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Variation in the *ADH1B* proximal promoter affects expression

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Abstract

The primary pathway of metabolism of dietary alcohol is via its oxidation in liver by alcohol dehydrogenases (ADH). Differences in the *ADH* enzyme activity or levels of enzyme present could affect the risk for alcoholism. Regulatory variations have been shown to affect the promoter activity and thereby affect the risk for alcoholism. In this study the functional effects of the two SNPs (rs1159918 and rs1229982) in the proximal promoter region of *ADH1B* that were associated with alcoholism were explored. We examined the effects of five naturally occurring haplotypes on the promoter activity. We observed that a C to A change at rs1229982 increased promoter activity 1.4-fold.

Keywords

alcohol dehydrogenase 1B; alcoholism; polymorphism; SNP; promoter; gene expression; haplotype

1. Introduction

Alcoholism is a complex disease affecting millions in the world. It is influenced by both genetic and environmental factors. Evidence that greater than 50% of the differences in risk can be attributed to genetic factors has been obtained from family, twin and adoption studies [1–10].

Alcohol dehydrogenases metabolize alcohol to acetaldehyde in the first step of alcohol metabolism. The rate at which ethanol is oxidized influences the concentration of ethanol and acetaldehyde. This is the hypothesized mechanism by which *ADH* variations affect the risk for alcoholism. There are seven alcohol dehydrogenases in humans: *ADH1A*, *ADH1B*, *ADH1C*, *ADH4*, *ADH5*, *ADH6*, and *ADH7* [11]. Class I *ADH*s (*ADH1A*, *ADH1B* and *ADH1C*) contribute to 70% of ethanol metabolism in the liver [12]. Coding variations in *ADH1B* have a strong effect on the risk for alcoholism; a single nucleotide polymorphism (SNP) that leads to an arginine to histidine change in the coding sequence (rs1229984;

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R48H) increases the catalytic rate of the enzyme and was shown to be protective against alcoholism [13–20].

The rate of alcohol metabolism can also be affected by regulatory variations that influence the amount of enzyme present. Several studies have reported regulatory variations of the *ADH*s. A SNP at position -136 (relative to the +1 translational start site) in the promoter of the *ADH4* gene affects the promoter activity in hepatoma cells, with the A allele having 2-fold higher activity than the C allele [21]. This variation has been associated with the risk for alcoholism in a Brazilian population [22]. Polymorphisms that affect expression levels were also identified in a regulatory element 3 kb upstream of *ADH1C* promoter [23]. Non-coding variations in and near other *ADH* genes have been associated with alcohol consumption and the risk for alcoholism [1,24–27].

Two SNPs in the *ADH1B* promoter region, rs1229982 and rs1159918, are associated with alcohol dependence defined by meeting both DSM-IIIR criteria for alcohol dependence plus criteria for Feighner definite alcoholism [25]. We tested the effect of the two polymorphisms and others in the *ADH1B* promoter region on promoter activity.

2. Materials and Methods

2.1 Cloning of test fragments

The region extending to 1484 bp upstream of the start of the *ADH1B* coding sequence (+1 CDS) was amplified by PCR using high fidelity platinum Pfx polymerase (Invitrogen, Carlsbad, CA), using HE3003 (CGAGATCTGTCTTCTCTGCCCACCAGC) and HE3116 (GTGGTACCCTGGGGCTATCTTCTTTCCG) as primers. PCR conditions were: 94C for 5 min, (94C for 15 s, 62C for 20 s, 68C for 90 s) × 10 cycles; (94C for 15 s, 60C for 20 s, 68C for 90 s) × 30 cycles, 68C for 7 min. DNA from five different individuals was used as template. Fragments were cloned into KpnI and BglII sites in the pXP2 luciferase reporter vector [28]. Clones were sequenced by BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, Santa Clara, CA); five different haplotypes were obtained.

