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Notes

Genotyping of Polymorphisms in Alcohol and Aldehyde Dehydrogenase Genes by Direct Application of PCR-RFLP on Dried Blood without DNA Extraction

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We have developed a simple, labor-saving, inexpensive, and rapid single nucleotide polymorphism (SNP) genotyping method that works directly on whole human blood. This single-tube genotyping method was used to successfully and reliably genotype *ADH1B* and *ALDH2* polymorphisms without DNA isolation using a 1.2-mm disc of dried blood and the KOD FX PCR enzyme kit. SNP genotyping was performed by a polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method. In addition to the labor and expense advantages, the possibility of sample contamination was considerably decreased, since the DNA extraction step was eliminated. In the post-genome era, a simple and inexpensive method for diagnostic analysis is in high demand, and this method will be very useful for genetic diagnoses in biological and medical laboratories.

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Introduction

The use of single nucleotide polymorphism (SNP) genotyping methods is expected to improve our understanding of the genetic basis of complex diseases, simplify diagnosis, and help to realize the potential of pharmacogenetics.[1,2] To meet the demands of medicine in the post-genome era, high-throughput, straightforward, and high-performance genotyping is required. Various assays have been developed to analyze SNP genotypes, including direct DNA sequencing after polymerase chain reaction (PCR) amplification,[3] PCR-restriction fragment length polymorphism (PCR-RFLP),[4] TaqMan PCR,[5] and allele-specific primer-PCR (ASP-PCR).[6] SNP genotyping can generally be divided into two steps: sample preparation, typically purification of DNA from biological specimens, such as blood and allele detection. The most basic method to isolate DNA from biological materials requires multiple steps, including Pro-K/detergent treatment, phenol/chloroform extraction, cold ethanol precipitation, and sample reconstitution in a buffer. This process is labor-intensive, time-consuming, costly; it also enhances the risk of cross-contamination. Usually, the time-consuming and expensive process of DNA extraction is unavoidable to perform PCR. To overcome this challenge, we recently detailed an improved ASP-PCR genotyping system that uses a direct PCR analysis of terminal hair root samples.[7] Kurihama National Hospital's standard PCR-RFLP methods were usually performed by PCR amplification using genomic DNA from general purification method. The PCR products were digested overnight with *Mae*III and *Mbo*II for *ADH1B* and *ALDH2*, respectively.[8,9] Protocols were recently introduced that allow PCR amplification from whole blood or dried blood spotted on filter paper without DNA extraction.[10-15] Here, we describe a simplified and rapid PCR-RFLP assay that can be performed on blood dried on paper.

In the first step, genomic DNA is amplified directly from whole blood using new designed PCR primers and an amplification enzyme kit, KOD FX, which uses the novel KOD1 DNA polymerase from *Thermococcus kodakaraensis*. This kit effectively neutralizes inhibitors present in biological specimens. As such, this system is also conducive for reproducible and high-throughput direct analysis of whole saliva (including buccal cells) dried on paper. KOD FX results in a much greater PCR yield because it has a higher efficiency and more rapid elongation than other Taq-based PCR enzymes. The PCR products are subsequently digested for 1 h with restriction enzymes, *Msl*I and *Acu*I for *ADH1B* and *ALDH2* which recognize a specific sequence of interest (*e.g.* a SNP site) in the DNA fragment. The digestion of fragments with a restriction fragment length polymorphism (RFLP) produces DNA fragments that vary based on the genotype. These fragments are then analyzed by an automated microchip electrophoresis detection system. Presented here is a specific example of a PCR-RFLP based SNP genotyping assay performed directly on blood dried on paper stored for over one year at room temperature without DNA purification. We also validated our results by comparing them with conventional sequencing and Kurihama's PCR-RFLP methods. To our knowledge, this is the first report of a cost-effective method that detects *ADH1B* and *ALDH2* gene polymorphisms without any prior DNA purification of the dried blood; also these data show that our new PCR-RFLP assay is a sensitive and reliable method.

