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HISTO-BLOOD GROUP LEWIS GENOTYPING FROM HUMAN HAIRS AND BLOOD

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Summary The expression of histo-blood group Lewis antigens is determined by the Lewis-type $\alpha 1\rightarrow 3/4$fucosyltransferase (Le enzyme) encoded by Fuc-TIII gene (*Le* gene). The genotyping of *Le* genes by the PCR-RFLP methods established recently and partly modified in this study was found to be useful not only for determining the genuine Lewis blood types of samples such as human hairs and blood stains but also for distinguishing non-genuine Lewis-negative phenotypes frequently observed in pregnant women from genuine ones. The availability of the present PCR-RFLP methods for the paternity tests was also discussed.

Key Words Lewis blood group genotyping, PCR-RFLP, non-genuine Lewis-negative phenotypes

INTRODUCTION

The histo-blood group Lewis antigens comprise Le^a and Le^b antigens and their antigenic determinants have been determined to be Gal$\beta 1\rightarrow 3$[Fuc$\alpha 1\rightarrow 4$]-GlcNAcβ and Fuc$\alpha 1\rightarrow 2$Gal$\beta 1\rightarrow 3$[Fuc$\alpha 1\rightarrow 4$]GlcNAcβ, respectively (Watkins, 1980). The Lewis blood group phenotypes have been determined by serological examinations of red blood cells and saliva through hemagglutination tests and hemagglutination inhibition tests using anti-Le^a and anti-Le^b antibodies. They are grouped as Le(a+b−) and Le(a−b+) (Lewis positive) and Le(a−b−) (Lewis negative) individuals. To date a large number of anti-Lewis monoclonal antibodies have been raised and some of them have been commercially available as is usually the case with monoclonal antibodies against most blood group antigens (Oriol *et al.*, 1990; Good *et al.*, 1992). However, the Lewis blood group pheno-

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types have been typed in error due to the specificities of anti-Le^a and anti-Le^b antibodies (Yazawa *et al.*, 1993, 1995). The Lewis blood group phenotypes of red blood cells have been reported to change in pregnancy (Hammer *et al.*, 1981), alcoholic cirrhosis and pancreatitis (Stigendal *et al.*, 1984), hydatid cysts (Makni *et al.*, 1987) and cancers (Ernst *et al.*, 1984; Limas, 1986; Hirano *et al.*, 1987; Yazawa *et al.*, 1988; Ørntoft *et al.*, 1991). The Lewis blood group antigens could also be found in Lewis-negative individuals (Limas, 1986; Ørntoft *et al.*, 1991). In these cases, the genuine Lewis blood types could not be always correctly determined from the serological tests.

Recently, molecular analyses of blood group related genes have been developed (Lutz and Dzik, 1992; Schachter, 1994), and our previous study on the genomic structures of *Le* genes from Lewis-positive and Lewis-negative individuals demonstrated that three missence mutations were present in *le* genes (Nishihara *et al.*, 1994). The *le* alleles were divided into two types, *le1* having the T59 to G and G508 to A mutations, and *le2*, having the T59 to G and T1067 to A mutations (Nishihara *et al.*, 1994) (Fig. 1). In addition, the first mutation (T59 to G) was found to occur unexceptionally in *le* genes and to become a genetic marker to distinguish between Lewis-positive and Lewis-negative individuals (Nishihara *et al.*, 1993, 1994; Yazawa *et al.*, 1995). We have also developed PCR-RFLP methods to detect the aforementioned three missence mutations in *le* genes (Nishi-

