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Association of the gastric alcohol dehydrogenase gene *ADH7* with variation in alcohol metabolism

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Seven alcohol-metabolizing enzymes are encoded by the human alcohol dehydrogenase (*ADH*) gene cluster on chromosome 4q22–23. One of these genes, *ADH7*, is uniquely expressed in the stomach mucosa and can influence metabolism of alcohol before its absorption into the blood. However, the contribution of *ADH7* to the overall genetic variation in alcohol oxidation *in vivo* is unknown. Data on *in vivo* alcohol metabolism were obtained for 206 Australian twin pairs of Caucasian ancestry, following ingestion of a standard dose (0.75 g kg^{-1} body weight) of alcohol. Twenty-five single nucleotide polymorphisms that cover the *ADH7* encoding region were genotyped. The patterns of linkage disequilibrium among these SNPs identified a recombinational hotspot within intron 7 of the *ADH7* gene. A model for the absorption and elimination of alcohol from the body led to the identification of haplotypes associated with inter-individual variation in the early stages of alcohol metabolism. These are within a 35 kb DNA tract contained in the region 5' of intron 7 in the *ADH7* gene. The region accounts for 18% of the linkage for alcohol concentration associated with the *ADH* region, or ~11% of the genetic variance.

INTRODUCTION

Variation in human alcohol (ethanol) metabolism is of interest because of known associations between alcohol dependence and polymorphisms in the alcohol-metabolizing enzymes alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). The disease associations of *ADH* gene variation have been the subject of extensive and continuing research (1–8), and the *ADH* allelic associations found are presumed to be mediated by effects on alcohol metabolism.

Linkage analysis using blood and breath alcohol concentration (BAC, BrAC) data from the Alcohol Challenge Twin Study (ACTS) (9) and subsequent microsatellite genotyping have shown strong evidence for a major quantitative trait locus (QTL) influencing alcohol metabolism, linked to the *ADH* gene region of chromosome 4. This QTL has been estimated to account for 64% of the genetic variance in alcohol metabolism (10). The coding polymorphisms *ADH1B* Arg47His and *ADH1C* Val349Ile, which are known to affect enzyme activity *in vitro*, accounted for only ~1% of the genetic variance in BAC *in vivo*. Therefore, this QTL must be attributable to other polymorphism(s) within or near the *ADH* gene cluster. Association studies with closely spaced single-nucleotide polymorphism (SNP) markers offer a way to locate and identify them.

The rate-limiting conversion of alcohol to acetaldehyde is catalysed by ADHs in the human stomach and liver. Seven *ADH* genes are in a cluster (5′-*ADH7-ADH1C-ADH1B-ADH1A-ADH6-ADH4-ADH5*-3′) on chromosome 4q22–23 (11–13). *ADH7* is only expressed in the oesophagus and gastric mucosa (14–18), whereas *ADH4, ADH6, ADH1A, ADH1B* and *ADH1C* are mainly expressed in the liver and account for ~80% of post-absorptive alcohol metabolism (19).

The *ADH*-related QTL affecting breath and blood alcohol levels (10) appears to act early in the time course of alcohol metabolism, soon after ingestion when ethanol concentrations are highest. This suggests that variation in ADH enzymes with a comparatively high K_m (low affinity) for alcohol, present in

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the stomach or liver, contributes to the QTL. Gastric ADH activity is primarily due to *ADH7*. The Class I genes *ADH1B* and *ADH1C* are also expressed in the gastric mucosa, and *ADH1C* is present in stomach muscle. However, the *in vitro* kinetic properties of ADH1B and ADH1C enzymes imply a smaller contribution to gastric metabolism than from ADH7, due to substrate inhibition of ADH class I enzymes by ethanol (20,21). Several authors have noted that at high alcohol concentration ADH7 has the highest activity of all stomach-expressed ADHs for ethanol (15,16,20–22), and therefore represents a good candidate for study. We have typed a total of 104 SNPs across the ADH gene cluster; this paper reports results from 25 SNPs within and flanking the *ADH7* gene. We have tested for association between these *ADH7* SNPs and variation in alcohol metabolism, and estimated the contribution of *ADH7* gene variation to the QTL previously described.

RESULTS

Linkage disequilibrium in the *ADH7* region

Two remarkably polarized haplotype blocks span the *ADH7* gene region, and separately include sequences flanking the 5′ and 3′ ends of the *ADH7* transcription unit, as shown in Figure 1. The short region between the haplotype blocks is marked by rs1154454 which is not in linkage disequilibrium (LD) with any other SNP. The 3′ haplotype block contains ten typed SNPs (rs1348276 to rs284784; see Table 1). SNPs rs1154458 to rs1583971 form a 5' block. This striking discontinuity of LD within the *ADH7* gene was also noted by Han *et al*. (22) in a study of 38 distinct populations. The 5′ and 3′ blocks of LD are consistent with a recombination hotspot within the transcribed *ADH7* gene. Since SNP rs1154454 (Intron 7) is not in LD with either the 5′ or 3′ blocks, it is likely that the hotspot is within Intron 7 where there would be no disruption to the coding sequences. Nevertheless, we cannot exclude Exon 7 and sequences in Intron 6 or the region 5′ of rs284784 in Intron 8 as boundaries of the hotspot. The preliminary scan of LD across the *ADH7* region showed a tendency for D′ values between SNPs located 5′ of Exon 1 (Fig. 1) to be less than unity, and the most 3′- located of the 25 genotyped SNPs, rs283406 through rs2032350, were part of another distinct haplotype block not involving *ADH7*. Cluster analysis [MERLIN 1.0.1 (23)] for all SNPs in the *ADH7* region also identified the two main 5′ and 3′ blocks, originally defined with HAPLOVIEW.

