INTRODUCTION

Alcohol dehydrogenase (EC1.1.1.1) β subunit is an enzyme that converts ethanol to acetaldehyde, whose gene, ADH2, located in 4q22, has a functional polymorphism Arg47His (Matsuo et al., 1989). The enzyme activity is higher in the 47His allele (ADH2*2) than in the 47Arg allele (ADH2*1) (Yin et al., 1984). Aldehyde dehydrogenase (EC1.2.1.3) converts acetaldehyde into acetic acid, whose gene, ALDH2 in 12q24.2, has a G-to-A polymorphism, Glu487Lys. The Glu487Glu (ALDH2*1) allele has full enzyme activity, whereas the 487Lys allele (ALDH2*2) has no activity, resulting in individuals with 487Lys/Lys genotype being unable usually to drink a glass of beer, because of the impairment of acetaldehyde detoxification.

Alcoholism was reported to have associations with ADH2 47Arg and ALDH2 487Glu in Chinese people (Muramatsu et al., 1995; Chen et al., 1999) and in Japanese people (Higuchi et al., 1995). The protective alleles, ADH2 47His and ALDH2 487Lys are more prevalent in Oriental people than in other ethnic groups (Goedde et al., 1992b; Hamajima et al., 2002a). Because alcohol tolerance depends on these genotypes, ADH2 and ALDH2 genotyping may be useful in realizing the dose–response of alcoholism.

Both polymorphisms have been simultaneously examined in several studies (Higuchi et al., 1995; Muramatsu et al., 1995; Wan et al., 1998; Chen et al., 1999; Chao et al., 2000; Wall et al., 2003). The common genotyping method for polymorphisms is polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP). For ADH2 Arg47His, the restriction enzyme is MboII (Goedde et al., 1992). ALDH2 Gly487Lys has been genotyped by a PCR–RFLP method using MboII (Yokoyama et al., 1998) or a mismatch primer making a restriction site of Ksp632I (Takeshita et al., 1994). We genotyped ALDH2 Gly487Lys with PCR with confronting two-pair primers (PCR–CTPP) (Hamajima et al., 2000; Matsuo et al., 2001). This paper reports a newly developed PCR–CTPP for allele-specific DNA amplification of ADH2 Arg47His and ALDH2 Gly487Lys simultaneously in one tube. The duplex PCR–CTPP was applied in hundreds of subjects to demonstrate that its use was practical and applicable.

MATERIALS AND METHODS

Study subjects

The subjects consisted of Japanese people who attended a health check-up programme supported by local government in 2000. The health-check examinees were inhabitants of Nagoya, Japan, mainly unemployed housewives and self-employed men. Written informed consent for the anonymous use of residual blood was obtained after blood draw from the examinees. On average, about 2 ml of blood was left after the routine health check-up tests. Of 489 examinees invited to the study, 468 agreed to provide their residual blood for genetic tests, together with their demographic information. Three of the residual blood samples were not sufficient for DNA extraction and 11 participants with a history of cancer were excluded. The remaining 454 persons (126 male, 328 female) aged 35–85 years were the subjects for this study.

Buffy coat was applied 72 h after blood draw on the residual blood (stored at 4°C after routine laboratory tests for health, and preserved at −80°C). DNA was extracted from theuffy coat using QIAamp DNA Blood Mini Kit (QIAGEN Inc., Valencia, CA, USA).

PCR–CTPP method

The basis of PCR–CTPP for single nucleotide polymorphisms is described in previous papers (Hamajima et al., 2000, 2002b; Hamajima, 2001). Four allele-specific primers
PCR was conducted as follows: 10 min of initial denaturation at 95°C; then 30 cycles of 1 min at 95°C, 1 min of 25°C × 10 PCR buffer including 15 mM MgCl₂. PCR was conducted as follows: 10 min of initial denaturation at 95°C; then 30 cycles of 1 min at 95°C, 1 min of 25°C × 10 PCR buffer including 15 mM MgCl₂. PCR can be performed. PCR–CTPP conditions were established for tens of polymorphisms (Hamajima et al., 2002). However, there are several limitations for PCR–CTPP designing; namely (i) the sequence around the targeted polymorphism should allow four primers with a similar melting temperature (Hamajima et al., 2002b); (ii) monotonous or repeated sequences make it difficult to amplify allele-specific DNA; and (iii) the existence of a pseudogene makes it difficult to find primers specific to the polymorphism under study, though this is a common problem for any PCR.

