Yuhua Liu,[1] *M.D., Ph.D.; Yoshiro Koda,*[1] *M.D., Ph.D.; Mikiko Soejima,*[1] *B.S.; Naomi Uchida,*[1] *M.D.; and Hiroshi Kimura,*[1] *M.D., Ph.D.*

PCR Analysis of Lewis-Negative Gene Mutations and the Distribution of Lewis Alleles in a Japanese Population

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ABSTRACT: Three mutations in the Lewis-negative gene, T59G, G508A and T1067A, have been detected by means of a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) in 149 unrelated Japanese individuals. We found three common Lewis alleles—*Le* (without the T59G, G508A, and T1067A mutations), *le1* (with the T59G and G508A mutations), and *le2* (with the T59G and T1067A mutations) in a Japanese population. In addition, we also found one rare Lewis-negative allele, *le3* (only with the T1067A mutation). The allele frequencies of *Le, le1, le2,* and *le3* were 0.607, 0.275, 0.114, and 0.003, respectively. Our results were in accordance with those expected by the Hardy-Weinberg equilibrium. Some statistical parameters of forensic interest were also calculated.

KEYWORDS: forensic science, polymerase chain reaction, Lewis-negative gene, population study, DNA polymorphism, FUT3, Japan

The Lewis blood group system has been of interest for a long time because the system is closely related to the ABO blood group system (1). Recent advance in Lewis-related antigens (Le^x, Le^y, sialyl Le^a, sialyl Le^x, etc.) has led to further interest in the regulation of the expression of the Lewis enzyme. The Lewis gene encodes the α1–3/4fucosyltransferase, which catalyzes the formation of Le^a and Le^b antigens. The formation of Le^b requires the presence of α1–2fucosyltransferase in addition to the Lewis enzyme. As a result, we have the three main phenotypes of the Lewis system, Le(a−b+), Le(a+b−), and Le(a−b−), a fourth phenotype Le(a+b+) has been found in Orientals (2–4), which are generally determined by a hemagglutination test. However, the Lewis phenotypes can not be directly genetically predetermined, as are all the other known human red cell antigens (1). It is well known that some complexities are present in the Lewis system because the Lewis antigens of erythrocytes are passively acquired from plasma (5). However, the red blood cells of neonates, of some pregnant women and of some cancer patients are known to give the reaction Le(a−b−) despite a presence of the Lewis antigens in the plasma (6–8). Further complexities have been reported recently involving the presence of Lewis antigens in plasma, small intestine, saliva, urothelium, and in various cancer tissues of individuals typed as Lewis-negative in hemagglutination assays (6–12). These complexities of the Lewis system have caused difficulties in phenotyping red blood cells because of weak hemagglutination and the specificity of anti-Le^a and -Le^b antibodies. Recent reports demonstrated that monoclonal anticarbohydrate antibodies which appear highly specific always have some degree of cross-reactivity with related structures (13,14). Almost all anti-Le^b antibodies are known to react with H type 1 (anti-Le^{bH}). Therefore, it is important to know the genotype of the Lewis system to resolve the inconsistency of the presence of Lewis antigens in some tissues of individuals phenotyped as Lewis-negative.

Lewis gene-encoded α(1–3/4)fucosyltransferase cDNA (*FUT3*) has been isolated by Kukowska-Latallo et al. (15). The DNA sequence analyses of *FUT3* alleles from the Lewis-negative individuals have revealed point mutations that lead to a loss of enzyme activity in transfected cells. We have found the T59G (Leu^{20} to Arg) and the G508A (Gly^{170} to Ser) mutations in a Japanese population (16), and the T1067A (Ile^{356} to Lys) mutation has been reported in an Indonesian (17) and in a Japanese population (18). Previously, we established the methods to detect the two missense mutations of T59G and G508A by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (19,20). In this study, we investigated the Lewis phenotypes and genotypes in a Japanese population by the PCR-RFLP method and found that one individual typed as Lewis-negative by a hemagglutination test was determined to be Lewis-positive from his genotype. We also calculated some parameters of forensic interest, such as the power of discrimination and the chance of exclusion in paternity analysis.

Materials and Methods

Typing of Lewis phenotypes: The 149 unrelated blood samples were supplied by the Japan Red Cross Blood Center in Fukuoka. Monoclonal anti-Le^a and anti-Le^b antibodies were obtained from Kokusai Reagents, Kobe, Japan. The Lewis phenotypings by a hemagglutination reaction were carried out after digestion of red blood cells by bromelin (Wako Chemicals, Osaka, Japan).

DNA samples: Genomic DNA was extracted from peripheral leukocytes with Genomix B kit (Talent, Trieste, Italy) according to the kit's instruction. DNA concentration was estimated by spectrophotometry.

