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Original Article

Simple and reliable genotyping of ALDH2 rs671 in the patients with acute alcoholism intoxication using Tetra-Primer ARMS PCR

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Abstract: Aldehyde dehydrogenase-2 (ALDH2) is the second enzyme of the major oxidative pathway of alcohol metabolism, and is critical for the effective breakdown of alcohol common single nucleotide polymorphism (SNP) *rs671* 1510G>A (Glu504Lys) in *ALDH2* which influences the enzymatic activity of the ALDH2 active protein. We hypothesized that there could be a link between this polymorphism and the occurrence of acute alcoholism intoxication (AAI). In order to study this, we developed a Tetra-Primer ARMS PCR that could identify genetic polymorphisms of aldehyde dehydrogenase-2 (ALDH2, rs671). We then used the Tetra-primer ARMS-PCR for *ALDH2 rs671* genotyping and characterize the distribution of a polymorphism in 287 AAI patients (239 male and 48 female) and 483 healthy controls. Tetra-Primer ARMS PCR was successful for genotyping *ALDH2 rs671*. The frequency of the A allele of rs671 in AAI patients was significantly lower than that of the healthy control group (P<0.01). The number of *ALDH2* A allele carrier in cases of severe poisoning was less than that observed in cases of mild poisoning (P<0.01). The A allele of *ALDH2* is a potential protective factor for AAI. Tetra-Primer ARMS PCR is a simple, reproducible and specific technique for clinical diagnosis by genotyping. It is also both time- and cost-effective.

Keywords: Tetra-primer, ARMS PCR, ALDH2, Rs671, acute alcoholism Intoxication

Introduction

Aldehyde dehydrogenase-2 (ALDH2) is the second enzyme of the major oxidative pathway of alcohol metabolism, which plays a critical role by detoxifying exogenous and endogenous acetaldehydes into acetic acid [1, 2]. The enzyme of ALDH2, encoded by the ALDH2 gene on chromosome 12, has the lowest Km for acetaldehyde, and is localized in mitochondrial matrix. The ALDH2 gene is about 44 kbp in length and contains at least 13 exons. It has been shown that a common single nucleotide polymorphism (SNP) rs671 1510G>A (Glu504-Lys) in *gene ALDH2*-denoted the *ALDH2 A* allele, leads to a dramatic decrease in enzymatic activity. Specifically, there is an alteration in acetaldehyde metabolism, which markedly reduces alcohol tolerance. Generally, this SNP only occurs in East Asian populations, with frequencies as high as 40% [3]. Some researchers have suggested that rs671 is responsible for several diseases other than alcoholism, such as cardiovascular disease, hepatocellular carcinoma, esophageal cancer, Alzheimer's disease and possibly more [4-9]. Therefore, it would be useful to identify patients with this specific genotype that could be at high risk of these diseases. In order for such *ALDH2* rs671 genotyping, there is a requirement for rapid, cost-effective and reproducible techniques that could be developed for clinical use. In this study, a Tetra-primer ARMS PCR was optimized and validated for use as a genotyping test, with the aim of having a short turn-around time and limited costs. Patients with ALDH2-deficient genotypes (*ALDH2 AA* homozygous and *ALDH2 AG* heterozygous) have been found to have a higher acetaldehyde accumulation in their blood [2, 3], and therefore may be more susceptible to the harmful influence of alcohol caused via toxic effects of acetaldehyde, leading to DNA damage. Therefore, we have also used this method in order to examine the distribution of this polymorphism in the patients with acute alcoholism intoxication (AAI).

