>T* allele. The 502C>T missense mutation predicts an amino acid alteration of Arg168Trp in the B transferase of the mutant gene product. As only a trace amount of the B antigenic determinant is present on the B_{el} RBCs, it is suggested that the Arg168Trp alteration to the B transferase results in reduced B-transferase activity. However, further investigation to express and analyse the transferase activity of the expressed product from the mutant *B* cDNA with the 502C>T substitution is still needed to provide

definite evidence of the effect of the Arg168Trp change on the B-transferase activity.

It has been shown that a single subgroup phenotype can result from a variety of molecular changes in the *ABO* alleles. The B^{el}*502C>T* allele, demonstrated in the present study, is the third B^{el} allele reported to date. Previously, two *B* suballeles with mutations of 641T>G and 669G>T, which lead to amino acid alterations of Met214Arg and Glu223Asp, respectively, were identified in Japanese individuals with the B_{el} phenotype [19].

It should be noted that a different mutation at the 502C nucleotide of the *ABO* gene has been identified in another suballele. Three unrelated individuals with the A_x phenotype were found to have an A^1 gene with a mutation on the 502C nucleotide [21]. However, it is a transversion mutation of C>G and occurred in the A^1 gene rather than the C>T transition mutation in the *B* gene, as identified in the present study. The 502C>G change in the A^x gene leads to an Arg168Gly alteration in the encoded A transferase. These two minor alleles – the B^{el}*502C>T* and A^x*502C>G* – have probably resulted from two individual mutation events on different alleles but occurring at the same nucleotide position of the *ABO* locus. A similar situation was also observed at another two nucleotide positions in the *ABO* gene. A *B(A)* allele was found to have a 700C>G mutation in the *B* gene [22], while the 700C nucleotide has changed to T in a *cis-AB* allele [23,24]. The 700C>G and 700C>T changes lead to the Pro234 to Ala and Pro234 to Ser amino acid alterations, respectively. Similarly, the 1054C nucleotide on the A^1 gene was shown to have changed to G and T nucleotides in two individual *A* suballeles, which were believed to be responsible for the A_2 phenotype [19]. The 1054C>G transversion mutation and 1054C>T transition mutation in these two A^2 suballeles result in Arg352 to Gly and Arg352 to Trp alterations, respectively. A 1054C>T change in the *B* gene has also been demonstrated in a B^3 allele [25].

The human *ABO* locus shows considerable polymorphism, and numerous *A*, *B* and *O* minor alleles have been identified. Several identical mutations or chimeric sequences have been found in different *ABO* minor alleles and thus it has been suggested that recombination and gene-conversion events contribute to the generation of *ABO* gene diversity [26]. However, the three pairs of suballeles mentioned above – the B^{el}*502C>T* and A^x*502C>G* alleles, the *cis-AB700C>T* and *B(A)700C>G* alleles, and the A^2*1054C>T* and A^2*1054C>G* alleles – which show different mutation events occurring on the same nucleotide position of the locus, represent a different feature. The A transferase encoded from the A^x*502C>G* cDNA, in which the Arg168 residue is replaced with Gly, is thought to maintain weak activity because the A_x RBCs possess weak A antigenic activity, while the B transferase encoded from the B^{el}*502C>T* cDNA, in which the Arg168 residue is replaced with Trp, might have only trace B-transferase activity as the B antigen on the B_{el} RBCs can only be demonstrated after adsorption and elution. Further study on the possible biophysical role played by residue 168 in A- or B-transferase activities, would seem to be warranted.

Acknowledgements

This work was supported, in part, by Grant CMRP 797-IV from the Chang-Gung Memorial Hospital, Grants NSC 91-2314-B-182-093 and NSC 91-2314-B-002-405 from the National Science Council, and Grant NSC 91-3112-B-002-028 from the National Research Program for Genomic Medicine of the National Science Council, Taiwan.

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