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TECHNICAL NOTE

CRIMINALISTICS

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Mismatched Multiplex PCR Amplification and Subsequent RFLP Analysis to Simultaneously Identify Polymorphisms of Erythrocytic *ESD*, *GLO1*, and *GPT* Genes*

ABSTRACT: *ESD* (esterase D), *GLO1* (glyoxalase I), and *GPT* (glutamate pyruvate transaminase) are human erythrocytic isoenzymes and have previously been applied in forensic medicine caseworks. The molecular bases of the polymorphic gene expression products have been demonstrated to be because of SNPs in respective coding regions. However, it has not been revealed whether the SNPs conferring the polymorphisms to the aforementioned erythrocytic isoenzymes could be simultaneously detected by using a simple PCR method. In this study, we used mismatched primers to simultaneously amplify three common isoenzyme loci so that all amplified products contained the same *Hph* I cleavage sites. The products were then digested with *Hph* I and electrophoretically separated and stained so that alleles were identified. The accumulated values for the probability of discrimination power and excluding the probability of paternity to the aforementioned systems attained 90.41% and 41.72%, respectively, in the Chinese Han population. This assay could be extremely valuable for future forensic medicine practices.

KEYWORDS: forensic science, isoenzyme, mismatched multiplex amplification, genotyping, esterase D, glutamate pyruvate transaminase, glyoxalase I

It is well known that there are many genetic markers, which include antigen differences on red and/or white blood cell membranes, serum protein polymorphisms, erythrocytic isoenzymes, etc., documented in human blood (1,2). The phenotypes from each polymorphic system may be characterized by its serological characteristics or proteins' separation through electrophoresis. In fact, these systems have previously been applied extensively in some forensic medicine caseworks, and they are still being used in some laboratories. So far, polymorphic analysis on single blood group locus based on DNA level has been performed, and a high-throughput typing blood group system for single nucleotide polymorphisms (SNPs) has been explored (3–5). However, simple methods for simultaneously identifying multiple SNP loci from human blood groups for application in forensic medicine in those laboratories without automatic analyzers for DNA have rarely been reported.

Restriction fragment length polymorphism (RFLP) is a technique in which a restriction endonuclease cleaves a DNA molecule in specific nucleotide sequence (depending on the particular enzyme used) and has previously been applied in forensic caseworks (6,7). Although RFLP analysis is easy to apply for the screening of known sequence variants, many common polymorphisms are the result of single-base substitutions that fail to create or remove any restriction sites, therefore, which cannot be easily typed by simple polymerase chain reaction (PCR) and RFLP analysis. However, the use of a mismatched PCR primer to synthetically create a restriction site in the amplified product makes it possible to solve this problem. The mismatched primer contains a single- or double-base mismatch near its 3′ end, so that the amplified product incorporates or removes a restriction site for the appropriate endonuclease in the presence of a base substitution (8,9). Based on these basic principles, we tried to perform multiplex mismatched PCR amplification and RFLP analysis to simultaneously identify multiple SNP sites. The prerequisites for potentially applicable biallelic SNP systems in this study are (i) an identical base from either wild or mutant alleles in the selected systems and (ii) an identical base located at the 3′ end from either forward or reverse primers in selected systems. The mismatched sequences in the primer should be as short as possible and should create an applicable restriction site around the SNP sites.

In this study, we selected erythrocytic *ESD* (esterase D), *GLO1* (glyoxalase I), and *GPT* (glutamate pyruvate transaminase) genes as test targets and successfully identified their genotypes, simultaneously, by using mismatched multiplex PCR amplification and subsequent RFLP analysis, so we recommend its usefulness for future forensic medicine caseworks.

Materials and Methods

Samples and DNA Extraction

Human venous blood was collected from 20 Chinese Han subjects. Ten of them, whose phenotypes of erythrocytic ESD, GLO1,

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and GPT had been determined from previous forensic medicine caseworks, were selected as the control group. Informed consent was obtained from each donor according to the ethical review board of China Medical University, P.R. China guidelines. DNA was extracted from the whole specimen using an SDS-proteinase K phenol-chloroform method and kept at −80°C until use.

Selection of the Loci from Human Blood Group Systems and Design of Mismatched PCR Primers

The selected loci in this study were first chosen from commonly used genetic markers in human blood group systems. Second, the population data had been reported, and the loci showed a high power of discrimination. Third, phenotypes of the polymorphic systems could be detected by serological or electrophoretic methods, and the molecular bases of the polymorphisms had been determined. In regard to primer design, the fragments size of PCR products, conditions for multiplex PCR amplification, and genotyping for PCR products had to be strictly considered. To meet the aforementioned criteria, a PCR–RFLP technique was designed to identify the erythrocytic isoenzyme polymorphisms at DNA level. As shown in Fig. 1 and Table 1, a few bases were mismatched in the forward primers (bases shown by arrows) to produce synthetic recognition sites for the restriction enzyme, *Hph* I [sequence of *Hph* I restriction site—GGTGA(N_8)/CCACT(N_7)].