The discontinuities of LD in the *ADH7* region were confirmed in data within the Hapmap project (http://www.hapmap.org/cgi-perl/gbrowse/hapmap_B35/; NCBI B25 assembly, dbSNP125). The region in intron 7 marked by rs1154454 coincided with a hotspot (estimated recombination rate, 3.5 cM/Mb), whereas the ~11.6 kb region between *ADH7* and *ADH1C* and respectively bounded by rs2032350 and rs1348276, corresponded to a further recombination hotspot (31.7 cM/Mb). In summary, the *ADH7* gene is characterized by two distinct regions of high LD which are genetically uncorrelated with each other, and the discontinuity of LD marking two haplotype blocks is within intron 7. In addition, the Hapmap data show very little LD between *ADH7* and *ADH1C*. This is consistent with the activity of recombination hotspots that have eroded the LD between *ADH7* and other members of the *ADH* gene family during recent primate evolution.

The presence of three segregating units within the *ADH7* gene—haplotypes for the 5′ and 3′ blocks and the intervening region marked by SNP rs1154454—allowed us to minimize the number of tests for association of haplotypes with alcohol metabolism. Haplotype frequencies within the 5′ haplotype block were estimated for the cluster of adjacent SNPs identified when $r^2 \geq 0.6$. Within this block, the most frequent haplotype (haplotype 5′a in Table 2) includes all of the common alleles and there are six rarer haplotypes (haplotypes 5′b to 5′g). Three haplotypes (5′e, 5′f and 5′g) had frequencies of $<5\%$ and their effects on alcohol metabolism were too small to be estimated.

Nine haplotypes (3′a through 3′i in Table 2) were identified for the nine SNPs in the 3′ haplotype block using a threshold value of $r^2=0.6$ for adjacent SNPs. Within this block, we observed a common haplotype including all common alleles (haplotype 3′a) and eight further haplotypes. Haplotypes 3′b, 3′d and 3′f had frequencies of $<5\%$ and their effects were not estimated in the analysis of phenotypic associations.

The detection of phenotypic effects associated with the *ADH7* gene/region was made with reference to the most frequent and clearly defined haplotypes within the 5′ and 3′ blocks, as well as individual SNPs.

Preliminary tests for effects of *ADH7* variation on alcohol metabolism

The constrained or time-averaged effect reached the 5% level of statistical significance (Fig. 2) for BAC levels with SNPs rs1154461, rs1154468 and rs894363 and for BrAC levels with these three and also rs1154470. All four of these SNPs are 5′ proximal to the hotspot for recombination within *ADH7*. The effects were generally largest at the first BAC or BrAC reading and were statistically consistent over time for BrAC level, but were heterogeneous for BAC levels and fell with time for rs1154468 and rs1154470. The non-synonymous coding SNP rs1573496, flanked by rs1154461 and rs1154468, is at a lower frequency than the remaining SNPs in the 5' haplotype block (Table 1), and was not significantly associated with alcohol levels. Similarly, SNP rs971074 formed part of the 5′ haplotype block, yet was not associated with alcohol levels. Evidence of phenotypic association was only seen for the four most frequent SNPs in the 5′ block. Association in the 3′ haplotype block was confined to rs994772 which is proximal to, but 3′ of, the *ADH7* transcription unit. Allelic association was also present for rs283406, located 3′ of the *ADH7* region in a further small haplotype block.

Goodness of fit to the pharmacokinetic model

The solutions for mean sex-adjusted BAC and BrAC values, using Eq. (1) (see Materials and Methods section), were:

$$\mathrm{BAC}(t) = 11.70(1 - \mathrm{e}^{-2.51t}) - 1.51t \quad \text{and}$$

$$\mathrm{BrAC}(t) = 9.80(1 - \mathrm{e}^{-2.99t}) - 1.30t$$

These blood and breath parameter estimates are in reasonable agreement for both the absorption rate constant, k_1 (-2.51 and -2.99), and the elimination rate k_2 (1.51 and 1.30). The difference in A_0 (11.7 against 9.8) is consistent with the BAC

