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A New Approach for the Rapid Detection of Common and Atypical Aldehyde Dehydrogenase Alleles

By *Guang-chou Tu*[1] and *Yedy Israel*[1,2]

[1] *Addiction Research Foundation of Ontario, Toronto, Ontario, Canada*
[2] *Departments of Pharmacology and Medicine, University of Toronto, Toronto, Ontario, Canada*

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Summary: A strong protection against the development of alcoholism is exerted by a point mutation in the gene coding for low K_m aldehyde dehydrogenase (ALDH), i.e. $ALDH_2$. We report a non-radioactive method for determining the common and atypical human mitochondrial aldehyde dehydrogenase ($ALDH_2$) genotypes. This method is based on the fact that the base change (G → A) in Exon 12 of the $ALDH_2$ gene abolishes an *Eco*57 I restriction site (CTGAAG → CTAAAG). A GC-clamp attached oligonucleotide was designed to yield a 176 base pair product by the polymerase chain reaction. After amplification, the resulting fragment containing the normal nucleotide sequence is cut by *Eco*57 I into two segments (131 base pairs + 45 base pairs) while the fragment containing the mutated sequence remains intact (176 base pairs). These are visualized by staining with ethidium bromide on agarose gels without blotting, hybridization or autoradiography.

Introduction

A single base substitution (G/A) in Exon 12 of mitochondrial aldehyde dehydrogenase ($ALDH_2$)[1]) gene causes the loss of the activity of the enzyme coded (1). Individuals carrying this gene mutation are unable to rapidly detoxify acetaldehyde produced in the metabolism of ethanol. When alcohol is consumed by these individuals, blood acetaldehyde concentrations are markedly increased, reaching values which lead to the development of facial flushing, hypotension, tachycardia, nausea and in some cases vomiting (2, 3).

Racial differences in $ALDH_2$ alleles between Caucasians and Orientals have been found. Approximately 30 – 50% of Orientals have the mutant gene, while virtually all Caucasians examined thus far have the usual alleles (4, 5).

The existence of the $ALDH_2$ gene mutation has been shown to drastically lower the prevalence of alcohol dependence. In Japan, only 5% of alcohol dependent patients present the mutation compared with about 40% for the normal population (6). Thus, the mutation exerts an 80 – 90% reduction in the risk of becoming an alcoholic. In Taiwan, the protection is of the order of 75% (7).

In order to elucidate the possible association between $ALDH_2$ genotypes, alcohol sensitivity, alcoholism, and other alcohol-related morbidities, it is important to develop a simple and reliable genotyping method.

In 1987, *Hsu* et al. (8) first developed a method for determining human $ALDH_2$ genotypes. These investigators used two 21-base synthetic oligonucleotides, one complementary to the usual $ALDH_2$ gene and the other complementary to the atypical $ALDH_2$ gene, as specific probes for in-gel hybridization analysis of human genomic DNA. Under appropriate hybridization conditions, these two probes hybridize to their specific complementary alleles and thus allow the genotyping of the $ALDH_2$ locus.

In 1989, *Crabb* et al. (9) developed a method for $ALDH_2$ genotyping based on the amplification of

[1]) Enzymes: Aldehyde dehydrogenase (aldehyde : NAD^+ oxidoreductase, EC 1.2.1.3)
Eco 57 I (restriction endonuclease, EC 00.00.00.0)

genomic DNA by the polymerase chain reaction (PCR). This method is faster and requires smaller amounts of DNA (2 μg or less) than methods that use probing of *Southern* blots of genomic DNA digested with restriction endonuclease. *Singh* et al. (5) and *Enomoto* et al. (10) improved the method by employing the dot-blot and slot-blot hybridization.

The above methods are time-consuming and require the use of radioactive phosphorous (^{32}P). We have developed a simple non-radioactive method in which the gene fragment containing either the mutated or the normal sequence is amplified by the PCR reaction and is then cut by a new restriction enzyme (*Eco*57 I)[1]) that recognizes *only* the normal (CT*G*AAG), but not the mutated (CT*A*AAG) nucleotide sequence. Following enzymatic digestion, separation by electrophoresis and staining by ethidium bromide, two distinct bands from the fragment containing the normal sequence, can be seen on agarose gels. The mutated sequence, not recognized by *Eco*57 I, yields only one band.

Materials and Methods

Whole blood and hair root samples

Samples of whole blood and hair roots were collected from 12 individuals working at the University of Toronto. The volunteer number is indicated by the number or the subscript in figures 2–4. [Chinese (#1–5, #8–9), Caucasians (#6, #12), Chileans (#7, #11), Indian (#10)].