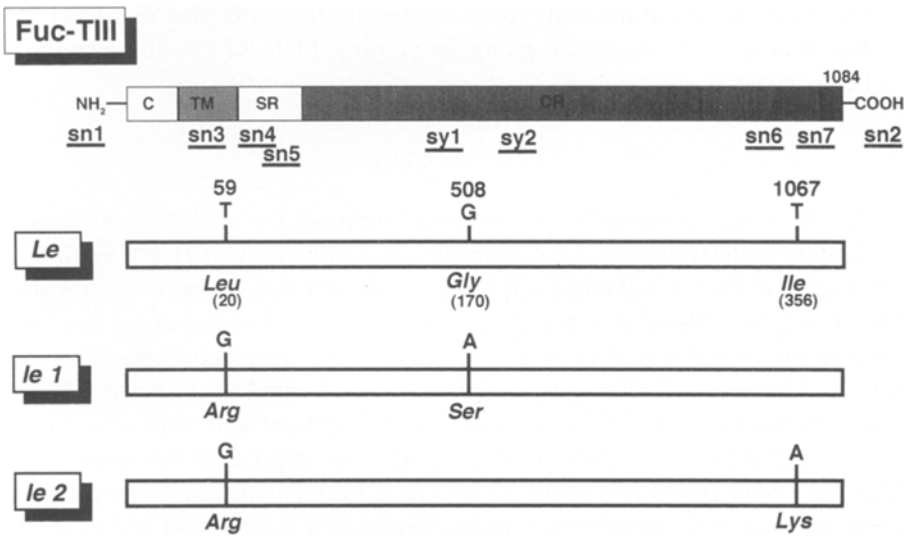

Fig. 1. Schematic comparison of Fuc-TIII nucleotide sequences from *Le* and *le* genes and the location of PCR primers. C, TM, SR and CR indicate cytoplasmic tail, transmembrane domain, stem region and catalytic region, respectively. sn1, sn2, sn3, sn4, sn5, sn6, sn7, sy1 and sy2 indicate location of PCR primers used in this study.

hara *et al.*, 1994). The incompatibility of Lewis phenotypes was observed in patients with cancers (Yazawa *et al.*, 1988), and the presence of Le enzyme activity in saliva from cancer patients seemed to be consistent with that of *Le* gene (Yazawa *et al.*, 1988, 1995). In order to determine the genuine Lewis blood types of such cancer patients, we analyzed their Lewis genotypes, and found that the patients whose salivas had Le enzyme activity possessed at least one *Le* gene even though the Lewis phenotypes of their erythrocytes and salivas were Lewis-negative (Yazawa *et al.*, 1995).

In this study, we determined the Lewis genotypes of samples obtained from human hairs and blood stains with the aid of PCR-RFLP methods previously developed and partly modified in this study. We also analyzed, for the first time, the Lewis genotypes of pregnant women with Lewis-negative erythrocytes and of the individuals in the paternity tests to evaluate the usefulness of the present PCR-RFLP methods for the determination of Lewis genotypes.

MATERIALS AND METHODS

Materials. GDP-L-[^{3}H]fucose (85.1 GBq/mmol) and Aquasol-2 were purchased from Du Pont (Boston, MA, U.S.A.). GDP-fucose was from Sigma (St. Louis, MO, U.S.A.). Sep-Pak plus C_{18} reverse-phase cartridges were from Waters (Milford, CT, U.S.A.). Anti-Le^a (2DG8) and anti-Le^b (TT42) monoclonal antibodies were obtained as described previously (Yazawa *et al.*, 1993). Anti-Le^a (lot AU2-070) and anti-Le^b (lot LAR-1018) monoclonal antibodies for hemagglutination test and hemagglutination inhibition tests were obtained from Chembiomed (Edmonton, Alberta, Canada). *Msp*I, *Pvu*II, *Hin*dIII enzymes, recombinant Taq DNA polymerase and deoxyribonucleoside triphosphates mixture were purchased from Takara (Ohtsu, Japan). Expand Long Template PCR System was from Boehringer Mannheim (Tokyo, Japan). 2′-*O*-Methyllacto-*N*-biose IβBn was prepared as described previously (Yazawa *et al.*, 1990a). Freshly plucked hairs from head were obtained from 12 healthy volunteers with different Lewis blood group phenotypes. Blood and salivas were also obtained from healthy individuals and pregnant women.

Genomic DNA. Genomic DNA was prepared from either peripheral blood leukocytes or human hairs and blood specimens by Proteinase K-SDS method (Sabrock *et al.*, 1992).