To date, we have reported duplex PCR–CTPP for two sets of polymorphisms (Hamajima et al., 2002c). However, there are several limitations for PCR–CTPP designing; namely (i) the sequence around the targeted polymorphism should allow four primers with a similar melting temperature (Hamajima et al., 2002b); (ii) monotonous or repeated sequences make it difficult to amplify allele-specific DNA; and (iii) the existence of a pseudogene makes it difficult to find primers specific to the polymorphism under study, though this is a common problem for any PCR.

The Hardy–Weinberg equilibrium tests indicated no discrepancy between allele and genotype frequencies, thus partly supporting correct genotyping. In addition, both polymorphisms distributed in similar to those reported in other studies on Japanese people. Yokoyama et al. (2002) reported that the genotype frequencies were 4.9% Arg/Arg, 34.7% Arg/His and 60.4% His/His for ADH2 Arg47His, and 53.8% Glu/Glu, 39.4% Glu/Lys and 6.8% Lys/Lys for ADH2 Glu487Lys, in 634 Japanese health check-up examinees. Takeshita et al. (1996) showed the frequencies to be 66.6% Arg/Arg, 31.8% Arg/His and 61.6% His/His for ADH2 Arg47His, and 55.4% Glu/Glu, 37.7% Glu/Lys and 6.8% Lys/Lys for ADH2 Glu487Lys among 424 male subjects.

PCR–CTPP is based on competitive DNA amplification, which usually gives a wider range of optimal annealing temperatures than allele-specific PCR for each allele (Hamajima et al., 2002b). The finding that the duplex PCR–CTPP made clearer the contrast of the amplified DNA for some polymorphisms than did single PCR–CTPP adds potential to the use of multiplex PCR–CTPP.

Because PCR–CTPP does not need incubation time with a restriction enzyme, the time and costs of the digestion of PCR products can be saved. This method is applicable for any laboratory where PCR can be performed. PCR–CTPP conditions were established for tens of polymorphisms (Hamajima et al., 2002c). However, there are several limitations for PCR–CTPP designing; namely (i) the sequence around the targeted polymorphism should allow four primers with a similar melting temperature (Hamajima et al., 2002b); (ii) monotonous or repeated sequences make it difficult to amplify allele-specific DNA; and (iii) the existence of a pseudogene makes it difficult to find primers specific to the polymorphism under study, though this is a common problem for any PCR.

To date, we have reported duplex PCR–CTPP for two sets of polymorphisms (Hamajima, 2001). One was for interleukin 1B C-31T and interleukin 1RN 86-bp variable number of tandem repeat polymorphisms. The other was complicated.

### RESULTS

Figure 2 shows the nine combinations of ADH2 Arg47His and ALDH2 Glu487Lys. The amplified DNA are 219 base pair (bp) for 47Arg (ADH2*1), 280 bp for 47His (ADH2*2), 119 bp for 487Glu (ALDH2*1), and 98 bp for 487Lys (ALDH2*2), as well as common bands with 459 bp for ADH2 and 176 bp for ALDH2. The bands were clear enough to genotype the samples correctly.

In the process of finding optimal conditions, it was evident that the duplex PCR–CTPP provides a clearer contrast than the PCR–CTPP solely for ADH2. As shown in Fig. 3, a weak band was observed for a spurious Arg allele band in His/His genotype (Lane 1) and for a spurious His allele band in Arg/Arg genotype (Lane 3); however, the bands disappeared in duplex PCR–CTPP (Lanes 4 and 6, respectively). A similar finding was not observed for single ALDH2 PCR–CTPP genotyping.

Table 2 shows the genotype distributions for 454 subjects, with two unsuccessful samples for ADH2 and one unsuccessful sample for ALDH2, through two independent PCR–CTPP for the unsuccessful samples. The observed genotype frequencies were within the Hardy–Weinberg equilibrium ($\chi^2 = 0.010$, $P = 0.922$ for ADH2 Arg47His; $\chi^2 = 0.345$, $P = 0.557$ for ALDH2 Glu487Lys).

### DISCUSSION

The Hardy–Weinberg equilibrium tests indicated no discrepancy between allele and genotype frequencies, thus partly supporting correct genotyping. In addition, both polymorphisms distributed in similar to those reported in other studies on Japanese people. Yokoyama et al. (2002) reported that the genotype frequencies were 4.9% Arg/Arg, 34.7% Arg/His and 60.4% His/His for ADH2 Arg47His, and 53.8% Glu/Glu, 39.4% Glu/Lys and 6.8% Lys/Lys for ADH2 Glu487Lys, in 634 Japanese health check-up examinees. Takeshita et al. (1996) showed the frequencies to be 66.6% Arg/Arg, 31.8% Arg/His and 61.6% His/His for ADH2 Arg47His, and 55.4% Glu/Glu, 37.7% Glu/Lys and 6.8% Lys/Lys for ADH2 Glu487Lys among 424 male subjects.