Genomic DNA

Genomic DNAs were prepared from either 50 μl of whole blood or 10 hair roots as described in a previous paper (11). The samples were dissolved in 20 μl of buffer (10 mmol/l Tris-HCl, pH 8.0 and 1 mmol/l EDTA).

Oligonucleotides

Two polymerase chain reaction (PCR) primers and two $ALDH_2$ allele-specific probes were synthesized on an Applied Biosystems 380B DNA synthesizer. The base sequences of two $ALDH_2$ allele-specific ^{32}P-probes are the same as these used by *Hsu* et al. (8), *Crabb* et al. (9) and *O'Dowd* et al. (11). The base sequence of PCR primer #1 (23 mer) and primer #2 (60 mer) are: 5′-CAAATTACAGGGTCAACTGCTAT-3′ and 5′-CCGCGCC CGCCGCCCCGCGCCCCCCCGCCCGCCCCGCGCTCCAC ACTCACAGTTTTCAC-3′, respectively.

Polymerase chain reaction

A 10 μl aliquot of the genomic DNA, extracted from either whole blood or hair roots as indicated above, was mixed with 100 pmol of each primer and submitted to the polymerase chain reaction using GeneAmp PCR Reagent kit with Ampli *Taq* DNA Polymerase (Perkin-Elmer Cetus). The amplification temperature conditions were as follows: 94 °C for 5 min followed by 30 cycles consisting of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C; the final extension was 7 min at 72 °C. After amplification, 10 μl of each reaction mixture were submitted to agarose gel electrophoresis analysis or enzymatic digestion.

*Eco*57 I digestion

A 10 μl aliquot of the PCR reaction mixture was mixed with 2 μl of buffer (100 mmol/l Tris-HCl, pH 7.5, 100 mmol/l $MgCl_2$), 1 μl of 0.2 mmol/l S-adenosyl-*L*-methonine, 1 μl of 2 g/l bovine serum albumin, 5.5 μl of distilled water and 0.5 μl of *Eco*57 I (4 units/μl, Fermentas, Lithuania; distributed by New England Biolabs). The digestion was carried out at 37 °C for 1 h and stopped by adding 1 μl of 0.5 mol/l EDTA. After digestion the fragments were separated on a 1.5% or 2% agarose gel and stained with ethidium bromide.

Slot-blot-hybridization

A 10 μl aliquot of the PCR reaction mixture was submitted to the slot-blot hybridization described by *Enomoto* et al. (10) on a Hybri-slot Manifold (BRL Life Technologies Inc., MD 20877, U.S.A.). After blotting, the duplicate nitrocellulose filters were treated in a Stratalinker UV Crosslinker (Stratagene, CA 92037, U.S.A.), then probed respectively with two allele-specific ^{32}P oligonucleotides, for $ALDH_2^1$ and $ALDH_2^2$, as described by *Hsu* et al. (8), using the prehybridization and hybridization conditions outlined by *Crabb* et al. (9). Each filter was exposed to Kodak XAR-5 film for several hours, with one intensifying screen.

Results and Discussion

Although the recognition sequence for *Eco*57 I is in Exon 12 where the Oriental point mutation occurs, the cutting point of the restriction enzyme is located 6 to 8 nucleotides downstream from the exon (fig. 1). In order to distinguish a digested PCR amplified fragment from an undigested one, it was necessary to amplify a PCR fragment at least 50 base pairs longer than Exon 12. Unfortunately, except for the first 10 bases, the sequence of intron 12 of the $ALDH_2$ gene is not available. Thus it is not possible to design a primer that is fully complementary to the sequence of intron 12.

To solve this problem we designed a primer by extending a 41 base pair of GC-clamp at the 5′-end of a 19 mer oligonucleotide which is complementary to the exon 12-intron 12 known sequence of the $ALDH_2$ gene (fig. 1). Using this primer (designated as Primer #2) and the other primer (designated as Primer #1, see fig. 1) a 176 base pair fragment was amplified from genomic DNA by the PCR and visualized with ethidium bromide on the agarose gel (fig. 2).

After digestion with *Eco*57 I, the 176 base pair fragment from 10 subjects (including 5 Orientals) were found to be cut into two segments (131 base pairs and 45 base pairs), while the fragment from subject 4 remained intact as one fragment and the fragment from subject 5 was partially (40%) cut as shown in figure 3 (the 45 base pair bands overlap with the 60 pair primer #2 bands).

