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ORIGINAL INVESTIGATION

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Allelic variation at alcohol metabolism genes (*ADH1B*, *ADH1C*, *ALDH2*) and alcohol dependence in an American Indian population

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Abstract Enzymes encoded by two gene families, alcohol dehydrogenase (*ADH*) and aldehyde dehydrogenase (*ALDH*), mediate alcohol metabolism in humans. Allelic variants have been identified that alter metabolic rates and influence risk for alcoholism. Specifically, *ADH1B*47His* (previously *ADH2-2*) and *ALDH2-2* have been shown to confer protection against alcoholism, presumably through accumulation of acetaldehyde in the blood and a resultant 'flushing response' to alcohol consumption. In the current study, variants at *ADH1B*(previously *ADH2*), *ADH1C* (previously *ADH3*), and *ALDH2* were assayed in DNA extracts from participants belonging to a Southwest American Indian tribe (n=490) with a high prevalence of alcoholism. Each subject underwent a clinical interview for diagnosis of alcohol dependence, as well as evaluation of intermediate phenotypes such as binge drinking and flushing response to alcohol consumption. Detailed haplotypes were constructed and tested against alcohol dependence and related intermediate phenotypes using both association and linkage analysis. *ADH* and *ALDH* variants were also assayed in three Asian and one African population (no clinical data) in order to provide an evolutionary context for the haplotype data. Both linkage and association analysis identified several *ADH1C* alleles and a neighboring microsatellite marker that affected risk of alcohol dependence and were also related to binge drinking. These data strengthen the support for *ADH* as a candidate locus for alcohol dependence and suggest further productive study.

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Introduction

The genes underlying human alcohol metabolism provide a rare example of how allelic variation contributes to a complex disease through intervening physiology and behavior. The process is best understood in terms of the simple two-step pathway that is responsible for the bulk of alcohol metabolism. Alcohol is first oxidized by alcohol dehydrogenase (ADH) to acetaldehyde, which is then oxidized to acetate by acetaldehyde dehydrogenase (ALDH). Both proteins occur in several isozyme forms encoded by multigene families. Specific alleles at the loci *ADH1B* (previously *ADH2*), *ADH1C* (previously *ADH3*), and *ALDH2* can increase the blood level of acetaldehyde (Bosron et al. 1983; Burnell et al. 1989; Farrés et al. 1994). This causes an adverse response to alcohol consumption characterized by elevated blood flow, dizziness, accelerated heart rate, sweating, and nausea (Wolff 1972; Goedde et al. 1979; Agarwal and Goedde 1990). These symptoms in combination define the 'flushing response'. Individuals who flush are protected by its unpleasantness from heavy drinking

and ultimately alcoholism (Wolff 1972; Schwitters et al. 1982; Suwaki and Ohara 1985).

ADH1B and *ADH1C* encode the primary ADH enzymes for alcohol metabolism in the liver. Both loci harbor functional polymorphisms (Yin et al. 1999). The *ADH1B*47His* allele (previously *ADH2-2*) results in enhanced catalytic activity (V_{max}), increased blood levels of acetaldehyde, flushing, and protection from alcoholism (Thomasson et al. 1991, 1993; Goedde et al. 1992; Nakamura et al. 1996). However, *ADH1B*47His* is present at significant frequencies only in Asian and Jewish populations, where its physiology and protective role appear similar (Agarwal et al. 1981; Neumark et al. 1997). Recently, low frequencies of *ADH1B*47His* have been detected in European, North African, and Middle Eastern populations and, in some cases, have been significantly associated with alcohol dependence (Whitfield et al. 1998; Borras et al. 2000; Osier et al. 2002). A protective role has also been proposed for *ADH1C*349Ile* (previously *ADH3-1*), although recent reports propose that the effect is a secondary consequence of linkage disequilibrium between *ADH1C*349Ile* and *ADH1B*47His* (Chen et al. 1999; Osier et al. 1999). Genetic variants of mitochondrial *ALDH2* have also been identified and *ALDH2-2* has been extensively studied. This variant is found mainly in Asian populations, as with *ADH1B*47His,* and blocks catalysis of acetaldehyde, resulting in its accumulation in the blood (Goedde et al. 1992; Novoradovsky et al. 1995; Peterson et al. 1999). Flushing is even more pronounced with *ALDH2-2* than with *ADH1B*47His* and protection against alcohol dependence is consequently stronger (Harada et al. 1982; Thomasson et al. 1991, 1993).

The *ADH1B*47His* and *ALDH2-2* alleles are virtually absent in most non-Asian populations, but there is tantalizing evidence that drinking patterns and alcohol dependence are influenced by other variations in these genes that control alcohol metabolism. Independent genome-wide linkage studies have been conducted in samples composed predominantly of Euro-Americans (Reich et al. 1998; Saccone et al. 2000) and the American Indian population analyzed in this study (Long et al. 1998). Both studies provided modest evidence for a locus contributing to alcohol dependence in the region of the *ADH* gene cluster on chromosome 4q, despite the fact that neither population possesses *ADH1B*47His* and *ALDH2-2*. Intriguingly, both populations are polymorphic for *ADH1C*349Ile*. Follow-up studies on the Euro-American sample demonstrated strong evidence for linkage in the 4q chromosomal region to a phenotype defined by the maximum number of drinks consumed on a single occasion (Saccone et al. 2000). This region has also been associated with illegal drug abuse through a genome-wide single nucleotide polymorphism (SNP) linkage disequilibrium scan (Uhl et al. 2001). The studied populations (Euro-Americans and Afro-Americans) typically lack *ADH1B*47His* and *ALDH2-2* and are polymorphic for *ADH1C*349Ile*. *ADH* was recently designated a replicated Substance Abuse (rSA) locus, reflecting the fact that multiple studies have demonstrated a relationship between the locus and substance abuse vulnerability (Uhl et al. 2002). There is additional evidence that *ADH1C*349Ile* may play a more general role related to alcohol use, health and disease. Hines et al. (2001) demonstrated that *ADH1C*349Ile* homozygous individuals are more protected from heart disease by moderate drinking than *ADH1C*349Val* homozygotes.

