nproved Methods for Genotype Determination of Human Alcohol Dehydrogenase (ADH) at 
DH 2 and ADH 3 Loci by Using Polymerase Chain Reaction-Directed Mutagenesis

ezio Groppi, Joel Begueret, and Albert Iron

The human gene for producing alcohol dehydrogenase (ADH; EC 1.1.1.1) is polymorphic at ADH 2 and ADH 3 loci. Until now, the study of this polymorphism required liver biopsy or allele-specific radioactive probes. We have used PCR to amplify and analyze the genotype of ADH 2 and ADH 3 loci. This allowed us to determine directly, and unambiguously, the complete genotype at these two loci by using a mix of radioactive and nonradioactive probes.

The rate-limiting step in the metabolism of ethanol is its oxidation to acetaldehyde, catalyzed by isoenzymes of alcohol dehydrogenase (ADH; alcohol:NAD+ oxidoreductase, EC 1.1.1.1). This cytosolic Zn-containing enzyme consists of a mixture of isoenzymes, which are composed of five subunits: \( \beta, \gamma, \pi, \) and \( \chi \), encoded, respectively, by the genes ADH 1, DH 2, ADH 3, ADH 4, and ADH 5. ADH 1, ADH 2, and DH 3 are closely related; their cDNA sequences are 94% identical (1–4). Polymorphism in ADH 2 and ADH 3 genes is due to three \( \beta \) subunits \((\beta_1, \beta_2, \beta_3)\) and to two \( \gamma \) subunits \((\gamma_1, \gamma_2)\). These polymorphisms are not due to the \( \beta \) subunits (5–7) and to two \( \gamma \) subunits (8). The \( \beta \) subunit, ADH 1, ADH 2, and ADH 3 differ by amino acid substitutions that affect the kinetic properties of the isoenzymes. Not all heavy drinkers (only 12–31%) develop cirrhosis (0). Perhaps this heterogeneity of response could be related to heterogeneity in the genetic background. Because the major role of ADH in the metabolism of ethanol, the udy of ADH genotype in each individual might establish linkage between genotype and the development of cirrhosis in heavy consumers of ethanol. Thus far, determination of this phenotype has required liver biopsy, followed by electrophoresis to determine the isoenzymes, an approach that does not allow studies of large populations. Recently, using polymerase chain reaction (PCR) (9) and radioactive probes, researchers have described methods to determine the genotype of ADH at the two loci, ADH 2 and ADH 3 (10, 12, 14). Here we report an improvement of these methods by which we can determine unambiguously the complete genotype at ADH 2 and ADH 3 loci, using a mix of radioactive and nonradioactive probes.

Materials and Methods

Blood Samples

Human whole-blood samples were stored at −20°C after addition of citrate, EDTA, or heparin. Venous blood was blotted onto 3MM filter paper (Whatman, Clifton, NJ), dried, and stored at room temperature.

PCR

Whole blood (with citrate, EDTA, or heparin). We performed PCR with 3 μL of blood. The erythrocytes were lysed by adding 100 μL of NaCl (37 mmol/L). The leukocytes were pelleted by centrifugation (1 min, 10,000 × g). The supernatant fluid was carefully removed and discarded. The pellet was suspended in 1.5 μL of proteinase K medium (14) containing, per liter, 5 mmol Tris · HCl (pH 7.6), 40 mmol of dithiothreitol, 3.4 μmol of sodium dodecyl sulfate, 50 mg of proteinase K (EC 3.4.21.14), and 0.1 μmol of each primer. We overlaid the mixture with 100 μL of mineral oil and incubated it at 37°C for 75 min. After adding 3 μL of water, we denatured the proteinase K by heat treatment (12 min at 95°C) and then cooled the mixture at room temperature. The volume of the reaction mixture was adjusted to 50 μL with PCR mixture containing, per liter, 67 mmol of Tris · HCl (pH 8.8), 12.5 mmol of (NH₄)₂SO₄, 2 mmol of MgCl₂, 2 g of bovine serum albumin, 10 mmol of β-mercaptoethanol, 50 mL of dimethyl sulfoxide, 200 μmol of each deoxynucleotide triphosphate, and 1 μmol of each primer. Genomic DNA was denatured by heating for 12 min at 95°C. The thermostable DNA polymerase extracted from Thermus aquaticus (Amersham Int., Amersham, Bucks, U.K.) was then added (0.5–1 U per 50 μL) and used for 35 cycles of enzymatic amplification (programmed times: 1 min at 55°C, 10 min at 70°C, and 1 min at 95°C in the Perkin-Elmer Cetus (Norwalk, CT) Thermal Cycler).

Blotted venous blood. We punched out 0.5-mm-diameter discs of filter paper onto which blood had been blotted. The discs were placed directly in the reaction mixture (100 μL) and overlaid with 100 μL of mineral oil. Enzymatic amplification by PCR was then performed as described above.

Allele Detection by Restriction Enzyme Digestion

For allele detection, we precipitated the amplification mixture with ethanol. Samples were digested for 2 h with Mae III for detection of ADH₃, with Alu I for detection of ADH₂, and with Sap I for differentiation of ADH₂ and ADH₃ under the supplier's (Boehringer Mannheim, Mannheim, F.R.G.) conditions. Aliquots were run on 10% polyacrylamide gels, stained with ethidium bromide, and photographed on Polaroid Type 665 film.