2.2 Transient transfections and Reporter gene assays

HepG2 human hepatoma cells (HB-8065; ATCC, Manassas, VA) were cultured in MEM (ATCC) with 10% FBS (Invitrogen, Carlsbad, CA), 4 mM glutamine (Thermo Scientific Hyclone, Waltham, MA) and 1X Penicillin and Streptomycin (Thermo Scientific Hyclone) on cell bind surface plates (Corning Inc, Corning, NY) at 37C. For transient transfection assays 8×10^5 cells were seeded per well in 6-well cell binding surface plates. 24 h after seeding, cells were transfected in serum free media with 2 μg of test DNA, along with 140 ng of CMV-galactosidase plasmid (Clontech, Mountain View, CA) and 1.2 μg of pUC19 DNA, using 3 μl Fugene HD (Roche, Indianapolis, IN). Complete medium was added 6 h after addition of DNA and cells were cultured for another 24 h. Cells were harvested 30 h after addition of DNA by scraping into ice-cold 1X PBS, pelleted by centrifugation and suspended in 100 μl of 1X Reporter lysis buffer (Promega, Madison, WI). Cell extracts were prepared by three repeated cycles of freeze-thawing followed by centrifugation at 16,000 × g for 1 min. Luciferase assays were carried out using 20 μl of the extract and the Luciferase assay system (Promega, Madison, WI). β-galactosidase assays were carried out using 5 μl of the extract and the Galacto-Light System (Tropix, Bedford, MA). Activities were measured on a Lmax Plate Luminometer (Molecular Devices Sunnyvale, CA).

All test constructs were transfected at least in triplicate in each individual experiment, with experiments repeated four times, on different days. Promoter activity was defined as luciferase activity normalized to β-galactosidase activity, to correct for the transfection

efficiency. A t-test assuming unequal variances was carried out in Microsoft Excel, considering each transfection as an independent data point.

3. Results and Discussion

Two SNPs in the non-coding region of *ADH1B* have been associated with alcohol dependence [25]; both rs1229982 and rs1159918 are located in the proximal promoter region. Allele and genotype frequencies of rs1229982 and rs1159918 are shown in Table 1. The Single Nucleotide Polymorphism database (dbSNP; www.ncbi.nlm.nih.gov/projects/SNP) reports a total of 12 SNPs in the region between −1484 bp and −10 bp (with respect to the coding sequence start site; Figure 1).

The effects of different alleles of rs1229982 and rs1159918, along with the other natural variations, were tested by transient transfection assays. The region was amplified from DNA of different individuals to generate the natural haplotypes, and cloned into the pXP2 reporter plasmid. Five different haplotypes (Table 2) were obtained; all five had the same allele for SNPs rs28913901, rs28913903, rs28913904, rs3076071 and rs28913905 (which have low minor allele frequencies); they differed in the remaining seven positions (Figure 1).

Five haplotypes were tested in HepG2 cells by transient transfections. The activity of each was normalized to that of haplotype 1, which had the sequence closest to the reference sequence in GenBank (accession number NT_016354.17). A significant decrease in the promoter activity relative to haplotype 1 was observed in haplotypes 3, 4 and 5 (p-value $\leq 5 \times 10^{-7}$), whereas no difference in activity was seen with haplotype 2 (p-value = 0.39; Figure 2). Haplotypes 1 and 2 share an A at rs1229982 (Table 2), suggesting that rs1229982 might be responsible for the decrease in promoter activity.

Data from the different haplotypes were therefore grouped based on the allele of each SNP (e.g., the average activities of all haplotypes with the rs1229982 A allele were compared to those of all haplotypes with C at that position) for analysis. A significant difference in expression was observed for only one SNP, rs1229982 (Table 3). Promoters with the A allele at rs1229982 have, on average, 39% more activity than those with C.

The *ADH1B* proximal promoter region extending to 270 bp upstream of the translational start site has binding sites for multiple transcription factors [29–30]. A putative CCAAT enhancer binding protein (C/EBP) family of transcription factor was predicted by PROMO [31] at rs1229982, which might explain the effect on promoter activity.

The A allele of rs1229982 is found at a low frequency (0.2) in European Americans (CEU); it is at high frequency in the Nigeria (YRI) population (60%) but is completely lost in the Asian populations (CHB, JPT). In the European-American population [25], the A allele was observed to be overtransmitted to alcoholics. This is somewhat surprising, given that higher ADH activity coding alleles are protective [13–20].

Because we did not detect a functional effect for rs1159918, its association with alcoholism could either be due to functional effects in other tissues or through linkage disequilibrium (LD) with another SNP that is functional. In the CEU population, rs1159918 is reported to be in high LD with rs6810842 ($r^2 = 0.949$), but rs6810842 also doesn't appear to have an effect on promoter activity (Table 3); the two SNPs are in different phase in 1 of the 5 haplotypes we tested. There are other SNPs in the ADH cluster which are in LD ($r^2 > 0.25$) with rs1159918 but have not been tested for function.

In summary, we have identified a functional effect of the variation rs1229982 in the *ADH1B* proximal promoter; the A allele at this position had higher promoter activity, and was

overtransmitted to alcoholics. These results support our hypothesis that regulatory variations could alter the levels of *ADH* enzymes and thus affect the risk for alcoholism.