This paper focuses on polymorphism at the *ADH1B*, *ADH1C*, and *ALDH2* loci in relation to flushing, drinking style, and alcoholism in a SW American Indian population. The population sample originally analyzed by Long et al. (1998) (Group 1) and a larger sample (Group 2) were investigated in the current study. The well-established protective alleles *ADH1B*47His* and *ALDH2-2* were not observed. Our strategy was to construct haplotypes composed of sets of closely linked SNPs embedded within the *ADH1B*, *ADH1C*, and *ALDH2* genes. The alleles and haplotypes were used in linkage and association studies designed to test allelic variation with alcoholism and two related intermediate phenotypes, flushing and binge drinking. Both linkage and association analysis identified several *ADH1C* alleles and a neighboring microsatellite marker that were related to an increased risk of alcohol dependence and binge drinking. Strong linkage disequilibrium was detected across all markers in all populations, but was enhanced in American Indians, presumably because of genetic drift and population bottlenecks associated with colonization of the New World, demonstrating that the pattern of linkage disequilibrium reflects each unique population history.

Materials and methods

Sampling strategy

Clinical data were collected from 582 adult members of a SW American Indian tribe. Blood samples were collected from the majority of these participants [*n*=499; 281 females and 218 males; mean (± SD) age = 36.0 (± 13) years]. A subsample of 152 subjects was previously investigated in an alcohol dependence genome scan study (Long et al. 1998). These subjects were genotyped as part of a long-term epidemiological study of another chronic disease. The sample from Long et al. (1998) (hereafter called Group 1) and the full set of DNA samples (hereafter called Group 2) were analyzed in the current study. Based on availability of DNA samples, final samples sizes for the two datasets were *n*=122 (Group 1) and *n*=490 (Group 2).

Subjects were recruited as putative members of large multigenerational pedigrees identified by knowledgeable elder tribal members. After verification by personal interview and genetic typing, the entire sample of 582 samples included one large pedigree of 422 members, a smaller pedigree of 104 members, nine small pedigrees with 2–15 members, and 13 isolated individuals. All participants were age ≥21 years and eligible for tribal enrollment (≥1/4 tribal heritage). Self-reported ancestry of all participants gave no indication of non-Indian admixture and 1/4 tribal heritage represented the minimum requirement of ancestry in that population for tribal membership. Williams et al. (1992) found a high correspondence between overall levels of stated ancestry and ancestry estimated from genetic markers and they found evidence for less than 5% non-American Indian admixture in the study population. Informed consent was obtained under a human subjects research protocol approved by the Tribal Council and the Institutional Review Board (IRB) of the National Institute on Alcohol Abuse and Alcoholism.

Native Siberian (Sakha), Mongolian (Uriankhai, Kazakh, Dervet), Chinese (Taiwanese), and Nigerian (Ibadan, Lagos) DNA samples

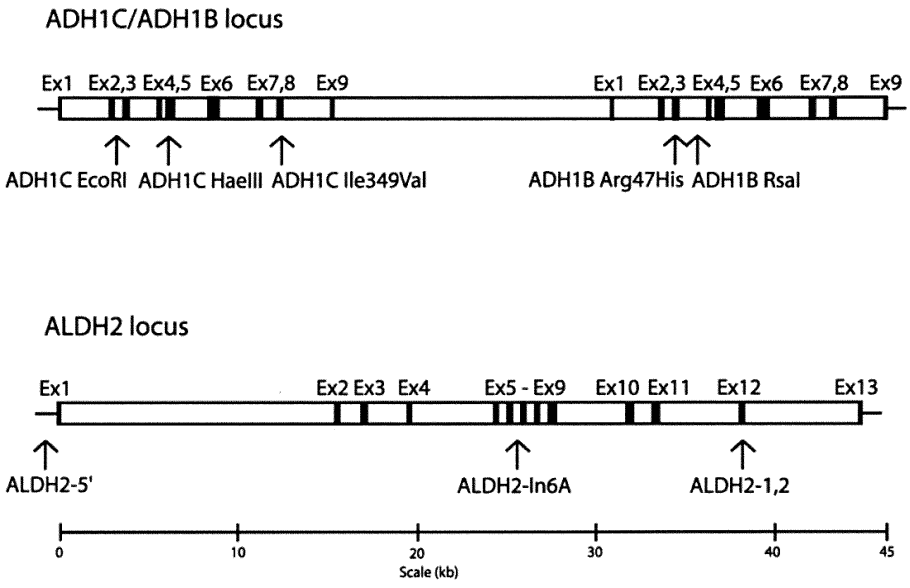

Fig. 1 Genomic organization of the *ADH1B*/*ADH1C* and *ALDH2* loci, with the location of assayed variants indicated. *Filled segments* represent exons and *open segments* represent introns. D4S1647 is not depicted because it has not been precisely located relative to the *ADH* variants; our analyses indicate it is roughly 1–2 cM distant from the *ADH* locus

had been previously collected by L. Goldfarb, N. Sambuughin, S.-J. Lin Tsai, and R. Kittles, respectively. Aliquots of DNA, with no identifying information, were provided for the current study. No clinical data were collected on these individuals. Exemption from review was obtained from the IRB of the University of Florida.

Testing instruments, interviews, and psychiatric diagnoses

Clinical data were collected on the American Indian population only. Focus groups comprised of tribal staff and community members reviewed testing instruments and questionnaires for potential cultural biases and general suitability to the population. Research diagnoses for alcohol dependence were based on: (1) semi-structured psychiatric interviews using the Schedule for Affective Disorders and Schizophrenia – Lifetime Version (SADS-L) with probes added to enable diagnoses using both Research Diagnostic Criteria and Diagnostic and Statistical Manual of Mental Disorders, Third Edition-Revised (DSM-III-R, American Psychiatric Association, 1987) criteria (Robin et al. 1998); (2) medical, educational, court, and other records; (3) corroborative information from family members. The SADS-L was administered to all subjects by a psychologist experienced with psychiatric assessment in this tribe and other American Indian populations. DSM-III-R diagnoses of alcohol dependence were made from the SADS-L by following operationally defined criteria and using the instructions of Spitzer et al (1989). Diagnoses were made from the SADS-L interview data independently by two raters: a clinical social worker and a clinical psychologist. Diagnostic differences were resolved in a consensus conference that included a senior psychiatrist experienced in diagnosis in American Indian people. Sampling strategy, interview procedure, and diagnosis protocol are summarized from Long et al. (1998) and Robin et al. (1998).