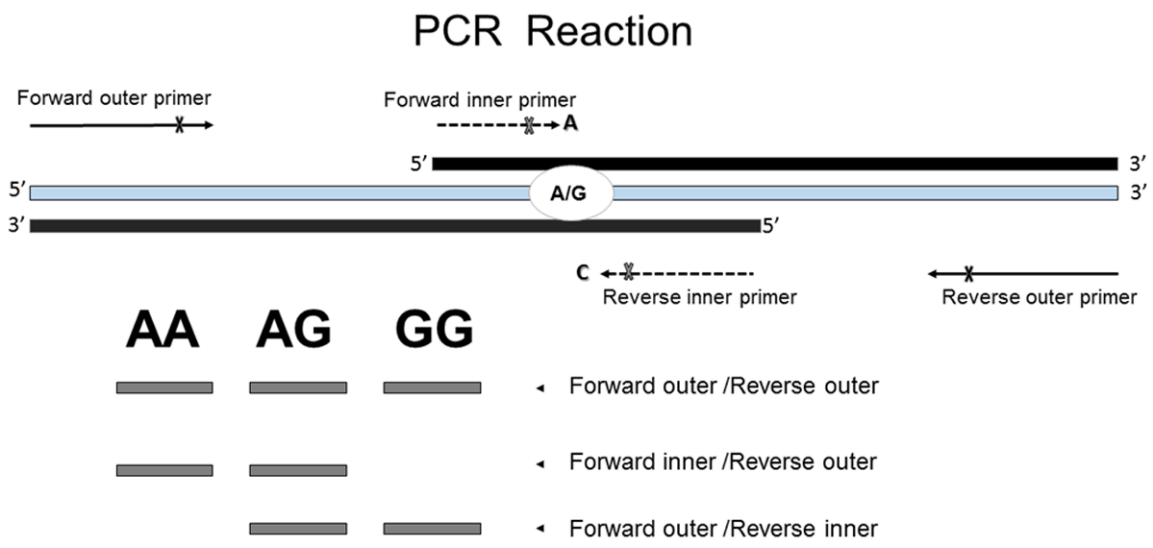

Figure 1. Schematic of the tetra-primer ARMS-PCR assay used for SNP genotyping to distinguish the three different genotypes. Two outer primers generate a 463 bp amplicon which serves as a (non-allele specific) control product. Forward inner and reverse outer primers generate a 290 bp product specific for the A allele, whereas forward outer and reverse inner primers generate a 221 bp amplicon specific for the G allele.

Materials and methods

Ethics statement

This study was approved by the Institutional Review Board of Renmin Hospital, Wuhan University. A written informed consent was obtained from each participant in accordance with the Ethics Committee of the Renmin Hospital of the Wuhan University.

DNA samples

A total of 770 blood samples from 287 AAI patients (239 male and 48 female) and 483 healthy controls (403 male and 80 female) were enrolled in the study at Renmin Hospital of Wuhan University. All participants were from the Han Chinese population of Wuhan city. Genomic DNA was extracted from peripheral blood samples using the TIANamp Blood DNA Kit (Tiangen Biotech, Beijing, China). DNA samples were then stored at -80°C in our laboratory.

Blood ethanol levels

Blood ethanol levels of AAI patients were measured using a colorimetric assay from the VITROS Chemistry Products Calibrator Kit, with quantification limits of 10-300 mg/dl. Severe poisoning was defined as measured blood ethanol levels higher than 200 mg/dl and anything below that was considered mild poisoning [10].

Sanger-sequencing

T-Primer PCR was evaluated by comparing with Sanger sequencing and the following primers were used for *ALDH2* PCR amplification: forward 5'-TGGTGGCTACAAGATGTCGG-3' and reverse 5'-CAGCAGACCCTAAATCCCTG-3'. The genotyping of ADH1B rs1229984 was also performed by Sanger sequencing using the following primers: forward 5'-GGTAGAGAAGGGCTTTAGACTG-3' and reverse 5'-CGGTACATAATGGTTGAAGGGT-3'. PCR product was purified using AxyPrep™ DNA Gel Extraction Kit (AXYGEN, USA). Sequencing was performed using an ABI 3500Dx machine (Applied Biosystems, USA) with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Results were analyzed with Sequence Scanner Software.

Tetra-Primer ARMS PCR

Figure 1 is a schematic representation of SNP identification using the technique of tetra-primer ARMS-PCR. To enhance the specificity in the ARMS PCR reaction, an additional mismatch was introduced at the third position from the 3' end of each of the primers [11]. In T-ARMS PCR, 2 pairs of primers in a single PCR tube [12], can simultaneously amplify both mutant and nor-

Table 1. Sequences of the Tetra-ARMS PCR primers designed for the genotyping of *ALDH2* rs671

Primer	Primer sequence	Tm	Amplicon size
Forward inner primer (A allele)	5'-GAGTACGGGCTGCAGGCATACA**A**TA-3'	64.70	A Allele 290 bp
Reverse inner primer (G allele)	5'-GGTCCCACACTCACAGTTTTCAC**G**TC-3'	65.84	G Allele 221 bp
Forward outer primer	5'-TAAAATAAAGACTTTGGGGCAATACAGGG**T**GT-3'	65.00	Outer amplicon 463 bp
Reverse outer primer	5'-ATTAGTAGGAAACACTGATGGCCTC**C**AG-3'	64.20	

Note: Tm, melting temperature; the mismatches of the primers were emphasized in bold; ARMS, amplification refractory mutation system; PCR, polymerase chain reaction.

