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Molecular Genetic Analysis of Variant Phenotypes of the ABO Blood Group System

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ABO is clinically the most important blood group system in transfusion medicine and includes many variant phenotypes. To understand the molecular genetic basis of this polymorphic system, we have analyzed genomic DNAs obtained from Japanese individuals possessing variant ABO phenotypes including A_2, A_x, A_{el}, *cis*-AB, B_x, and B_{el}. By polymerase chain reaction–single-strand conformation polymorphism (SSCP) and nucleotide sequence analyses, we identified 11 different alleles. These alleles had nucleotide sequences different from those of the previously described 13 different alleles responsible for the common ABO phenotypes. Analysis of the nucleotide sequences of the alleles responsible for those variant phenotypes showed that the amino acid residues at positions 266 and 268 may be crucial for transferase specificity, whereas those at positions 214, 216, 223, 291, and 352 may be critical for the activity level. Nine of the 11 alleles, responsible for the A_2, A_x, A_{el}, *cis*-AB, B_x, and B_{el} phenotypes, were presumed to be generated from common *ABO* alleles by single nucleotide mutations such as nonsynonymous substitution, deletion, or insertion. Two other alleles, responsible for the A_2 and A_{el} phenotypes, may have originated by recombination, gene conversionlike events or accumulation of nucleotide substitutions. Our data indicate that different alleles could cause the same ABO variant phenotypes, and that these alleles do not necessarily belong to a single evolutionary lineage.

THREE MAJOR ALLELES (A^1, B, and O) encoding transferases of the ABO blood group system were first cloned and sequenced by Yamamoto et al.[1,2] The A^1 and B alleles encode 354-amino acid–long proteins, which are predicted to be type II transmembrane glycosyltransferases comprised of a short N-terminal domain, a hydrophobic transmembrane domain, and a long C-terminal catalytic domain. The O allele has a single nucleotide deletion at position 261 compared with the A^1 allele, which results in a frame shift to create a different amino acid sequence and a stop codon at amino acid position 118, and thus the O allele encodes a catalytically inactive protein.

In a previous study,[3] we determined the sequences of 13 different alleles detected in 262 healthy Japanese individuals possessing common ABO phenotypes and clarified the existence of several alleles responsible for single phenotypes. These alleles were classified into three major lineages in terms of their evolutionary relationships. In addition, we established a polymerase chain reaction (PCR)–single-strand conformation polymorphism (SSCP) method which allowed us to identify the common *ABO* alleles as well as to detect unknown *ABO* alleles.

In the present work, we have extended our study to variant ABO phenotypes. We report here eight newly sequenced alleles. The results enabled us to predict amino acid residues critical for the specificity and activity level of transferases in the catalytic domain. The evolutionary lineages of these alleles and other *ABO* alleles are also discussed.

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MATERIALS AND METHODS

ABO grouping. ABO phenotypes including A_2, A_x, A_{el}, B_x, B_{el}, and *cis*-AB were determined by agglutination and adsorption-elution tests using murine monoclonal anti-A and anti-B IgM antibodies (Ortho, Raritan, NJ), human polyclonal anti-A, anti-B (Dade, Miami, FL), and anti-A,B antibodies (Ortho), and plant lectins (*Dolichos biflorus* for anti-A_1, *Ulex europaeus* for anti-H; Sanko Junyaku Co, Tokyo, Japan). The phenotypes A_1 and A_2 were discriminated by anti-A_1: strong agglutination (4+) for A_1 and no agglutination (0) for A_2. The weak phenotypes A_x and A_{el} were determined using monoclonal anti-A and polyclonal anti-A,B antibodies, weak agglutination (1-2+) for A_x and 0 for A_{el}, and the presence of the A determinants on the A_{el} red blood cells (RBCs) was shown using an adsorption-elution technique with polyclonal anti-A antibodies. The phenotypes B_x and B_{el} were determined in the same manner using anti-B and anti-A,B antibodies. The *cis*-AB positive individuals were selected from two families. In total, we selected 19 A_2, 3 A_x, 4 A_{el}, 2 *cis*-AB, 1 B_x, and 2 B_{el} positive individuals from among healthy Japanese people.

