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약-B형과 같은 *ABO* 아형의 문제해결을 위한 알고리즘 접근법: *B101/O04-variant* 대립유전자를 가진 약-B형 증례

An Algorithm to Work-up *ABO* Subgroups Presenting as Weak B in a Real-world Laboratory: A Case with a Weak B Phenotype Harboring *B101/O04-variant* Alleles

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Weaker ABO subgroups are the main cause of ABO discrepancies, and ABO genotyping studies are crucial to identify them. We performed ABO genotyping to determine the cause of a weak B phenotype in a Korean family, and aimed to develop a practical algorithmic approach to work-up ABO subgroups. ABO genotyping, along with serological ABO typing, was performed on exon 6 and exon 7 sites, sequentially from exon 2 to intron 6, exon 1 and the ABO promoter region, CBF/NF-Y enhancer region, +5.8-kb site in intron 1 using long PCR, and the +22.6-kb enhancer region. A single nucleotide variant (c.579T>C) known to be associated with the *O04* allele was observed in exon 7, and an insertion variant (c.203+1622_1623insC) was observed in intron 4, which was confirmed to have originated from the *O* allele using allele-specific sequencing. Based on these results, we made a tentative determination of the *O04-variant* allele. No remarkable variants were observed at other sites in our study. We were unable to reveal the genetic cause of the weak B phenotype, but detected a new *O04-variant* allele. This stepwise algorithmic approach to work-up ABO subgroups may be a practical alternative method to whole-genome sequencing.

Key Words: Weak B, ABO discrepancy, ABO subgroups, ABO genotyping

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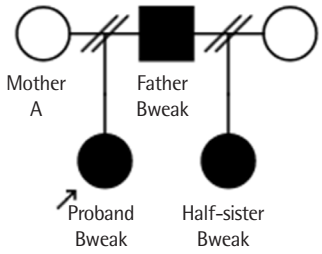
Discovered by Karl Landsteiner in 1900, the ABO blood type is the most important blood type for transfusions [1]. For safe blood transfusions, cell type and serum type test results of the patient should match. If the results of the tests are inconsistent, we call it ABO discrepancy. It is essential to resolve ABO discrepancies in clinical laboratories for safe blood transfusion [2]. Weak ABO subgroups are the main cause of ABO discrepancy. Various genotyping methods can be used to confirm the ABO blood group. Recently, next-generation sequencing has been used for blood grouping. However, this technique is expensive [3]. Here, we describe a weak B phenotype harboring *B101/O04-variant* alleles observed in a Korean family, and suggest a practical algorithm to work-up ABO subgroups without using whole-genome sequencing. The Institutional Review Board of Chonnam National University Hwasun Hospital (CNUHH), Hwasun, Korea (CNUHH-2022-096) ap-



	Phenotype					
	Cell typing				Serum typing	
	anti-A	anti-B	anti-A1	anti-H	A1 cell	B cell
Father	-	+/-	-	3+	4+	-
Mother	4+	-	4+	-	-	4+
Half-sister	-	+/-	-	3+	4+	-
Proband	-	+/-	-	1+	4+	-