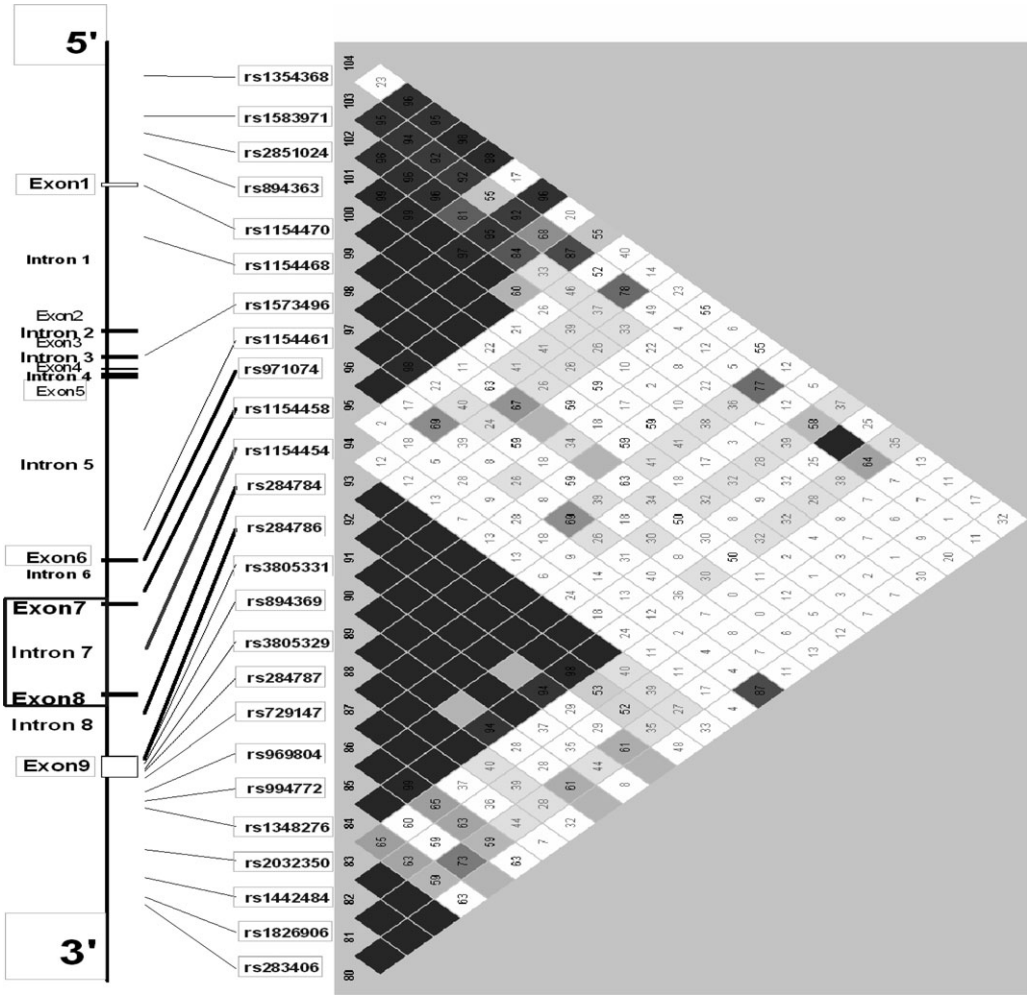

Figure 1. Pairwise patterns of linkage disequilibrium (D′) in the *ADH* region. SNP's rs971074, rs1154454, rs284784 and rs2872486 flank the region characterized by an absence of LD and marked by rs1154454 within the *ADH7* gene.

and BrAC means; the calibration of the breath analyser is known to produce under-estimation of the blood alcohol concentrations. Comparison of the goodness of fit of this model with an analysis in which only the means are estimated by maximum likelihood resulted in $\chi^2_3 = 4.73$; $P = 0.19$ for blood and $\chi^2_7 = 45.46$; $P = 1.1 \times 10^{-7}$ for breath alcohol readings. While the kinetic model did not yield a poorer goodness of fit than the empirical analysis of means at each time point for BAC readings, a much poorer fit to the observed readings was found for BrAC readings. This discrepancy may in part be due to the greater power to detect a departure from the model-free estimates of means provided by the longer time span of readings covered by the ten BrAC time points, and remained when the model was extended to include the large differences due to sex (Fig. 3). The deviation between observed and expected means in BrAC readings is seen for both sexes during the first three readings, and for males during the linear stage of alcohol elimination. Nonetheless, the magnitude of the discrepancy between observed and expected readings is small (see Fig. 3) and the kinetic model is a good approximation to the BAC and BrAC curves.

Parameter estimates for effects of sex and age on BAC and BrAC curves following the kinetic model (Table 3) show the effects of age are small and confined to A_0. Although the subjects were within a narrow age range (18–34 years, mean 23 years), there was a small but significant positive regression of age on A_0 for both BAC and BrAC (Table 3). An effect of sex upon BAC and BrAC was detected for A_0, while the rate of absorption also depends upon sex for BrAC but not for BAC profiles. The kinetic model was therefore parameterized in the model for the means as:

$$\mathrm{BAC}(t) = (A_0 + A_{0\mathrm{A}} + A_{0\mathrm{S}})(1 - \mathrm{e}^{k_1 t}) + k_2 t$$

$$\mathrm{BrAC}(t) = (A_0 + A_{0\mathrm{A}} + A_{0\mathrm{S}})(1 - \mathrm{e}^{(k_1 + k_{1\mathrm{S}})t}) + k_2 t$$

where $A_{0\mathrm{A}}$ and $A_{0\mathrm{S}}$ are the effects of age and sex (male deviation) and $k_{1\mathrm{S}}$ is the male deviation for the rate of absorption. These adjustments for age and sex were included in the estimation of SNP and haplotype effects in the *ADH7* region.

