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Abstract

A method of genotyping IgA2 alleles in the human immunoglobulin alpha 2 heavy chain constant region (C alpha 2 gene) was developed by using the polymerase chain reaction (PCR). By this method, the genotype was determined by discriminating base substitution in the 3'-flanking region of alleles, A2m*1 and A2m*2, which manifest A2m serum types, by nested PCR using allele-specific primers. Three types, IgA2*1/IgA2*1, IgA2*2/IgA2*1, and IgA2*2/IgA2*2, were detected from DNA extracted from lymphocytes. Genotyping was possible from 100 pg of DNA by this method. The estimated allele frequency in 318 Japanese subjects was 0.561 for IgA2*1 and 0.439 for IgA2*2. Analysis of 29 cases of paternity tests suggested that the data follow Mendel's law of inheritance. This genotype could also be detected in whole blood, blood stains, saliva stains, and various organs and tissues. These results suggest the usefulness of the present method for paternity testing and individual identification in forensic medicine.

KEYWORDS: polymorphism, deoxryibonucleic acid(DNA), immunoglobulin alpha 2, polymerase chain reaction(PCR), allele-specific amplificartion

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IgA2 Genotyping by Polymerase Chain Reaction (PCR) Using Allele-Specific Amplification Primers

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A method of genotyping IgA2 alleles in the human immunoglobulin alpha 2 heavy chain constant region (Cα2 gene) was developed by using the polymerase chain reaction (PCR). By this method, the genotype was determined by discriminating base substitution in the 3'-flanking region of alleles, A2m*1 and A2m*2, which manifest A2m serum types, by nested PCR using allele-specific primers. Three types, IgA2*1/IgA2*1, IgA2*2/IgA2*1, and IgA2*2/IgA2*2, were detected from DNA extracted from lymphocytes. Genotyping was possible from 100 pg of DNA by this method. The estimated allele frequency in 318 Japanese subjects was 0.561 for IgA2*1 and 0.439 for IgA2*2. Analysis of 29 cases of paternity tests suggested that the data follow Mendel's law of inheritance. This genotype could also be detected in whole blood, blood stains, saliva stains, and various organs and tissues. These results suggest the usefulness of the present method for paternity testing and individual identification in forensic medicine.

Key words: polymorphism, deoxyribonucleic acid (DNA), immunoglobulin alpha 2, polymerase chain reaction (PCR), allele-specific amplification

Immunoglobulin A (IgA) is divided into two subclasses, IgA1 and IgA2. Subclass IgA2 has genetic polymorphism called alpha 2 marker (A2m). Vyas and Fudenberg (1) bound myeloma IgA protein to erythrocytes by the chromium chloride method (2) and performed hemagglutination inhibition using the erythrocytes as the indicator. They showed that the serum anti-IgA antibody of a patient who showed anaphylactic symptoms after blood transfusion contained the anti-IgA allotype antibody (1). On the other hand, Kunkel *et al.* (3) discovered an allotype of IgA2 by precipitin reaction. Thereafter, these two allotypes were revealed to be the same, and are presently referred to as A2m(1). van Loghem *et al.* (4) found that the serum anti-IgA of a patient with IgA deficiency, who had received administrations of immunoglobulin, reacted with the second allotype A2m(2).

Phenotypes of A2m are divided into 3 types, A2m(1), A2m(1,2) and A2m(2). The majority of Caucasians have A2m(1) (4-12). In Japanese (13) and Chinese (4, 14-16), there is only a slight difference between the distribution of A2m(1) and A2m(2). A2m is considered a useful genetic marker for paternity testing and individual identification in these Mongolians.

Phenotypes of serum A2m are controlled by two types of alleles A2m*1 (IgA2*1) and A2m*2 (IgA2*2) of the Cα2 gene which is present in the Igα gene locus of chromosome 14 and codes for an amino acid sequence in the immunoglobulin α2 chain constant region. In the Cα2 chain amino acid sequence, the CH3 domain including Glu428, Ile458 and Ala467 is involved in A2m(2) specificity and the CH1 domain is involved in non-A2m(2) specificity. The S-S bond is necessary for expression of non-A2m(2) specificity (17). Base substitution is observed in these sites involved in serological specificity in two types of alleles of the Cα2 gene. The nucleotide sequence of these two types of alleles has been well presented (18).

We performed the nested polymerase chain reaction (PCR), in which each allele is amplified by PCR using allele-specific primers, after specific amplification of the Cα2 gene by PCR, and IgA2 genotyping was possible by this method. However, the sites of base substitution between alleles used in this method is not in agreement with the site of the base substitution controlling serol-

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ogical reaction specificity of A2m (17).

Materials and Methods

Materials. Lymphocytes isolated from peripheral blood of 318 unrelated Japanese individuals and 81 individuals in 29 cases of paternity testing were used for population and genetic study. Blood in sealed test tubes and blood stains on cotton cloths were left at room temperature in a dark place up to 3 months. Old blood stains on filter papers which had been stored at room temperature for 20 years in a dark place in our laboratory served as materials. Blood stains were heated for 30 min at 100°C, 150°C, 200°C and 250°C. Saliva stains were prepared on filter paper. Organ and tissue blocks were collected from 5 subjects during forensic autopsies. These materials were used for forensic study.