Determination of Lewis blood phenotypes and levels of Lewis antigens. Lewis blood phenotypes of samples were determined by a standard test of erythrocytes and saliva, under standard serological techniques (American Association of Blood Banks, 1981). Serum levels of Le^a and Le^b antigens were measured by enzyme-linked immunosorbent assay using anti-Le^a (2DG8) and anti-Le^b (TT42) monoclonal antibodies and expressed in an arbitrary unit (U/ml) by comparison with standard Le^a and Le^b antigens prepared from human meconium (Yazawa *et*

al., 1990b) (details of the method will be published elsewhere).

Assay of Le enzyme activity. The standard reaction mixture for Le enzyme (α1→3/1→4fucosyltransferase) assay contained the following components in a final volume of 100 μl: 25 μM GDP-[^{3}H]fucose, 50 mM Tris-HCl (pH 7.2), 10 mM NaN_3, 10 mM $MnCl_2$, 5 mM ATP, 10 μl saliva and 50 mM 2′-*O*-methyllacto-*N*-biose IβBn. After incubation at 37°C for 5 hr, an equal volume of absolute ethanol was added and the mixture was centrifuged. The enzyme activity of the supernatant was measured by the method described previously using Sep-Pak plus C_{18} (Yazawa *et al.*, 1992).

PCR-RFLP methods. The PCR primers to obtain the full-length open reading frame of the Fuc-TIII gene (Nishihara *et al.*, 1994) and to detect the mutations are listed in Table 1. Genomic DNA (~500 ng) was amplified in 50 μl of solution containing sn1 and sn2 primers (Nishihara *et al.*, 1994) (1 μM), 200 μM each dNTP, 50 mM Tris-HCl (pH 9.2), 14 mM $(NH_4)_2SO_4$, 2.25 mM $MgCl_2$, 2% (v/v) DMSO, 0.1% (v/v) Tween 20 and 2.6 units Taq (Expand Long Template PCR System containing thermostable Taq and Pwo DNA polymerases). Thirty cycles (2 min at 94°C, 2 min at 60°C, 2 min at 72°C) were run to obtain the full-length open reading frame of the Fuc-TIII gene. The product of the first PCR amplifications was subsequently used for the following PCRs as a template to detect the three missence mutations. To detect the T59G mutation, the first PCR product was then amplified in 50 μl of solution containing sn3 and sn4 primers (1 μM), 200 μM each dNTP, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin and 2.5 units of Taq polymerase. The first cycle (3 min at 94°C, 2 min at 62°C, and 3 min at 72°C) was run, then followed by 39 cycles (1 min at 94°C, 1.5 min at 62°C, and 1.5 min at 72°C). To detect the G508A mutation, the first PCR product was amplified in 50 μl of the same solution except sy1 and sy2 primers (1 μM). One cycle (3 min at 93°C) and 30 cycles (1 min at 93°C, 1.5 min at 55°C, 1.5 min at 72°C) and one cycle (5 min at 72°C) were run. To detect the T1067A

Table 1. PCR primers for the amplification of Fuc-TIII genes.

Primer set[1]	Annealing temperature (°C)	Product size (bp)	Sequence
sn1	60	1,615	5′-CTCGAATTCTAAGCAGGAGATTGTCATCAC-TGACC-3′
sn2			5′-CTCAAGCTTCGTGCCGTGATGATCTCTCTG-CAC-3′
sn3	62	93	5′-CCATGGCGCCGCTGTCTGGCCGCCC-3′
sn4			5′-AGTGGCATCGTCTCGGGACACACG-3′
sy1	55	202	5′-GAAGCCCTGGACAGATACTTCA-3′
sy2			5′-GCAGGCTCTGGTAGTAGCGCA-3′
sn6	60	109	5′-CGCTCCTTCAGCTGGGCACTGGA-3′
sn7			5′-CGGCCTCTCAGGTGAACCAAGAAGCT-3′

[1] Primers except sy1 and sy2 were shown in J Biol Chem **269**: 29271-29278, 1994.

mutation, the first PCR product was amplified in 50 μl of the same solution except sn6 and sn7 (1 μM). Thirty cycles (1 min at 94°C, 2 min at 60°C, 2 min at 72°C) were run. The second PCR products for detection of T59G, G508A and T1067A mutations were digested by *Msp*I, *Pvu*II and *Hin*dIII, respectively, and then analyzed by 1.5% Synergel (BioCraft, Tokyo, Japan)+0.7% agarose gel electrophoresis.