Table 1. Typed SNPs within and flanking the *ADH7* gene

SNP ID[a]	Chromosome Position (bp)[b]	Distance next SNP (bp)	Ancestral allele[c]	Poly morphism [d]	MAF	Functionality/placement[e]
rs1354368	100 799 065	34 864	G	G/A	0.350	DKFZP434G072
rs1583971	100 764 201	15 918	T	A/T	0.107	Upstream ADH7
rs2851024	100 748 283	14 258	T	T/G	0.480	Upstream ADH7
rs894363	100 734 025	20 509	C	C/T	0.403	Upstream ADH7
rs1154470	100 713 516	2080	G	G/A	0.335	Intron 1
rs1154468	100 711 436	4589	A	A/T	0.335	Intron 1
rs1573496	100 706 847	6766	C	C/G	0.100	Exon 3 (Ala:Gly)
rs1154461	100 700 081	1041	—	G/C	0.336	Intron 5
rs971074	100 699 040	1339	G	G/A	0.110	Exon 6 (Arg:Arg)
rs1154458	100 697 701	2180	C	C/G	0.413	Intron 6
rs1154454	100 695 521	2468	T	T/C	0.182	Intron 7
rs284784	100 693 053	1897	G	G/T	0.236	Intron 8
rs284786	100 691 156	45	T	A/T	0.309	3′-UTR
rs3805331	100 691 111	86	A	A/G	0.073	3′-UTR
rs894369	100 691 025	234	C	C/G	0.229	3′-UTR
rs3805329	100 690 791	56	T	T/C	0.074	3′-UTR
rs284787	100 690 735	289	C	C/T	0.236	3′-UTR
rs729147	100 690 446	3574	A	G/A	0.228	Downstream ADH7
rs969804	100 686 872	2029	—	T/A	0.392	Downstream ADH7
rs994772	100 684 843	2034	G	G/A	0.118	Downstream ADH7
rs1348276	100 682 809	11 568	T	T/G	0.394	Downstream ADH7
rs2032350	100 671 241	7886	C	C/T	0.197	Downstream ADH7
rs1442484	100 663 355	5128	T	T/C	0.200	Downstream ADH7
rs1826906	100 658 227	2577	C	T/C	0.314	Downstream ADH7
rs283406	100 655 650	0	C	C/T	0.052	Downstream ADH7

[a]From dbSNP, http://www.ncbi.nlm.nih.gov/SNP.
[b]NCBI Build 36.1.
[c]Based on chimpanzee (NCBI)—indicates unavailable.
[d]Major allele→Minor allele.
[e]From dbSNP, http://www.ncbi.nlm.nih.gov/SNP.

Table 2. Haplotype frequencies for the *ADH7* 5′- and 3′-haplotype blocks

Haplotype Name	Frequency	rs971 074 0.110	rs1154 461 0.336	rs1573 496 0.100	rs1154 468 0.335	rs1154 470 0.335	rs894 363 0.403			
5′a	0.478	1	1	1	1	1	1			
5′b	0.330	1	2	1	2	2	2			
5′c	0.112	2	1	2	1	1	1			
5′d	0.067	1	1	1	1	1	2			
5′e	0.010	2	1	1	1	1	1			
5′f	0.001	1	2	1	2	2	1			
5′g	0.001	1	2	1	1	1	1			
5′-Sub-block[a]		A	A	A	B	B	B			
		rs1348 276 0.394	rs994 772 0.118	rs969 804 0.392	rs729 147 0.228	rs284 787 0.236	rs3805 329 0.074	rs894 369 0.229	rs3805 331 0.073	rs284 786 0.309
3′a	0.377	1	1	1	1	1	1	1	1	1
3′b	0.001	1	1	1	1	1	2	1	2	2
3′c	0.117	1	1	1	2	1	1	2	1	1
3′d	0.001	1	1	2	1	1	2	1	2	2
3′e	0.116	1	2	1	2	1	1	2	1	1
3′f	0.001	2	1	1	1	1	1	1	1	1
3′g	0.086	2	1	2	1	1	1	1	1	1
3′h	0.067	2	1	2	1	1	2	1	2	2
3′i	0.233	2	1	2	1	2	1	1	1	2
3′-Sub-block[a]		A	A	A	B	B	B	B	C	C

Haplotypes depicted in terms of major (1) and minor (2) alleles as shown in Table 1.
[a]Haplotype sub-blocks revealed with $r^2 > 0.6$.

