SUBTYPES OF ADH2 GENE IN ALCOHOLICS

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ABSTRACT

Alcoholism is the multifactorial disease influenced by genetic environmental interaction and genetic variation of the genes may be associated with alcohol dependence due to its modified function in behavioral and physiological responses. In the present study, genetic variation was detected in the subtypes of gene, coding for the alcohol metabolizing enzyme Alcohol Dehydrogenase 2 (ADH2). Blood samples were collected from the alcoholic and non alcoholic subjects and genotyping was performed using PCR-RFLP (Polymerase Chain Reaction - Restriction Fragment Length Polymorphism), analysis to determine genetic polymorphisms in the ADH2 gene subtypes. The three subtypes of ADH2 gene (ADH2.1, ADH2.2 and ADH2.3) were found in both alcoholics (Family History Positive and Family History Negative) as well as non alcoholics.

KEY WORDS

Alcohol dehydrogenase 2 gene subtypes, Familial alcoholics, Non familial alcoholics.

INTRODUCTION

Alcohol is the most common psychoactive drug used throughout the world. Alcoholism is a chronic disorder marked by uncontrolled intake of alcoholic beverages that influences physical or mental health, social and familial relationships, and occupational responsibilities. Accumulating evidence supporting a genetic etiology in at least some forms of alcoholism have an interest in Children of Alcoholics (COA) as a group at high risk for the development of drinking problems. Family studies consistently find a high prevalence of alcoholism in the first degree relatives of alcoholics. (Kenneth J Sheer, 1998). Recent studies have shown that males who are at risk for familial alcoholism have different reactions to alcohol on a variety of physiological variables.

Most of the workers in the field believe that the principal influence of genetic factors is to affect the frequency and amount of drink consumed. Simple Mendelian inheritance has however, been considered unlikely because of studies showing similar alcoholism rates in first degree and second degree alcoholics. Family studies have repeatedly confirmed that the risk

of alcoholism is higher among first-degree relative (i.e. parents, siblings, children) of alcoholics as compared with the general population (Goodwin, 1989, 1990). With male first -degree relatives of alcoholics having a higher incidence (ranging from 27 to 54 percent) than female first degree relative (6 to 17 percent) as compared to first -degree relatives of non alcoholics (20 percent of males 4 percent of females) (Jaffe, 1990). An interesting "risk marker" for alcoholism has been revealed by studies of individual who are Family History Positive (FHP) vs. Family History Negative (FHN) for alcoholism. FHP individuals when tested showed lower subjective response to alcohol than FHN controls, and a follow -up study showed that low initial responders (whether of FHP or FHN background) went on to develop alcoholism at higher rates than high responders (Schuckit, 1996).

To search the human genome for specific genes related to alcoholism, researchers employ two experimental techniques. The first, the candidate gene approach, involves hypothesizing that particular genes are related to the physiology of alcoholism and then individually testing these genes for linkage (Goldman, 1990). The second approach, scanning of the human genome, involves characterizing, piece by piece, the entire length of DNA and finding genes that relate to alcoholism without proposing candidate genes. The discovery of a specific genetic effect on the development of alcoholism would be beneficial for at least three reasons. First, it would lead to the identification of some people at risk, who could act to avoid developing alcohol related problems. Second it may help us to understand the role of environmental

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factors that are critical in the development of alcoholism. Third, it may lead to better treatments, based on new understandings of the physiological mechanisms of alcoholism. Evidence for linkage with susceptibility to developing alcohol dependence was reported on chromosomes. Linkage analysis of an "unaffected but exposed" phenotype (individuals who drank but had few, if any, symptoms of alcohol dependence) suggested that a protective locus exists on chromosome 4 near the loci for Alcohol Dehydrogenases (ADHs). "Protective" ADH alleles that reduce alcohol consumption have been found in Asian populations (Reich, 1998).