These results show that sample 4 was from an individual who carries a homozygous atypical $ALDH_2$

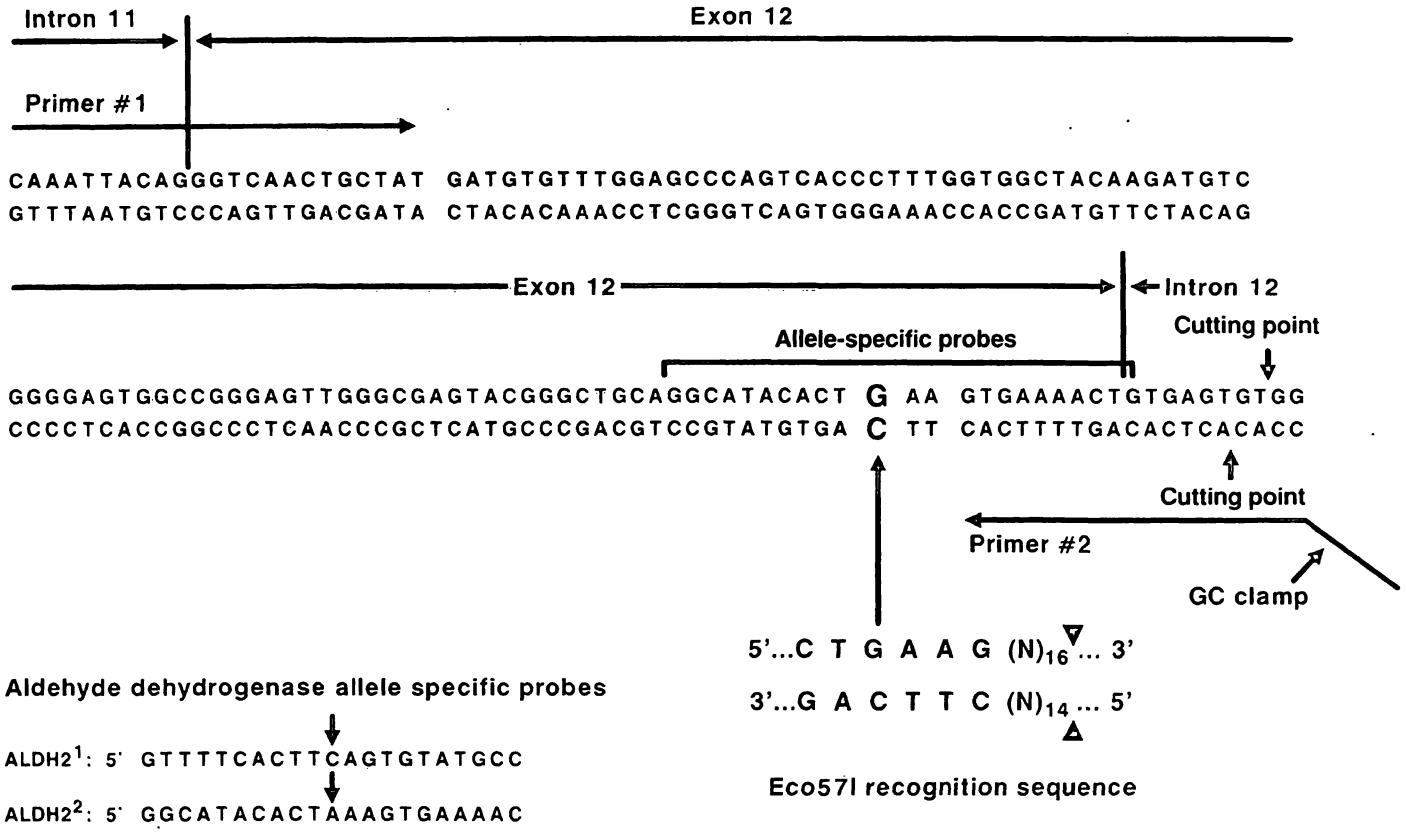

Fig. 1. Structure of the common $ALDH_2$ gene in the region amplified by the polymerase chain reaction. Two oligonucleotides, primer #1 and primer #2, serve to initiate DNA synthesis of the 176 base pair fragment. The nucleotide bases in bold type indicate the position of the mutation in the atypical allele. The two allele-specific probes are those described by *Hsu* et al. (8) and by *Crabb* et al. (9). The site of the single base change in the oligonucleotide $ALDH_2^2$ is indicated by an arrow. The oligonucleotides anneal to complementary DNA strands.

