Duplex PCR-RFLP for the Simultaneous Genotyping of Single Nucleotide Polymorphisms in *ADH1B* and *ALDH2* Genes

Naoto SHIRASU[†] and Shin'ichiro YASUNAGA

Department of Biochemistry, Faculty of Medicine, Fukuoka University, 7-45-1 Nanakuma, Jonan, Fukuoka 814-0180, Japan

Single nucleotide polymorphisms (SNPs) in *ADH1B* and *ALDH2*, which encode alcohol dehydrogenase 1B and aldehyde dehydrogenase 2, respectively, are responsible for ethanol-metabolizing activity and alcohol-related disease. We developed a simple and rapid polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) method for the simultaneous genotyping of the genes in a single-tube reaction. Using an alkaline lysate of a human hair root as a template, SNP-containing regions of each gene were PCR-amplified simultaneously. The PCR products were directly applied to double restriction digestion, followed by agarose gel band analysis. The duplex PCR-RFLP allows for the simultaneous and accurate determination of the SNP genotypes of *ADH1B* and *ALDH2* within 1.2 h.

Keywords Alcohol dehydrogenase, aldehyde dehydrogenase, genotyping, restriction fragment length polymorphism, single nucleotide polymorphism

(Received August 1, 2016; Accepted August 25, 2016; Published December 10, 2016)

Introduction

Single nucleotide polymorphisms (SNPs) constitute the most common form of genetic variation between individuals. SNPs have been demonstrated to be highly associated with the response to drug treatment, the susceptibility to disease, and other phenotypic variations, and offer great potential as diagnostic markers for precision medicine.¹ Various methods for characterizing SNPs have been developed, including the direct sequencing of genomic DNA fragments amplified by the cycle polymerase reaction, polymerase chain reaction-restriction fragment length polymorphism analyses (PCR-RFLP),^{2,3} allele-specific hybridization,⁴ oligonucleotide ligation assays,⁵ real-time PCR using displacing probes,⁶ and TaqMan[®] PCR.^{7,8} However, these methods are generally time-consuming, and require specialized probes and expensive instruments. The allele-specific PCR is a relatively simple and cost-effective method compared with other genotyping techniques,^{9,10} however, multiple reaction parameters must be optimized for effective allele-specific amplification, which is often labor-intensive.¹¹ Moreover, the allelic specificity is easily affected, and sometimes lost by subtle changes of the reaction conditions.

We herein report on a novel PCR-RFLP method for rapidly determining a pair of SNPs (rs1229984 and rs671) in two genes: *ADH1B* encoding alcohol dehydrogenase 1B and *ALDH2* encoding aldehyde dehydrogenase 2. These SNPs have been comprehensively examined as significant risk factors for alcohol use disorders and other alcoholism-related diseases.¹²⁻¹⁴ In this method, we used the MightyAmp[®] system, purchased from TaKaRa (Shiga, Japan); this system includes a powerful DNA

polymerase with an improved PCR buffer for resistance to PCR-inhibiting materials, and can effectively amplify the target genes directly from crude biological specimens. Using one pair each of optimized primers specific to the SNP-containing regions in *ADH1B* and *ALDH2*, a duplex PCR reaction was performed using an alkaline lysate of a human hair root as a template. Subsequently, the restriction digestion of the PCR products was carried out in the same reaction tube by directly applying the FastDigest[®] enzymes obtained from Thermo Scientific (Rockford, IL, USA), which were advanced restriction enzymes that enabled a complete DNA digestion within 10 min in the supplied universal buffer. The FastDigest Green Buffer containing a density reagent and tracking dyes also allowed for direct loading of the reaction mixtures onto an agarose gel. Thus, the fragment size of double-digested samples indicating the concerned SNPs was determined *via* standard electrophoresis without any troublesome steps. To our knowledge, this is the first report of the single-tube PCR-RFLP method for the rapid simultaneous genotyping of the SNPs in *ADH1B* and *ALDH2*.