A

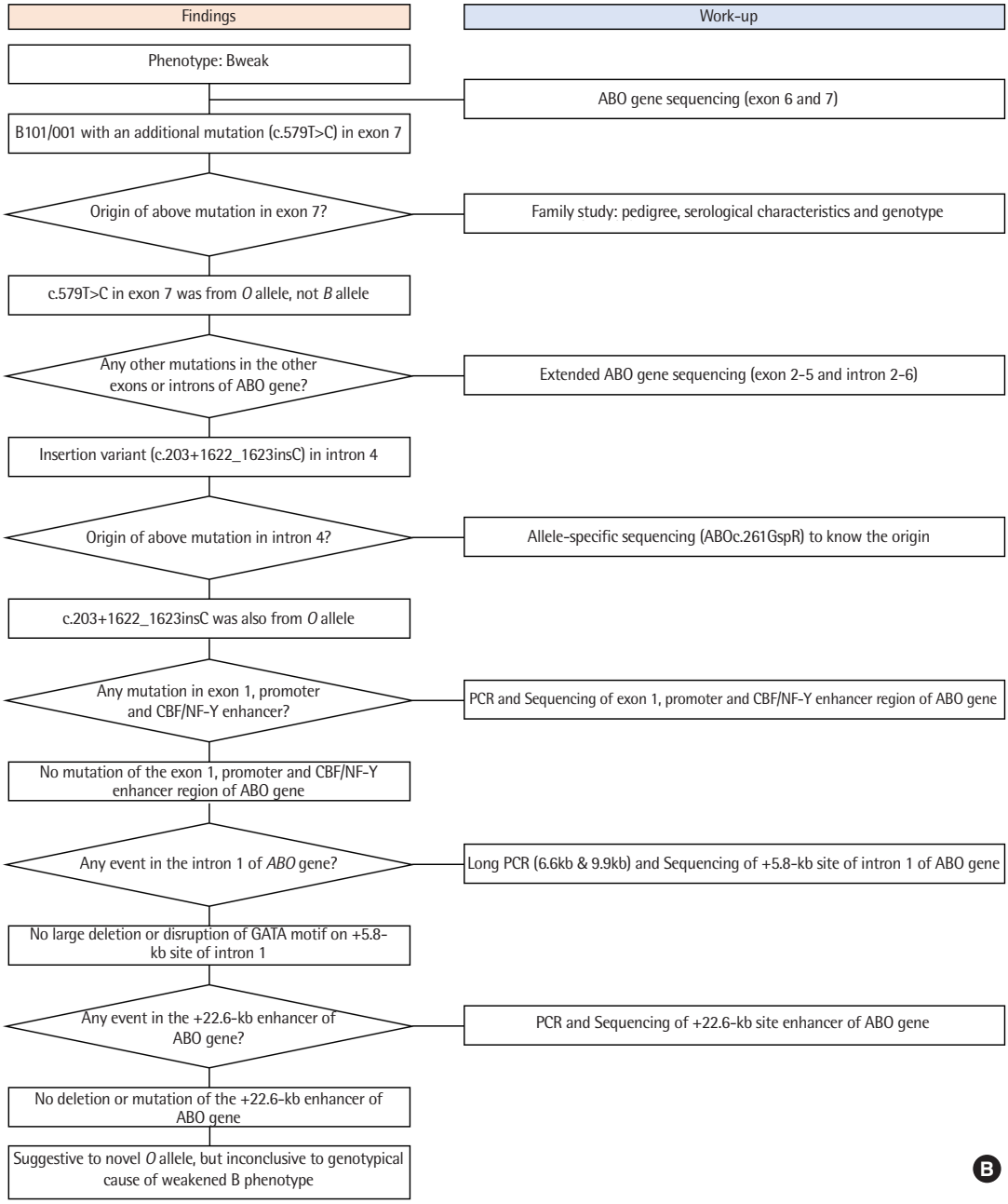


Fig. 1. Summary of our work. (A) The ABO phenotypes and genotypes of the family members with the family tree and table. The ABO phenotypes are indicated below each symbol. The arrow indicates the proband. (B) A stepwise algorithm to work-up the ABO subgroups.