DNA extraction. One hundred microliters of lysis buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.1 % Triton X-100, 200 μg/ml proteinase K) was added to lymphocyte pellet (about 1,000,000 cells) and 500 μl to other materials. The mixture was heated at 60°C for 3–5 h for lymphocytes and overnight for other materials. After heating, DNA was extracted with phenol/chloroform, and recovered by isopropanol precipitation. After being air dried, the DNA recovered was dissolved in TE buffer solution (10 mM Tris-HCl, 1 mM Na_2EDTA pH 8.0), and used as the sample for the first PCR. DNA extracted from lymphocytes was quantified by the absorbance at 260 nm with a spectrophotometer (Beckman Instruments Inc., Irvine, CA, USA). The concentration of DNA was adjusted to 100 μg/ml with TE buffer solution. The minimum level of DNA necessary for identification of each type was determined.

Table 1 Oligonucleotide primers synthesized for IgA2 genotyping by nested PCR

Primer Name	Primer Symbol[a]	Sequence (5'–3')	Notes[b]
AR-101	Ⓕ	GGGCACGTGGGCACACATG	5'Cα2 primer
AR-102	Ⓡ	TTCTGATGCCACCCCACGC	3'Cα2 primer
AR-111	Ⓒ	GCCTGAGCCCTGCAGGAGT	5'Cα2 primer
AR- 11	Ⓣ	TGTGGAGTTGGTGCCTGAT	ASA(IgA2*1) primer
AR- 12	Ⓖ	TGTGGAGTTGGTCCCTGAG	ASA(IgA2*2) primer

a: Symbols for the primers are used in Table 2 and Fig 1. *b*: ASA represents allele-specific amplification.
PCR: polymerase chain reaction.

Table 2 Primer sets for the first and second PCR

PCR	Primer set	Predicted product (bp)	Target gene or allele
First PCR	Ⓕ—Ⓡ	309	Cα2
Second PCR			
Reaction Ⓐ	Ⓒ—Ⓣ	156	IgA2*1
Reaction Ⓑ	Ⓒ—Ⓖ	156	IgA2*2

PCR: See Table 1.

Primers. Table 1 shows the primers prepared for IgA2 genotyping by nested PCR. Ⓕ (AR-101) and Ⓡ (AR-102) are primers for amplification of the 309-bp fragment of the Cα2 gene. The base at the 3'-terminal of each of these primers is complementary to the IgA2 gene, but is not complementary to the IgA1 gene (18). Ⓒ (AR-111), Ⓣ (AR-11) and Ⓖ (AR-12) are allele-specific primers for allele detection, which amplify the 156-bp fragment. Ⓒ is an upstream primer that is complementary to the common site of the nucleotide sequence of IgA2*1 and IgA2*2. Base substitutions A and C are observed for IgA2*1 and IgA2*2, respectively, which correspond to the 2401st base of the IgA1 gene (18). The base at the 3' terminal of primer Ⓣ is complementary to the site of the base substitution of IgA2*1 and the base at the 3' terminal of primer Ⓖ is complementary to the site of the base substitution of IgA2*2 (18). These primers were synthesized by a DNA Synthesizer 380B (Applied Biosystems, Foster City, CA, USA), and used after simple purification with a Sep-Pak C18 (Millipore, Milford, MA, USA).

The principle of IgA2 genotyping. The present method consisted of the first PCR and second PCR (reactions Ⓐ and Ⓑ). Table 2 shows the primer sets used for the present nested PCR. The table also shows the target DNA fragment (target allele) and its size. Fig. 1 shows a schema of amplification of the IgA2 allele. In the first PCR using upstream primer Ⓕ and downstream primer Ⓡ, the 309-bp fragment including the site of the base substitution specific for the allele of the IgA2 gene was amplified. The second PCR consisted of reaction Ⓐ using primers Ⓒ and Ⓣ and reaction Ⓑ using primers Ⓒ and Ⓖ. In reaction Ⓐ, the 156-bp DNA fragment of IgA2*1 was amplified, and in reaction Ⓑ the 156-bp DNA fragment of IgA2*2 was amplified. The second PCR products in reactions Ⓐ and Ⓑ were