Experimental

Reagents and subjects

MightyAmp[®] DNA polymerase Ver. 3 was purchased from TaKaRa. Oligonucleotide primers were synthesized by Eurofins Genomics (Tokyo, Japan). Gene fragments containing each allele of *ADH1B* (*ADH1B**1 or *ADH1B**2) and *ALDH2* (*ALDH2**1 or *ALDH2**2) were custom synthesized and cloned into the pIDTSMART-Amp vector (Integrated DNA Technologies, Coralville, IA, USA).¹⁰ FastDigest *MspI* and *TscAI* were purchased from Thermo Scientific. Agarose S was purchased from Nippon Gene (Toyama, Japan). GelRed[™] DNA

[†] To whom correspondence should be addressed.
E-mail: shirasu@fukuoka-u.ac.jp

Fig. 1 Schematic representation of the primers and their binding sites in the duplex PCR-RFLP for simultaneous SNP-genotyping of *ADH1B* and *ALDH2*. (A) *ADH1B* alleles. (B) *ALDH2* alleles. The recognition sequences by restriction enzymes are indicated by white letters on a black background. The cutting sites are indicated by open triangles. The potential *MspI* site located in *ALDH2* is indicated by a dashed box. The bases at the SNP site are underlined. The artificial mismatch base for deletion of *MspI* site is indicated in the sequence of the *ALDH2_RFLP-R6* primer by a capital letter.

stain was purchased from Biotium (Hayward, CA, USA). Hair samples were obtained from 21 healthy Japanese volunteers, who provided their informed consent. The Medical Ethics Committee of Fukuoka University approved of the study protocol.

Template preparation

The PCR template was prepared as described.¹⁰ Briefly, 18 μ l of 50 mM NaOH was added to a microcentrifuge tube containing a human hair root (approximately 1 cm in length). The sample was incubated at 95°C for 10 min, and then 2 μ l of 1 M Tris-HCl (pH 8.0) was added. After brief mixing with a vortex mixer and centrifugation, the resulting debris-free supernatant was used as a template for PCR. To validate the accuracy of our method, each allelic gene-specific pIDTSMART-Amp plasmid DNA was mixed at a final total concentration of 200 fg/ μ l to provide the control for determining a specific genotype among the nine genotypes as follows: *ADH1B**1/1, *ALDH2**1/1; *ADH1B**1/1, *ALDH2**1/2; *ADH1B**1/1, *ALDH2**2/2; *ADH1B**1/2, *ALDH2**1/1; *ADH1B**1/2, *ALDH2**1/2; *ADH1B**1/2, *ALDH2**2/2; *ADH1B**2/2, *ALDH2**1/1; *ADH1B**2/2, *ALDH2**1/2; and *ADH1B**2/2, *ALDH2**2/2.

Genotyping by duplex PCR-RFLP

The target SNP regions of *ADH1B* and *ALDH2* were simultaneously amplified via duplex PCR using the following four carefully designed primers: *ADH1B_RFLP-F4*: 5'-AGGAATAGTAGGGATTAGTAGCAAAACCCTCA-3', *ADH1B_RFLP-R4*: 5'-GATGGTGAACCACACGTGTTC-3', *ALDH2_RFLP-F6S2*: 5'-GAGTTGGGCGAGTACGGGCTGCA-3', and *ALDH2_RFLP-R6*: 5'-AGCAGGTCCCACACTCACAGTTTT-GACTT-3' (Fig. 1). Each 10- μ l PCR reaction mixture contained 5 μ l of 2 $\times$ MightyAmp buffer Ver. 3 (TaKaRa), 0.2 μ l (0.25 U) of MightyAmp DNA polymerase, 0.5 μ l of *ADH1B* primer mixture (6 μ M *ADH1B_RFLP-F4*, 6 μ M *ADH1B_RFLP-R4*), 0.5 μ l of *ALDH2* primer mixture (6 μ M *ALDH2_RFLP-F6S2*, 6 μ M *ADH1B_RFLP-R6*), 2.8 μ l of H₂O, and 1 μ l of template (hair lysate or 200 fg of plasmid mixture). PCR amplification was performed using a thermal cycler GeneAtlas G (ASTEC, Fukuoka, Japan) with the following two-step thermal cycles: 2 min at 98°C, followed by 35 cycles of 98°C for 10 s and 68°C for 8 s. In validation experiments, 3 μ l of the PCR products was added to a new PCR tube containing 6 μ l of 1.5 $\times$ RE solution (4.5 U FastDigest *MspI* and 4.5 U FastDigest *TscAI* in 1 $\times$ FastDigest Green Buffer; TaKaRa). Separately, when performing single-tube genotyping, 20 μ l of the RE solution was directly added to tubes containing 10 μ l of the PCR