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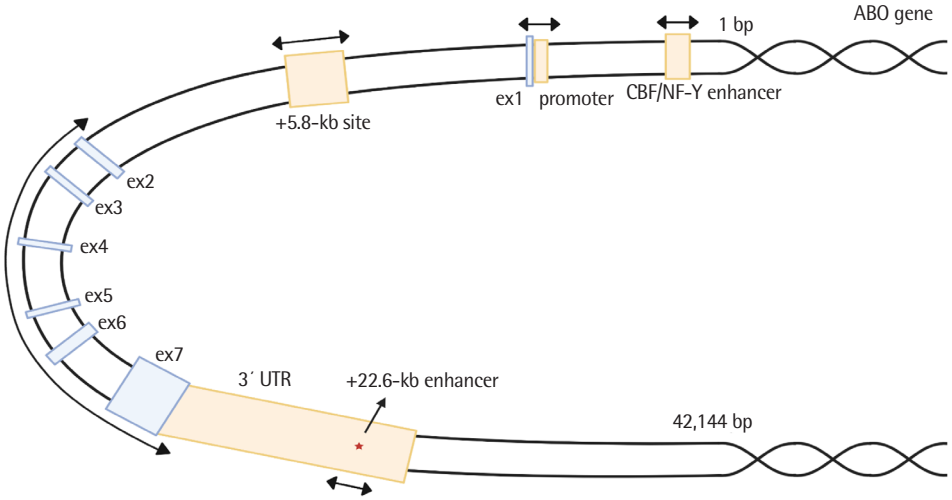

	Sequencing results of ABO gene															Provisional genotype
	Exon 6		Exon 7													
	261	297	467	526	579	646	657	681	703	771	796	803	829	930	1096	
Father	G/del	G/G	C/C	G/C	T/T	T/A	T/C	G/A	A/G	C/T	A/C	C/G	G/A	A/G	A/G	*B101/002*
Mother	G/del	A/A	T/C	C/C	T/C	T/T	C/C	G/G	G/G	C/C	C/C	G/G	G/G	G/G	G/G	*A102/004*
Half-sister	G/del	G/A	C/C	G/C	T/T	T/T	T/C	G/G	A/G	C/C	A/C	C/G	G/G	A/G	A/G	*B101/001*
Proband	G/del	G/A	C/C	G/C	T/C	T/T	T/C	G/G	A/G	C/C	A/C	C/G	G/G	A/G	A/G	*B101/004*

C

Fig. 1. (Continued) (C) Schematic of the genetic targets used to determine ABO genotypes and the ABO phenotypes of all family members and their ABO sequencing results (exons 6 and 7).

proved this study.

A healthy 24-year-old woman visited the Chonnam National University Hospital to confirm her blood type. We carried out serological ABO typing for her and her family. ABO cell type test was performed using the tube method with anti-A, anti-B, anti-A1, and anti-H agents (Shinyang Diagnostics, Siheung, Korea). Serum type test was performed using the tube method with A1 and B cells (Shinyang Diagnostics). The proband, her father, and her half-sister presented a weak B phenotype: trace positivity (+/–) for anti-B antibodies in cell typing and strong positivity (4+) for A1 cells in serum typing (Fig. 1A). The mother of the proband presented an A phenotype. To clarify the genetic cause of the weak B phenotype, we performed a stepwise molecular work-up. For sequencing, we extracted DNA from the EDTA sample tube with QIA symphony DSP DNA mini kit (Qiagen, Hilden, Germany). PCR products were purified using QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions.

ABO genotyping of exons 6 and 7 was performed first. Then, the other regions were genotyped sequentially (Fig. 1B). We performed PCR amplification using the primers ABOe6F and ABOe7R, and sequenced the PCR products using the primers ABOe6R, ABOe7F, ABOe7SF1, and ABOe7SF2. PCR amplification protocol and the cycling conditions described by Kim et al. were followed [4]. In exons 6 and 7 of the *ABO* gene, we found a common *B101* allele in the proband, her father, and half-sister. A rare single nucleotide variant (c.579T>C) known to be associated with the *O04* allele was observed in the proband and her mother (Fig. 1C).