Associations between haplotypes or SNPs and BAC or BrAC levels

The effects of the rare haplotypes 5′e, 5′f and 5′g in the 5′ haplotype block (Table 2) were not estimated. Haplotypes 5′c and 5′d differed from the consensus haplotype 5′a by two and one nucleotides respectively and were lower in frequency than haplotype 5′b (Table 2). Haplotypes 5′c and 5′d were not associated with BAC or BrAC levels (Table 4). Haplotype 5'b was more strongly associated with BAC than with BrAC

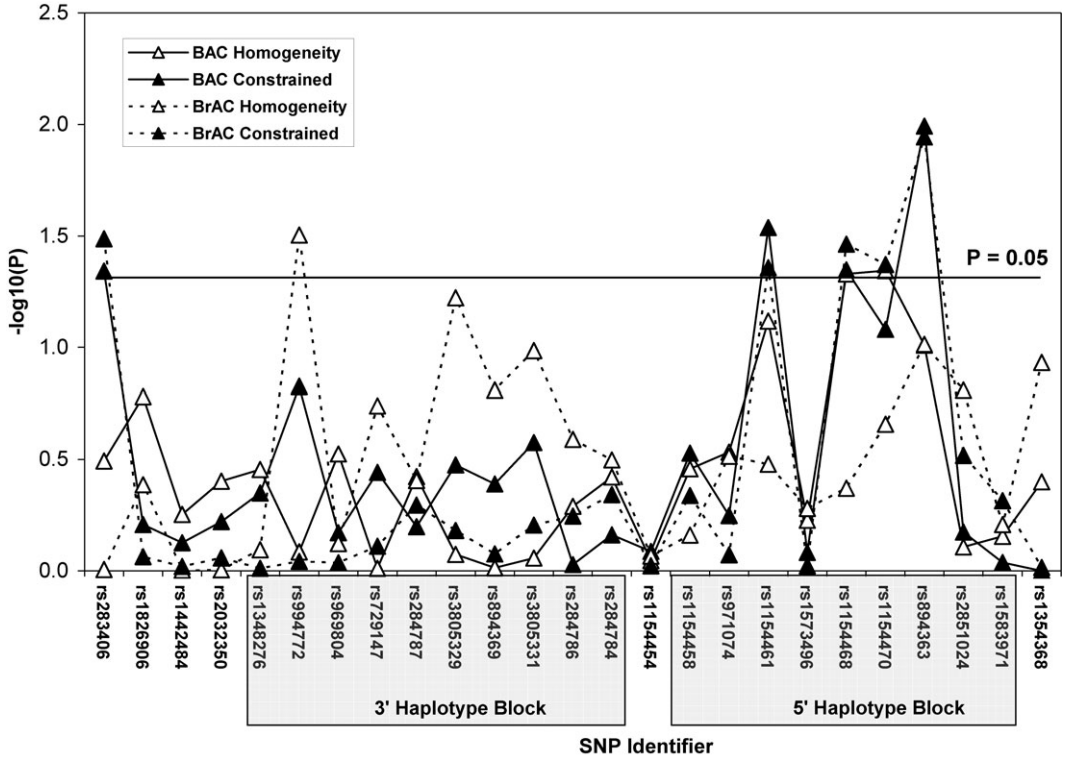

Figure 2. Probabilities for tests of SNP association with BAC and BrAC levels across the *ADH7* region. See Table 1 for SNP details.

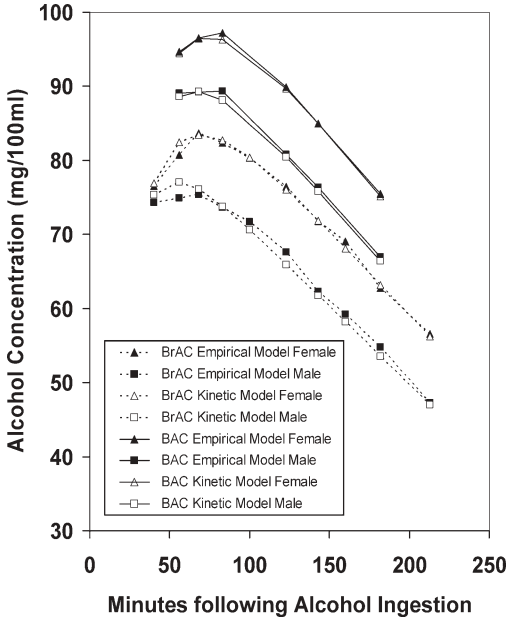

Figure 3. Time course for mean BAC and BrAC levels by sex. The means for the 'Empirical model' of mean BAC and BrAC levels are compared to the estimates from the 'Kinetic model' [Eq. (1)].

levels (Table 4, Fig. 4). The signs of the effect sizes of haplotype 5′b upon A_0 and k_1 (level and rate of absorption, respectively) were the same, in accordance with expectations of a first-order kinetic process. The joint effect of the two parameters A_0 and k_1 for 2 df, measuring the effects of the early stages of absorption, and the rate of elimination k_2 (1 df) were used to compare haplotype effects. No effects for alcohol elimination were detected for k_2 but A_0 and k_1, were jointly significant for BAC level and, less strongly, for BrAC (Table 4). Compared to haplotype 5′a, haplotype 5′b is associated with the rare allele at the four most common SNPs rs1154461, rs1154468, rs1154470 and rs894363. Haplotype 5′c is complementary to haplotype 5′b (Table 2) and was not associated with alcohol levels; its effect size is not significantly different from that of haplotype 5′a. The effect on BAC and BrAC levels is most likely associated with rs1154461, rs1154468, rs1154470 or rs894363.