Interview data were also used to evaluate subjects for binge drinking (≥three episodes of consuming a case of beer or equivalent/day for at least three days) and presence of the "flushing response" symptoms [occurrence of facial flushing, flushing elsewhere, itchiness, dizziness, drowsiness, anxiety, headaches, head pounding, sweating, accelerated heart rate, nausea, shortness of breath, and chills after ingestion of alcohol (see Higuchi et al. 1992 for a complete description of the flushing questionnaire)]. Flushing score was determined by averaging the responses (1=never, 2=sometimes, 3=always) to ten out of 13 questions (headaches, shortness of breath and chills were eliminated due to insufficient responses). Flushing score could vary from 1 to 3 with higher scores indicating more intense flushing. Wall et al. (1995) reported that investigator-observed flushing was a more accurate predictor of *ALDH2* genotype than self-reported flushing based on a dichotomous variable that measured facial flushing only. To compensate for possible subject bias, our measure of flushing included three possible responses to ten questions resulting in a continuously distributed variable.

Genetic typing

Eight restriction site polymorphisms were assayed. Location of the variants is presented in Fig. 1 and conditions for PCR analysis of the variants are presented in Table 1. *ADH1B Arg47His* and *ADH1C Ile349Val* were assayed two times using two different primer pairs, while all other polymorphisms were assayed using a single protocol (Table 1). Restriction digests were performed with the restriction enzymes (New England Biolabs, Beverly, Mass.) listed in Table 1 and were analyzed by electrophoresis on 2% agarose or 4% Metaphor (FMC BioProducts, Rockland, Me.) gels. Microsatellite marker D4S1647 was typed as described in Urbanek et al. (1996). These *ADH* variants (plus one additional site) were recently assayed in 40 populations distributed worldwide (Osier et al. 2002). Osier et al. (2002) presented ancestral states for all variants and used the same nomenclature as the current study with the exception of *ADH1C Ile349Val*, in which the Val allele was designated as allele 2. *ADH1B Arg369Cys* has been identified in Mission Indians with known non-American Indian admixture (Wall et al. 1997), but was not polymorphic in a subset of the samples analyzed here (Osier et al. 2002) and was not assayed in the current study.

Haplotype determination

Allele frequencies were determined by direct gene counting. Genotype distributions for each site were evaluated for departure from Hardy-Weinberg equilibrium (HW equilibrium) using a contingency table test. Haplotypes for the *ADH1B*/*ADH1C* (five sites) and *ALDH2* (three sites) loci were estimated from phase-unknown multi-site genotypes (Weir 1996) using an E-M algorithm [multiple locus haplotype analysis (MLOCUS) (Long et al. 1995)]. The MLOCUS program provides all possible phase-known genotypes compatible with an individual's phase-unknown genotype, and assigns relative probabilities to each phase-known possibility. The program allows one to test the model of proposed haplotypes by removing inferred haplotypes (not directly observed in multi-site homozygotes or single site heterozygotes) and recalculating the log

Table 1 *ADH1B*/*ADH1C* and *ALDH2* variants with corresponding amplification conditions and oligonucleotide sequences. All PCRs were performed using 200 ng of genomic DNA, 0.5 μM each primer, 200 μM each dNTP, 2 μl GeneAmp buffer [PE Applied Biosystems, Foster, Calif., 1x=10 mM Tris (pH 8.3), 50 mM KCl], 1.5–2.0 mM $MgCl_2$ and 0.4–1.0 U Amplitaq Gold (PE Applied Biosystems) in a final volume of 20°μl. Amplifications were performed in a Perkin Elmer 9600 Thermocycler with an initial 10 min 95 °C denaturation step and a final 5 min 72 °C extension step

Locus	Primer name	Primer sequence	PCR conditions[a]	RE
ADH1B Arg47His	#247 #303	GAAGGGGGGTCACCAGGTTG ATTCTGTAGATGGTGGCTGT	94 °C (30 s), 60 °C (30 s), 72 °C (30 s); 35 cycles; 2.0 mM $MgCl_2$ [ref 1]	*Mae*III
ADH1B Arg47His (alternate)	A2FXNFOR A2FXNREV	ATTCTAAATTGTTTAATTCAAGAAG ACTAACACAGAATTACTGGAC	95 °C (30 s), 56 °C (30 s), 72 °C (60 s); 35 cycles; 2.0 mM $MgCl_2$ [ref 2]	*Msl*I
ADH1B RsaI	A2IN3DW3 A2IN3UP2	ATATTTATTTTACCCTAAACTTATG GAGCTAAAACATACTTTGGATAG	94 °C (30 s), 60 °C (30 s), 72 °C (30 s); 35 cycles; 1.5 mM $MgCl_2$ [ref 4]	*Rsa*I
ADH1C EcoRI	A3EX2DW A3EcoUP2	TTGCACCTCCTAAGGCTC TCTAATGCAAATTGATTGTGAAC	94 °C (15 s), 51 °C (15 s), 72 °C (75 s); 40 cycles; 2.0 mM $MgCl_2$; 5% DMSO [ref 2]	*Eco*RI
ADH1C HaeIII	A3EX5FOR2 A3EX5REV1	TGAGTTTGCACATTAGTTATGG TGCTCTCAGTTCTTTCTGGG	94 °C (40 s), 56 °C (30 s), 72 °C (60 s); 35 cycles; 2.0 mM $MgCl_2$ [ref 2]	*Hae*III
ADH1C Ile349Val	#321 #351	GCTTTAAGAGTAAATATTCTGTCCCC AATCTACCTCTTTCCGAAGC	94 °C (30 s), 55 °C (30 s), 72 °C (30 s); 35 cycles; 2.0 mM $MgCl_2$ [ref 1]	*Ssp*I
ADH1C Ile349Val (alternate)	A3FXNFOR1 A3FXNREV3	TTGTTTATCTGTGATTTTTTTTGT CGTTACTGTAGAATACAAAGC	94 °C (15 s), 51 °C (15 s), 72 °C (75 s); 40 cycles; 2.0 mM $MgCl_2$ [ref 2]	*Ssp*I
ALDH2-5'	5′.for 5′.rev	GCAGTGCCGTCTGCCCCATCCATGT GGCCCGAGCCAGGGCGACCCTGAGCT	94 °C (30 s), 60–62 °C (30 s), 72 °C (30 s); 40 cycles; 1.5 mM $MgCl_2$ [ref 3]	*Sac*I
ALDH2-In6A	In6A.For In6A.Rev	AAATATTGCTCTAGGCCAGGC TGGGAATTCTAAATGGGACGG	94 °C for 10 cycles/89 °C for 30 cycles(30 s), 55 °C (30 s), 72 °C (30 s); 2.0 mM $MgCl_2$ [ref 3]	*Hae*III
ALDH2-1,2	L12 R12	TTTGGTGGCTAGAAGATGTC CACACTCACAGTTTTCTCTT	94 °C (30 s), 57 °C (30 s), 72 °C (30 s); 40 cycles; 2.0 mM $MgCl_2$ [ref 4]	*Mbo*II