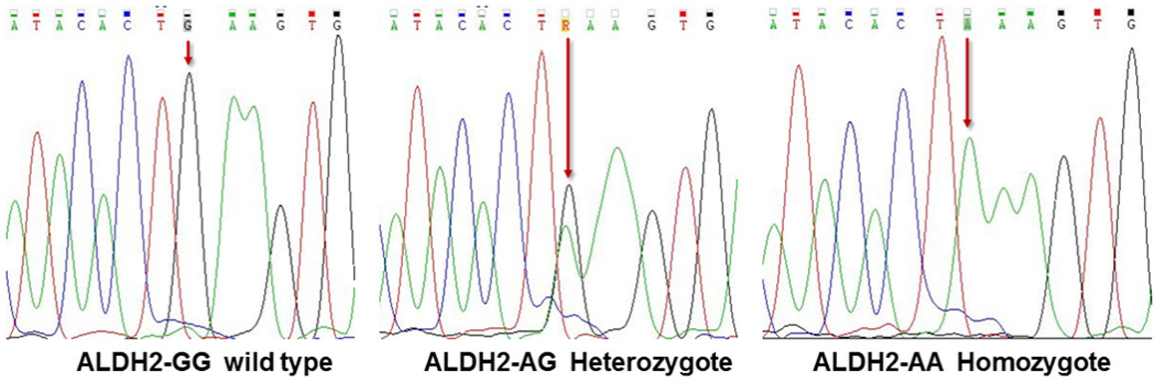

Figure 2. ALDH2 genotyping by Sanger sequencing.

mal alleles, as well as allow amplification of an internal DNA control (**Figure 1**). The inner primers were designed to amplify the two allelic states (e.g., in a G->A transition, one primer will amplify the A allele and the other the G allele).

The PCR primers were designed using an online primer designing tool-Primer 3 and primer synthesis was carried by Takara. (Takara Biotechnology Co. Ltd., Dalian, China). The primer sequences are shown in **Table 1**.

The ARMS PCR was performed in a final volume of 20 μL containing 1.5 μL of DNA template (50 μg), 10 μL 2×Taq PCR Master Mix (Lifefeng, China), 6.5 μL H_2O and 2 μL primers (5 μM). The cycling conditions included an initial denaturation at 95°C for 3 min, followed by 32 cycles of 30 sec at 94°C, 60 sec at 66°C and 30 sec at 72°C, followed by a final extension step at 72°C for 5 min. The reaction was performed in a Veriti® 96-well thermal cycler (Life Technology, USA). After amplification, PCR products were separated by standard electrophoresis on 2% agarose gels containing Gel-Red.

Statistical analysis

Statistical analysis was performed with SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). The performance of Tetra-Primer ARMS PCR for the genotyping of *ALDH2* rs671 was evaluated by comparing with Sanger sequencing. The chi-square (χ^2) test was used to assess deviation from the Hardy-Weinberg equilibrium, as well as comparison of the allelic and genotype frequencies between AAI patients and healthy controls. Differences in sex, poisoning time and *ALDH2/ADH1B* genotype in AAI were tested using the χ^2 statistical test. Age-related differences were evaluated using a t-test. A *P*-value<0.05 was considered statistically significant.

Results

Sanger sequencing of ALDH2

To verify the genotype of *ALDH2* rs671, Sanger sequencing was carried out as a reference method. **Figure 2** illustrates how the three genotypes of rs671: GG, AG and AA, can be clearly distinguished using Sanger-sequencing.

Specificity and sensitivity of Tetra-Primer ARMS PCR genotyping

In this study, both inner primers and outer primers were designed with mismatched bases. Inner-outer primer ratio-different ratios of inner and outer primers *viz*., 1:1, 1:2, 1:3, 1:4 were