PCR amplification and analysis of the mutations: PCR amplification was performed as previously described (18–20). Briefly, the PCR was carried out in a reaction mixture of 25–50 μL containing 0.1–0.5 μg genomic DNA, 25 mol of each pair of primers, 200 μM of dNTPs and 1.2 U of *Taq* DNA polymerase (Toyobo, Osaka,

[1]Visiting assistant professor, assistant professor, lecturer, postgraduate student, and professor, respectively, Department of Legal Medicine, Kurume University School of Medicine, Kurume, Fukuoka 830, Japan.

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Japan). The PCR condition and primer sequences for the detection of these mutations and for DNA sequence analysis are listed in Table 1. The PCR primers were designed to amplify only the Lewis gene but not to amplify other homologous genes for α1–3fucosyltransferases (19). For detection of the T59G mutation, resulting PCR products (120 bp) were digested by 12 U *Msp*I endonuclease for 2 h, and then were separated on an 8% polyacrylamide gel. PCR products from alleles containing the T59G mutation were cleaved into 97 and 23 bp fragments by *Msp*I digestion (20). For the G508A mutation, the PCR products (273 bp) were cleaved with *Pvu*II enzyme, and subjected to separation through 2% agarose gel by electrophoresis. The digestion by *Pvu*II of PCR products from alleles containing the G508A mutation produced 169 and 104 bp fragments (19). For the T1067A mutation, the PCR products with the No. 5 and No. 6 primers (Table 1) were used as the template for the second PCR amplification by a pair of 1067 primers (No. 7 and No. 8) as previously described (18). The second PCR products (109 bp) were digested by *Hind*III endonuclease to cleave into 85 and 24 bp fragments from PCR products containing the T1067A mutation, and the mutations were analyzed on 2% agarose or 8% polyacrilamide gel by electrophoresis.

For analysis of the DNA sequence of the Lewis gene, PCR products with the No. 9 and No. 6 primers (Table 1) were digested by *Hind*III and *Xba*I and then ligated into pRc/CMV (Invitrogen, San Diego, CA). The DNA sequence was determined by the didoxynucleotide chain termination method using an AutoRead DNA Sequencing kit and an ALF DNA sequencer (Pharmacia, Uppsala, Sweden).

Results

The phenotype of erythrocytes and the genotype of the Lewis gene from 149 unrelated Japanese individuals were examined by an hemagglutination test and PCR-RFLP, respectively. The PCR products for detection of the T59G, G508A, and T1067A mutations were digested with *Msp*I, *Pvu*II, and *Hind*III, respectively, and analyzed by gel electrophoresis. Figure 1 shows the electrophoresis patterns of these three mutations for the homozygous mutation and for the heterozygous mutation of each allele. Recent study indicated that only two Lewis-negative alleles (*le,*) *le1* and *le2,* were found from Lewis-negative individuals in a Japanese population (16,18). The *le1* allele had two missense mutations—T59G and G508A. The *le2* also had two mutations—T59G and T1067A. In our present study, 23 of 24 Lewis-negative individuals were homozygous for the T59G mutation. Fifteen of them were homozygous for the G508A mutation, possessing the *le1/le1* genotype. Five of them were heterozygous for the G508A and T1067A, and the genotype was assigned to *le1/le2.* Three of them were homozygous for the T1067A, possessing the genotype *le2/le2.* Only one individual (No. 129) with a Lewis-negative phenotype on the erythrocytes lacked the T59G mutation in double dose. To examine haplotypes of the Lewis gene, PCR products of the Lewis gene from this individual (No. 129) were ligated into pRc/CMV. As judged by PCR analysis for detection of T59G and T1067A mutations, some clones contained both T59G and T1067A mutations, and the other clones contained neither T59G nor T1067A mutation. In addition, we did not find any mutation in the clones without the T59G and T1067A mutations by DNA sequencing.

These results indicated that No. 129 individual had *Le/le2* genotype, and we had mistyped this individual despite careful determination of the Lewis phenotype. Previous results in our laboratory indicated that 5 of 12 individuals with the Lewis-negative phenotype on their erythrocytes did not have the T59G mutation in the Lewis gene in double dose (20). In addition, these five individuals had the *Le/le1* genotype, which was revealed by PCR and DNA sequence analyses as mentioned above. These results further suggested that these five individuals were nongenuine Lewis-negative individuals (10).