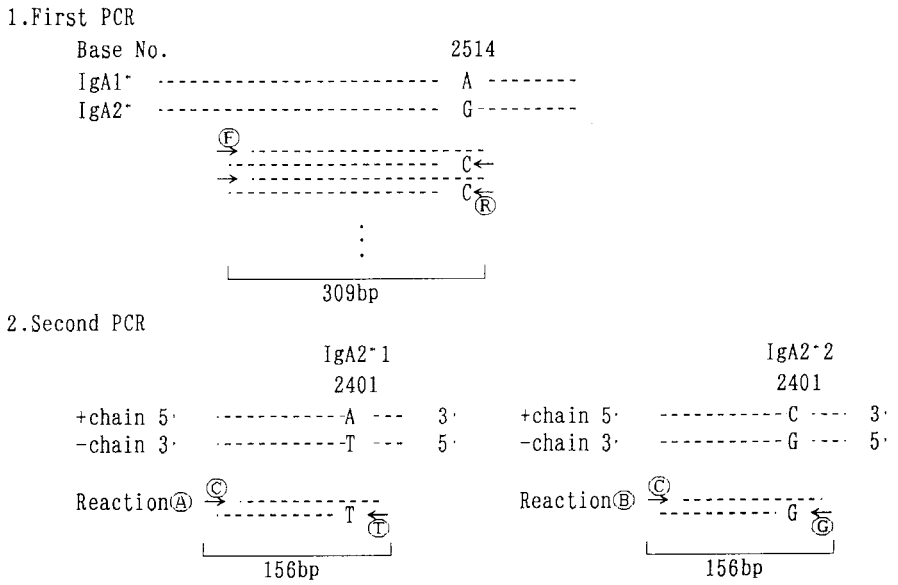

Fig. 1 Strategy for identifying IgA2 alleles by nested polymerase chain reaction (PCR).

separated by electrophoresis and stained with ethidium bromide, then the presence or absence of bands of the amplified DNA fragment of each allele was examined under ultraviolet light for genotyping.

First PCR. PCR procedures were performed as described previously (19) except for using primers Ⓕ and Ⓡ at 0.5 μM each. In principle, 1 μl of each sample was used. When samples other than DNA extracted from lymphocytes were used, bovine serum albumin was added to the reaction solution at 100 μg/ml, to suppress the influence of PCR inhibitors (20–23). After preheating at 95 °C for 3 min, 35 thermal cycles of PCR (each cycle: 40 sec at 94 °C; 1 min at 70 °C) were performed in a Program Temp Control System PC-700 (Astec, Fukuoka, Japan). The reaction included a final extension at 70 °C for 10 min.

Second PCR. Using the primers of each set of reactions Ⓐ and Ⓑ, PCR procedures were performed as described previously (19). In principle, 1 μl of the 10-fold diluted solution of the first PCR product was used as the sample. After preheating at 95 °C for 3 min, 25 cycles of PCR (each cycle: 40 sec at 94 °C; 40 sec at 66 °C) were performed in a Program Temp Control System PC-700. The reaction included a final extension at 66 °C for 10 min.

Electrophoresis. The products (8 μl) of each of the first and second PCRs were electrophoresed at 350V for 40 min on an 8 % polyacrylamide gel. After staining with ethidium bromide following electrophoresis, bands appearing under ultraviolet light were observed for IgA2 genotyping.

Restriction enzyme analysis. First PCR products of 3 samples which were typed as having each of the three genotypes by the present method were digested with 4 units each of *Eco*RII and *Sty* I (Life Technologies, Inc., Gaithersburg, MD, USA) at 37 °C for 6 h. Digested first PCR products were electrophoresed at 350 V for 40 min on 8 % polyacrylamide gels. Second PCR products were digested with 4 units of *Eco*RII at 37 °C for 6 h, and electrophoresed at 350 V for 40 min on 8 % polyacrylamide gels.

Results

IgA2 genotyping. Fig. 2 shows the results of electrophoresis of the first PCR products in 3 subjects. All samples showed the 309-bp fragment bands derived from IgA2 genes. Fig. 3 shows the results of electrophoresis of the second PCR products. The 156-bp fragment bands were observed in the lanes of products of reaction Ⓐ in sample 1, reactions Ⓐ and Ⓑ in sample 2, and

reaction Ⓑ in sample 3. These samples were determined to be the IgA2*1/IgA2*1, IgA2*2/IgA2*1 and IgA2*2/IgA2*2 types, respectively.

Results of restriction enzyme analysis. Digestion of the first PCR products with *Eco*RII yielded fragments of 110-bp, 94-bp, 84-bp and 21-bp, and no digestion with *Sty* I was observed in all three genotypes, as expected from the reported sequences (18) of IgA2*1 and IgA2*2. This observation shows that IgA1* was not amplified and that IgA2* was amplified. As expected from the sequences (18), the digestion of the second PCR products with *Eco*RII gave rise to fragments of 102-bp and 54-bp in the products of reaction Ⓐ in those samples which were typed as IgA2*1-positive and in the products of reaction Ⓑ in samples typed as IgA2*2-positive.

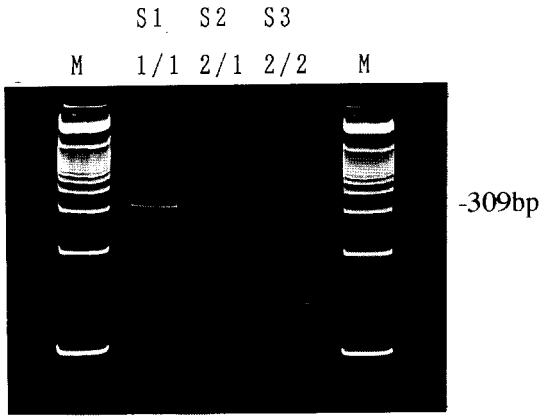

Fig. 2 Electrophoretic separation of the first PCR products. Products of the first PCR were separated by means of 8% polyacrylamide gel electrophoresis at 350 V for 40 min, then stained with ethidium bromide. PCR: See Fig. 1.
S1: Sample 1; S2: Sample 2; S3: Sample 3;
M: 100 base-pair ladder DNA marker (Pharmacia).