Extensive tests were performed for effects of individual haplotypes and combinations of haplotypes within the 3′ block on the various parameters of alcohol metabolism, but no significant effects were found (data not shown).

Table 3. Maximum likelihood estimates (θ) of the effects of sex and age upon the parameters of the kinetic equation A_0, k_1 and k_2

Covariates	Parameter	BAC θ	χ^2_1	P	BrAC θ	χ^2_1	P
Age	A_0	0.079	4.48	0.034	0.045	4.77	0.029
	k_1	0.009	0.12	0.735	0.035	1.77	0.184
	k_2	−0.023	1.53	0.217	−0.003	0.21	0.649
Sex	A_0	−1.187	13.83	2.00e-04	−1.281	47.61	5.21e-12
	k_1	0.345	2.35	0.125	0.996	17.61	2.71e-05
	k_2	0.143	0.89	0.347	0.094	3.19	0.074

Table 4. Effects of haplotypes in the 5′ Haplotype Block of *ADH7* upon BAC and BrAC levels

	Model	versus	BAC −2LL	df	χ^2	df	*P*	BrAC −2LL	df	χ^2	df	*P*
1	Haplotypes b, c and d		5489.64	2038				7206.80	3367			
2	Drop haplotypes c and d	1	5500.33	2044	10.696	6	0.0982	7211.20	3373	4.400	6	0.6227
3	Drop haplotype b	2	5516.96	2047	16.628	3	0.0008	7220.38	3376	9.185	3	0.0269
	Effects of Haplotype b											
4	A_0	2	5500.34	2045	0.010	1	0.9203	7215.63	3374	4.437	1	0.0352
5	k_1	2	5509.65	2045	9.321	1	0.0023	7212.83	3374	1.633	1	0.2013
6	k_2	2	5500.55	2045	0.213	1	0.6444	7212.73	3374	1.536	1	0.2152
7	Drop A_0, k_1	2	5513.69	2046	13.358	2	0.0013	7220.03	3375	8.833	2	0.0121
8	Drop k_2	7	5515.96	2047	2.270	1	0.1319	7220.38	3376	0.352	1	0.5530

All effects are deviations from the baseline value of haplotype 5′a.

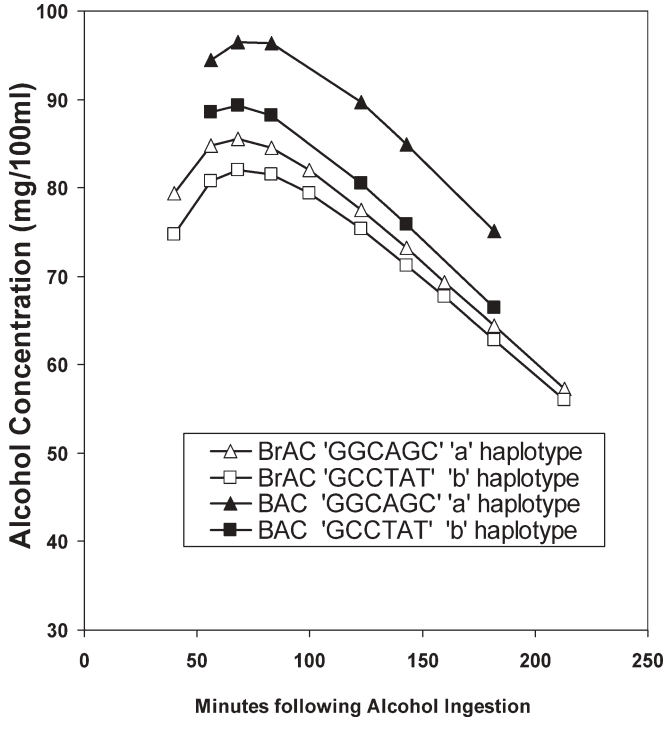

Figure 4. Effect of haplotypes a and b from the 5′ haplotype block on BAC and BrAC levels.

Contribution of haplotypes in the 5′ block of ADH7 to linkage in the *ADH* gene region

The models for the effects of the 5' block upon mean BrAC or BAC levels were used to estimate the contribution of haplotypes in the 5′*ADH7* region to linkage of *in vivo* metabolism to the *ADH* region on chromosome 4. The effect of haplotype 5′b (Table 2) was estimated as a deviation from the baseline effects of all remaining haplotypes. Since the effects of haplotypes 5′c through 5′g (Table 2) were homogenous with those of the most common haplotype 5′a, our statistical comparison of haplotype effects is as previously described, a deviation of the effect of haplotype 5′b from the baseline haplotype 5′a. In the bivariate simplex model, the covariance due to the QTL and the effect of haplotypes in the means part of the equation are jointly estimated, in the model for the multivariate normal distribution.