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**Table 1. Primers for alcohol dehydrogenase 2 (ADH2) Arg47His and aldehyde dehydrogenase 2 (ALDH2) Glu487Lys genotyping**

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ADH2 Arg47His</strong></td>
<td>F1: 5′ GGG CTT TAG ACT GAA TAA CCT TGG R1: 5′ AAC CAC GTG GTG TGC ATC TGG GGC</td>
</tr>
<tr>
<td></td>
<td>F2: 5′ GGT GGC TGT AGG AAT CTA TGA R2: 5′ AGG GAA AGA AGA AAC TCC TGA A</td>
</tr>
<tr>
<td><strong>ALDH2 Glu487Lys</strong></td>
<td>F1: 5′ TGC TAT GAT GTG TTT GGA GCC R1: 5′ CCC ACA CTC ACA GTT TCC ACT TGC</td>
</tr>
<tr>
<td></td>
<td>F2: 5′ GGG CTG CAG GCA TAC ACT A R2: 5′ GGC TCC GAG CCA CCA</td>
</tr>
</tbody>
</table>

Underlining indicates the polymorphic base.
PCR–CTPP for Se, sej, and se5 alleles of fucosyltransferase 3. They are not duplex for two independent PCR–CTPP, but the combination of PCR–CTPP and ordinary PCR. These successful examples provide further possible inexpensive and time-saving methods with which to genotype a set of polymorphisms. With ADH2 Arg47His and ALDH2 Glu487Lys, separated genotyping with PCR–RFLP is common. As the elimination of the incubation time shortens the overall

Fig. 2. Representative gels for alcohol dehydrogenase 2 (ADH2) Arg47His and aldehyde dehydrogenase 2 (ALDH2) Glu487Lys by polymerase chain reaction with confronting two-pair primers (PCR–CTPP). Lane M: 100 bp ladder. Lanes 1–9 are: His/His and Glu/Glu; His/His and Glu/Lys; His/His and Lys/Lys; Arg/His and Glu/Glu; Arg/His and Glu/Lys; Arg/His and Lys/Lys; Arg/Arg and Glu/Glu; Arg/Arg and Glu/Lys; and Arg/Arg and Lys/Lys.

Table 2. Genotype frequencies of alcohol dehydrogenase 2 (ADH2) Arg47His and aldehyde dehydrogenase 2 (ALDH2) Glu487Lys for 454 Japanese health check-up examinees

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotype</th>
<th>Allele</th>
<th>n</th>
<th>%</th>
<th>n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADH2 Arg47His</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Arg</td>
<td></td>
</tr>
<tr>
<td>Arg/Arg</td>
<td>19</td>
<td>4.2</td>
<td>Arg</td>
<td>187</td>
<td>20.7</td>
<td></td>
</tr>
<tr>
<td>Arg/His</td>
<td>149</td>
<td>33.0</td>
<td>His</td>
<td>717</td>
<td>79.3</td>
<td></td>
</tr>
<tr>
<td>His/His</td>
<td>284</td>
<td>62.8</td>
<td>Total</td>
<td>904</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>452</td>
<td>100</td>
<td></td>
<td></td>
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<tr>
<td>Hardy–Weinberg equilibrium: P = 0.922</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ALDH2 Glu487Lys</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Glu</td>
<td></td>
</tr>
<tr>
<td>Glu/Glu</td>
<td>221</td>
<td>48.8</td>
<td>Glu</td>
<td>629</td>
<td>69.4</td>
<td></td>
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<tr>
<td>Glu/Lys</td>
<td>187</td>
<td>41.3</td>
<td>Lys</td>
<td>277</td>
<td>30.6</td>
<td></td>
</tr>
<tr>
<td>Lys/Lys</td>
<td>45</td>
<td>9.9</td>
<td>Total</td>
<td>906</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>453</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hardy–Weinberg equilibrium: P = 0.557</td>
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</table>

Fig. 3. DNA bands. Bands shown are those for ADH2 Arg47His (Lanes 1–3), and those for ADH2 Arg47His and ALDH2 Glu487Lys by duplex polymerase chain reaction with confronting two-pair primers (PCR–CTPP) (Lanes 4–6); Lane M: 100 bp ladder.
genotyping time to less than half, the present duplex PCR–CTPP reduces the overall time to less than one-quarter.

Genotyping of both polymorphisms could be applied to alcoholism, as well as to the risk estimation of alcohol-related diseases (Yokoyama et al., 2002). Persons aware of their own genotypes could adjust alcohol intake to avoid risk. Behaviour changes resulting from the recognition of one’s genotype would be an interesting theme for examination by those involved in health promotion.

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REFERENCES


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