[a]References for PCR protocols in *square brackets*: *1* Groppi et al. (1990), *2* http://info.med.yale.edu/genetics/kkidd and Osier et al. (2002), *3* Peterson et al. (1999), *4* Dandre et al. (1995)

likelihood value. For each of the five studied populations, all inferred haplotypes were removed if they did not significantly improve the model as assessed by a log likelihood ratio. Based on this criterion, the following numbers of unconfirmed haplotypes were removed from each population: American Indian, 3; Siberian, 2; Mongolian, none; Chinese, none; Nigerian, none. The American Indian population had more unconfirmed haplotypes than the others, most likely because it had the largest sample size and contained relatives. However, all of the unconfirmed haplotypes were rare and increased the likelihood of particular individuals only trivially. For each individual, in the SW American Indian population, only one phase-known genotype was possible based on the final set of confirmed haplotypes at both *ADH* and *ALDH2*. The phase-known genotype inferred for each American Indian participant was confirmed by transmission through the pedigree. Some individuals and variants were selected for retyping for the following reasons: improbable genotype frequencies, absence of HW equilibrium, pedigree violations, and/or inconsistent linkage phase. Overall, the PCR success rate was 95% and typing accuracy was 99.5% based on retypings.

Linkage analysis

Linkage analysis was conducted by means of the nonparametric sib-pair regression method of Haseman and Elston (1972) using the SIBPAL module of the S.A.G.E. package (S.A.G.E. 1994). In the Haseman-Elston method, the squared trait difference between siblings is regressed on the estimated proportion of marker alleles *P* shared identical by descent (IBD). A negative slope is indicative of linkage because siblings who resemble each other in the trait of interest tend to share alleles that are IBD. Since the accuracy of sib-pair linkage analysis depends on large sampling approximations, *P* values were verified by computer simulations as described in Lappalainen et al. (1998). Parallel linkage analyses were performed that included age and gender as covariates. The results of these analyses did not differ from those reported and only the simpler models are reported.

Association analysis

Association was measured by a contingency table χ^2 statistic as described in Lappalainen et al. (1998). Basically, an empirical sample distribution was generated by estimating population allele frequencies from the sample and then simulating genotypes for the pedigree founders based on the population frequencies. The genotypes were transmitted to the offspring and the contingency table χ^2 statistic was computed for the simulated data set. After >1,000 replications, a null distribution for the χ^2 statistic was generated and was used to determine the significance of observations in the original contingency table.

Nested cladistic analysis

Cladograms for the haplotypes were constructed using the principle of maximum parsimony. The nesting design was determined following Templeton et al. (1987). Association between discrete phenotypes (alcohol dependence and binging) and genetic typings was evaluated using a series of nested two (affected and unaffected) $\times n_i$ likelihood ratio χ^2 contingency analyses, where n_i was the number of clades in nesting category i. The continuously distributed phenotype (flushing) was analyzed by calculating the average excess of each haplotype and tested for significance by permuting the haplotype count data 1,000 times over the entire dataset as described by Templeton et al. (1988). When age and gender were included as covariates, the results did not differ significantly and are not reported.

Results

Clinical diagnoses

Alcohol dependence is highly prevalent in the study population and is more common in men than women (Table 2). Approximately 80% of the men and 55% of the women in this sample met the DSM-III-R criteria for alcohol dependence at some point in their lives. While many of these participants were in remission at the time of examination, these data emphasize the heavy burden of alcohol-related problems in this population. In light of the high prevalence, it is noteworthy that the average age at participation exceeded the age-of-onset of alcohol dependence by 15–20 years (Table 2). Binging is also highly prevalent with an even stronger bias towards men compared with a diagnosis of alcohol dependence. Average flushing scores showed no difference between men and women. Groups 1 and 2 exhibited equivalent prevalence of alcohol dependence and binging and similar average flushing scores, before stratification by sex.

Allele frequencies

Frequencies of the five *ADH1C*/*ADH1B* and three *ALDH2* alleles are shown in Table 3. *ADH1C EcoRI* and *ADH1C HaeIII* showed relatively high levels of polymorphism (21% and 40% site absent alleles, respectively). *ADH1C Ile349Val* showed levels of polymorphism (40% *ADH1C*349Val*) similar to those previously reported for American Indians (Osier et al. 2002). Typically, *ADH1B*47His* is not seen in American Indian populations and was not detected in our study population. *ADH1B RsaI* was virtually monomorphic as had been previously reported in a study sample with overlap to the current sample (Osier et al. 1999). *ALDH2-5′*, *ALDH2-In6A* and *ALDH2-2* had been assayed previously in a subset of the American Indian samples analyzed here (Peterson et al. 1999). The *ALDH2* results presented here are similar to previous results including absence of the deficiency allele (*ALDH2-2*) as expected for an American Indian population.

Allele distributions at all eight loci in Group 2 were tested for departure from HW equilibrium and are presented in Table 3. The three *ADH1C* alleles showed significant departure from HW equilibrium. This effect is most likely due to the family structure of the sample, which will result

Table 2 Clinical phenotypes in American Indian population (*n.d.* indicates calculations that were not performed)

	n	Average age ± SE (years)	Prevalence of alcohol	Average age of onset ± SE[a] dependence	Prevalence of binging	Average flushing score ± SE[b]
Group 1	121	41.4 ± 1.2	66.9%	n.d.	41.3%	1.29 ± 0.02
Females	78	43.5 ± 1.5	60.2%	23.8 ± 1.4	26.9%	1.31 ± 0.03
Males	43	37.6 ± 1.8	79.1%	17.5 ± 0.7	67.4%	1.25 ± 0.04
Group 2	490	36.0 ± 0.6	66.3%	n.d.	42.2%	1.31 ± 0.01
Females	276	37.0 ± 0.8	52.5%	22.0 ± 1.4	25.4%	1.32 ± 0.02
Males	214	34.7 ± 0.8	84.1%	16.9 ± 0.4	64.0%	1.29 ± 0.02

[a]Average age of onset was determined for the original sample sets with larger numbers of individuals (Group 1, *n*=152 and Group 2, *n*=582)

[b]Flushing score was determined by averaging the responses (1= never, 2=sometimes, 3=always) to flushing questions. Flushing score could vary from 1 to 3 with higher scores indicating more intense flushing.