The vast majority of ethanol metabolism occurs in the liver (Li, 1977). Within this organ, the major pathway of ethanol destruction is via Alcohol Dehydrogenase (ADH). There are at least seven different ADH-related genes producing at least eight forms of this enzyme, with ADH2 (for which there are at least three different alleles) and ADH3 (for which there are at least two different alleles) having greatest clinical relevance. The mammalian medium-chain alcohol dehydrogenases (ADHs), a group of dimeric zinc metalloenzymes with subunits of approximately 40 kDa. Mammals have multiple medium-chain ADHs that arose by repeated gene duplication. These ADHs catalyze the reversible oxidation of alcohols to aldehydes, acting on a wide range of substrates, from methanol to long-chain alcohols and sterols. They are believed to play important roles in detoxification pathways. The human class I ADH genes lie in head-to-tail array within 80 kb, in the order 5'-ADH3-ADH2-ADH1 (Yasunami, 1990). The class I gene cluster is on chromosome 4q22-23 (Smith and Duester, 1985). Groppi (1990) could determine easily and unambiguously, the complete genotype at these loci by using a micro sample of blood and restriction fragment length polymorphism after DNA amplification by PCR. Some forms of ADH2 (ADH2-2) and ADH3 (ADH3-1) are associated with a faster rate of metabolism of alcohol. A negative relationship between ADH2-2 and alcohol consumption has been reported in Asians and Israelis.

Polymorphic ADH2, ADH3 and ALDH2 genes can affect the propensity for alcohol drinking in Chinese. The alleles of ADH2.2, ADH3.1 and ADH2.2 are most likely to play a protective role against excessive consumption. ADH2.2 and ADH2.2 may contribute to susceptibility for alcohol dependence. Matsuo *et al.* (1989) showed that the typical and atypical forms of ADH2 differ in only a single amino acid. In the ADH2.2 (atypical) allele, CAC codes for histidine at residue 47; in the corresponding codon of the ADH2.1 (typical) allele, CGC codes for arginine. A high preponderance of heterozygosity for the ADH2.1/2.2 genes and a high frequency of ADH2.1 allele were found in heavy

drinkers compared with moderate drinkers (Tanaka, 1997).

MATERIALS AND METHODS

Subjects between the ages of 25 - 55 years were alcoholics consuming at least 150 gms / day for 2 years {Daily intake of greater than 150 ml of ethanol (120 g) have been considered as excessive (Glatt, 1977)} was included for this study. Subjects with no previous history of alcoholism, between the ages of 25 - 55 years and no family history of alcoholism for at least two generations were taken as controls. Subjects were selected by the psychiatric physician among persons with an identified alcoholism from Kasthurba Gandhi Memorial De-addiction and Rehabilitation Center, Coimbatore, Tamil Nadu. The subjects underwent a formal diagnostic assessment. A structured interview was done to classify the subjects as familial alcoholics (Family History Positive) and non familial alcoholics (Family History Negative). 50 alcoholic subjects out of which, 25 samples of familial alcoholic subjects were obtained from a group of known alcoholics of alcoholic fathers (Family History Positive), 25 samples of non familial alcoholic subjects were obtained from a group of known alcoholics of nonalcoholic fathers (Family History Negative), (Schuckit, 1988) and 25 Healthy nonalcoholic subjects of nonalcoholic fathers were chosen as controls.

3 mL of sterile blood was collected from subjects in EDTA coated tubes and stored at -2°C for DNA analysis. DNA extraction from the whole blood was done by using Genei reagents of Cat No. KT23 in Bangalore Genei Pvt.Ltd., Bangalore, India. The ADH2 gene has 3 subtypes - ADH2.1, ADH2.2 and ADH2.3. ADH2 gene is an 1125 base pair gene present on chromosome # 4 of human being. Three alleles of the subtype are present on the same chromosome # 4 approximately 20kb distances from each other. Base counts on chromosome # 4 starts from base pair 49, 62,099 for ADH1, 49, 93, 320 for ADH2 and 50, 22, 280 for ADH3 [National Center for Biotechnology Informations (NCBI)]. Multiplex PCR Detection System was developed for the determination of ADH2 genotyping in human individuals with known family history of drinking habits. In this technique multiple primer sets were used simultaneously to amplify several different DNA loci.