The serum transferase activity levels were assayed by estimating the degree (agglutination score[4]) of the conversion of group O RBCs into A or B cells[5] using the monoclonal anti-A and anti-B antibodies.

Discrimination of ABO alleles. Genomic DNAs were prepared from peripheral blood samples of the above individuals by standard phenol-chloroform extraction.[6] The PCR-SSCP analysis, group-specific PCRs, and direct sequencing were performed as described previously.[3] Exons 6 and 7 of the *ABO* gene,[7,8] which encode most of the catalytic domain of the transferase, were amplified using four primer pairs under appropriate conditions and the four fragments obtained (I-IV) were subjected to SSCP analysis for detecting sequence differences.

SSCP analysis was performed using a 12.5% to 15% polyacrylamide gel (acrylamide:bisacrylamide = 49:1) with or without 5% (vol/vol) glycerol. Briefly, 1 μL of amplified DNA solution was mixed with 7 μL of denaturing solution (95% formamide, 20 mmol/L EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol FF), heated at 95°C for 5 minutes, and immediately chilled on ice, and 1 μL of the mixture was subjected to polyacrylamide gel electrophoresis at 12°C to 30°C. The separated single-strand DNA fragments

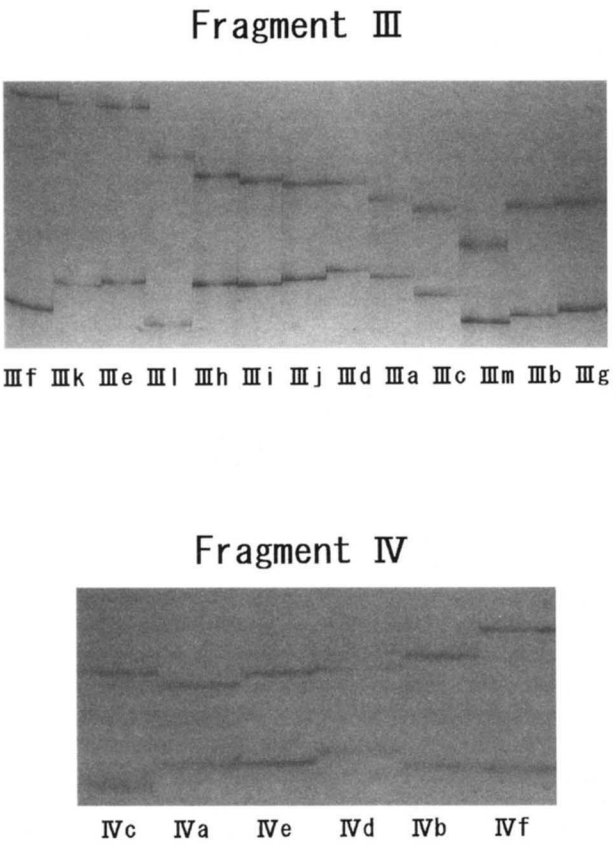

Fig 1. PCR-SSCP analysis of fragments III and IV of *ABO* blood group gene. Only the patterns of single alleles obtained either by group-specific PCR or from homozygous samples are shown.

in the gel were visualized by silver staining (Daiichi Pure Chemicals, Tokyo, Japan).

Group-specific PCRs were then performed to prepare the DNA templates for sequence analysis. The prepared DNAs were directly sequenced at least twice for each direction by a cycle-sequencing method with the same primers as those used for PCR-SSCP using an automated DNA sequencer (373A; Applied Biosystems, Foster City, CA).

The observed alleles were provisionally named according to the guidelines for human gene nomenclature.[9] When an identified allele was presumed to be generated by recombination, R was used as the first letter of the allele name. In the case of an allele responsible for the *cis*-AB phenotype, the letter C was used.