Amplification of DNA Fragments

The triplex PCR was performed in a volume of 20 μL *AmpliTaq* Gold buffer II (Mg^{2+} free) containing 50–100 ng genomic DNA, 1.25 mM dNTP, 0.5 mM $MgCl_2$, 0.05–1.2 μM of each primer

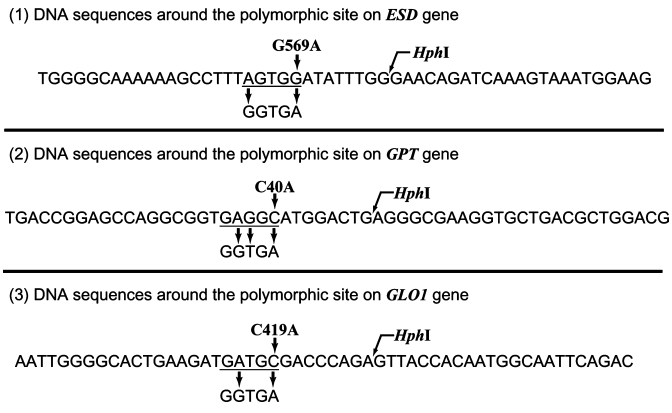

FIG. 1—*DNA sequences around the polymorphic sites in the three isoenzyme genes. Arrows below the underlined bases represent polymorphisms (right one each sequence) and mismatched bases (left all) that were created on all forward primers. The anamorphic arrows stand for the cutting site for the restriction enzyme Hph* I.

(primer sequences and applied concentration were shown in Table 1), and 0.7 U *AmpliTaq* DNA polymerase (Applied Biosystems, Foster City, CA). PCR amplification was performed with initial denaturing at 95°C for 5 min followed by 38 cycles of denaturing at 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 min and final extension at 72°C for 7 min.

Restriction Enzyme Digestion and Genotype Detection

The PCR products were digested at 37°C for more than 2 h in 10 μL NEBuffer 4 containing 3 μL PCR product and 2.5 U *Hph* I (New England Biolabs, Beijing, China). Separation of the digested products was carried out using polyacrylamide gel electrophoresis (T = 8%, C = 5%). Electrophoresis was conducted at room temperature for 1 h with a voltage gradient of 10 V/cm. Subsequently, the gel was placed into 20 mL of 1× TBE buffer with 2 μL 10,000× Genefinder (Bio-V, Xiamen, China) for staining for about 10 min and then washed in distilled water for 1 min. The bands were observed using UV MiniBis BioImaging System (DNR Bio-Imaging Systems Ltd, Jerusalem, Israel).

Direct Sequencing

To validate the formation of synthetically mismatched sequences, direct DNA sequencing around the SNP sites was performed with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) using reverse primers.

Results and Discussion

To validate the mismatched sequences from PCR products of the three isoenzyme loci, *ESD*, *GLO1,* and *GPT* in humans, direct DNA sequencing was performed using the reverse PCR amplification primers. The results showed that the mismatched sequences containing the restriction site for *Hph* I for the three loci were correctly generated (as shown in Fig. 1). After DNA sequencing confirmation, we focused on mismatched multiplex PCR amplification and subsequent RFLP analysis to be able to simultaneously identify the polymorphisms of the isoenzyme genes. To obtain complete PCR products from the aforementioned loci, it was also important that the size of target amplicons, specificity of the primer sequences, PCR amplification conditions, and appropriate primer concentration were considered (Table 1). The concentration of the primers used here had to be varied to obtain each band clarity and at a similar intensity on the gel (10,11). With regard to the concentration of the amplification primers, GLO1 > GPT > ESD were used as 1.2, 0.6, and 0.05 μM, respectively.