Fifty-five of 125 Lewis-positive individuals were *Le/Le* genotype. The other 70 possessed a heterozygous genotype with *Le/le1* or *Le/le2.* Only one Lewis-positive individual had a heterozygous genotype with *Le* and *le* which contained the T1067A mutation, but not the T59G mutation. The genotype was designated as *Le/le3.* Recently, Mollicone et al. (17) have reported the presence of *le3* in some Indonesian pedigrees, which was defined as L2. Our present results indicated that *le3* was a rare allele of the *le* genes in this Japanese population. Among the 117 *le* alleles studied., there were 82 (70.1%) *le1* and 34 (29.1%) *le2* alleles.

The frequencies of alleles and genotypes are listed in Table 2. The frequencies of *Le, le1, le2,* and *le3* alleles in the Japanese

TABLE 1—*Primers and the conditions for PCR amplification of Lewis genes.*

Primers	Sequences	Mutations	Conditions		
			Temperature (°C)	Time (min.)	Cycles
No. 1	5′-ATGGCGCCGCTGTCTGGCCGCCC	T59G	94	1	30
No. 2	5′-GAGGACCCACTGGGAGCCCT		65	0.5	
			72	0.5	
No. 3	5′-ATGTCCAACCCTAAGTCACG	G508A	94	1	35
No. 4	5′TGAGTCCGGCTTCCAGTTGGA		58	1	
			72	1	
No. 5	5′-ATGATGGAGACGCTGTCCCGGTACAAGTT	(First PCR) T1067A	94	1	30
No. 6	5′-CTCTCTAGACGTGCCGTGATGATCTCTCTGCAC *Xba*I		58	1	
			72	1	
No. 7	5′-CGCTCCTTCAGCTGGGCACTGGA	Second PCR) T1067A	94	1	25
No. 8	5′-CGGCCTCTCAGGTGAACCAAGAAGCT		60	0.5	
			72	0.5	
No. 9	5′-CTCAAGCTTAAGCAGGAGATTGTCATCACTGACC *Hind*III	(For DNA sequencing)	94	1	35
			60	1	
			72	2	

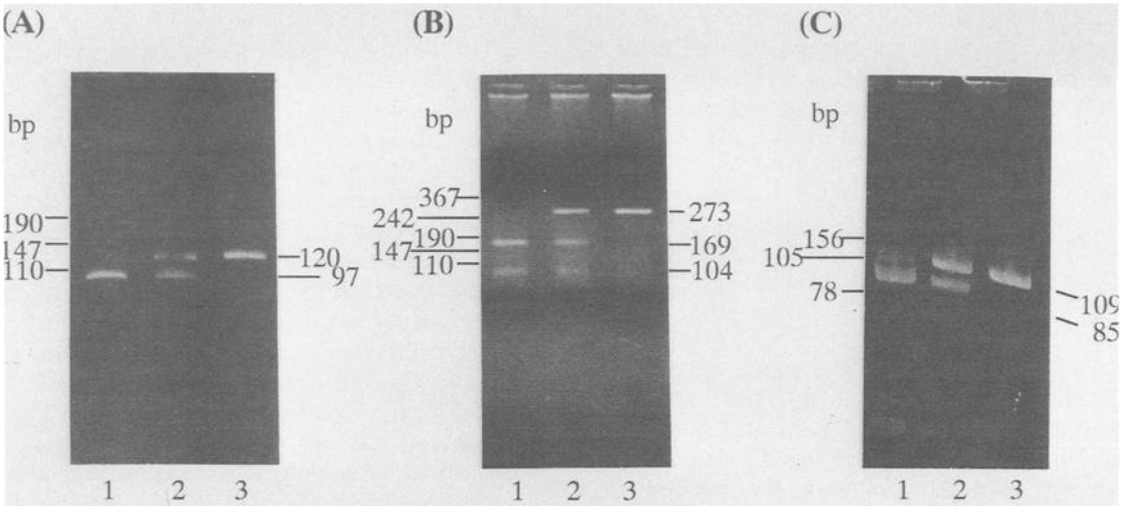

FIG. 1—*Restriction endonuclease digestion of the Lewis allele PCR products. (A) PCR for detection of the T59G mutation. The fragment amplified was digested by* MspI *(12U) for 2 h followed by analysis on 8% polyacrylamide gel. (B) PCR for detection of the G508A mutation. The fragment amplified was cleaved by* PvuII *enzyme and separated on 2% agarose gel. (C) PCR for detection of the T1067A mutation. The fragment amplified was digested by* HindIII *for 2 h, then subjected to be separated on 8% polyacrylamide gel. Lane 1, homozygote for Lewis-negative gene; lane 2, heterozygote with Lewis-positive and -negative genes; lane 3, homozygote for Lewis-positive gene. The expected DNA fragments are indicated on the right of each figure.*