RESULTS AND DISCUSSION

The Fuc-TIII gene is a member of the α1→3fucosyltransferase gene family and determines the expression of Lewis antigens (Kukowska-Latallo *et al.*, 1990). The genomic structure analyses of Fuc-TIII genes from Le-negative individuals in our previous studies (Nishihara *et al.*, 1993, 1994) demonstrated that the three missense mutations, *i.e.*, the T59G, the G508A and T1067A were present in *le* genes. To detect these three mutations found in *le* genes, PCR-RFLP methods were also developed (Nishihara *et al.*, 1994) and the usefulness of the methods was demonstrated when the genuine Lewis blood types were determined in cancer patients whose salivas contained Le enzyme even though they were phenotyped as Lewis-negative from their erythrocytes (Yazawa *et al.*, 1995).

While the methods for Lewis genotyping previously reported were simple, it happened occasionally that the PCR amplification using sn5 and sn2 primers (Nishihara *et al.*, 1994; Yazawa *et al.*, 1995) was incomplete to obtain the 1.4-kbp fragment of Fuc-TIII which was necessary for the analyses of both G508A and T1067A mutations (Nishihara *et al.*, 1994; Yazawa *et al.*, 1995). Therefore, we modified the previously reported methods as follows. In order to obtain the adequate amount of the template for second PCRs encompassing positions 59, 508 and 1067, we used the expanded long template PCR system with sn1 and sn2 primers. Using the first PCR products as the template, the 93-bp PCR fragment was amplified with sn3 and sn4 primers as described in "MATERIALS AND METHODS." As demonstrated previously (Nishihara *et al.*, 1994), both the artificial change at position 57 of Fuc-TIII sequence from A to C with sn3 primer and T59G mutation created an *Msp*I site, CCGG. The 93-bp PCR fragment was then cleaved into two fragments, 68 and 25 bp (Fig. 2, upper panel). For the detection of the G508A mutation, we synthesized a new primer set, sy1 and sy2 (Table 1). The sy1 primer had the sense nucleotide sequence corresponding to the sequence from position 439 to 460 of the Fuc-TIII gene. On the contrary, the sy2 primer had the antisense nucleotide sequence corresponding to the sequence from position 592 to 602. The change at position 508 created a *Pvu*II site, CAGCTG and the 202-bp fragment was then cleaved into two fragments, 131 and 71 bp (Fig. 2, middle panel). The sn7 primer had the antisense nucleotide sequence to the sequence from positions 1067 to 1093 of the Fuc-TIII gene with the mismatched position 1071 and 1072. The amplified 109-bp PCR fragment of the template possessing the T1067A mutation

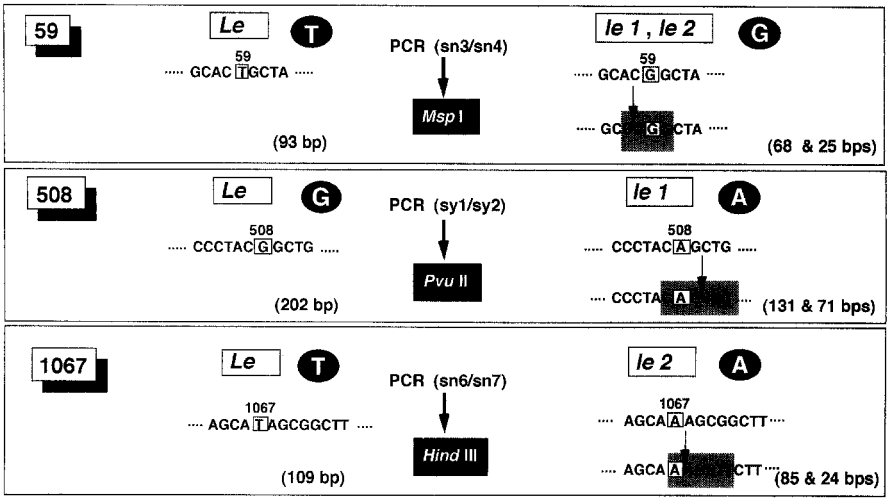

Fig. 2. Schematic illustration of PCR-RFLP methods detecting three missence mutations found in *le* genes. Shaded area shows new sites created for the action of the restriction enzymes.