As shown in Fig. 2, the results demonstrated that amplified PCR products were 284 bp (*GLO1*), 218 bp (*GPT*), and 161 bp (*ESD*). After the PCR products were digested by restriction enzyme *Hph* I and electrophoretic separation was carried out, the fragments that

TABLE 1—*Information of isoenzyme loci and parameters for mismatched multiplex PCR amplification.*

SNP loci	Fragment Size (bp)	Allele	Serological Type	Primer Direction	Primer Sequence (5′ → 3′)	Primer Concentration (μM)
ESD	161 (130, 31)	G/A	G = *ESD*[1]	F	TGGGGCAAAAAAGCCTTTGGTG	0.05
			A = *ESD*[2]	R	AGCTCTAGAGCCCTAACATGAGCA	0.05
GPT	218 (187, 31)	C/A	C = *GPT*[1]	F	TGACCGGAGCCAGGCGGTGGTG	0.6
			A = *GPT*[2]	R	TTATTAGTGGGGCTGGGGCCAACTCG	0.6
GLO1	284 (253, 31)	C/A	C = *GLO1*[1]	F	AATTGGGGCACTGAAGATGGTG	1.2
			A = *GLO1*[2]	R	TCATGGTGAGATGGTAAGTGTAAT	1.2

ESD, esterase D; GLO1, glyoxalase I; GPT, glutamate pyruvate transaminase.

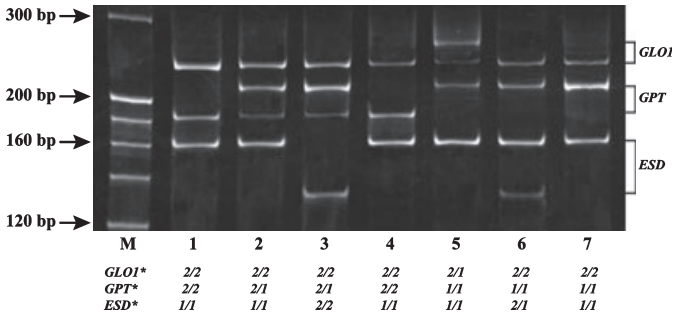

FIG. 2—*The electrophoretic pattern of the mismatched multiplex PCR–RFLP on the three isoenzyme loci in a polyacrylamide gel stained by Genefinder. M stands for a DNA ladder that ranged from 120 to 300 bp as a molecular size marker. Lanes 1–7 are results from seven participants in this study. Genotypes are listed below the corresponding lanes.*

retained their original positions represented *GLO1*1*, *GPT*1,* and *ESD*1* alleles, respectively, while the fragments 253, 187, and 130 bp corresponded to alleles *GLO1*2*, *GPT*2,* and *ESD*2,* respectively. The short fragments of 31 bp ran off the gel under the electrophoretic conditions. Thus, we were able to successfully develop a mismatched multiplex assay using the SNP sites to determine the common phenotypes of the three erythrocytic isoenzymes in humans.

Traditionally, polymorphic erythrocytic isoenzymes could be phenotyped by electrophoretic separation and specific substrate–enzyme-catalyzed reactions—a zymogram (12). Even now it may still be used in some laboratories. However, there are certain weak points in the course of isoenzymes' phenotyping. One of them is the band's diffusion during color development caused by long staining time and interaction between isoenzyme and substrate. As a result, ambiguous or misjudging phenotype may occur. Another is the erythrocytic isoenzymes' activity may be affected by environmental factors including temperature, humidity, and holding time of sample. These factors may result in degradation and loss of enzyme activity so that isoenzymes' phenotypes may not be determined owing to the weak bands or the bands disappearing. With the advent of molecular techniques and our current comprehensive understanding of the molecular basis of the forensically relevant genetic marker antigens, there has been a recent paradigm shift toward determining blood group antigen phenotype by DNA analysis (13). The molecular bases of all the significant erythrocytic isoenzyme polymorphisms are known, and many have previously been described (14), which means that it is possible to predict erythrocytic isoenzyme phenotypes from tests on genomic DNA with a high degree of accuracy. The molecular identification and characterization of erythrocytic isoenzymes reveals that the majority of forensically important polymorphic isoenzymes are encoded by SNPs (4,15). This permits the design of simple PCR assays based either on allele-specific amplification or on RFLP that can discriminate between erythrocytic isoenzyme alleles (16). In this study, three types of erythrocytic isoenzymes phenotypes were identified by applying a DNA level genotyping method. At the same time, we developed a multiplex PCR technique based on sequence character around the SNP sites in the isoenzymes so that the SNP sites for determining phenotypes of erythrocytic ESD, GLO1, and GPT could be simultaneously detected. The accumulated values for the probability of discrimination power and excluding probability of paternity to the aforementioned systems attained 90.41% and 41.72%, respectively, in the Chinese Han population. This method can be extremely valuable in practicing forensic medicine. In addition, genotyping the isoenzyme polymorphism will also supply data for linkage analyses, including human genetics and disease relationships.

Conflict of interest: The authors have no relevant conflicts of interest to declare.

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