Primers were developed which specifically amplifies particular ADH2 polymorph. The primer sets reproductively amplified specific products from blood genomic DNA samples. Six individual PCR primers were developed from sequence data downloaded from the human genome sequence homepage in NCBI. The primers were typically around 30 bases long with 50% AT /GC frequencies and were different from each other at the 3' - end of the primers. Two out of 3 bases at the

3' - end of the specific primers were typical to individual ADH isotypes. ADH2 subtypes differ from the other subtypes in only a single amino acid coding base,

ADH with Arginine (R) → ADH2.1,

ADH with Histidine (H) → ADH2.2

ADH with Cysteine (C) → ADH2.3

DNA amplification

Three Forward primers and three Reverse primers were synthesized and used for the amplification of the three gene subtypes ADH 2.1, ADH 2.2 and ADH 2.3 by Polymerase Chain Reaction (PCR) [Refer Table 1, Table 2, Table 3 and Table 4].

Through gel electrophoresis (using 5 µl of amplified product on 1.5% agarose gel), banding patterns reflecting the genotypes of the ADH2 were clearly and unambiguously distinguished. The gene subtypes were detected by comparing the bands of the subjects to the control (having 100 bp ladder which was run

along with the amplified products in gel electrophoresis).

RESULTS

In the present study, 3 different alleles of ADH2 gene (ADH2.1, ADH2.2 & ADH2.3) were found and the frequency of the gene subtypes in the subjects was analyzed. The frequency of ADH 2.1 allele was shown as 86.6 percent in familial alcoholic group, 56.7 percent in non familial alcoholic group and 56.7 percent in non alcoholic group respectively. This supports the report of Matsuo *et al.* (1989) that Vmax of ethanol oxidation is increased by 100 fold in ADH 2.1 homozygote when compared to ADH 2.2 homozygote.

The frequency of ADH 2.3 allele was shown as 6.7 percent in familial alcoholic group, 23.3 percent in non familial alcoholic group and 20.0 percent in non alcoholic group respectively. Frequency of ADH 2.3 was significantly low when compared with ADH 2.1 and ADH 2.2. In contradiction to this study, ADH 2.3 was not found among the population of Japanese, both

Table 1. Forward Primers: (Same as the gene sequence)

F I'	5' TCT GTA GAT GGT GGC TGT AGG AAT CTG ACG 3' (PI)
F III'	5' TCT GTA GAT GGT GGC TGT AGG AAT CTG CCA 3' (PII)
N3	5' TAC TTC TAC AAA TCC TTA AAC AAC CGT A 3' (PIV)
F I':	This primer is Arginine specific (R) (CGC). The 3 rd base from 3' end is changed from T → A. This primer binds on Exon III.
F II':	This primer is Histidine specific (H) (CAC). The 3 rd base from 3' end is changed from T → C. This binds on Exon III.
N3:	This primer is sequence specific primer binding on Intron VIII.

Table 2. Reverse Primers: (Reverse complementary sequence to gene sequences)

R II'	5' TCT ATT GCC TCA AAA CGT CAG GAC GGT GCG 3' (PV)
R III'	5' TCT ATT GCC TCA AAA CGT CAG GAC GGT GCA 3' (PVI)
N2	5' TAC TTT TTT TCC CTC CTC CCG TTT CTA CTT CTA 3' (PIII)
R III':	This primer is Arginine specific (R) (CGT). The 3 rd base is changed from A → G. This binds on Exon IX.
R II':	This primer is Cystine specific (R) (TGT). The 3 rd base is changed from A → C. This binds on Exon IX.
N2:	This is sequence specific primer binding on Intron III.
The ARMS primers were designed with respect to the difference in the base sequence between ADH 2.1/2.2./2.3. ARMS primers have the 3 rd base mismatch from 3' end to ensure specificity of product. The designed primers were PAGE purified.	

Table 3. PCR Protocol for DNA amplification

(Template) Genomic DNA	100 ng
Primer mix	800 ng (200 ng each)
Assay buffer	5 µl
10Mm dNTP mix	2 µl
Taq DNA Polymerase	1 µl (3 units)
Water	-
Total volume	50 µl

Table 4. PCR cycle condition

TEMP :	94°C	94°C	64°C	72°C	94°C	55°C	72°C	72°C
TIME :	2.0	0.30	0.30	0.30	0.30	0.30	0.30	0.00
<div style="display: flex; justify-content: space-around; align-items: center;"> <div style="text-align: center;"> Add DNA polymerase ↓ Initial Denaturation </div> <div style="text-align: center;"> 10 cycles </div> <div style="text-align: center;"> 20 cycles </div> <div style="text-align: center;"> Final Extension </div> </div>								