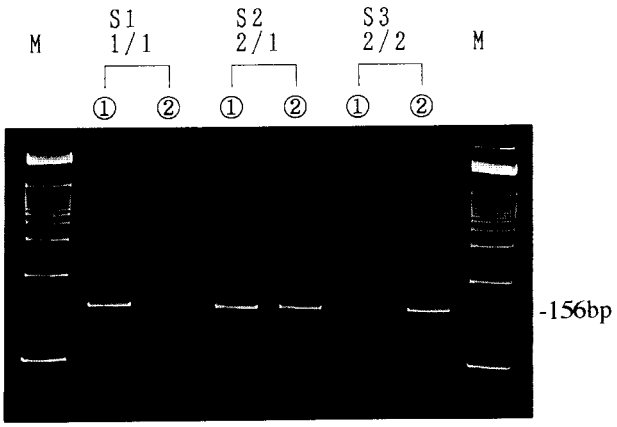

Fig. 3 Electrophoretic separation of the three IgA2 genotypes. Products of the second PCRs were separated by means of 8% polyacrylamide gel electrophoresis at 350 V for 40 min, then stained with ethidium bromide. PCR: See Fig. 1.
S1: Sample 1; S2: Sample 2; S3: Sample 3; M: 100 base-pair ladder DNA marker (Pharmacia); ①: Product of reaction Ⓐ; ②: Product of reaction Ⓑ.

Table 3 Distribution of the IgA2 genotypes in Okayama Prefecture, Japan

Genotype	Number observed (%)	Number expected (%)	Allele frequency
IgA2*1/IgA2*1	102 (32.1)	100.2 (31.5)	IgA2*1=0.561
IgA2*2/IgA2*1	153 (48.1)	156.6 (49.3)	IgA2*2=0.439
IgA2*2/IgA2*2	63 (19.8)	61.2 (19.2)	$\chi^2=0.168$
Total	318 (100.0)	318.0(100.0)	d. f.=1
			$0.50<P<0.75$

Table 4 Results of IgA2 genotyping in the samples of nonexcluded paternity cases

Mating		Number of cases	Child		
Mo	AFa		1/1	2/1	2/2
1/1	1/1	2	2	0	0
	2/1	4	1	3	0
	2/2	2	0	2	0
2/1	1/1	4	2	2	0
	2/1	4	1	0	3
	2/2	2	0	1	1
2/2	1/1	1	0	1	0
	2/1	1	0	1	0
	2/2	0	0	0	0

Mo: Mother; AFa: Alleged Father.

Sensitivity. Each type of DNA extracted from lymphocytes and quantified with a spectrometer (Beckman Instruments Inc.) at a concentration adjusted to 100 μg/ml, was diluted stepwise. Genotyping was possible from 1 μl of the 10^3-fold diluted solution (100 pg of DNA).

Genotype and allele frequency in Japan. Table 3 shows the results of IgA2 genotyping by the present method in blood samples collected from 318 unrelated Japanese in Okayama Prefecture. The IgA2*1/IgA2*1 type was observed in 102 (32.1%) subjects,

Table 5 Results of IgA2 genotyping in the samples of excluded paternity cases

Mating		Number of cases	Child		
Mo	AFa		1/1	2/1	2/2
1/1	1/1	0	0	0	0
	2/1	2	0	2	0
	2/2	0	0	0	0
2/1	1/1	2	1	0	1*
	2/1	1	1	0	0
	2/2	1	0	0	1
2/2	1/1	1	0	0	1*
	2/1	2	0	1	1
	2/2	0	0	0	0

Mo: Mother; AFa: Alleged Father.
*: Excluded by IgA2 genotyping

of father to child was excluded by 2-5 conventional blood group tests.

Fresh and putrefied blood. Table 6 shows the results of genotyping of fresh and putrefied blood. The results obtained on 50 μl of fresh blood on the first day of storage and 50 μl of putrefied blood for 1, 2 and 3 months of storage at room temperature were in agreement with each genotype determined preliminarily in the 6 subjects.

Blood stain. As Table 7 shows, the results of genotyping 25 mm^2 (corresponding to about 5 μl of blood) of fresh and 3-month-old blood stains are in agreement with each genotype, which was preliminarily determined from the DNA extracted from lymphocytes in 6 subjects.

Twenty-year-old blood stain. IgA2

Table 6 Detection of IgA2 genotypes by PCR from whole blood left at room temperature

Genotype of subjects	Specific sequence	The 1st day	Stored whole blood		
			1month	2months	3months
IgA2*1/IgA2*1 (n=2)	IgA2*1	+	−	+	+
	IgA2*2	−	−	−	−
IgA2*2/IgA2*1 (n=2)	IgA2*1	−	+	−	+
	IgA2*2	+	+	+	+
IgA2*2/IgA2*2 (n=2)	IgA2*1	−	−	−	−
	IgA2*2	−	−	+	+

+: A distinct band was observed; −: No specific band was observed. PCR: See Table 1.