The contribution of the major haplotype 5′b to the QTL for alcohol concentration was estimated by dropping its effects from the bivariate simplex model. The change in the log-likelihood was $\chi^2_6 = 22.7$ ($P = 0.0009$) for the kinetic parameters, A_0, k_1 and k_2, corresponding to the effects of haplotypes upon breath alcohol (almost entirely due to A_0) and blood alcohol level (almost entirely due to k_1). Direct calculation of the joint effect of allelic deviations (in the means part of the multivariate likelihood equation) for both blood and breath alcohol levels gave an estimate of 18% for the effect of the 5′ haplotypes, and the difference in means between haplotypes 5′a and 5′b at the first blood alcohol measurement time was 5.8 mg/100 ml.

DISCUSSION

There are a number of practical reasons to study inter-individual variation in alcohol pharmacokinetics. The peak concentration achieved after any specified dose and the rate of metabolism will affect the duration and intensity of intoxication, and the rate of production and peak concentration of alcohol metabolites such as acetaldehyde. There is some evidence to suggest that variation in alcohol pharmacokinetics affects alcohol dependence risk (24), and a large amount of evidence implicating alcohol-metabolizing enzymes in alcohol dependence risk and in the development of other alcohol-related disease (25–28). Many of the published twin studies have been under-powered to detect a significant genetic component to variation in alcohol metabolism (29–32), but Martin *et al*. (9) showed heritability of 0.62 for peak blood alcohol and 0.49 for rate of elimination. So far, there is little information on the exact genes and polymorphisms involved. Studies on the most obvious candidates, the long-recognized polymorphisms in *ADH1B* (Arg47His and Arg369Cys) and *ADH1C* (Ile 349Val/ Arg271Gln) have revealed only minor *in vivo* effects (10,33,34), although *in vitro* kinetic properties are substantially different (35). Despite the negative results with known *ADH* variants, we were able to show (10) that a substantial QTL for alcohol metabolism exists at the *ADH* gene cluster on chromosome 4. This could of course be due to a single polymorphism or to additive or interaction effects of several; the latter would be harder to detect but should be approachable through haplotype association studies.

We have now shown that SNP haplotypes across approximately 35 kb of DNA in *ADH7*, spanning a region just 5′ of exon 1 and extending into intron 6 of the *ADH7* coding region, are related to blood and breath alcohol concentrations *in vivo*. Results for blood and breath data differ in some details, but Table 4 shows that although the 5′ haplotype effect is associated with k_1 for blood alcohol and A_0 for breath alcohol, the joint effects are significant for both. This is evident in Figure 4, which illustrates that early alcohol concentrations differ by 5' haplotype for both blood and breath measurements. Although blood and breath alcohol measurements are both subject to measurement error, they point to a similar conclusion.

This result defines a new region of significance for the *in vivo* metabolism of alcohol, although our best estimate is that it accounts for only 18% of the previously reported linkage to the *ADH* gene region, or 11% of the total genetic variance. This is consistent with the expectation of multiple additive or interaction effects at several loci within or close to the *ADH* gene cluster.

Definition of the boundaries of the region responsible for this effect is aided by the pattern of LD between adjacent SNPs across the *ADH7* gene. We identified two major haplotype blocks, which show a marked discontinuity of LD in a region centered on SNP rs1154454, within intron 7, and flanked by rs1154458 (5′) and rs248784 (3′). The most likely explanation for this is that there is a recombination hotspot within intron 7 of *ADH7*. This was also observed by Han *et al*. (22) in a world-wide study of patterns of allele frequency and LD. They used different markers, but included rs971074 and rs1154458 (3′) and rs284784 and rs284786 (5′). The 5′ haplotype block was observed by Luo *et al*. (12), in D′ values for rs971074, rs1573496 and rs1154470 in Americans of European descent. While only one SNP (rs284786) in the 3′ block was genotyped by Luo *et al*., it was not in LD with any of the 5′ located *ADH7* SNPs. D′ values for SNPs in the *ADH7* region chosen by Edenberg *et al*. (8) also identified the 5′ LD block, but their coverage of the 3′ block adjacent to rs1154454 was less complete.

Two major haplotypes in the 5′ haplotype block were found to be associated with *in vivo* metabolism. The nucleotide composition of these haplotypes was, in 5′–3′order, GGCAGC (frequency 0.48) and GCCTAT (0.33), which are distinguished by four nucleotide substitutions. Of these, we can tentatively exclude the effect of GGCAGC as compared to GCCTAT since the effect of the rare haplotype AGGTAT was no different from that of GGCAGC in the analysis of 5′ block effects. Because this haplotype has a frequency of only 0.11, we should regard this conclusion with some caution. However, the second nucleotide in the two most frequent haplotypes GGCAGC and GCCTAT differs. The relevant SNP is rs1154461 within intron 5, which has a minor allele frequency (MAF) of 0.3. This is immediately 5′ of rs1573496 which is responsible for the non-synonymous substitution *ADH7Ala80Gly* (with a MAF∼0.1) which is exclusively part of haplotype 5′c. However, SNP rs1573496 is the same (C) in both haplotypes 5′a and 5′b so cannot account for the effect of the 5′ haplotype block on *in vivo* metabolism.