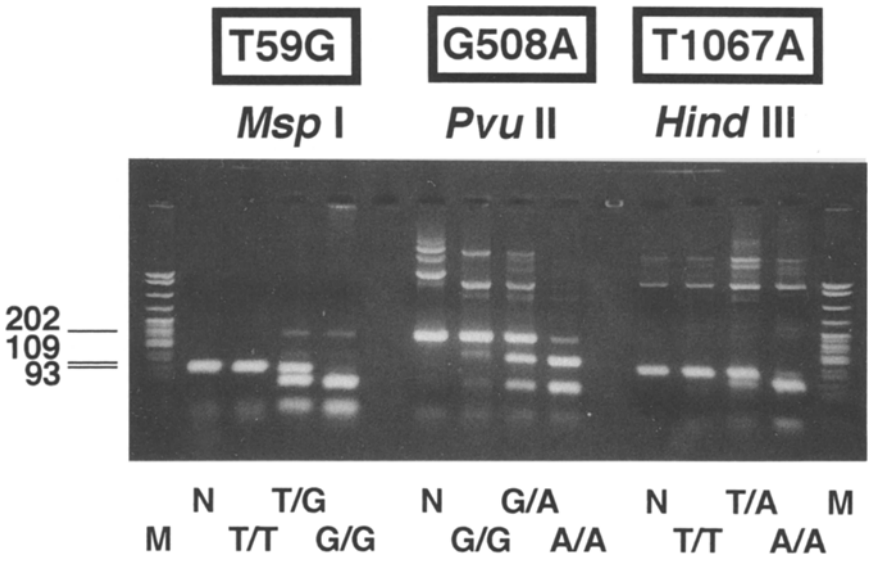

Fig. 3. PCR-RFLPs for detection of three single base substitutions found in Lewis-negative Fuc-TIII genes. The PCR-amplified DNAs of each genotype were tested with restriction enzyme and analyzed by a mixture of 1.5% Synergel and 0.7% agarose electrophoresis. The sizes were determined according to pBR322 DNA-*Msp*I digest (M) run in parallel. N, no digestion.

was then cleaved into two fragments, 85 and 24 bp by *Hin*dIII (Fig. 2, lower panel). The standard PCR-RFLPs to show individual genotypes were prepared using genomic DNAs obtained from peripheral blood leukocytes (Fig. 3). After Lewis genotyping of more than 100 samples, two individuals having homozygous T1067A missense mutation were found, for the first time, and their genotypes were assigned to *le2/le2* (described below).

Twelve saliva samples comprising of 4 Le(a−b+), 4 Le(a+b−) and 4 Le(a−b−) individuals were examined serologically with anti-Le^a and anti-Le^b antibodies. The activity of α1→4fucosyltransferase (Le enzyme) was also examined with saliva samples at the same time (Table 2). The genomic DNAs of these 12 individuals were prepared from their hairs and PCR-RFLPs to detect three point mutations in the *Le* genes were carried out as described above (Fig. 4) and their Lewis genotypes were determined as summarized in the right column of Table 2. The Lewis-positive individuals whose saliva had Le enzyme activity and Le^a and/or Le^b antigen were found to possess at least one *Le* allele, and their genotypes were *Le/Le*, *Le/le1* or *Le/le2*. On the other hand, the Lewis-negative individuals whose saliva didn't contain Lewis antigen nor Le enzyme activity were found to possess *le* genes homozygously and their genotypes were *le1/le1*, *le1/le2* or *le2/le2* (Table 2). It was therefore demonstrated that the presence of Lewis antigen and Le enzyme activity was completely consistent with that of the *Le* gene. Furthermore, the first mutation in the *Le* gene (T59 to G) was observed unexceptionally in *le* genes and then seemed to become a genetic marker to distinguish between Lewis-positive and Lewis-negative individuals. No individual has been observed who

Table 2. Salivary α1→4fucosyltransferase activity and nucleic acid polymorphisms of *Le* genes from individuals with blood group Lewis-positive and -negative phenotypes.