Table 7 Detection of IgA2 genotypes by PCR from blood stains left at room temperature

Genotype	Specific sequence	Blood stains	
		Fresh blood stains	3-month-old blood stains
IgA2*1/IgA2*1 (n=2)	IgA2*1	+	−
	IgA2*2	−	−
IgA2*2/IgA2*1 (n=2)	IgA2*1	−	−
	IgA2*2	+	+
IgA2*2/IgA2*2 (n=2)	IgA2*1	−	−
	IgA2*2	−	−

+: A distinct band was observed; −: No specific band was observed. PCR: See Table 1.

IgA2*2/IgA2*1 type in 153 (48.1 %), and IgA2*2/IgA2*2 type in 63 (19.8 %). The estimated allele frequency was 0.561 for IgA2*1 and 0.439 for IgA2*2. Thus, the results were in good agreement with Hardy-Weinberg's law of autosomal inheritance.

Heredity. Tables 4 and 5 show the results of the test by this method in 81 subjects of 29 cases of paternity testing. In the 20 cases whose relations of father to child were confirmed at a high probability (99.62-99.999 %) by the conventional blood groups test, there was no discrepancy regarding a relation of father to child in the results of the present test. A relation of father to child was excluded by this method in 2 of the 9 cases whose relation

Table 8 Detection of IgA2 genotypes by PCR from blood stains heated for 30 min at various temperatures

Genotype	Specific sequence	Heating temperature (°C)			
		100	150	200	250
IgA2*1/IgA2*1 (n=2)	IgA2*1	+	+	−	−
	IgA2*2	−	−	−	−
IgA2*2/IgA2*1 (n=2)	IgA2*1	−	−	−	−
	IgA2*2	+	+	−	−
IgA2*2/IgA2*2 (n=2)	IgA2*1	−	−	−	−
	IgA2*2	+	+	−	−

+: A distinct band was observed; −: No specific band was observed. PCR: See Table 1.

Table 9 Detection of IgA2 genotypes by PCR from tissues and organs of autopsy cases

Case No.	Sex	Age	Postmortem time	Cause of death	Specific sequence	Tissues and organs									
						Blood	Muscle	Skin	Cerebrum	Heart	Lung	Liver	Kidney	Spleen	Pancreas
1.	♀	77y	2days	Cardiac tamponade	IgA2*1	+	+	+	±	±	+	+	±	±	+
					IgA2*2	+	+	+	−	±	+	+	−	−	−
2.	♀	38y	1.5-2days	Fatal loss of blood	IgA2*1	−	+	+	+	±	+	+	±	±	±
					IgA2*2	−	−	−	−	−	−	−	−	−	−
3.	♀	51y	2days	Acute heart failure	IgA2*1	−	−	−	−	−	−	−	−	−	−
					IgA2*2	±	−	±	+	−	±	+	−	+	−
4.	♂	51y	1.5-2days	Fatal loss of blood	IgA2*1	+	±	±	+	±	±	+	±	+	±
					IgA2*2	−	−	−	−	−	−	−	−	−	−
5.	♂	56y	1-1.5days	Death from fire	IgA2*1	+	±	+	+	+	±	+	±	+	±
					IgA2*2	+	+	+	−	+	−	+	−	+	+

+: A distinct band was observed. −: No specific band was observed. PCR: See Table 1.

genotypes were detected from 25 mm^2 of 20-year-old blood stains of 20 subjects with unknown IgA2 type. In all specimens, the target DNA fragment could be amplified, and IgA2*1/IgA2*1, IgA2*2/IgA2*1 and IgA2*2/IgA2*2 types were observed in 4, 11 and 5 subjects, respectively.

Heated blood stain. As shown in Table 8, genotyping was possible in all 6 subjects from 25 mm^2 (corresponding to about 5 μl of blood) of blood stains heated at 150 °C for 30 min. The results are in agreement with the genotypes determined preliminarily with DNA extracted from lymphocytes. No amplified band was observed when blood stains were heated at 200 °C or at 250 °C for 30 min.

Saliva stain. IgA2 genotypes were detected from 25 mm^2 (corresponding to about 4 μl of saliva) of saliva stains. In all 9 subjects, the results are in agreement with the genotypes determined preliminarily with DNA extracted from lymphocytes.

Organs and tissues. Table 9 shows the results of the test of DNA extracted from of 50 μl blood, each 8 mm^3 organ (cerebrum, heart, lung, liver, kidney, spleen and pancreas) and tissue (muscle and skin) block from 5 cadavers. When the amount of template DNA is large, 1 μl of 10- to 100-fold-diluted solution of DNA sample was used as the sample in the first PCR and 1 μl of 100-fold-diluted solution of the first PCR product was used as the sample in the second PCR. Genotypes were determined in all these specimens, and were in agreement in the same person.