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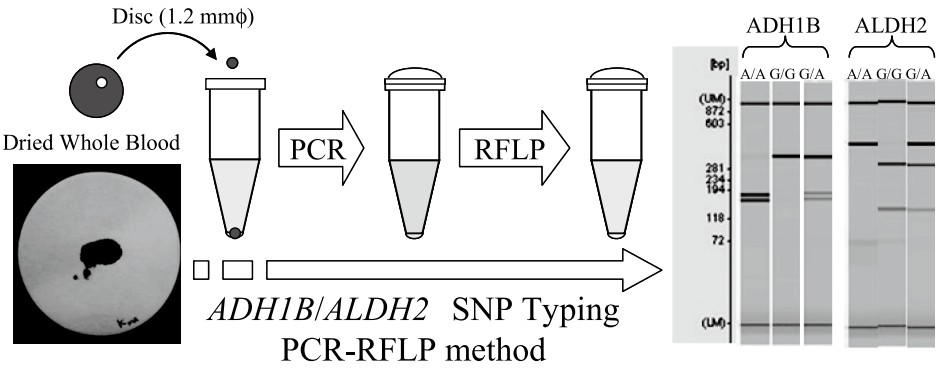

Fig. 1 Outline of the PCR-RFLP genotyping method using human dried whole blood on filter paper.

Experimental

Subjects

We validated our direct PCR-RFLP assay on dried blood discs against samples previously analyzed at the National Institute on Alcoholism, Kurihama National Hospital. In total, samples from 300 Japanese alcoholic patients whose blood had been analyzed by the hospital's standard PCR-RFLP method, were used, with fifty representative patients for each of the combinations of *ADH1B* and *ALDH2* genotypes (*ADH1B**1/*1 and *ALDH2**1/*1, *ADH1B**1/*1 and *ALDH2**1/*2, *ADH1B**2/*2 and *ALDH2**1/*1, *ADH1B**2/*2 and *ALDH2**1/*2, *ADH1B**1/*2 and *ALDH2**1/*1, *ADH1B**1/*2 and *ALDH2**1/*2). The proposed study was reviewed and approved by the ethics committee of this institute, and informed consent was obtained from each participating patient.

Study design

The aim of the present study is to demonstrate an inexpensive and high-throughput genotyping method by detecting polymorphisms of alcohol dehydrogenase subunit β (*ADH1B*)- and aldehyde dehydrogenase 2 (*ALDH2*)-related SNPs by PCR-RFLP using dried whole blood as the starting material. We developed the SNP genotyping method that analyzes blood dried on filter paper by direct PCR-RFLP. As shown in Fig. 1, the procedure is very simple, since all of the necessary PCR reagents as well as a 1.2-mm disc of dried blood were added directly combined in one tube before thermocycling. The direct use of blood dried filter paper considerably decreases the possibility of sample contamination.

Genotyping

Standard SNP genotyping of the *ADH1B* (Arg47His) and *ALDH2* (Glu487Lys) genes was performed using a PCR-RFLP assay on a Gene Amp PCR System 9700 (Applied Biosystems). The amplification conditions for both genes were as follows: an initial denaturing step of 95°C for 5 min, followed by 40 cycles of 98°C for 10 s, 60°C for 30 s, and 74°C for 45 s and a final elongation step of 74°C for 2 min. PCR primers were designed for SNPs of the *ADH1B* and *ALDH2* genes. *ADH1B*-specific allele was amplified with primer set (ADH1B_RFLP_F: 5′-CCTTGGGGATAAACTGAATCTT-3′; ADH1B_RFLP_R: 5′-GAAATCCTGGATGGTGAACC-3′; amplicon 348 bp) and *ALDH2*-specific allele with primer set (ALDH2_RFLP_F: 5′-TCAAATTACAGGGTCAACTGCT-3′; ALDH2_RFLP_R: 5′-GGCTGGGTCTTTACCCTCTC-3′; amplicon 430 bp). The reaction mixture contained 7.5 µL of distilled water, 12.5 µL of 2X PCR buffer for KOD FX, 2.5 µL of 2 mM dNTPs, 10 pmol each primers for *ADH1B* or *ALDH2*, and 0.5 U of KOD FX DNA polymerase (1 U/µL, KFX-101, TOYOBO) in a total volume of 25 µL.