RESULTS

ABO alleles identified by PCR-SSCP. Typical SSCP patterns of the alleles obtained from healthy Japanese with variant ABO phenotypes are shown in Fig 1. The newly observed SSCP patterns were IIIg, IIIh, IIIi, IIIj, IIIk, IIIl, and IIIm for fragment III, and IVc, IVd, IVe, and IVf for fragment IV. As for fragments I and II, all the alleles identified in this study showed SSCP patterns identical to some of those reported previously for common ABO phenotypes[3] (data not shown).

As shown in Table 1, we identified 11 different alleles from the SSCP patterns which were different from the previously identified *ABO* alleles.[3] Assay of serum transferase showed that all the samples possessing these alleles had no detectable activity, except for the sample possessing *ABO*R101*. The alleles responsible for specific phenotypes were often heterogeneous: 4 alleles (*ABO*A105*, **A106*, **A107*, and **R101*) for A_2, 2 alleles (*ABO*A109* and **A110*) for A_{el}, and 2 alleles (*ABO*B105* and **B106*) for B_{el}. In A_2 phenotype samples, the relative frequencies of *ABO*A105*, **A106*, **A107*, and **R101* were 5%, 53%, 37%, and 5%, respectively. In addition, 2 of 3 alleles obtained from the A_x phenotype samples were *ABO*A108*, but the other allele showed SSCP patterns identical to those of the previously described *ABO*A102* (for A_1)[3] for fragments I-IV (data not shown). Similarly, 2 of 4 alleles obtained from the A_{el} phenotype samples were different from the alleles responsible for common ABO phenotypes and thus named *ABO*A109* and **A110*, whereas the other 2 alleles could not be discriminated from *ABO*A102* in the present SSCP analysis. In contrast, we found the same allele, *ABO*C101*, in the two unrelated families with the *cis*-AB phenotype (Fig 2). Although we examined only one B_x phenotype sample, the identified allele, *ABO*B104*, showed a specific SSCP pattern (IVf) as shown in Fig 1.

Nucleotide sequences of variant ABO alleles. The nucleotide sequences of the 11 different alleles* responsible for the variant ABO phenotypes are summarized in Table 2. One of the four alleles responsible for the A_2 phenotype, *ABO*A105*, had a nonsynonymous substitution, CCG (Pro) to CTG (Leu), at codon 156, and a nucleotide deletion (one of the cytosines at nucleotide positions 1059-1061) compared with *ABO*A101*, and thus had a sequence identical to that of the previously described allele responsible for the A_2 phenotype.[10] The other three alleles responsible for the A_2 phenotype, *ABO*A106*, **A107*, and **R101*, had sequences different from those of the previously described *ABO* alleles. Both *ABO*A106* and **A107* had a single nucleotide substitution (C to T and C to G, respectively) at position 1054 compared with *ABO*A101* resulting in an amino acid substitution at position 352 (Arg to Trp and Arg to Gly, respectively). *ABO*R101* had six nucleotide differences, at positions 297, 526, 657, 703, 771, and 829, compared with *ABO*A101* resulting in three amino acid substitutions, at positions 176, 235, and 277.

*ABO*C101* responsible for the *cis*-AB phenotype had two nonsynonymous substitutions, at codons 156 (CCG coding for Pro to CTG for Leu) and 268 (GGG coding for Gly to GCG for Ala), as compared with *ABO*A101*, and the nucleotide sequence was identical to that of the previously described allele responsible for the *cis*-AB phenotype.[11] *ABO*A108* responsible for the A_x phenotype also had a sequence identical to that of the previously described allele[12]

* The nucleotide sequences in this report have been submitted to the DDBJ/EMBL/GenBank data bases (accession nos. D82835-D82845).