Table 3 *ADH* and *ALDH* allele frequencies in American Indian population (*n.d.* indicates tests not performed because of insufficient heterozygosity)

	Site absent	Site present	No. of chromosomes	HW equilibrium[a]
ADH1C EcoRI	0.21	0.79	988	0.006
ADH1C HaeIII	0.40	0.60	984	0.034
ADH1C Ile349Val	0.40 (Val)	0.60 (Ile)	992	0.034
ADH1B Arg47His	1.00 (Arg)	-0- (His)	990	n.d.
ADH1B RsaI	0.99	0.01	974	n.d.
ALDH2–5'	0.53	0.47	980	0.457
ALDH2-In6A	0.74	0.26	982	0.570
ALDH2-1,2	-0-	1.00	990	n.d.

[a]*P* values for departure from Hardy-Weinberg equilibrium

Table 4 *ADH* and *ALDH* haplotype frequencies in American Indian and Asian populations. Haplotypes are based on the chromosomal configuration of assayed variants: *ADH1C EcoRI*/*ADH1C HaeIII*/*ADH1C Ile349Val*/*ADH1B Arg47His*/*ADH1B RsaI* and *ALDH2-5′*/*ALDH2-In6A*/*ALDH2-1,2*. A *1* designates an allele from the first column of Table 2 and a *2* designates alleles from the second column of Table 2

Haplotypes	American Indian	Chinese	Mongolian	Siberian	Nigerian
ADH1C/*ADH1B*					
Ht1 (11111)	21%	3.1%	2.1%	8.0%	1.7%
Ht2 (21111)	19%	3.0%	14%	16%	-0-
Ht3 (22211)	58%	3.3%	44%	52%	82%
Ht4 (22212)	1.3%	4.7%	9.2%	14%	3.2%
Ht5 (22222)	-0-	78%	30%	10%	-0-
Ht6 (21122)	-0-	8.0%	-0-	-0-	-0-
Ht7 (21211)	-0-	-0-	-0-	-0-	4.5%
Ht8 (21212)	-0-	-0-	-0-	-0-	1.8%
Ht9 (22111)	-0-	-0-	-0-	-0-	2.0%
Ht10 (12111)	-0-	-0-	-0-	-0-	5.3%
ALDH2					
Ht1 (222)	26%	26%	37%	40%	20%
Ht2 (212)	21%	34%	28%	26%	49%
Ht3 (112)	53%	12%	32%	34%	31%
Ht4 (211)	-0-	26%	3.6%	-0-	-0-
No. of chromosomes	978	64	54	50	50

in an excess of homozygosity as seen here. *ALDH2-5′* and *ALDH2-In6A* exhibited no departure from HW equilibrium. *ADH1B Arg47His*, *ADH1B RsaI* and *ALDH2-2* had insufficient levels of variation to adequately test for HW equilibrium.

Haplotype frequencies and linkage disequilibrium

Four haplotypes (Hts) based on the five *ADH1C*/*ADH1B* variants were directly observed in the American Indian dataset (Table 4). Hts1–3 were observed at polymorphic frequencies, but Ht4 was observed on only 13 chromosomes out of 978 tested. The *ADH1C*/*ADH1B* variants were in linkage disequilibrium with $D'=1.0$ for all pairs of sites (Weir 1996). This indicates the absence of recombination over the entire region of approximately 40 kb extending from *ADH1C EcoRI* to *ADH1B RsaI*. A single cladogram could be drawn that connected all haplotypes with no evidence of recombination (Fig. 2). The order of *ADH1C HaeIII* and *ADH1C Ile349Val* could not be determined based solely on the American Indian data because of complete cosegregation of these markers. However, Ht7 in the Nigerians fit the cladogram most parsimoniously between Hts 2 and 3, which placed *ADH1C Ile349Val* after *ADH1C HaeIII* when moving outward in the cladogram (Fig. 2). Alternatively, Osier et al. (2002) inferred a haplotype in two American Indian populations that would reverse the order of *ADH1C HaeIII* and *ADH1C Ile349Val*. Osier et al. (2002) also inferred five additional *ADH* haplotypes present at low frequencies in four American Indian populations. Two of Osier and co-worker's (2002) haplotypes, including the one that would reverse the order of *ADH1C HaeIII* and *ADH1C Ile349Val,* were removed from our dataset based on insignificant improvement of the model, suggesting that a more minimal set of haplotypes may exist for the populations investigated in Osier et al. (2002).

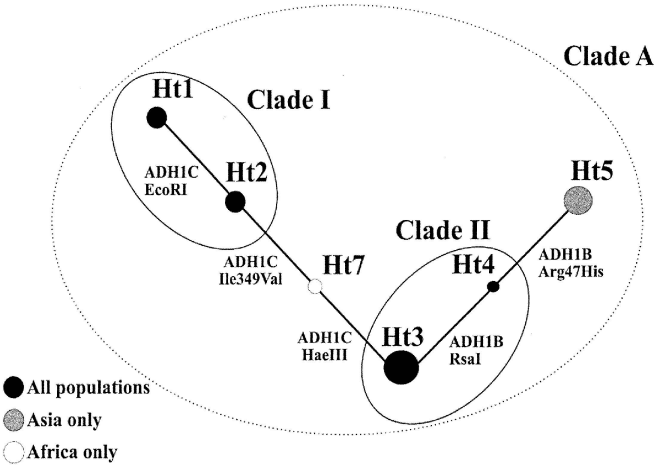

Fig. 2 Cladogram illustrating the relationship between *ADH1B*/*ADH1C* haplotypes. Recombinant haplotypes (Hts 6, 8–10) are not depicted. The *size* of each circle reflects the frequency of that haplotype in the American Indian population, with the exception of the haplotypes absent in the American Indian population in which case circle size reflects the average frequency of the haplotype in the population(s) in which they occur. Clades tested in the nested clade analysis are labeled

ADH1C/*ADH1B*/Hts1–4 were also observed in the three Asian populations (Table 4), along with two additional haplotypes, Ht5 and Ht6. Both of these haplotypes possessed the *ADH1B*47His* allele, which was not detected in the American Indian population. One of the haplotypes, Ht5, was present in all three Asian populations and showed a striking south-to-north frequency cline; 78% (Chinese) to 30% (Mongolian) to 10% (Siberian) to 0% (American Indian). This haplotype fit the original cladogram by adding one step. The remaining haplotype, Ht6, appeared to be a possible recombinant or gene convertant based on its restricted presence in only three Chinese individuals. In the African samples, Ht2 was not detected and exhibited a geographic frequency cline from 0% (Nige-