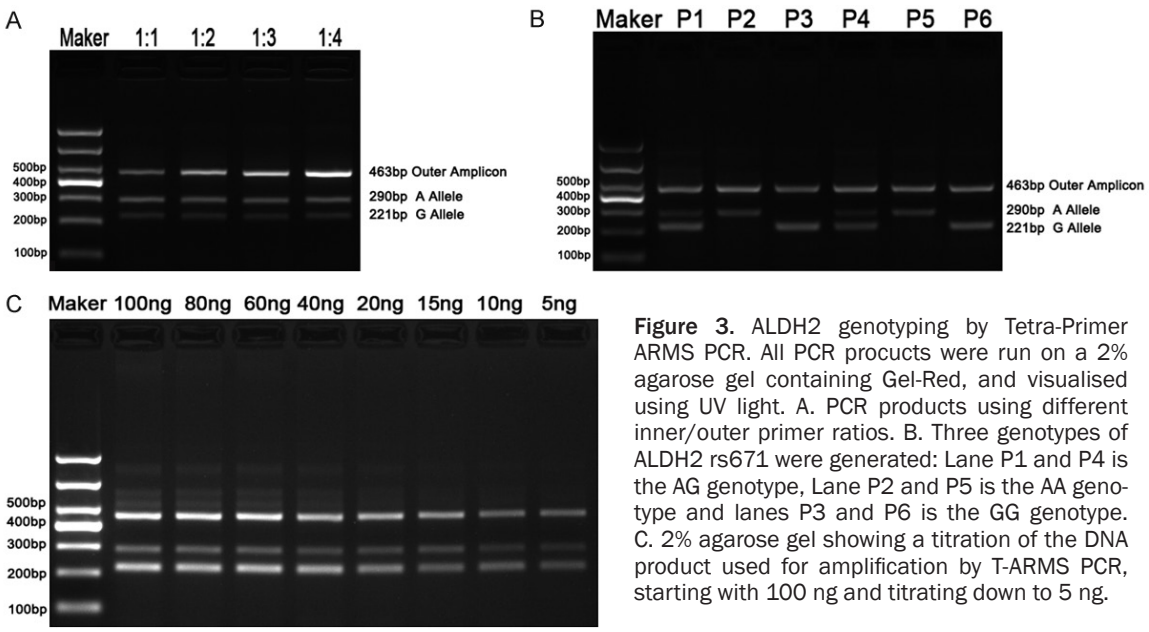

Figure 3. ALDH2 genotyping by Tetra-Primer ARMS PCR. All PCR procucts were run on a 2% agarose gel containing Gel-Red, and visualised using UV light. A. PCR products using different inner/outer primer ratios. B. Three genotypes of ALDH2 rs671 were generated: Lane P1 and P4 is the AG genotype, Lane P2 and P5 is the AA genotype and lanes P3 and P6 is the GG genotype. C. 2% agarose gel showing a titration of the DNA product used for amplification by T-ARMS PCR, starting with 100 ng and titrating down to 5 ng.

Table 2. Comparative results for Tetra-Primer ARMS PCR and Sanger sequencing

Genotype	Number of tests		Accuracy, %
	T-ARMS PCR	Sanger Sequencing	
ALDH2 GG	30	10	100
ALDH2 AG	30	10	100
ALDH2 AA	16	8	100
Total	76	28	100

Note: T-ARMS, Tetra-Primer amplification refractory mutation system; PCR, polymerase chain reaction.

tested (**Figure 3A**). The inner/outer primer ratio of 1:2 was chosen as the optimum ratio for use in further experiments. Three genotypes of *ALDH2* rs671: AG, GG and AA, could be identified (**Figure 3B**). To explore the detection limits of Tetra-Primer ARMS PCR, a serial dilution (ranging from 100 ng to 5 ng) of the P1 DNA was used as the PCR template (**Figure 3C**).

The sensitivity and specificity of Tetra-Primer ARMS PCR for the genotyping of *ALDH2* rs671 was evaluated by comparing with Sanger sequencing. Our results indicated 100% concordance between Sanger sequencing and T-ARMS PCR detection (**Table 2**).

Genotyping and frequency of ALDH2 genetic polymorphisms

The *ALDH2 rs671* genotype was examined in 287 AAI cases and 483 healthy controls. The genotype distributions and allelic frequencies for ALDH2 rs671 polymorphisms among the AAI cases and healthy controls are displayed in **Table 3**. The genotype distributions were all within the Hardy-Weinberg Equilibrium. The genotypes of *ALDH2 rs671*: GG, AG, and AA were 79.44%, 19.86% and 0.70% in the AAI cases and 62.73%, 33.33% and 3.93% in the healthy controls, respectively. The frequency of the A allele of rs671 in patients with AAI was significantly lower than that in healthy controls ($P<0.01$). The clinical characteristics of patients with mild poisoning and severe poisoning shown in **Table 4**. No significant differences were observed between patients with a different sex, age or poisoning time. However, there was a notable genotype difference between patients presenting with mild poisoning and severe poisoning ($P>0.01$). The number of *ALDH2 A* allele carrier in severe poisoning cases was less than that in mild poisoning cases, whereas the *ALDH2 G* allele was more closely associated with severe poisoning.