Table 1. SSCP Patterns and Serum Transferase Activity Levels for Alleles Obtained From Blood Samples of Each Phenotype

Allele*	Phenotype of Blood Sample	Serum Transferase†		n	SSCP Pattern of Each Fragment			
		A	B		FI	FII	FIII	FIV
*ABO*A101*	A_1	90 (89-91)		4	Ia	IIa	IIIa	IVa
**A102*	A_1	82 (76-89)		4	Ia	IIb	IIIa	IVa
**A105*	A_2	0		1	Ia	IIb	IIIa	IVc
**A106*	A_2	0		10	Ia	IIa	IIIa	IVd
**A107*	A_2	0		7	Ia	IIa	IIIa	IVe
**R101*	A_2	75		1	Ib	IId	IIIg	IVa
**C101*	*cis*-AB	0	0	2	Ia	IIb	IIIh	IVa
**A108*	A_x	0		2	Ia	IIa	IIIi	IVa
**A109*	A_{el}	0		1	Ia	IIa	IIIj	IVa
**A110*	A_{el}	0		1	Ia	IIb	IIIk	IVa
*ABO*B101*	B		79 (75-89)	4	Ib	IId	IIIb	IVb
**B104*	B_x		0	1	Ib	IId	IIIb	IVf
**B105*	B_{el}		0	1	Ib	IId	IIII	IVb
**B106*	B_{el}		0	1	Ib	IId	IIIm	IVb

* Because all the samples, except for one of the *cis*-AB phenotype samples, examined possessed an *O* allele (**O101*, **O201*, or **O202*[3]), only the SSCP patterns of the alleles presumed to be responsible for each variant phenotype are shown. *ABO*A101* and **A102* responsible for A_1 phenotype and **B101* responsible for B phenotype are also shown.

† A and B transferase activity levels are indicated by agglutination score, mean, and (range). Sera of one of the *cis*-AB and two of the A_2 (**A106*) samples were not available.

with a nonsynonymous substitution at codon 216 (TTC coding for Phe to ATC for Ile) compared with *ABO*A101*.

One of the alleles responsible for the A_{el} phenotype, *ABO*A109*, had a single nucleotide insertion (G) in the guanine repeat at positions 798-804 resulting in a frame shift as compared with *ABO*A101*. In addition, *ABO*A110*, responsible for the A_{el} phenotype, had three nucleotide differences, at positions 467 (C to T), 646 (T to A) and 681 (G to A), resulting in two amino acid substitutions, at positions 156 (Phe to Leu) and 216 (Phe to Ile), compared with *ABO*A101*. The nonsynonymous substitution at nucleotide position 467 was also detected in *ABO*A105*, **C101* and the previously described *ABO*A102*,[3] and the other two nucleotides, at positions 646 and 681, were the same as those in *ABO*O201*.

All three *B* variant alleles (*ABO*B104*, **B105*, and **B106*) and *ABO*B101* shared seven nucleotide differences, at positions 297, 526, 657, 703, 796, 803, and 930, compared with *ABO*A101*. In addition, *ABO*B104* responsible for the B_x phenotype had a single nonsynonymous substitution at codon 291 (GAC coding for Asp to AAC for Asn) compared with *ABO*B101*. *ABO*B105* and **B106* responsible for the B_{el} phenotype also had single nonsynonymous substitutions, at codons 214 (ATG coding for Met to AGG for Arg) and 223 (GAG coding for Glu to GAT for Asp), respectively, compared with *ABO*B101*.

In total, 8 of the above 11 alleles (*ABO*A106*, **A107*, **R101*, **A109*, **A110*, **B104*, **B105*, and **B106*) were newly sequenced *ABO* variant alleles.

DISCUSSION

The amino acid differences of the transferase for each variant phenotype deduced in the present nucleotide sequence analysis and the previous report[13] are summarized in Table 3. Because the amounts of A or B determinants on erythrocytes are, in order of quantity, $A_1 > A_2 > A_3 > A_x > A_{el}$ or $B > B_3 > B_x > B_{el}$, it is predictable that the most critical amino acids for the enzymatic activity level are found at positions 214 (Met or Arg), 216 (Phe or Ile) and 223 (Glu or Asp). The amino acid substitutions at positions 291 (Asp