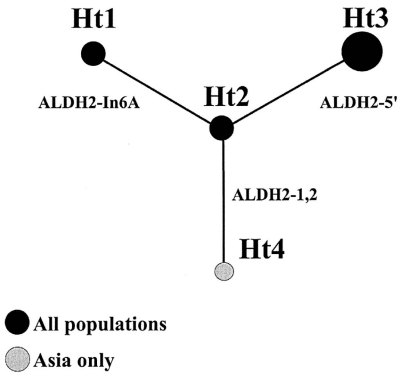

Fig. 3 Cladogram illustrating the relationship between *ALDH2* haplotypes. The *size* of each circle reflects the frequency of that haplotype in the American Indian population, with the exception of the Asia-only haplotype that reflects the average frequency of that haplotype in three Asian populations

Three haplotypes based on the three *ALDH2* variants were directly observed in the American Indian dataset (Table 4). The *ALDH2* variants exhibited complete linkage disequilibrium (D'=1.0) over the region of approximately 40 kb extending from *ALDH2-5′* to *ALDH2-2*. A single cladogram could be drawn that connected all haplotypes with no evidence of recombination (Fig. 3). *ALDH2*/Hts1–3 were also observed in the Asian and African populations (Table 4). One additional haplotype that exhibited the *ALDH2-2* variant was detected in the Chinese and Mongolians. This haplotype showed a marked geographic cline with frequencies of 26% in the Chinese, 4% in the Mongolians, and 0% in the Siberians and American Indians. The *ALDH2-2* haplotype fit the original cladogram by adding one additional step so that the Asian and African data confirmed the topology of the cladogram based solely on the American Indian data.

rians) to 3% (Chinese) to 14–16% (Mongolians and Siberians) to 19% (American Indians). Four additional rare haplotypes (Ht7–10) were detected only in the African samples. Osier et al. (2002) assayed the same *ADH* sites in 40 worldwide populations. They found no individuals with Hts 6, 8, or 10 and inferred six additional haplotypes present at low frequencies in Asian and African populations. This difference in haplotype distribution may reflect the fact that different Asian and African populations were analyzed by Osier et al. (2002).

Linkage analysis – linkage of *ADH*/*ALDH* variants with alcohol dependence and binging

Sib-pair linkage analysis was conducted to test for linkage between the *ADH*/*ALDH* loci and a quantitative trait locus that influences alcohol dependence or binge drinking (Table 5). Variants *ADH1B Arg47His*, *ADH1B RsaI*, and *ALDH2-1,2* were eliminated from the analysis because of insufficient levels of polymorphism. The remaining variants were analyzed individually and also as haplotypes in

Table 5 Linkage analysis of *ADH*/*ALDH* loci with alcohol dependence and binging. Probabilities of allelic identity by descent (IBD), as computed by SIBPAL, are given individually for the three sib-pair classes. Nominal *P* values are from the Haseman-Elston sib-pair regression, which combines information over all three affected/unaffected classes of sib-pairs. *Bold type* indicates variants that were significant in both sib-pair linkage and Haseman-Elston regression analyses

Marker	Alcohol dependence				Binging			
	IBD in sib-pairs			Nominal regression *P* value	IBD in sib-pairs			Nominal regression *P* value
	Unaffected	Discordant	Affected		Unaffected	Discordant	Affected	
Group 1, *n*=122								
ADH1C EcoRI	0.5189	0.5112	0.5178	0.41877	**0.5487***	**0.4774**	**0.5272**	**0.02106**
ADH1C HaeIII	0.5417	0.4891	0.4923	0.39305	**0.5376**	**0.4399***	**0.5272**	**0.00392**
ADH1C Ile349Val	0.5417	0.4890	0.4967	0.35390	**0.5376**	**0.4468***	**0.5273**	**0.00676**
ADH1C haplotype	0.5389	0.5071	0.4940	0.55876	0.5428	0.4541	0.5230	0.03815
D4S1647	**0.6447**	**0.3929***	**0.5624**	**0.00832**	0.5624	0.4301	0.5171	0.06300
ALDH2-5'	0.4533	0.5431	0.5225	0.70728	0.6003	0.4767	0.4569	0.06221
ALDH2-In6A	0.4803	0.5511	0.5012	0.86891	0.5544	0.5244	0.4121	0.59728
ALDH2 haplotype	0.4545	0.5398	0.5302	0.62067	0.5981	0.4981	0.4260	0.21314
Group 2, *n*=490								
ADH1C EcoRI	0.5019	0.4959	0.5108	0.20662	0.5086	0.4960	0.5125	0.18231
ADH1C HaeIII	0.5149	0.4917	0.4980	0.28725	0.5119	0.4818	0.5107	0.05400
ADH1C Ile349Val	0.5131	0.4911	0.4993	0.26623	0.5112	0.4827	0.5106	0.06136
ADH1C haplotype	0.4927	0.4930	0.4929	0.50235	0.5081	0.4789	0.4969	0.12613
D4S1647	**0.5085**	**0.4742**	**0.5485***	**0.03398**	0.4827	0.5122	0.5720	0.58056
ALDH2-5'	0.5335	0.5027	0.5142	0.23282	0.5336	0.5158	0.4523	0.61198
ALDH2-In6A	0.5195	0.5048	0.5039	0.43603	0.5302	0.4990	0.4720	0.22910
ALDH2 haplotype	0.5324	0.4907	0.5174	0.12440	0.5309	0.5121	0.4465	0.59455

*$P<0.05$.