Table 5. Primer pairs {(P I / P III and P IV / PV) and (P II / PIII and P IV / PVI) were multiplexed for PCR amplification and interpretation of the data

PIV / PV PI / PIII				PIV / PVI PII / PIII							
+	-	+	+	+	+	+	-	+	-	-	+
+	+	+	+	+	-	+	+	-	+	+	-
2.1/2.2		2.2/2.3		2.1/2.3		2.1/2.1		2.2/2.2		2.3/2.3	
+ Presence of the bands in gel electrophoresis pattern - Absence of the bands in gel electrophoresis pattern											

Table 6. Percentage of ADH2 gene expressions in alcoholics and non alcoholics

Type of alcoholics	ADH 2.1	ADH 2.2	ADH2.3
Alcoholic persons of alcoholic parents (Family history positive)	86.6%	6.7%	6.7%
Alcoholic persons of non alcoholic parents (Family history negative)	56.7%	20.0%	23.3%
Non alcoholic persons of non alcoholic parents (control)	56.7%	23.3%	20.0%
Family History Positive (FHP)			
Family History Negative (FHN)

Fig. 1. Position of Bands and Subtypes of Gene ADH2

9, 16	-	100 bp Ladder
3, 4	-	2.1/2.1
5, 6	-	2.1/2.1
7, 8	-	2.2/2.3
10, 11	-	2.1/2.1
12, 13	-	2.1/2.1
14, 15	-	2.1/2.1
17, 18	-	2.1/2.1
19, 20	-	2.1/2.1

in alcoholics as well as in non alcoholic group (Higuchi, 1994).

Heterozygosity of ADH 2.1 / 2.2 was rare in alcoholics. ADH 2.1 / 2.2 was found in non familial alcoholic group and non alcoholic group and was not found in familial alcoholic group. Frequency of ADH 2.2 / 2.3 gene subtypes were significantly very low in familial alcoholic group compared with the non familial alcoholic group and non alcoholic group.

There was significant difference in the frequency of ADH2.1 and ADH2.2 in alcoholic groups. This offers evidence that genetic polymorphism in ethanol metabolizing enzyme may in part explain the difference in susceptibility to alcoholism. Polymorphism of ADH2 gene may affect the propensity of alcohol drinking. The alleles of ADH2 are most likely to play a protective role against excessive consumption. ADH 2.2 or ADH 2.3 may contribute to susceptibility for alcohol dependence, since the ADH 2.2 and ADH 2.3 alleles were present in higher

8 9 10 11 12

Fig. 2. Position of Bands and Subtypes of Gene ADH2

5, 12	-	100 bp ladder
1, 2	-	2.1/2.3
3, 4	-	2.1/2.3
6, 7	-	2.1/2.3
8, 9	-	2.1/2.2
10, 11	-	2.1/2.1

percentage in non alcoholic and non familial alcoholic group when compared to familial alcoholic group. This study supports the statement of Thomason (1994) stating that polymorphism at the ADH2 loci of ADH has been shown to have an effect on the predisposition to alcoholism in Asian individual.

DISCUSSIONS

The most interesting aspect of the ADH2 variants is their connection to different ethnic groups.

ADH2.1 - Predominate allele among most world populations (90%)

ADH2.2 - Allele in East Asian populations (70%)

ADH2.3 - Allele in African origins (20%)

(Jenny Lake, 1999)

Frequency of the predominate allele ADH2.1 found in both alcoholics (71.6%) and nonalcoholics (56.7%) was significantly high in familial alcoholics (86.6%)

than in non familial alcoholics (56.7%), revealing that the ADH2.1 may be established as a candidate gene subtype for familial alcoholism (FHP).

ADH2.2 is the allele that seems to have the ability to completely prevent alcoholism. No individual with ADH2.2 homozygosity has been found among the alcoholics. Similar findings have been obtained with individuals of Jews (Jenny Lake 1999).

ADH2.3 has been documented only in people of African descent and certain Native American tribes. The ADH2.3 allele may be associated with a lowered risk for the development of alcoholism. Our results demonstrate that the ADH2.3 allele is associated with a negative family history of alcoholism and hence reveal that there is an association between the presence of ADH2.3 alleles in Indian adults and a Negative family history of alcoholism.

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