Fig. 2 Duplex PCR-RFLP using control plasmid mixtures. The lanes with odd numbers indicate the undigested PCR products, while the lanes with even numbers are the *MspI*/*TscAI*-digested products. Lane M: DNA size marker; lanes 1–2: *ADH1B**1/1, *ALDH2**1/1; lanes 3–4: *ADH1B**1/1, *ALDH2**1/2; lanes 5–6: *ADH1B**1/1, *ALDH2**2/2; lanes 7–8: *ADH1B**1/2, *ALDH2**1/1; lanes 9–10: *ADH1B**1/2, *ALDH2**1/2; lanes 11–12: *ADH1B**1/2, *ALDH2**2/2; lanes 13–14: *ADH1B**2/2, *ALDH2**1/1; lanes 15–16: *ADH1B**2/2, *ALDH2**1/2; lanes 17–18: *ADH1B**2/2, *ALDH2**2/2.

products. Each tube was placed in a thermal cycler and incubated at 37°C for 5 min and at 65°C for another 5 min. The digested samples were electrophoresed on 2% agarose gel containing GelRed dye in 1× TAE (Tris/acetate/EDTA) buffer. Gel images were acquired using an UV transilluminator FAS III (Toyobo, Tokyo, Japan). The genotypes of the *ADH1B* and *ALDH2* loci in each subject were previously determined using direct sequencing of PCR-amplified genomic DNA.¹⁰

Results and Discussion

To achieve rapid and simultaneous genotyping of the SNPs of *ADH1B* and *ALDH2*, we developed a novel PCR-RFLP strategy for duplex amplification and rapid restriction digestion in a single-tube reaction. The key findings of this study were that: 1) the size of the two PCR amplicons (for *ADH1B* and *ALDH2*) should be appropriate for sufficient separation in order to differentiate the bands of pre- or post-digested fragments; 2) restriction enzymes able to single-cut only the fragment derived from the specific allele should be adopted; and 3) the digestion should produce two fragments of the same length (but different between *ADH1B* and *ALDH2* fragments), not only making the discrimination of the bands simple and easy, but also making the signals of shortened fragments very clear. We cautiously chose the restriction enzymes and set primers to meet the requirements described above (Fig. 1).

We selected the sequence of the primers for *ADH1B* while taking care not to obtain any undesirable amplifications from the highly homologous genes, *ADH1A* and *ADH1C*. Since the target sequence of the SNP region in *ALDH2* actually contains an *MspI* recognition sequence meant to digest the *ADH1B* gene fragment, the *ALDH2*_RFLP-R6 primer has a C to G mutation at the 5th position from the 3'-end in order to abolish the site. Thus, the PCR product amplified by the *ALDH2* primer pair is insensitive to *MspI*. To directly amplify the target genes from crude templates, such as hair lysates, MightyAmp DNA polymerase was utilized in the PCR reaction. We also adopted the FastDigest enzymes and their buffer system, which enabled double digestion in the same reaction tube within 10 min. The optimized PCR thermal cycles and the protocol for the digestion

Fig. 3 Genotyping of *ADH1B* and *ALDH2* SNPs in Japanese subjects ((A) #1–10, (B) #11–21) by duplex PCR-RFLP. The genotypes previously determined by direct sequencing are shown within the box outlined by a dashed line. Lane M: DNA molecular marker; lane C: the *MspI*/*TscAI*-digested products derived from the PCR reaction using the control plasmid mixture correspond to an *ADH1B**1/2, *ALDH2**1/2 genotype.

reaction allowed us to complete the whole genotyping procedure in a single tube within 70 min.