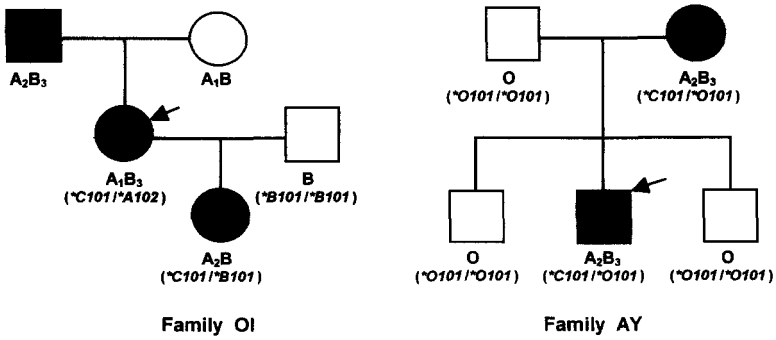

Fig 2. Schematic diagram of the inheritance of *cis*-AB in two families. The phenotype and genotype determined are indicated below each symbol. Arrows indicate propositi.

Table 2. Comparison of Nucleotide and Deduced Amino Acid Sequences of the *ABO* Alleles Identified by PCR-SSCP

	Nucleotide and Amino Acid Position																		
Allele	nt (aa)	261	297	467 (156)	526 (176)	641 (214)	646 (216)	657	669 (223)	681	703 (235)	771	796 (266)	803 (268)	798-804	829 (277)	871 (291)	930	1054 (352)	1059-61
*A101		G	A	C (Pro)	C (Arg)	T (Met)	T (Phe)	C	G (Glu)	G	G (Gly)	C	C (Leu)	G (Gly)	GGGGGGG	G (Val)	G (Asp)	G	C (Arg)	CCC
*A105		—	—	T (Leu)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	CC (→)
*A106		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	T (Trp)	—
*A107		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	G (Gly)	—
*C101		—	—	T (Leu)	—	—	—	—	—	—	—	—	—	C (Ala)	—	—	—	—	—	—
*A108		—	—	—	—	—	A (Ile)	—	—	—	—	—	—	—	—	—	—	—	—	—
*A109		—	—	—	—	—	—	—	—	—	—	—	—	—	GGGGGGGG (→)	—	—	—	—	—
*A110		—	—	T (Leu)	—	—	A (Ile)	—	—	A	—	—	—	—	—	—	—	—	—	—
*O201		del (→)	G	—	—	—	A	—	—	A	—	T	—	—	—	A	—	—	—	—
*R101		—	G	—	G (Gly)	—	—	T	—	—	A (Ser)	T	—	—	—	A (Met)	—	—	—	—
*B101		—	G	—	G (Gly)	—	—	T	—	—	A (Ser)	—	A (Met)	C (Ala)	—	—	—	A	—	—
*B104		—	G	—	G (Gly)	—	—	T	—	—	A (Ser)	—	A (Met)	C (Ala)	—	—	A (Asn)	A	—	—
*B105		—	G	—	G (Gly)	G (Arg)	—	T	—	—	A (Ser)	—	A (Met)	C (Ala)	—	—	—	A	—	—
*B106		—	G	—	G (Gly)	—	—	T	T (Asp)	—	A (Ser)	—	A (Met)	C (Ala)	—	—	—	A	—	—

Only the difference from the nucleotide and amino acid sequences of **A101* are indicated. **O201* and **B101,* responsible for common ABO phenotypes,[2,3] are also indicated.

Abbreviations: nt, nucleotide; aa, amino acid; →, start of frame shift due to single-base deletion or insertion.

to Asn) and 352 (Arg to Gly/Trp) may also lead to a decrease in the activity level, but amino acid changed at positions 156 (Pro to Leu) and 277 (Val to Met) may have little affect. The serum transferase activity levels of these variant samples (Table 1) support the above prediction.