No.	Donor	Blood type	HAI[1] titer of saliva		α1→4FT[2] activity (pmol/ml/hr)	PCR-RFLP, position			Geno-type
			anti-Le^a	anti-Le^b		59	508	1067	
Lewis-positive									
1.	M.S.	Le(a−b+)	16	128	52.33	T,T	G,G	T,T	*Le/Le*
2.	M.M.	Le(a−b+)	4	128	44.35	T,G	G,G	T,A	*Le/le2*
3.	A.N.	Le(a−b+)	4	64	29.15	T,T	G,G	T,T	*Le/Le*
4.	H.O.	Le(a−b+)	32	512	8.98	T,T	G,G	T,T	*Le/Le*
5.	Y.N.	Le(a+b−)	128	8	12.80	T,G	G,A	T,T	*Le/le1*
6.	A.M.	Le(a+b−)	128	4	92.69	T,T	G,G	T,T	*Le/Le*
7.	K.K.	Le(a+b−)	512	4	29.21	T,G	G,A	T,T	*Le/le1*
8.	H.M.	Le(a+b−)	128	8	16.05	T,G	G,G	T,A	*Le/le2*
Lewis-negative									
9.	N.F.	Le(a−b−)	2	2	0	G,G	A,A	T,T	*le1/le1*
10.	K.N.	Le(a−b−)	<2	<2	0	G,G	A,A	T,T	*le1/le1*
11.	A.T.	Le(a−b−)	<2	4	0	G,G	G,A	T,A	*le1/le2*
12.	T.T.	Le(a−b−)	<2	<2	0	G,G	G,G	A,A	*le2/le2*

[1] HAI, hemagglutination inhibition; [2] FT, fucosyltransferase.