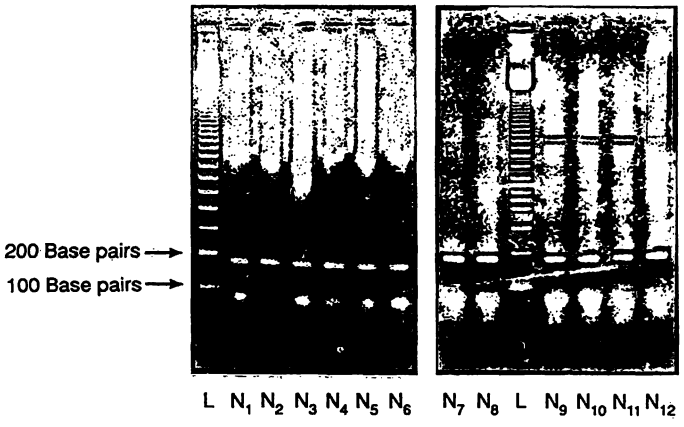

Fig. 2. The genomic DNA from whole blood or hair root samples was amplified, using the primers described in Materials and Methods, and subjected to electrophoresis in 2% agarose gel, followed by ethidium bromide staining. Lane L is 100 base pair ladder. Lanes N_{1-12} are samples from subjects 1–12 (not digested with restriction enzyme). The major band generated corresponds to the 176 base pair product.

gene ($ASDH_2^2/ALDH_2^2$), sample 5 from the individual who carries the heterozygous $ALDH_2$ gene ($ALDH_2^1/ALDH_2^2$) and samples 1–3 and 6–12 were taken from the individuals who carry the common homozygous $ALDH_2$ gene ($ALDH_2^1/ALDH_2^1$).

In order to confirm the new method, the PCR products were transferred to nitrocellulose by the slot-blot technique and the filters were hybridized with the two specific $ALDH_2$ allele ^{32}P-labelled probes. After washing, the filters were exposed to X-ray film. As shown in figure 4 the DNA amplified from sample 4 was only recognized by probe $ALDH_2^2$, the DNA amplified from sample 1–3 and 6–12 were only recognized with probe $ALDH_2^1$, and the DNA amplified from sample 5 was recognized with both the probe for $ALDH_2^1$ and the probe for $ALDH_2^2$. The new method gives the same results as the *Southern* blot radioactive method (5, 8–11).

According to the *Eco57* I manufacturer's information, the cleavage of DNA by this restriction enzyme is never quantitative. In addition to the 131 base pair band, all subjects (except 4 and 5) show a very weak undigested band at the location of the 176 base pair band on the agarose gel (fig. 3). This may be the reason for the fact that for sample 5 (heterozygote) about 40% of the 176 base pair fragment was found to be cut instead of 50%. However, this characteristic of the enzyme does not affect the genotype of the samples.

Fig. 3. The polymerase chain reaction (PCR) products were digested with restriction enzyme *Eco*57 I as described in Materials and Methods, subjected to electrophoresis in 2% agarose gel (or 1.5% on the extreme right), followed by ethidium bromide staining. Lane L is a 100 base pair ladder. N = non-digested PCR product, D = *Eco*57 I digested PCR product.

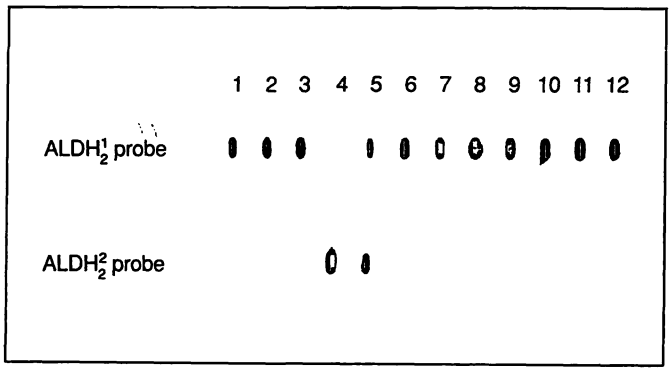

Fig. 4. The polymerase chain reaction (PCR) products were slot blotted on nitrocellulose filters as described by *Enomoto* et al. (10), hybridized with two ^{32}P-labelled allele-specific probes, ALDH$_2^1$ and ALDH$_2^2$, described in figure 1, followed by autoradiography.

In conclusion, we have developed a new approach for genotyping the common and the atypical aldehyde dehydrogenase ($ALDH_2$) alleles, which

(i) is considerably faster and simpler than presently available methods,

(ii) does not use a radioactive isotope and

(iii) requires very minute amounts of DNA, which can be obtained from a drop of whole blood or from hair roots.

This new approach should be of value in the genotyping of populations for epidemiological and genetic studies.

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Dr. Yedy Israel
Primary Mechanisms Department
Addiction Research Foundation
33 Russell Street
Toronto, Ontario M5S 2S1
Canada