Discussion

According to conventional studies using indirect hemagglutination inhibition, there is a marked difference among races in the distribution of IgA2 genetic marker A2m allotypes. The majority of Caucasoids show A2m (1) allotype (4-12). Many Negroids show the A2m(2) allotype (4). In Mongolians, on the other hand, which include Japanese (13), Chinese (4, 14-16), Vietnamese (24) and Thais (25), the frequencies of A2m(1) and A2m (2) types are relatively similar. In these countries, the A2m type is considered a useful marker for paternity testing and individual identification. However, in Japan, it is very difficult to obtain antiserum as the reagent; it has not been possible to use the A2m type for the practical work in forensic medicine. Therefore, we developed a method of IgA2 genotyping using nested PCR without any serological procedures for the utilization of IgA2 type for paternity testing and individual identification in forensic medicine.

Lefranc and Rabbitts (26) and Hammarström *et al.* (27) reported a method of determining the A2m type by using restriction fragment length polymorphisms (RFLPs) of the Cα2 gene. Soua *et al.* (28) and Fakhfakh *et al.* (29) have applied this method to a study of population genetics. However, the method using RFLPs generally requires a relatively large amount of sample (about 5 μg of DNA) (30, 31). It can be applied to population genetics study and paternity testing, but it was difficult to apply it to the practical work in forensic

medicine, in which sometimes only trace specimens collected at a crime scene are available for testing.

In the IgA2 genotyping using nested PCR that we established in this study (32), genotyping was possible with 100 pg of DNA. When the amount of template DNA is large in this IgA2 genotyping, thin 156-bp fragment bands were sometimes observed in the lanes of products of 'mismatched' allele-specific primers whose base at the 3'-terminal is not complementary to an allele of samples; for example, in lane ② in the IgA2*1/IgA2*1 type and in lane ① in the IgA2*2/IgA2*2 type. Even in these cases, the thin 156-bp fragment bands were almost undetectable when 1 μl of 100-fold-diluted solution of DNA sample was used as the sample in the first PCR and when 1 μl of 100-fold-diluted solution of the first PCR product was used as the sample in the second PCR. Fujita *et al.* (33) studied IgA2 typing using the site of base substitution corresponding to serological reaction specificity by PCR-RFLPs (using RFLPs following amplification with PCR), but they did not describe the sensitivity. Nested PCR is highly sensitive (34) and is currently being used for some kinds of DNA typing in forensic medicine (35-37).

There was no significant difference between the estimated allele frequency obtained by IgA2 genotyping in 318 Japanese subjects by the present method (IgA2*1 = 0.561, IgA2*2 = 0.439) and by the serological method (13, 38) (A2m*1 = 0.557, A2m*2 = 0.443) on χ^2 test ($\chi^2 = 0.004$, d. f. = 1, $0.9 < P$). The results obtained from genotyping of 81 subjects in 29 paternity cases suggest that this genotype follows Mendelian hereditary law. The sites of base substitution used in the present method were not in agreement with the site of base substitution contributing to the serological reaction specificity of A2m (17), but the IgA2*1 allele among the IgA alleles detected by our present method seems to correspond to the A2m*1 allele and the IgA2*2 allele seems to correspond to the A2m*2 allele.

The DNA fragment amplified by the present method is relatively small (309-bp), and amplification is relatively easy even though the DNA is fragmented. By the present method, genotyping was possible not only in fresh blood but also in putrefied blood that had been stored for 3 months. This indicates that DNA fragments of hundreds of base pairs necessary for IgA2 genotyping remain even in decayed blood that has been left at room temperature.

A2m typing from blood stains seems to be difficult by conventional serological methods, whereas genotyping from 3-month-old blood stains was possible by the present method. Even with 20-year-old blood stains, IgA2 genotypes were detected in all 20 cases. The distribution of the genotype has no significant difference on χ^2 test from that on the test of DNA extracted from lymphocytes in 318 subjects ($\chi^2 = 1.128$, d. f. = 1, $0.25 < P < 0.5$). This suggests that the IgA2 genotype had been correctly determined from 20-year-old blood stains.

IgA2 genotyping was possible from blood stains heated at 150°C for 30 min. This result shows that DNA was highly stable against heat in dried blood stains. Semba *et al.* (39) have also reported that sex determination was possible by PCR of sex chromosomal DNA from the blood stains heated at 150°C for 30 min.

IgA is present in body fluids such as saliva (40). Vyas *et al.* (1) determined A2m types with 10-fold-concentrated saliva by hemagglutination inhibition. By our method, IgA2 genotyping was possible from only one 25 mm^2 saliva stain (corresponding to 4 μl of saliva). Saliva contains cells derived from the oral mucosa. The amount of DNA in saliva is similar to that in blood (41, 42). Saliva stains are useful not only as evidence samples but also as control samples in forensic medicine, because saliva can be collected noninvasively.

Genotyping of DNA extracted from each organ and tissue block collected during forensic autopsy was possible by the present method. Unlike serological methods, specimens other than serum and saliva can be tested by this method. Genotyping from blood and organs and tissues, which were collected from burned bodies was also possible. It was clarified that genotyping is possible even with the burned body, when blood, organs and tissues are not influenced much by heat and remain intact to some extent.