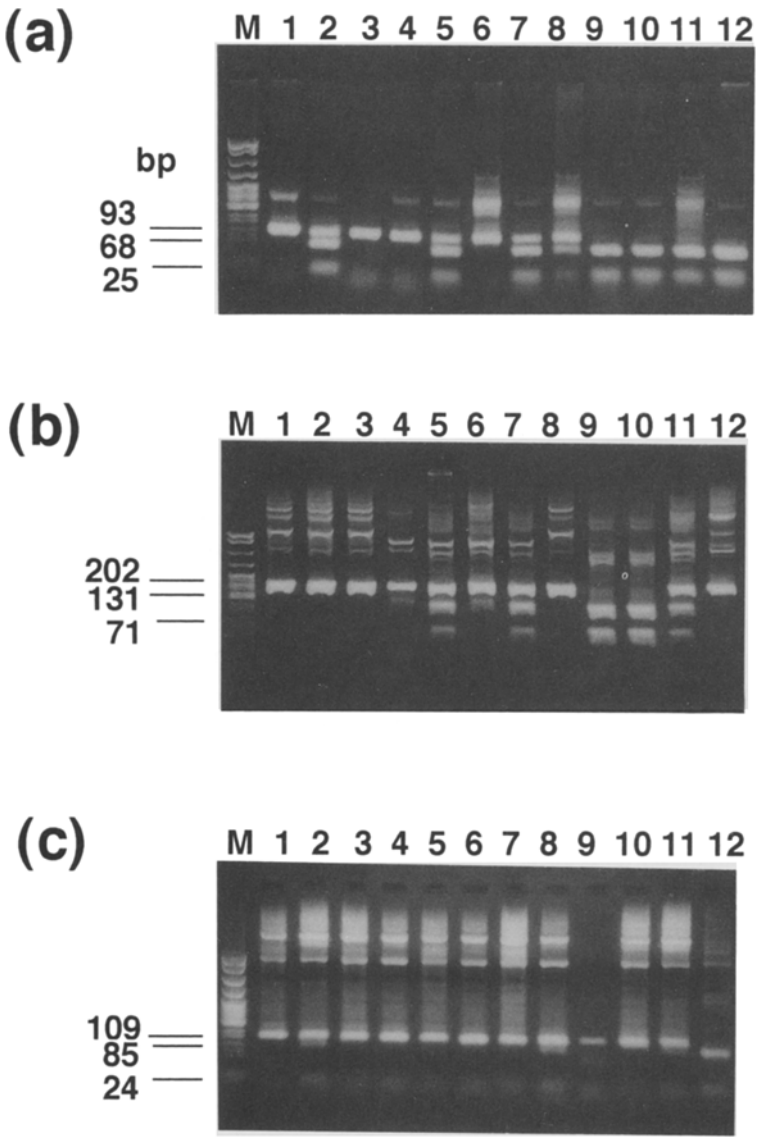

Fig. 4. PCR-RFLPs of *Le*, *le1* and *le2* genes. PCR-RFLP for detection of the T59G mutation (a), the G508A mutation (b) and T1067A (c) after cleavage of *Msp*I, *Pvu*II and *Hin*dIII restriction enzymes, respectively. Gel electrophoresis was done as in Fig. 3. Lane numbers correspond to samples described in Table 2.

had only the T59G mutation without the G508A or the T1067A mutation after the Lewis genotyping of more than 200 specimens (data not shown).

Twelve of the 50 pregnant women showed Lewis-negative erythrocytes. Seven

women among them showed the presence of Le^a and/or Le^b antigen in their plasma inconsistently (Table 3). The Lewis blood phenotypes have been reported to change from Lewis-positive to Lewis-negative during pregnancy (Hammer *et al.*, 1981). It was then supposed that the seven women with Lewis-negative phenotypes whose plasma contained Lewis antigens were Lewis-positive, *i.e.*, Le(a+b−) or Le(a−b+).

The Lewis genotyping of these 12 women to detect T59G mutation demonstrated that seven women who had Le^a and/or Le^b antigen in plasma possessed at least one *Le* allele (Table 3). Two women (M.W. and M.Y.) whose saliva were available were found to have Le^a antigen and Le^b antigen, respectively and Le enzyme activity (data not shown). Recently, we determined the Lewis genotypes of Lewis-negative patients with cancers whose erythrocytes and saliva were found to show inconsistent Lewis phenotypes. The genotyping of their *Le* genes demonstrated that some of them were non-genuine Lewis-negative and that patients who had Le enzyme activity were genuine Lewis-positive irrespective of their Lewis phenotypes (Yazawa *et al.*, 1995). These results showed that the Lewis phenotypes from patients with cancer and pregnant women could not provide accurate information regarding the genuine Lewis blood types. However, it was clear that the single PCR-RFLP experiment to detect T59G mutation could be helpful for the determination of the genuine Lewis blood types and, in particular, for distinguishing Lewis-negative individuals from Lewis-positive ones correctly in such cases. More recently, molecular genetic analysis of *Se* and *se* genes demonstrated the presence of *Se* gene (FUT2 gene) encoding $\alpha 1 \rightarrow 2$fucosyltransferase which relates to the synthesis of $\alpha 1 \rightarrow 2$fucosyl residue of H type 1 and Le^b antigens in the secretor system (Rouquier *et al.*, 1995; Kelly *et al.*, 1995). It must be possible to determine Le(a−b+) and Le(a+b−) types from sequential genotypings of both the *Le* and

Table 3. Levels of plasma Lewis antigens, and their Lewis genotypes in pregnant women with Lewis-negative erythrocytes.