Conclusions

ALDH2, encoded by the *ALDH2* gene, is the major enzyme for the oxidation and detoxification of the ethanol metabolite acetaldehyde. ALDH2 is also involved in the oxidation of a wide spectrum of endogenous and exogenous aliphatic and aromatic aldehydes. The *ALDH2* gene is located at chromosome 12q24.2 and

Table 3. ALDH2 rs671 distribution in AAI and control groups

Groups	Genotype frequencies (%)			Allele frequencies (%)	
	GG	AG	AA	G	A
AAI cases	228 (79.44%)	57 (19.86%)	2 (0.70%)	513 (89.37%)	61 (10.63%)
Healthy controls	303 (62.73%)	161 (33.33%)	19 (3.93%)	767 (79.40%)	199 (20.60%)
χ^2	25.747			25.512	
P	<0.0001			<0.0001	

Table 4. Clinical characteristics in mild poisoning and severe poisoning cases

	Mild Poisoning	Severe Poisoning	P
Sex			
Male	104	135	0.409
Female	24	24	
Age	34.5±11.7	36.4±12.9	0.216
Poisoning time			
Before midnight	69	98	0.091
After midnight	51	45	
Day	8	16	
ALDH2 rs671			
AA+AG	51	8	<0.001
GG	77	151	
ADH1B rs1229984			
AA+AG	119	141	0.216
GG	9	18	

ALDH2 rs671 is the most common genetic variation.

Genetic variation, or SNP genotyping can be performed by methods such as PCR-restriction fragment length polymorphism (PCR-RFLP), amplification refractory mutation system PCR (ARMS-PCR) and TaqMan genotyping followed by Sanger sequencing [13, 14]. PCR-RFLP is a simple technique, but is relatively time consuming due to its requirement for specific restriction endonucleases. TaqMan genotyping assays use two fluorescent probes to detect specific PCR products, which is accurate and rapid, but increases assay labor and material costs. Sanger sequencing is reliable and highly accurate and is therefore universally considered the "gold standard" for genotyping and mutation detection. However, it still requires a longer process time than regular PCR.

In our protocol for Tetra-Primer ARMS PCR, a new mismatch at the -3 position of the outer primers was designed in order to decrease their efficiency. However, careful consideration as given to calculating equal annealing temperatures of allelic fragments as this can help facilitate a more efficient PCR reaction. Overall, this assay only took 2 hours and was highly cost effective, which is an important factor in a diagnostic setting.

In this study, we used Tetra-primer ARMS PCR to analyze *ALDH2* G1510A genotypes in 770 samples, and validated the result via Sanger sequencing. Genotyping of all the samples was performed successfully by these two methods, which generated completely consistent results, with 100% sensitivity and specificity. Additionally, all amplifications could be distinguished with a template concentration as low as 5 ng. Therefore, Tetra-primer ARMS-PCR is a cost-effective, rapid and specific technique for the genotyping of *ALDH2* rs671.

Our study reports the association between the *ALDH2 rs671* polymorphism and acute alcoholism intoxication. Importantly, our study revealed that the frequency of the *ALDH2* c.1510G>A polymorphism was significantly higher in patients presenting with AAI when compared with healthy controls. In addition, the number of ALDH2 A allele carriers in cases of severe poisoning was less than that in cases of mild poisoning. Perhaps this is because the low-activity ALDH2 protein resulting from the c.1510G>A polymorphism, is better able to breakdown alcohol following consumption, and therefore protects carriers from developing alcoholism [15, 16]. However, no significant difference was found in the distribution of the *ADH1B* genotype between these two cases. Contrary to this study, there was a previous report suggested that higher blood ethanol levels persisted for longer periods in the *ADH1B GG* carriers, than in the *ADH1B A* allele carriers [17]. The two studies showed inconsistent

results, which could be attributed to a low frequency of the *ADH1B G* allele in China [18].

In summary, we performed a diagnostic validation of Tetra-Primer ARMS PCR for genotyping of *ALDH2* rs671 in a clinical setting. The *A* allele of *ALDH2 rs671* is a potential protective factor for AAI in the local residents of Wuhan in China. Tetra-Primer ARMS PCR is simple, time-saving, cost-effective, specific and reproducible and therefore an ideal assay for genotyping as a means of clinical diagnosis.

Disclosure of conflict of interest

None.

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