Table 3. Relationship Between Amino Acid Sequences of Transferases and Produced ABO Characters Deduced From Nucleotide Sequences

ABO Character	Amino Acid Position and Residue						
	156	214	216	223	277	291	352
A_1 or B	Pro	Met	Phe	Glu	Val	Asp	Arg
A_1	Leu	—	—	—	—	—	—
A_2	—	—	—	—	—	—	Gly
A_2 or B_3*	—	—	—	—	—	—	Trp
A_2	—	—	—	—	Met	—	—
A_3* or B_x	—	—	—	—	—	Asn	—
B_{el}	—	—	—	Asp	—	—	—
A_x	—	—	Ile	—	—	—	—
A_{el}	Leu	—	Ile	—	—	—	—
B_{el}	—	Arg	—	—	—	—	—

* Described by Yamamoto et al.[13]

Interestingly, we found a single nucleotide insertion (G) in the guanine repeat at positions 798-804 in one of the alleles obtained from the A_{el} phenotype sample, *ABO*A109*, which resulted in a frame shift to create a different amino acid sequence and a stop codon at a different position. This frame shift resulted in a greater decrease in the level of the enzymatic activity than that of *ABO*A105* (for A_2) possessing a single nucleotide deletion between positions 1059 and 1061. According to the sequence of a genomic clone described by Yamamoto et al,[8] the new stop codon (TGA) is postulated to be at nucleotide positions 1170-1172. Very recently, Olsson et al[14] detected the same allele in A_{el} phenotype samples.

Concerning the alleles obtained from A_2 individuals, it is interesting to note the difference in the allele frequencies described by us and others.[10] Yamamoto et al[10] reported that all of the eight alleles from A_2 phenotype blood samples obtained from the American Red Cross (Portland, OR) had a single nucleotide deletion between positions 1059 and 1061, which was identical to that of the *ABO*A105* in the present study. In contrast, we found three other alleles from 19 Japanese possessing the A_2 phenotype. Among them, *ABO*A106* and **A107* were predominant at relative frequencies of 53%

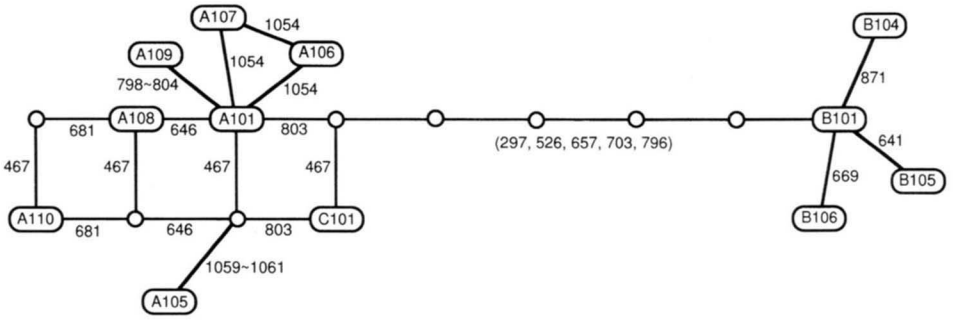

Fig 3. A phylogenetic network for 12 alleles described in Table 2. Open circles designate intermediate states, and numbers on each branch are nucleotide positions where mutations occurred. The five numbers in parentheses between the **A* and **B* lineages, 297, 526, 657, 703, and 796, could not be assigned to specific branches.

and 37%, respectively. These results clearly indicate that multiple alleles could cause a certain ABO phenotype and that their frequencies vary among various populations.

Yamamoto et al[2] were the first to report that there were seven nucleotide differences between *A* and *B* alleles, at nucleotide positions 297, 526, 657, 703, 796, 803, and 930, resulting in four amino acid differences, at positions 176, 235, 266, and 268. They showed that the last two amino acid residues at positions 266 and 268 are critical for A/B transferase sugar specificity in gene reconstruction and expression studies.[15] An allele encoding a bifunctional transferase for sugar specificity was subsequently identified in *cis*-AB individuals.[11] This allele encodes a transferase with an amino acid sequence identical to that of A_1 transferase except for a substitution at position 268 which is shared with the B transferase. As a result of this amino acid substitution, the RBCs of *cis*-AB individuals may express both A and B determinants. In the present study, we also identified an allele, provisionally called *ABO*C101*, in two unrelated *cis*-AB families by PCR-SSCP analysis, and its nucleotide sequence was identical to that of the previously described allele responsible for the *cis*-AB phenotype.[11]