Specimen	Lewis phenotype	Lewis antigen (U/ml)		Nucleotides of Fuc-TIII position 59	Probable Lewis genotype
		Le^a	Le^b		
M.W.	Le(a−b−)	54.37	2.00	T,T	*Le/Le*
M.Y.	Le(a−b−)	n.d.	28.51	T,T	*Le/Le*
E.M.	Le(a−b−)	48.01	91.70	T,G	*Le/le*
O.M.	Le(a−b−)	37.81	5.22	T,G	*Le/le*
Y.Y.	Le(a−b−)	254.81	8.08	T,T	*Le/Le*
I.M.	Le(a−b−)	88.66	25.53	T,T	*Le/Le*
K.M.	Le(a−b−)	39.24	1.59	T,G	*Le/le*
M.S.	Le(a−b−)	3.41	2.01	G,G	*le/le*
S.T.	Le(a−b−)	3.83	3.88	G,G	*le/le*
W.N.	Le(a−b−)	3.12	5.82	G,G	*le/le*
F.U.	Le(a−b−)	1.42	6.14	G,G	*le/le*
Y.K.	Le(a−b−)	3.41	2.01	G,G	*le/le*

Se genes which encodes the Lewis-dependent α1→4fucosyltransferase demonstrated by us and the secretor-dependent α1→2fucosyltransferase, respectively. The genotyping of both *Le* and *Se* genes, therefore seems to be indispensable to determine genuine Le(a−b−), Le(a+b−) and Le(a−b+) types.

To evaluate the usefulness of the present Lewis genotyping for the paternity test, three cases under examination were picked up and Lewis genotyping of individuals was carried out using their blood stains (Fig. 5). In case 1, a daughter (A.S.) whose Lewis phenotype was Le(a+b−) possessed *le1* and *Le* genes. However, neither the putative father (N.K.) nor the mother (M.S.) possessed *le1* gene and they were determined to be homozygote of *Le* genes. Although the Lewis phenotypes of the individuals in this case did not contribute to the test, it was clear that the putative father could be excluded from the candidate of her father from their Lewis genotypings. In cases 2 and 3, each daughter possessed *le1* (N.G.) and *le2* (N.K.) genes, respectively, which probably came from the putative fathers but not from their mothers whose Lewis genotypes were determined to be homozygote of *Le* genes. In these cases, the putative fathers could not be excluded from the candidate. These results demonstrated that *le1*, *le2* as well as *Le* genes determined by Lewis genotyping could be useful for the paternity tests and in some cases the Lewis genotyping could provide a conclusive result (as in case 1) which is hard to be obtained from the Lewis phenotyping.

While the conventional determination of Lewis blood types has been carried out mainly by serological examinations using anti-Le^a and anti-Le^b antibodies, there are problems for Lewis phenotyping with antibodies, in particular, in case when fresh and adequate samples are not available, and it is impossible to determine genuine Lewis blood types of the aforementioned individuals by the conventional analyses.

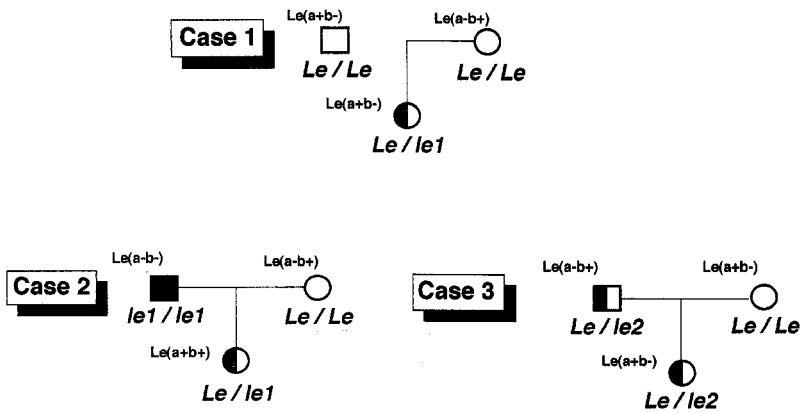

Fig. 5. Lewis genotyping of three cases of paternity tests. A slash separates two alleles. Filled, half-filled and open symbols indicate *le/le*, *Le/le* and *Le/Le*, respectively.

In conclusion, we demonstrated not only the availability of the newly developed PCR-RFLP methods for Lewis genotypings but also the usefulness of the genotyping of *Le* genes whose genuine Lewis blood group types were hard to be determined with the conventional methods.

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