Results and Discussion

Genotype at the ADH 2 locus. Three alleles are known for the ADH 2 locus: ADH₂₁, ADH₂₂, and ADH₂₃, coding for the \( \beta_1, \beta_2, \) and \( \beta_3 \) subunits, respectively. Therefore, there are six possible genotypes for this locus. We have devised a method for detection of each allele, based on restriction fragment length polymorphism (RFLP), i.e., on the absence or presence of a restriction site characteristic of each allele after PCR amplification of specific regions. With this method we can determine whether the allele is present in a
homzygous or a heterozygous state. The complete genotype at the ADH 2 locus is determined by the compilation of the results for each allele analysis (Table 1).

Identification of the ADH₂ allele. The ADH₂ detection strategy is shown in Figure 1. The β₂ subunit differs from both β₁ and β₃ by the presence of His instead of Arg at position 47 (15, 16). This single amino acid substitution results from the substitution of a guanine (in ADH₂) and ADH₃ by an adenine (in ADH₂) in the coding sequence of the third exon (Figure 1). This modification creates a Mae III restriction site. The location of the primers for amplification of this region was chosen for the following reasons. First, 35 basepairs (bp) from this ADH₂ characteristic Mae III site is another Mae III site, present in all ADH genes (Figure 1). Including this second site in the amplified fragment provided an internal control for the digestion procedure (incomplete digestion of the amplified DNA fragment can lead to confusion between β₁β₂ and β₂β₃ genotypes). Thus, including this internal control for digestion allows us to determine the genotype unambiguously. Moreover, taking into account the homology between the three loci—ADH 1, ADH 2, and ADH 3—we chose the primers in regions of ADH 1, ADH 2, and ADH 3 that differ, to avoid amplification of the other ADH genes. This specificity was controlled by the digestion of the amplified DNA with Kpn I, which has a site only in ADH 1, and with Pst I, which has a site only in ADH 3 (data not shown). Figure 2 shows the result of the ADH₂ detection after digestion of the amplified region with Mae III.

Identification of the ADH₂ allele. The ADH₂ detection strategy is summarized in Figure 3. The β₂ subunit differs from both β₁ and β₃ by the presence of Cys instead of Arg at position 369 (7). This single amino acid substitution results from the substitution of a cytosine (in ADH₂) and ADH₃ by a thymine (in ADH₂) in the coding sequence of the ninth exon. But in this case, this modification does not give rise to an RFLP. This particular situation led us to develop a new strategy. Using PCR-directed mutagenesis, we created a restriction site specific for the ADH₂ sequence. First we amplified the region surrounding the codon for amino acid 369, producing a 232-bp fragment (Figure 3). The location of the primers for this amplification was chosen according to Xu et al. (12) for the primer 424 and to amplify only the ADH 2 gene for the primer 290. A second amplification was then performed with 1/40 000 of the first amplification mixture as template and the primers 352 and 290 (see Figure 3). The primer 352 contains a G/A mismatch. Thus, this PCR-directed mutagenesis creates the sequence AGCT for ADH₁ and ADH₂ and the sequence AGCT for ADH₂, which is the ALU I recognition site (see Figure 3). Moreover, there are two other ALU I restriction sites in the amplified segment, which provide an internal control for the digestion procedure. Because one of these ALU sites is present only in the ADH 2 gene, the digestion with ALU I verifies that only the ADH 2 gene was amplified.

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<th>Table 1. Genotype at the ADH 2 Locus</th>
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<td>Possible genotypes</td>
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<td>ADH₂ (β₂)</td>
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<td>ADH₃ (β₃)</td>
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Digestion profile H indicates the homzygous presence of an allele; h, the heterozygous presence of an allele; and 0, the absence of an allele.

Figure 4 shows the result of the ADH₂ detection after digestion with ALU I of the amplified segment.

Genotype at the ADH 3 locus: detection of ADH₁ and ADH₂ allele. The strategy for the identification of ADH and ADH₂ is summarized in Figure 5. There are two allele at the ADH 3 locus: ADH₁ and ADH₂ coding, respectively, for γ₁ and γ₂ subunits. The γ₁-subunit differs from γ₂ by two amino acid substitutions, Arg → Gin at position 271 and I → Val at position 349 (6, 17). We have chosen to amplify 145-bp segment of the eighth exon surrounding the code...
iterozygous products with mutagenesis. This modification creates an Ssp I site in ADH2. But in this case, there is no other Ssp I site in this region. To provide an internal control for digestion, we have created a new Ssp I site, using PCR-directed mutagenesis. Thus, the primer 321 contains two mismatches, giving rise to the sequence AATT, which is recognized by the enzyme Ssp I. Moreover, the location of the two primers 321 and 351 was chosen to amplify only the ADH3 gene. This specificity was controlled by digestion of the amplified DNA with Nco III, which has one site in ADH2 and ADH3, but none in ADH3 (data not shown). Figure 5 shows the result of the differentiation between ADH2 and ADH3 after Ssp I digestion of the 145-bp amplified segment.

In summary, we can now determine unambiguously and very quickly the genotype at ADH2 and ADH3 loci. This method presents improvements with good reliability. Particularly, the use of an internal control for digestion avoids confusions from incomplete digestions. Second, this method requires only a microsample of blood, either treated with citrate, EDTA, or heparin, or blotted on paper (results obtained with these methods are identical). In the last case, the method of sampling offers great advantages in storage and handling. The genotype can be determined unambiguously by PCR-directed mutagenesis without use of allele-specific oligonucleotidic probes, which require more manipulations and are less easy to use than RFLP. Using these strategies, we will compare the genotype at ADH2 and ADH3 loci of a healthy Caucasian population with a cirrhotic Caucasian population. We will also study the polymorphism of ADH in three healthy populations—Caucasians, Asians, and Africans—to estimate frequency variations of different alleles between these three populations. Studies are in progress to define a strategy to study the genotype at the ADH5 locus.

This work was supported by grants from INSERM and from Conseil Régional d’Aquitaine.

References