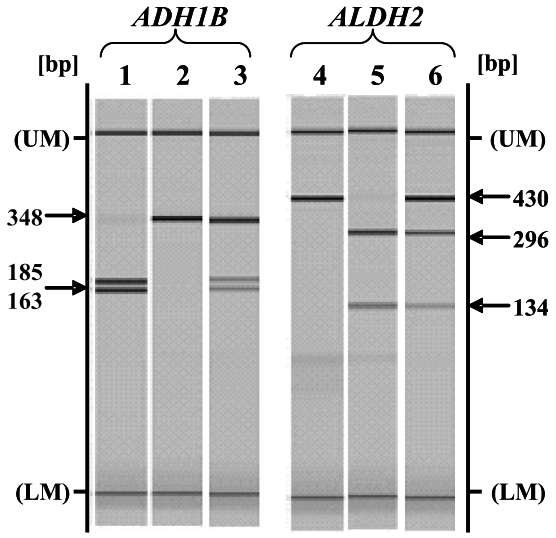

Fig. 2 Automated microchip electrophoresis detection of *ADH1B* and *ALDH2* SNP genotypes. Lane 1, *ADH1B**2/*2 (185, 163 bp); Lane 2, *ADH1B**1/*1 (348 bp); Lane 3, *ADH1B**1/*2 (348, 185, 163 bp); Lane 4, *ALDH2**2/*2 (430 bp); Lane 5, *ALDH2**1/*1 (296, 134 bp); Lane 6, *ALDH2**1/*2 (430, 296, 134 bp). LM, lower marker; UM, upper marker.

After PCR, reactions were centrifuged at 1000*g* for 2 min and supernatants were collected for restriction digestion. Digestions were prepared directly with 6 µL of supernatant with *Msl*I (*ADH1B*) or *Acu*I (*ALDH2*) according to the manufacturer's instruction (New England Biolabs Inc.). The 20 µL reactions were incubated for at least 1 h at 37°C. For the *ADH1B* gene, the 348 bp mutant fragment (2*/2*) was cleaved into two fragments of 185 and 163 bp. The wild-type allele (1*/1*) could not be cut. For the *ALDH2* gene, the 430 bp wild-type fragment (1*/1*) was cleaved into two fragments of 296 and 134 bp. The mutant allele (2*/2*) could not be cut. The resulting fragments were analyzed by automated microchip electrophoresis detection on MultiNA (MCE-202, Shimadzu).

Table 1 Genotype distribution of *ADH1B* and *ALDH2* in all patients

Group	*ADH1B*	*ALDH2*	This work	Kurihama
1	*1/*1	*1/*1	52	50
2	*1/*2	*1/*1	49	50
3	*2/*2	*1/*1	53	50
4	*1/*1	*1/*2	48	50
5	*1/*2	*1/*2	50	50
6	*2/*2	*1/*2	48	50
Total			300	300

Results and Discussion

The results of our study, shown in Fig. 2, demonstrate that direct PCR amplification using a dried blood as a template could be successfully performed in a single tube. Table 1 shows the performance of our direct PCR-RFLP method in determining the *ADH1B* and *ALDH2* genotypes of 300 Japanese alcoholic patients; also the distribution of called genotypes compared with Kurihama data is given in Table 1. Our results showed a slightly different distribution from those of Kurihama. All of the different results were carefully examined by a conventional direct sequencing method. It was suggested that all of our results were exactly correct. Overall, direct PCR-RFLP analysis of dried blood was a highly reliable genotyping method, and it was also faster and less expensive than the commonly used RFLP method or direct sequencing following DNA isolation. In addition, this PCR-RFLP assay was so sensitive that dried whole blood on filter paper was sufficient to obtain successful typing. This PCR assay eliminated the DNA extraction process, which is too laborious, costly, and time-consuming for practical use. In terms of transportation and storage, it is very convenient to use dried whole blood on filter paper as a DNA source because no refrigeration is required. As such, dried blood samples can be collected in remote areas and subsequently analyzed.

In conclusion, we have developed a highly accurate SNP genotyping method that uses only conventional equipment: a PCR thermocycler, an electrophoresis instrument, and an illuminometer. When amplifying DNA directly from dried whole blood, the PCR product contains blood- and PCR-derived components that may interfere with the subsequent digestion. However, we show that the use of the KOD FX kit allows accurate amplification and digestion of DNA; thereby, allowing genotype determination does not require DNA isolation. This PCR-RFLP assay can detect both point mutations and large structural alterations in less than 3 h. Consequently, PCR using a commercially available DNA polymerase kit, KOD FX, is a true single-tube SNP typing method because all of the reagents and the unprocessed dried blood samples are added simultaneously, and no additional steps are required before the PCR. Our genotyping method therefore has great potential for practical use in clinical settings as well as in academic laboratories.

Acknowledgements

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