Then, we performed PCR amplification of ABO exon 2 to coding region of exon 7 using primers ABOe27longF and ABOe27longR, and sequenced ABO exon 2 to intron 6. PCR amplification protocol and the cycling conditions described by Huh et al. [5] were followed. Further sequencing from exon 2 to intron 6 showed an insertion variant in intron 4 (c.203+1622_1623insC), which was confirmed to have originated from the *O* allele using allele-specific sequencing using a specific primer for ABOc.261GspR (5′-CAA TGG GAG CCA GCC AAG GGG TCA-3′). A base 'C', two steps

away from the 3′ end, combines with a base 'G' at the locus c.261, which would bind specifically to the *B* allele. As a result, no insertion variant was found in this sequence using the specific primer. Based on these results, we made a tentative determination of the *O04-variant* allele (Table 1).

Table 1. Several substitutions of the *ABO* gene found in the proband indicating the *B101/O04-variant* allele

Exon/ Intron	Location	Site number in the Reference*	Nucleotide in the Reference allele (*A101*)	Nucleotide substitution in the template allele	Tentative origin
Intron 2	c.98+362	18478	C	T	*O04-variant* allele
	c.98+369	18485	C	G	*O04-variant* allele
	c.98+396	18512	T	C	*O04-variant* allele
Intron 3	c.155+377	19274	C	T	*O04-variant* allele
	c.155+969	19866	T	C	*O04-variant* allele
	c.155+1357	20254	C	T	*O04-variant* allele
Intron 4	c.203+28	20424	G	C	*O04-variant* allele
	c.203+74	20470	A	T	*O04-variant* allele
	c.203+215	20611	A	G	*O04-variant* allele
	c.203+216	20612	A	C	*O04-variant* allele
	c.203+673	21069	A	T	*O04-variant* allele
	c.203+738	21134	T	G	*O04-variant* allele
	c.203+1467	21863	G	A	*O04-variant* allele
	c.203+1496	21892	T	C	*O04-variant* allele
	c.203+1511	21907	T	G	*O04-variant* allele
	c.203+1622_1623	22020	C	insC	*O04-variant* allele
Intron 5	c.239+530	22648	A	G	*B101* allele
Exon 6	c.261	22694	G	delG	*O04-variant* allele
	c.297	22730	A	G	*B101* allele
Intron 6	c.374+42	22849	G	T	*B101* allele
	c.374+163	22970	C	T	*B101* allele
	c.374+179	22986	T	C	*B101* allele
	c.374+271	23078	A	G	*B101* allele
	c.374+280	23087	C	T	*B101* allele
	c.374+446	23253	A	G	*B101* allele
	c.374+628	23435	A	G	*B101* allele
	c.374+686	23493	C	A	*B101* allele
	c.374+786	23593	A	G	*B101* allele
	c.374+891	23698	A	G	*B101* allele
	c.374+901	23708	G	A	*B101* allele
	c.374+950	23757	A	G	*B101* allele
Exon 7	c.526	24011	C	G	*B101* allele
	c.579	24064	T	C	*O04-variant* allele
	c.657	24142	C	T	*B101* allele
	c.703	24188	G	A	*B101* allele
	c.796	24281	C	A	*B101* allele
	c.803	24288	G	C	*B101* allele
	c.930	24415	G	A	*B101* allele
	c.1096	24581	G	A	*B101* allele

*The nucleotide sequence of GenBank accession no. NG_006669.2 was used as a refeFtrence.

Then, we performed PCR amplification, including exon 1, adjacent ABO promoter site, and CBF/NF-Y enhancer site using primers ABOenhe1longF and ABOenhe1longR and sequenced each region. We did not find any remarkable variants for these regions [5, 6]. We followed the PCR amplification protocol and the cycling conditions described by Huh et al. [5].