Table 6 Association analysis of *ADH/ALDH* markers with alcohol dependence and binging. *Bold type* indicates significant results

Marker	Alcohol dependence		Binging	
	Chi-square	*P* value	Chi-square	*P* value
Group 1, *n*=122				
ADH1C EcoRI	0.90	0.34	0.02	0.88
ADH1C HaeIII	**6.27***	**0.01**	0.01	0.91
ADH1C Ile349Val	**6.16***	**0.01**	0.01	0.93
ADH1C haplotype	**7.41***	**0.02**	0.11	0.94
D4S1647	6.26	0.28	6.04	0.30
ALDH2-5'	0.30	0.58	0.38	0.54
ALDH2-In6A	0.01	0.92	1.43	0.23
ALDH2 haplotype	0.54	0.76	1.30	0.52
Group 2, *n*=490				
ADH1C EcoRI	0.28	0.60	0.09	0.77
ADH1C HaeIII	0.69	0.41	0.18	0.67
ADH1C Ile349Val	0.80	0.37	0.17	0.68
ADH1C haplotype	1.45	0.69	1.78	0.62
D4S1647	11.18	0.08	7.27	0.30
ALDH2-5′	0.03	0.85	1.54	0.21
ALDH2-In6A	0.01	0.93	0.28	0.60
ALDH2 haplotype	0.13	0.94	1.54	0.46

*0.01<*P*<0.05. The odds ratio for these alleles/haplotype and alcohol dependence is 2.0. These results are perfectly correlated due to the fact that *ADH1C HaeIII* and *ADH1C Ile349* exist on only one haplotype

Table 7 Nested clade analysis of *ADH1B/ADH1C* for alcohol dependence and binging. Haplotypes are based on the five assayed *ADH1B/ADH1C* variants in their chromosomal configuration, *ADH1C EcoRI/ADH1C HaeIII/ADH1C Ile349CVal/ADH1B Arg47His/ADH1B RsaI*, and are defined as follows: Ht1=11111, Ht2=21111, Ht3=22211, Ht4=22212 (haplotypes as defined in Table 3). *Bold type* indicates significant results

Marker	Alcohol dependence		Binging	
	Chi-square	*P* value	Chi-square	*P* value
Group 1, *n*=120				
Zero-step clades				
Ht1-Ht2	1.04	0.3070	0.10	0.7508
One-step clade				
Clade I-Clade II	**6.27***	**0.0123**	0.01	0.9097
Group 2, *n*=489				
Zero-step clades				
Ht1-Ht2	0.02	0.8940	0.38	0.5355
Ht3-Ht4	0.24	0.6278	1.96	0.1611
One-step clade				
Clade I-Clade II	1.08	0.2989	0.00	0.9983

**P*<0.05

order to maximize the power of the analysis (D4S1647 was not included in a haplotype). Results were considered significant only if the following criteria were met: *P* value <0.05 in both sib-pair and Haseman-Elston regression analysis, number of sib-pairs in each category >25 (only in Group 2), allele sharing >0.5 for concordant sib-pairs and allele sharing <0.5 for discordant sib-pairs. Significant evidence for linkage was detected between alcohol dependence and D4S1647 in Groups 1 and 2 (Table 5). Significant evidence for linkage was detected between binge drinking and *ADH1C EcoRI, ADH1C HaeIII*, and *ADH1C Ile349Val* in Group 1 only (Table 5). Although not significant, evidence for linkage was also detected in Group 2 between binging and *ADH1C HaeIII* and *ADH1C Ile349Val*, the two markers with the highest significance values for linkage to binging in Group 1.

Association analysis – association of *ADH/ALDH* variants with alcohol dependence and binging

ADH/ALDH variants were tested for association with alcohol dependence or binge drinking (Table 6). As in the linkage analysis, variants *ADH1B Arg46His*, *ADH1B RsaI*, and *ALDH2-1,2* were eliminated from analysis because of insufficient polymorphism. The remaining variants were analyzed individually and also as haplotypes in order to maximize the power of the analysis (D4S1647 was not included in a haplotype). When Group 1 (*n*=122) was analyzed, significant evidence for association was identified between alcohol dependence and *ADH1C HaeIII*, *ADH1C Ile349Val* and the *ADH1C* haplotype (Table 6). These results are perfectly correlated due to the fact that *ADH1C HaeIII* and *ADH1C Ile349* exist on only one haplotype.

Nested clade analysis – association of *ADH/ALDH* variants with alcohol dependence, binge drinking, and flushing

Clades were identified, in a nested manner, in the *ADH* and *ALDH* cladograms using the algorithm of Templeton et al. (1987) and are shown in Fig. 2 and Fig. 3, respectively. The number of zero-step clades is simply the number of haplotypes and, for the *ADH* cladogram, was equal to four (American Indian data only). There were also two one-step and one two-step clades for the *ADH* locus (Fig. 2). For *ALDH*, there were three zero-step clades that are all included in a single one-step clade (American Indian data only; Fig. 3).

Alcohol dependence and binging were treated as discrete phenotypes and were evaluated using likelihood ratio χ^2 statistics for each level of nesting. A significant χ^2 value was detected only in Group 1 for alcohol dependence between Clades I and II, which are distinguished by variants *ADH1C HaeIII* and *ADH1C Ile349Val* (*P*<0.05; Table 7). Specifically, the *ADH1C HaeIII*-site-present allele and the *ADH1C*349Ile* were associated with an increase in alcohol dependence.

Since there was only a single one-step clade in the *ALDH* cladogram, nested clade analysis was not performed. Instead, all combinations of haplotype frequencies were tested for association with alcohol dependence, binging, and flushing. No significant χ^2 values were detected (data not shown). The highest χ^2 values were detected with binging. Additional analyses of binging were performed by

Table 8 Linear regression analysis of *ADH*/*ALDH* loci with flushing. *Bold type* indicates significant results

Marker	*df*	Slope	*P* value
Group 1, *n*=122			
ADH1C EcoRI	49	–198.75	0.08383
ADH1C HaeIII	48	–131.08	0.15782
ADH1C Ile349Val	48	–134.16	0.14882
ADH1C haplotype	48	–70.89	0.23692
D4S1647	26	61.42	0.79916
ALDH2-5'	49	–47.73	0.28921
ALDH2-In6A	48	–155.47	0.06090
ALDH2 haplotype	49	–47.90	0.25846
Group 2, *n*=490			
ADH1C EcoRI	210	–2.17	0.48964
ADH1C HaeIII	211	61.90	0.81698
ADH1C Ile349Val	213	62.13	0.81646
ADH1C haplotype	214	36.71	0.73902
D4S1647	142	15.57	0.62089
ALDH2-5'	208	–37.01	0.26334
ALDH2-In6A	213	**–135.90***	**0.02164**
ALDH2 haplotype	212	–64.87	0.09356

*$P<0.05$

stratifying the Group 2 dataset by sex and by age. Again, no significant χ^2 values were detected (data not shown).

Flushing was a continuously distributed phenotype and was analyzed by calculating the average excess of each haplotype and tested for significance by permuting the haplotype count data 1,000 times over the entire dataset as described by Templeton et al. (1988). Significant evidence for association was detected between flushing and *ALDH2-In6A* in Group 2 (Table 8).