TABLE 2—*The distribution of the Lewis genotypes and allele frequencies in a Japanese population.*

Genotypes	Observed		Expected		χ^2*	Allele frequencies
	Number	%	Number	%		
Le/Le	55	36.9	55.0	36.9	0.0000	*Le* = 0.607
Le/le1	47	31.5	49.8	33.4	0.1574	*le1* = 0.275
Le/le2	23	15.4	20.7	13.9	0.2256	*le2* = 0.114
Le/le3	1	0.7	0.6	0.4	0.2667	*le3* = 0.003
le1/le1	15	10.1	11.5	7.6	1.0652	
le1/le2	5	3.4	9.4	6.3	2.0596	
le2/le2	3	2.0	1.9	1.3	0.6368	

*χ^2 = 6.7417; df = 6; $0.2 < p < 0.5$.

population we studied were 0.607, 0.275, 0.114, and 0.003, respectively. The distribution of alleles in the Japanese population was in good agreement with that suggested by the Hardy-Weinberg equilibrium (χ^2 = 6.74, df = 6, $0.2 < p < 0.5$). The power of discrimination (PD) was 0.728 and the exclusion of chance was 0.278. Comparing our results with those obtained in another laboratory (18), no significant difference was observed in allele frequencies between a southern Japanese population and a central Japanese population (χ^2 = 8.44, df = 6, $0.2 < p < 0.5$).

Discussion

The mechanism of the adsorption of Lewis antigens on red cells from plasma is unclear. Therefore, typing of the Lewis blood group by a hemagglutination assay is difficult in some cases. Hammer et al. (7) found that Le(a−b+) women became Le(a−b−) during pregnancy, and suggested that the increased ratio of lipoprotein to red cell mass disturbed the repartition of Lewis fucolipids to red blood cells. Moreover, Le(a−b+) has sometimes been mistyped as Le(a−b−) by the conventional hemagglutination tests due to poor antisera. In the present study, we have examined the Lewis phenotypes and genotypes of 149 unrelated individuals, and have mistyped one Le(a−b+) as Le(a−b−) as judged from the genotype which was determined by DNA sequence analysis. Eleven individuals with weak hemagglutination against anti-Le^b were observed and found to be heterozygous for *Le/le* except for two who were homozygous for *Le*.

Among 149 genotypes, we found 55 homozygous for *Le*, 71 heterozygous for *Le/le*, and 23 homozygous for *le* (Table 2). Our previous studies demonstrated that transfection of the gene with only the T59G mutation expressed Lewis antigens on the surface of COS cells (16,18). However, all the *le* genes except one gene had the T59G mutation with either the G508A mutation (*le*1) or the T1067A mutation (*le*2). A role for the T59G mutation on the *le* genes is not known at present. We found only one *le* gene with the T1067A but without the T59G (*le*3) in the Japanese population studied. However, Mollicone et al. (17) reported a few *le* genes without the T59G mutation in an Indonesian population. They also reported some *le* genes without the T1067A but with the T59G, although they did not mention the presence of the G508A mutation in their *le* genes. Elmgren et al. (21) have reported the homozygous C314T (Thr^{105} to Met) mutation in 5 of 18 Swedish individuals with Lewis-negative phenotype on their erythrocytes. However, we and another laboratory in Japan (15,17) did not find the C314T mutation in the *le* genes among our Japanese population.

Hirano et al. (8) reported a high frequency of Le(a−b−) on the erythrocytes of patients with pancreatic carcinoma, colorectal cancer, and bile duct carcinoma, who also had a high serum CA19-9 level. They determined the Lewis phenotype in serum using a

dot-immunobinding assay and found that most patients with a Lewis-negative phenotype on their red cells were Lewis-positive in serum. They also demonstrated negative immunohistochemical staining for Le^a antigen and for CA19-9 in both noncancerous and cancerous pancreatic tissue in a patient who had Le-negative phenotypes in both erythrocytes and serum. Yazawa et al. (22) also reported a similar result by determination of α1–4fucosyltransferase activity in saliva samples from cancer patients and recently by genotyping of the Lewis gene from cancer patients (23).

Although the Lewis blood group system has been of interest for a long time, it has not been used as one of genetic markers in forensic practice because of some complexities and difficulties in phenotyping of the Lewis system. Thus, determination of the genotype of the Lewis gene will be required for better understanding the presence of Lewis antigens in some tissues of individuals typed as Le-negative in both red cells and saliva, and will be useful for personal identification, as well as for paternity testing in forensic field.

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Address requests for reprints and additional information to
Hiroshi Kimura, M.D., Ph.D.
Department of Legal Medicine
Kurume University School of Medicine
Kurume, Fukuoka 830, Japan