Next, we focused on intron 1. Long PCR using the primers ABO+4419S and ABO+11078AS was performed as described by Sano et al. [7]. PCR amplification was performed in a final reaction volume of 50 μL, containing 25 μL of KOD One™ PCR Master Mix (TOYOBO, Osaka, Japan), 1.0 μM of each primer, and 3 μL of genomic DNA. The PCR amplification was carried out in Veriti 96-well Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA). The cycling conditions were: initial denaturation at 98˚C for 180 seconds, 35 cycles of 98˚C for 10 seconds, and 68˚C for 60 seconds, followed by a final elongation at 68˚C for 180 seconds. The PCR product was electrophoresed on 0.7% agarose gel. It revealed no deletion of the +5.8-kb site, including the erythroid cell-specific regulatory element in intron 1. Sequencing using the primers ABO+5.8kbseqF and ABO+5.8kbseqR showed no remarkable variants on this site. We also amplified the 9.9 kb region that has CBF/NF-Y enhancer site, the binding site of the ABO+4419S, and the GATA motif in the +5.8-kb site. The heterozygosity of the 41st nucleotide in *ABO* minisatellite in the PCR product was maintained, confirming that the binding site of the PCR primer and the GATA motif was not deleted.

Lastly, we focused on the +22.6-kb enhancer region within the 3′ UTR region. PCR amplification was performed using the primers ABO+22.6kbpcrF and ABO+22.6kbpcrR, and the PCR product was sequenced using the primers ABO+22.6kbseqF and ABO+22.6kbpcrR as described by Sano et al. [8]. PCR amplification was performed in a final reaction volume of 50 μL, containing 25 μL of 2x Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific), 1.0 μM of each primer, and 3 μL of genomic DNA. The PCR amplification was carried out in Veriti 96-well Thermal Cycler (Thermo Fisher Scientific). The cycling conditions were: initial denaturation at 98˚C for 180 seconds, 35 cycles of 98˚C for 15 seconds, and 72˚C for 60 seconds, followed by a final elongation at 72˚C for 180 seconds. The PCR product was electrophoresed on 1.0% agarose gel. We did not find any deletion or nucleotide variants, or large deletion in the amplified sequences. During sequencing, the nucleotide sequence of GenBank accession no.

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NG_006669.2 was used as a reference. Sequencher software (Gene Codes, Ann Arbor, MI, USA) was used for sequence analysis. We described primer sequences and their targets in the *ABO* gene used in this study (Table 2).

Whole-genome sequencing may be ideal for determining *ABO* genotypes [3], but it is not readily available in routine practice yet. Here, we used a stepwise procedure to identify genetic variations in the *ABO* gene based on previously reported causes of a weak phenotype [6-9]. We made a tentative determination of the *B101/O04-variant*, which harbored c.203+1622_1623insC in addition to c.579T>C in this case. The *O04* allele is rarely observed, although a case of *Ael02/O04* presenting as a typical O phenotype and a chimera with *A102/B101/O04* have been reported in Korea [10]. A recent Chinese report suggested the potential effect of the *O04* allele associated with the expression of weak A antigen in a case of *B101/O04* with a weak AB phenotype [11]. However,