To prove the feasibility, we first carried out validation experiments using control plasmid mixtures as a template

Table 1 *ADH1B* and *ALDH2* genotypes determined using duplex PCR-RFLP and direct DNA sequencing

<i>ADH1B</i>	<i>ALDH2</i>	Subject genotyped in this study	Subject genotyped by sequencing
*1/1	*1/1	10	10
*1/1	*1/2	13	13
*1/1	*2/2	—	—
*1/2	*1/1	1, 7, 9, 11, 16	1, 7, 9, 11, 16
*1/2	*1/2	2, 5, 6, 8, 18, 20	2, 5, 6, 8, 18, 20
*1/2	*2/2	—	—
*2/2	*1/1	21	21
*2/2	*1/2	3, 12, 14, 15, 17, 19	3, 12, 14, 15, 17, 19
*2/2	*2/2	4	4

corresponding to each *ADH1B/ALDH2* genotype. The duplex PCR reaction specifically and simultaneously amplified the gene fragments of *ADH1B* and *ALDH2* (Fig. 2). The double digested samples with *MslI/TscAI* clearly demonstrated the correct number and size of bands expected from each genotype (Fig. 2). The *TscAI*-digested *ALDH2**1 fragment showed slightly slower migration than that expected from the molecular size, possibly due to the fact that *TscAI* tends to remain associated with the cleaved DNA. However, this is little problematic in distinguishing between the cleaved and uncleaved fragments.

Next, we determined the *ADH1B* and *ALDH2* genotypes of Japanese individuals ($n = 21$) using the hair root lysates as PCR templates in a single-tube reaction (Fig. 3). No non-specific products, which complicate the band-assignment for SNP-genotyping, were observed. Table 1 summarizes the results of the genotyping by the duplex PCR-RFLP in a single-tube reaction and the previous sequencing analysis. The newly determined genotypes completely matched the previous sequencing results,¹⁰ suggesting that our single-tube PCR-RFLP method is highly accurate, and may be practically applicable in genetic diagnosis.

In conclusion, we developed a simple, rapid, and accurate SNP-genotyping method for the *ADH1B* and *ALDH2* genes in a single-tube reaction. This duplex PCR-RFLP is easy to perform without extensive practice, and it requires only minimal lab

equipment and a short handling and execution time compared with other genotyping procedures, suggesting its suitability for use in small-scale clinical laboratories and for training biomedical students.

Acknowledgements

The authors wish to thank Ms. Hiromi Yamada for her helpful technical assistance.

References

1. D. S. Wishart, *Nat. Rev. Drug Discov.*, **2016**, doi:10.1038/nrd.2016.32.
2. M. Hayashida, K. Iwao-Koizumi, S. Murata, A. Yokoyama, and K. Kinoshita, *Anal. Sci.*, **2010**, *26*, 503.
3. S. Vatansever, F. Tekin, E. Salman, E. Altintoprak, H. Coskunol, and U. S. Akarca, *Bosn. J. Basic Med. Sci.*, **2015**, *15*, 37.
4. W. M. Howell, M. Jobs, and A. J. Brookes, *Genome Res.*, **2002**, *12*, 1401.
5. M. A. Iannone, J. D. Taylor, J. Chen, M. S. Li, P. Rivers, K. A. Slentz-Kesler, and M. P. Weiner, *Cytometry*, **2000**, *39*, 131.
6. Y. Gu and Q. Li, *Clin. Biochem.*, **2007**, *40*, 1325.
7. F. M. De la Vega, K. D. Lazaruk, M. D. Rhodes, and M. H. Wenz, *Mutat. Res.*, **2005**, *573*, 111.
8. M. Hayashida, T. Ota, M. Ishii, K. Iwao-Koizumi, S. Murata, and K. Kinoshita, *Anal. Sci.*, **2014**, *30*, 427.
9. M. Hayashida, K. Iwao-Koizumi, S. Murata, and K. Kinoshita, *Anal. Sci.*, **2009**, *25*, 1487.
10. N. Shirasu and M. Kuroki, *Anal. Sci.*, **2014**, *30*, 1093.
11. S. Little, "Current Protocols in Human Genetics", **2001**, Chap. 9, Unit 9.8.
12. A. J. Birley, M. R. James, P. A. Dickson, G. W. Montgomery, A. C. Heath, N. G. Martin, and J. B. Whitfield, *Hum. Mol. Genet.*, **2009**, *18*, 1533.
13. S. Higuchi, S. Matsushita, T. Muramatsu, M. Murayama, and M. Hayashida, *Alcohol. Clin. Exp. Res.*, **1996**, *20*, 493.
14. R. Li, Z. Zhao, M. Sun, J. Luo, and Y. Xiao, *Life Sci.*, **2016**, *147*, 59.