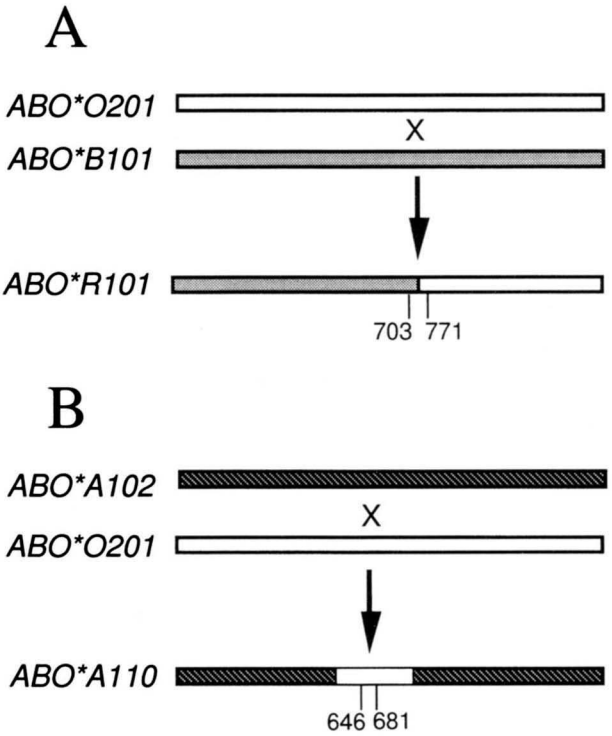

Fig 4. Schemes for generation of (A) *ABO*R101* and (B) *ABO*A110* alleles.

On the other hand, in an A_2 individual we detected a new allele (*ABO*R101*) that had six nucleotide differences resulting in three amino acid differences, at positions 176, 235, and 277, compared with the common A_1 transferase encoded by *ABO*A101*. Although the transferase encoded by *ABO*R101* has amino acid residues at positions 176 and 235 identical to those of the B transferase, those at positions 266 and 268 are identical to those of the A_1 transferase. Thus, the amino acid residues at positions 176 and 235 do not affect A/B transferase specificity, whereas those at positions 266 and 268 are concluded to be critical.

In a previous study,[3] we classified the alleles responsible for common ABO phenotypes into three major lineages, **A/*O1*, **B*, and **O2*, in terms of their evolutionary relationships. Six of the 11 different alleles from the variant phenotypes determined in this study, *ABO*A105*, **A106*, **A107*, **C101*, **A108*, and **A109*, have only a single nucleotide difference compared with *ABO*A101* or **A102*. Thus, these six variant alleles may belong to the *ABO*A* lineage. Similarly, three variant *B* alleles (*ABO*B104*, **B105*, and **B106*) have a single nucleotide difference compared with *ABO*B101*, and thus may belong to the *ABO*B* lineage.

We constructed phylogenetic networks to delineate the relationships among the nucleotide sequences determined in this study, as we did in our previous study.[3] A phylogenetic network can contain many equally parsimonious trees,[16] and can be considered to be a generalization of the discordancy diagram proposed by Fitch.[17] Figure 3 shows a phylogenetic network for all the alleles shown in Table 2 (except for *ABO*O201* and *ABO*R101*). It is clear that there are two distinct **A* and **B* lineages.

In contrast, *ABO*R101* has six nucleotide differences, at positions 297, 526, 657, 703, 771, and 829, compared with *ABO*A101*. The nucleotide sequence of *ABO*R101* is identical to that of *ABO*B101* upstream of nucleotide position 703 and to that of **O201* downstream of nucleotide position 771. Based on these findings, *ABO*R101* may have originated by recombination between *ABO*B101* and **O201* at

around positions 703-771, and may belong to a lineage intermediate between *ABO*B* and **O2* (Fig 4A). Similarly, *ABO*A110* has a nucleotide sequence identical to that of *ABO*A102*,[3] except for the nucleotides at positions 646 and 681 which are the same as in **O201*. Thus, *ABO*A110* is likely to have originated both by a double recombination or a gene conversion-like event (Fig 4B) and by accumulation of nucleotide substitutions.

These results indicate that there are different alleles responsible for the same ABO variant phenotypes, and that their evolutionary lineages are not necessarily the same.

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