Table 2. Primer sequences and their targets in the *ABO* gene used in this study

Name	Primer sequence	Primer binding sites*	Purpose	Reference
ABOe6F	5′-GCTGAGTGGAGTTTCCAGGT-3′	Intron 5, 22573–22592	PCR amplification	[4]
ABOe7R	5′-AACAGGACGGACAAAGGAAA-3′	Exon 7, 24633–24652	PCR amplification	[4]
ABOe27longF	5′-TACTCACCTATTATTGGCCTTTGGTT-3′	Intron 1, 17421–17446	PCR amplification	[5]
ABOe27longR	5′-TAGGCTTCAGTTACTCACAACAGGAC-3′	Exon 7, 24645–24670	PCR amplification	[5]
ABOenhe1longF	5′-CTTACCAAAGGAGTCACACCCTCAAA-3′	Pre-enhancer region, 42–67	PCR amplification	[5]
ABOenhe1longR	5′-GAACTCAGCGATACTGAACACAGTGC-3′	Intron 1, 6119–6144	PCR amplification	[5]
ABO+4419S	5′-TGGAATTGCTGTCTCCTCTTTTAGTCC-3′	Intron 1, 9458–9484	PCR amplification	[7]
ABO+11078AS	5′-GGTCCCTCCTGACCCTGACAA-3′	Intron 1, 16070–16090	PCR amplification	[7]
ABO+22.6kbpcrF	5′-CAAGGACGAGGGCGATTTCTACTAC-3′	Exon 7, 24256–24280	PCR amplification	[8]
ABO+22.6kbpcrR	5′-CTCTGACACCCGATTGCTGCT-3′	Exon 7, 28089–28109	PCR amplification	[8]
ABOenhsF	5′-GCTCTTGCTCCTAGATGAT-3′	Enhancer, 1065–1083	CBF/NF-Y enhancer	[5]
ABOenhsR	5′-CAGGGAAGGACTTGGTTCAG-3′	Enhancer, 1591–1610	CBF/NF-Y enhancer	[5]
ABOe1sF	5′-GGCGCCGTCCCTTCCTAG-3′	Promoter, 4838–4855	Promoter, Exon 1	[5]
ABOe1sR	5′-CGAGGAGAGGCTGGAGAC-3′	Intron 1, 5350–5367	Promoter, Exon 1	[5]
ABO+5.8kbseqF	5′-TCATGTATTGCTGCGGGATAAT-3′	Intron 1, 10558–10579	+5.8-kb site	[7]
ABO+5.8kbseqR	5′-ACCATGTTCGCCAGGCTAGT-3′	Intron 1, 11257–11276	+5.8-kb site	[7]
ABOe2sF	5′-ACCATCTTGGCAGATGAAGG-3′	Intron 1, 17923–17942	Exon 2	[5]
ABOi2sR	5′-CCCCAGACTCCACACTTAG-3′	Intron 2, 18341–18359	Intron 2	[5]
ABOi2sF	5′-TTAGTCGCTTCCAGACACAG-3′	Intron 2, 18431–18450	Intron 2	[5]
ABOe3sF	5′-ACCAACAGGCAGTCTTCGTT-3′	Intron 2, 18785–18804	Exon 3	[5]
ABOi3sF1	5′-TCTTTCCAGAACATAAGGTAGG-3′	Intron 3, 19388–19409	Intron 3	[5]
ABOi3sF2	5′-GCTGGGTGGTTCACTTTGGG-3′	Intron 3, 19718–19737	Intron 3	[5]
ABOe4sF	5′-TGCCCTAAATCCTGCTCCTA-3′	Intron 3, 20294–20313	Exon 4	[5]
ABOi4sF1	5′-CCTGGGCTCAAGTGATTCTC-3′	Intron 4, 20717–20736	Intron 4	[5]
ABOi4sF2	5′-CTGTTGTTATGAGTCTGCTAC-3′	Intron 4, 21269–21289	Intron 4	[5]
ABOe5sF	5′-GCTGAATCAGAGACTCTGAG-3′	Intron 4, 21869–21888	Exon 5	[5]
ABOe5sR	5′-AAGAGACGCAAGTCAGAGAAAG-3′	Intron 5, 22290–22311	Exon 5	[5]
ABOi5sF1	5′-GAAGGTATTAGAGGGCGGTT-3′	Intron 5, 22170–22189	Intron 5	[5]
ABOi5sF2	5′-GGGTTTGTTCCTATCTCTTTG-3′	Intron 5, 22403–22423	Intron 5	[5]
ABOc.261GspR	5′-CAATGGGAGCCAGCCAAGGGGTCA-3′	Exon 6, 22693–22716	c.203+1622_1623insC	This study
ABOe6R	5′-CCACCCCACTCTGTCTTGAA-3′	Intron 6, 22884–22903	Exon 6	[4]
ABOi6sF1	5′-CGAGTGACTGTGGACATTGAG-3′	Intron 6, 22850–22870	Intron 6	[5]
ABOi6sR1	5′-CTGCCGAGAAGTCAAGTATGTGT-3′	Intron 6, 23347–23369	Intron 6	[5]
ABOi6sF2	5′-GAATGACTTACTCTTAGGAATAG-3′	Intron 6, 23408–23430	Intron 6	[5]
ABOi6sR2	5′-GGTGAAGACATAGTAGTGGAC-3′	Exon 7, 23924–23944	Intron 6	[5]
ABOe7F	5′-TCTGCTGCTCTAAGCCTTCC-3′	Exon 7, 23746–23765	Exon 7	[4]
ABOe7SF1	5′-TCCTCAGCGAGGTGGATTAC-3′	Exon 7, 24084–24103	Exon 7	[4]
ABOe7SF2	5′-ACGAAGAGAGCCACCTGAA-3′	Exon 7, 24387–24405	Exon 7	[4]
ABO+22.6kbseqF	5′-ATGGCTATTCCTGACCGTTG-3′	Exon 7, 27547–27566	+22.6-kb enhancer	[8]
ABO+22.6kbpcrR	5′-CTCTGACACCCGATTGCTGCT-3′	Exon 7, 28089–28109	+22.6-kb enhancer	[8]