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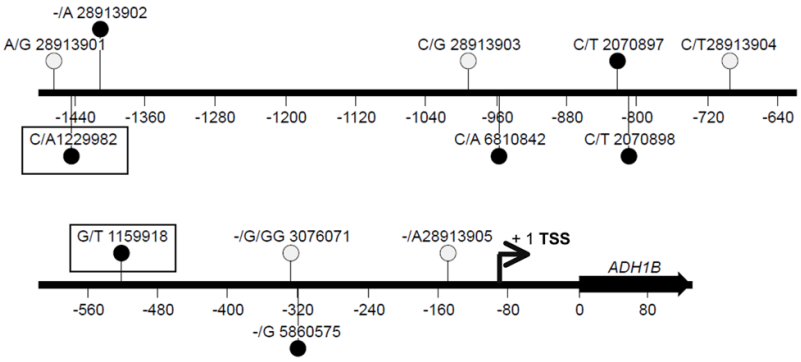

Figure 1. Variations in the *ADH1B* proximal promoter region

Twelve variations in the proximal promoter are shown as vertical lines with the SNP ID (rs number) and the two alleles to the left of the rs number. Filled circles represent those variations that were tested in the transfection assays. Variations associated with the risk for alcoholism are outlined by rectangular boxes. For some variations (shown as open circles), the same allele was present in all the haplotypes tested. Numbering is relative to the *ADH1B* translational start site (+1). The transcriptional start site (TSS) is indicated by an arrow.

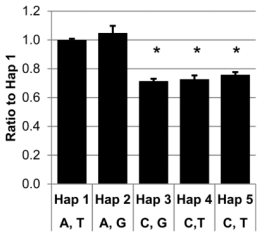

Figure 2. Variations in the *ADH1B* promoter affect activity

Five different haplotypes of the *ADH1B* proximal promoter region were tested in transient transfections in HepG2 cells (n = 12). The promoter activity of each haplotype was normalized to the promoter activity of Haplotype 1. Alleles of rs1229984, and rs1159918, respectively are shown on the x-axis. The error bars indicate standard errors of the mean. * p-value 7le;7×10^{-4}.

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Table 1

Allele and genotype frequencies for two SNPs in the *ADH1B* proximal promoter region

Allele and genotype frequencies in different populations for the two SNPs that were associated with alcoholism were obtained from HapMap (release 27, Phase II and III; hapmap.ncbi.nlm.nih.gov). Populations are Utah residents with ancestry from northern and western Europe (CEU), Han Chinese in Beijing, China (CHB), Japanese in Tokyo, Japan (JPT), Yoruba in Ibadan, Nigeria (YRI).

	rs1229982				rs1159918					
	Allele		Genotype		Allele			Genotype		
HapMap Population	A	C	A/A	C/A	C/C	T	G	T/T	T/G	G/G
CEU	0.2	0.8	0.0	0.3	0.7	0.3	0.7	0.1	0.4	0.5
CHB	0.0	1.0	0.0	0.1	0.9	0.2	0.8	0.0	0.3	0.6
JPT	0.0	1.0	0.0	0.1	0.9	0.2	0.8	0.0	0.3	0.7
YRI	0.6	0.4	0.3	0.5	0.2	0.9	0.1	0.8	0.2	0.0

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Table 2

Tested haplotypes of the *ADH1B* proximal promoter

Five haplotypes with seven SNPs that were obtained from cloning the proximal promoter region are shown; they were identical at all other positions. Alleles of the two SNPs that were associated with alcoholism are shown in bold.

Haplotype	rs1229982	rs28913902	rs6810842	rs2070897	rs2070898	rs1159918	rs5860575
1	**A**	-	A	C	C	**T**	-
2	**A**	T	C	T	C	**G**	-
3	**C**	T	C	T	T	**G**	-
4	**C**	T	C	C	C	**T**	-
5	**C**	T	A	C	C	**T**	G

Table 3

Analysis of the haplotypes grouped by the allele at each SNP

The promoter activities (β-galactosidase normalized luciferase activity) of different haplotypes were grouped by the alleles of four SNPs; the other three SNPs could not be compared as there was only one representative haplotype for one of the alleles. The ratio of the activity of the two groups was determined and the significance of this ratio was calculated by t-test.

	Allele 1	Allele 2	Ratio of allele 1/allele 2	p-value
rs1229982	A	C	1.39	6.2×10^{-6}
rs6810842	C	A	0.95	0.46
rs2070897	C	T	0.94	0.42
rs1159918	T	G	0.94	0.42