Discussion

In the present study, allelic variation at *ADH* and *ALDH* genes was studied in an American Indian population with respect to alcohol dependence and two related intermediate phenotypes, flushing and binge drinking. As previously reported for American Indian populations, neither of the well-established protective alleles, *ADH1B*47His* and *ALDH2-2*, were detected in the study population. A high prevalence and high heritability of alcohol dependence in American Indians suggested that additional genetic factors increasing vulnerability to alcohol dependence were present. Therefore, the study population was chosen because of the unique combination of absence of *ADH1B*47His* and *ALDH2-2*, high prevalence of alcohol dependence, presence of flushing, and availability of related physiological, behavioral, and clinical data. The presence of related individuals and a large sample size allowed both linkage and association analyses to be conducted. Flushing was utilized as a physiological response to drinking that had been previously identified as protective against alcoholism. Binge drinking was utilized as a drinking behavior that increases risk of alcoholism.

Low levels of allelic and haplotypic variation detected in the American Indian population likely reflects the unique population history of New World indigenous groups, including founder effects and possible population bottlenecks related to New World colonization (Kolman et al. 1995; Kolman and Bermingham 1997). The complete association of alleles at *ADH1C HaeIII* and *ADH1C Ile349Val* also supports this interpretation. An intermediate haplotype was detected in the African population suggesting that the haplotype was lost from New World and Asian populations through founder effects and/or genetic drift. Greater allelic and haplotypic variation in Africans is consistent with the greater genetic diversity and greater time depth that has been previously detected in African populations (Tishkoff et al. 1996; Jorde et al. 1997; Harris and Hey 1999; Hammer et al. 2001).

No evidence of recombination was detected at the *ADH* or *ALDH* loci in the American Indian population. One Chinese and three Nigerian *ADH* haplotypes appear to have arisen by recombination and/or gene conversion, a result that is consistent with a deeper evolutionary history in these populations compared with American Indians. Conversely, no evidence of recombination at the *ALDH* locus was detected in the Asian or African populations, a result that mirrors that found in the American Indian population. With respect to current efforts to define haplotype blocks or linkage disequilibrium maps in the human genome (Gabriel et al. 2002; Dawson et al. 2002), these data suggest that haplotype structure is likely to be extremely sensitive to chromosomal location and population history.

Both association and linkage analysis identified multiple *ADH1C* markers that affect alcohol dependence and binge drinking. Two *ADH1C* variants (*ADH1C HaeIII* allele 2 and *ADH1C*349Ile*) were associated with an increase in alcohol dependence (only in Group 1). The neighboring microsatellite marker, D4S1647, demonstrated linkage to alcohol dependence (Groups 1 and 2). Association analysis is statistically powerful over very short physical distances, which may explain the evidence for association between alcoholism and the *ADH* alleles, but not with the more distant microsatellite marker. On the other hand, the power of linkage analysis is enhanced by highly polymorphic markers, which may explain the evidence for linkage to alcoholism with the microsatellite marker but not with the biallelic markers. The combined weight of the linkage and association analyses strengthens the role of *ADH* as a candidate locus for alcohol dependence. Specifically, *ADH1C*349Ile* and *ADH1C HaeIII* allele 2 are highlighted as conferring a slight increase in risk of alcoholism. These markers may have a direct effect on risk of alcoholism or they may be in linkage disequilibrium with an unassayed marker that affects alcoholism. Recent studies have proposed that previously reported protective effects of *ADH1C*349Ile* were due to linkage disequilibrium with *ADH1B*47His* (Chen et al. 1999; Osier et al. 1999). However, the effect of *ADH1C*349Ile* typically has been investigated in the presence of *ADH1B*47His*. It is possible that the small increase in risk due to *ADH1C* variants detected in the current study was overwhelmed by the larger,

protective effect of *ADH1B*47His* in previous studies (Thomasson et al. 1993; Chen et al. 1996; Nakamura et al.1996). Furthermore, the large sample size assayed in the current study afforded greater statistical power to detect small effects on disease vulnerability when compared to previous studies.

Significant effects were also detected for binge drinking and flushing. Three *ADH1C* sites (*ADH1C EcoRI, ADH1C HaeIII,* and *ADH1C Ile349Val*) exhibited linkage to binge drinking (Group 1 only). *ALDH2-In6A* demonstrated linkage with flushing (Group 2 only). The fact that the binging phenotype highlighted the same locus (and some of the same variants) as alcohol dependence offers a tantalizing indication that this endophenotype may be used to represent alcohol dependence and provides further support for the importance of *ADH1C* in alcohol dependence.

Significant effects of *ADH1C* alleles were detected in Group 1, but not in Group 2. Since the two samples were collected to represent alcoholism in the same population, it is difficult to identify specific factors that vary between the samples and could account for the difference in results. Prevalence of alcohol dependence and frequency of tested genetic variants did not differ significantly. Two factors did vary significantly between the two samples: sex ratio and average age (Table 1). First, there were more females in Group 1 relative to Group 2 (64.5% vs 56.3%). Females in this population show a higher heritability for alcoholism than males (Long et al. 1998). Therefore, increased numbers of females may increase the genetic loading for alcoholism in Group 1 and tip the balance in favor of a significant genetic effect. Second, members of Group 1 were, on average, six years older than members of Group 2 (41.4 vs 36.0 years old). Members of both groups were older than the average age of onset of alcohol dependence (16.9 years and 22.0 years for males and females, respectively, in Group 2). Combined with a similar prevalence of alcohol dependence in both pedigrees, it seems unlikely that there are nascent cases of alcohol dependence in Group 2 that would alter the results. On the other hand, there could be cohort effects between the two samples. Specifically, if the younger participants experienced easier access to alcohol and drugs than the older participants, environmental effects, and not heritability, may have played a larger role in development of alcoholism in Group 2.

In sum, our results reinforce support for *ADH1C* as a candidate gene that affects vulnerability to alcoholism. Two *ADH1C* variants (*ADH1C HaeIII* allele 2 and *ADH1C*349Ile*) were associated with an increase in alcohol dependence (Group 1) and a neighboring microsatellite marker, D4S1647, demonstrated linkage to alcohol dependence (Groups 1 and 2). These data also highlight the sensitivity of linkage and association analyses to population history and demographics. In complex diseases, it has generally been assumed that individual genetic markers will have small, but relatively uniform, effects throughout human populations. Our results suggest that the effect of individual genetic variants may be much more population-specific and, consequently, more difficult to detect than previously thought.

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