The IgA2 genotyping reported in the present study is expected to become a useful testing method for practical work in forensic medicine.

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References

1. Vyas GN and Fudenberg HH: Am (1), the first genetic marker of human immunoglobulin A. Proc Natl Acad Sci USA (1969) **64**, 1211-1216.
2. Gold ER and Fudenberg HH: Chromic chloride: A coupling reagent for passive hemagglutination reactions. J Immunol (1967) **99**, 859-866.
3. Kunkel HG, Smith WK, Joslin FG, Natvig JB and Litwin SD: Genetic

marker of the γA2 subgroup of γA immunoglobulins. Nature (1969) **223**, 1247-1248.
4. van Loghem E, Wang AC and Shuster J: A new genetic marker of human immunoglobulins determined by an allele at the α2 locus. Vox Sang (1973) **24**, 481-488.
5. Helal AN, Rivat-Peran L, van Loghem E, de Lange G, Langaney A and Lefranc G: Further contribution of common Gm*-Am*haplotypes and Km*alleles in the characterization of the Tunisian population. Exp Clin Immunogenet (1988) **5**, 1-14.
6. Lefranc G, Rivat L, Serre J-L, Lalouel J-M, Pison G, Loiselet J, Ropartz C, de Lange G and van Loghem E: Common and uncommon immunoglobulin haplotypes among Lebanese communities. Hum Genet (1978) **41**, 197-209.
7. Lefranc G, de Lange G, Rivat L, Langaney A, Lefranc M-P, Ellouze F, Sfar G, Sfar M and van Loghem E: Gm, Am and Km immunoglobulin allotypes of two populations in Tunisia. Hum Genet (1979) **50**, 199-211.
8. van Loghem E, de Lange G, Matsumoto H, Miyazaki T and Ohkura K: Immunoglobulin haplotypes of two population groups in Iran. J Immunogenet (1977) **4**, 385 390.
9. Schanfield MS, Herzog P and Fudenberg HH: Immunoglobulin allotypes of European populations: II. Gm, Am and Km (Inv) allotypic markers in Czechoslovakians. Hum Hered (1975) **25**, 382-392.
10. Piazza A, van Loghem E, de Lange G, Curtoni ES, Ulizzi L and Terrenato L: Immunoglobulin allotypes in Sardinia. Am J Hum Genet (1976) **28**, 77 86.
11. Stevenson JC and Schanfield MS: Immunoglobulin allotypes in European populations: III. Gm, Am and Km allotypes in people of European ancestry in the United States. Hum Biol (1981) **53**, 521 542.
12. Stevenson JC, Schanfield MS and Sandler SG: Immunoglobulin allotypes in Jewish populations living in Israel and the United States. Am J Phys Anthropol (1985) **67**, 195-207.
13. van Loghem E, Natvig JB and Matsumoto H: Genetic markers of immunoglobulins in Japanese families. Inheritance of associated markers belonging to one IgA and three IgG subclasses. Ann Hum Genet (1970) **33**, 351-359.
14. Jeannet M, Schapira M, Gervasoni C, Metaxas-Bühler M, Bütler R and van Loghem E: Study of the HL-A system and other polymorphisms in the Tibetan population; in Histocompatibility Testing 1972, Munksgaard, Copenhagen (1972) pp241-250.
15. de Lange G, Fa-Ming Z, Henke J, Feng Z, Bernhardt R, van Leeuwen F and van Eede P: Immunoglobulin allotypes in a Chinese population: Comparison of haplotype frequencies with other Asian groups. J Immunogenet (1985) **12**, 191-195.
16. Wu G, Liu D, Fang G, Hu W, Jia H, Zhang Q, Fukada K, Yoshimura K, Saji H and Lee TD: The detection of an antibody against IgA allotype A2M2 and a study of the *Am* genetic marker among the Han Chinese population. Transfusion (1989) **29**, 337-340.
17. van Loghem E and Biewenga J: Allotypic and isotypic aspects of human immunoglobulin A. Mol Immunol (1983) **20**, 1001 1007.
18. Flanagan JG, Lefranc M-P and Rabbitts TH: Mechanisms of divergence and convergence of the human immunoglobulin α1 and α2 constant region gene sequences. Cell (1984) **36**, 681-688.
19. Yamamoto Y and Ishizu H: Km genotyping by polymerase chain reaction (PCR) using allele-specific amplification primers. Forensic Sci Int (1995) **75**, 85-93.
20. de Franchis R, Cross NCP, Foulkes NS and Cox TM: A potent inhibitor of *Taq* polymerase copurifies with human genomic DNA. Nucleic Acids Res (1988) **16**, 10355.
21. Akane A, Matsubara K, Takahashi S, Nakamura H and Shiono H: Polymerase chain reaction analysis of contaminated and degraded DNA from forensic specimens; in DNA Polymorphism, Misawa S and Harada S eds, Vol. 1, Bunkodo, Tokyo (1993) pp31-35 (in Japanese).