*The nucleotide sequence of GenBank accession no. NG_006669.2 was used as a reference.

the *O04-variant* allele was unlikely to be the main cause of the weak B phenotype in our case, since the father and half-sister had the common *O* allele, rather than the *O04-variant* allele, but still expressed a weak B phenotype. Regrettably, we could not clarify the cause of the weak B phenotype as it is difficult to find the exact cause of the *ABO* mismatch based on general PCR techniques. However, we found a new *O04-variant* allele that has both c.579T>C and c.203+1622_1623insC. In conclusion, we described a stepwise algorithm to work-up ABO subgroups, which can be used instead of whole-genome sequencing in a real-world laboratory.

요 약

ABO 아형은 ABO 불일치의 주요 원인이며, 이를 식별하기 위해서는 ABO 유전형 검사가 필요하다. 저자들은 weak-B 표현형을 보이는 한 한국인 가족의 원인을 파악하고, ABO 아형에 대한 실용적인 알고리즘 접근법을 제시하고자 ABO 유전형 검사를 수행하였다. 혈청학적 혈액형 검사와 함께, 먼저 엑손 6번 및 7번 부위에 대해 유전형 검사를 시행한 후 이어서 엑손 2번에서 인트론 6번 부위, ABO promoter 영역, CCAAT 결합 인자/NF-Y (CBF/NF-Y) enhancer 영역, long PCR을 이용한 인트론 1번의 +5.8kb 영역, 그리고 +22.6kb enhancer 영역에 대해 유전형 검사를 시행하였다. 엑손 6번 및 7번 부위에서 발단자와 그녀의 어머니에게서 *O04*형 대립유전자와 관련된 것으로 알려진 단일염기변이(c.579T>C)가 관찰되었다. 또한 인트론 4번 부위에서 발견된 삽입 변이(c.203+1622_1623insC)는 *O04*형 대립유전자에서 관찰되지 않는 변이이나, 대립유전자 분리 후 염기서열분석에 의해 *O*형 대립유전자 기원임을 확인하였다. 이러한 결과를 바탕으로 저자들은 *O04-variant* 대립유전자를 잠정 결론지었다. 이 외에 저자들이 조사한 다른 영역에서는 주목할 만한 변이를 관찰하지 못하였다. 저자들은 weak-B 표현형의 정확한 유전적 원인을 밝힐 수 없었지만 새로운 *O04-variant* 대립유전자를 검출하였고, ABO 아형에 대한 단계적 알고리즘을 제시하였으며 이러한 접근법은 전장 유전체 분석의 대안이 될 수 있을 것이다.

Conflicts of Interest

None declared.

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