22. Hagelberg E, Sykes B and Hedges R: Ancient bone DNA amplified. Nature (1989) **342**, 485.
23. Hochmeister MN, Budowle B, Jung J, Borer UV, Comey CT and Dirnhofer R: PCR-based typing of DNA extracted from cigarette butts. Int J Legal Med (1991) **104**, 229-233.
24. de Lange G, Wright P, van Eede P, van Leeuwen F, Long HT and Hong NTD: Association between leprosy and immunoglobulin allotypes: Gm-A2m and Km frequencies in Vietnamese. J Immunogenet (1984) **11**, 173 180.
25. van Loghem E, Chandanayingyong D and Douglas R: Immunoglobulin genetic markers in the Thai population. J Immunogenet (1975) **2**, 141 145.
26. Lefranc M-P and Rabbitts TH: Human immunoglobulin heavy chain A2 gene allotype determination by restriction fragment length polymorphism. Nucleic Acids Res (1984) **12**, 1303-1311.
27. Hammarström L, de Lange GG and Smith CIE: IgA2 allotypes determined by restriction fragment length polymorphism in IgA deficiency: Re-expression of the silent A2m(2) allotype in the children of IgA-deficient patients. J Immunogenet (1987) **14**, 197 201.
28. Soua Z, Ghanem N, Salem MB, Lefranc G and Lefranc M-P: Frequencies of the human immunoglobulin IGHA2*M1 and IGHA2*M2 alleles corresponding to the A2m(1) and A2m(2) allotypes in the French, Lebanese, Tunisian and Black African populations. Nucleic Acids Res (1989) **17**, 3625.
29. Fakhfakh F, Rouis S, Makni H and Ayadi H: Détermination de la fréquence de l'allèle A2M(2) des immunoglobulines A dans la population tunisienne par la technique de RFLP. Rev Fr Transfus Hémobiol (1991) **34**, 441-447.
30. Shimmoto M, Tsuji A and Suzuki Y: Restriction fragment length polymorphisms on the short arm of X chromosome among the Japanese population. Jpn J Hum Genet (1988) **33**, 333-338.
31. Taga T, Shirahase W, Shimada M, Kurokawa K and Ueyama H: Restriction fragment length polymorphisms on the q24-q28 region of X chromosome among Japanese population. Jpn J Hum Genet (1989) **34**, 123-128.
32. Takata S, Yamamoto Y and Ishizu H: IgA2 gene polymorphism detected by PCR; in DNA Polymorphism, Yamanouchi H and Dewa K eds, Vol. 2, Toyo Shoten, Tokyo (1994) pp155-159 (in Japanese).
33. Fujita K, Miyazaki T, Matsumoto H and Mizoi Y: Detection of specific base changes for Am allotype by PCR-RFLP technique; in DNA polymorphism, Sawaguchi A and Nakamura S eds, Vol. 3, Toyo Shoten, Tokyo (1995) pp174-177 (in Japanese).
34. Erlich HA, Gelfand D and Sninsky JJ: Recent advances in the polymerase chain reaction. Science (1991) **252**, 1643 1651.
35. Akane A, Seki S, Shiono H, Nakamura H, Hasegawa M, Kagawa M, Matsubara K, Nakahori Y, Nagafuchi S and Nakagome Y: Sex determination of forensic samples by dual PCR amplification of an X-Y homologous gene. Forensic Sci Int (1992) **52**, 143 148.
36. Honda K, Sugiyama E, Tsuchikane A, Katsuyama Y, Harashima N, Ota M and Fukushima H: Nested amplification of COL2A1 3' variable region in skeletal remains. Jpn J Legal Med (1994) **48**, 156-160.
37. Uchihi R, Yamamoto T, Kojima T, Tamaki K and Katsumata Y: Efficient amplification of the HLA-DQA1 gene in single genomes using a semi-nested polymerase chain reaction. Jpn J Legal Med (1994) **48**, 329-335.
38. Matsumoto H, Miyazaki T and Fong JM: Further data on the Gm and Am allotypes of the Takasago in Taiwan. Jpn J Legal Med (1973) **27**, 273-277.

39. Semba S, Yamamoto Y and Ishizu H: Sex determination from blood and bloodstains by polymerase chain reaction (PCR). Jpn J Legal Med (1994) **48**, 7-18.
40. Mestecky J and Russell MW: IgA subclasses; in Monogr. Allergy, Dukor P, Kallós P, Trnka Z and Waksman BH eds, Vol. 19, Karger, Basel (1986) pp277-301.
41. Kojima T, Uchihi R, Yamamoto T, Tamaki K and Katsumata Y: DNA typing of the three HLA-class II loci from saliva stains. Jpn J Legal Med (1993) **47**, 380 386 (in Japanese).
42. Sakai I, Kasai K, Yoshida K and Mukoyama H: An improved method for DNA isolation from human bloodstains and typing of the isolated DNA using single locus VNTR probes. Rep Natl Res Inst Police Sci Res Forensic Sci (1991) **44**, 36-49 (in Japanese).

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