Another SNP in this region, which we did not type, may affect gene expression. A T/C substitution at +25 bp in the 5′-untranslated region changes a TATA sequence to TACA (36,37). This variant is within the 5′ haplotype block and probably in strong LD with our typed SNPs. It may account for the variation in alcohol metabolism, but the individual effects of SNPs in strong LD are difficult to differentiate by allelic association studies.

In contrast to the 5′-haplotype block, the 3′-haplotype block shows very little evidence of association with *in vivo* metabolism. Although tests of haplotypes did reveal indications of effects, they were not consistent for BAC and BrAC and we conclude that any effects are smaller than those seen for the 5′-haplotype block.

Turning to the issue of pre-absorptive alcohol metabolism, the contribution of gastric ADH has been recognized in the literature, but with variable results that may be confounded by study method (38,39). As noted above, gastric ADH is primarily coded by the *ADH7*, *ADH1B* and *ADH1C* genes which are expressed in the gastric mucosa, with *ADH1C* expressed in stomach muscle. *In vitro* kinetic properties imply a smaller contribution of ADH1B and ADH1C enzymes to gastric metabolism than of ADH7, due to substrate inhibition of ADH class I enzymes (20,21).

As well as *in vitro* study of gastric isoenzymes and gene expression, there has been extensive study *in vivo* of 'first pass metabolism' (FPM) of ethanol. This after absorption, or occurring before absorption, and assessed from the difference in blood alcohol concentrations between oral and intravenous alcohol administration. Its contribution to total alcohol metabolism in Caucasians ranges from 1 to 10% (40). Ethnic differences between Caucasians and Asian peoples support a major role of ADH7 in FPM in Caucasians; FPM is very low in Japanese because of an absence of expressed ADH7 enzyme (39,41). At very high alcohol concentrations, Japanese and Caucasian subjects both show an additional contribution to FPM from ADH5, which has a poor substrate affinity for ethanol (39). The contribution of gastric alcohol metabolizing enzymes (ADH7 and ADH5) to FPM in both Japanese and Caucasian subjects has been estimated to reach 40% at high alcohol concentration (39), although this estimate has been criticized as too large (19,40,42). Since FPM almost disappears on gastrectomy (43), gastric rather than hepatic ADHs must account for the bulk of FPM in Caucasians (44).

Our study shows that *ADH7* variation has a significant impact on *in vivo* alcohol metabolism, commencing at the early times of blood alcohol measurement and carrying forward to the later times (Fig. 4). Since the SNPs on *ADH7* 5′ haplotype block are not associated through LD with those in the 3′ block, it is unlikely that we are measuring correlated effects due to LD elsewhere in the *ADH7* region. The result also has implications for ethnic differences in the role of alcohol oxidation to acetaldehyde. Since *ADH7* apparently is not expressed in a substantial proportion of people from East Asian populations (20,21,41) the effects of genetic variability in *ADH7* upon initial acetaldehyde levels and consequences for alcohol dependence may be more important in Caucasians.

The magnitude of this effect has been estimated at 18% of the QTL or 11% of the genetic variance. Comparing the effect size for haplotype 5′b with the standard deviation for the peak blood alcohol concentration, we can express it as a z-score of 0.36. We conclude that although the contribution of genetic variation at

ADH7 blood and breath alcohol concentrations is sizeable, it accounts for only part of the effects due to linkage in the *ADH* region as a whole. A set of 104 SNPs across the entire *ADH* gene cluster has been typed in these subjects, and results are being assessed (manuscript in preparation).

Further investigation of the effects of genetic variation in other ADHs on blood and breath alcohol concentrations in these Alcohol Challenge subjects must be complemented by SNP typing in our larger cohorts with data on alcohol use and dependence, and by existing published reports on related topics. As mentioned previously, there are few genetic linkage or association studies on alcohol metabolism or pharmacokinetics. There are, however, a substantial number which test for effects of variation in or near *ADH* genes on related phenotypes, including alcohol dependence or *ADH* gene expression. These may be useful as a guide to genes, haplotypes or polymorphisms which affect alcohol metabolism. Until recently, most studies examined *ADH1B* Arg47His and *ADH1C* Val349Ile, but other suggestive or significant reports for alcohol dependence include effects within *ADH1A, ADH1B, ADH5* and *ADH7* (12); *ADH1A, ADH1B* and *ADH4* (8); *ADH7* (45) and the broad region of chromosome 4 spanning the ADH cluster (28). The report on *ADH7* suggested interaction between a polymorphism in this gene and the *ADH1B* Arg47His polymorphism, producing differences in risk of alcohol dependence among Chinese subjects. It will be more difficult to test for such interaction in European subjects, because of the lower frequency of the *ADH1B*47His* allele, but interactions across the *ADH* gene region need to be considered more generally when adequate data become available.

Expression of *ADH* genes has been shown to be affected by sites within the *ADH* gene cluster (46–48), or elsewhere on chromosome 4 (49). These diverse reports suggest that additive or epistatic effects of sequence variation in the *ADH* gene cluster combine to produce the effect found in our previous linkage analysis, and evaluating these will require multiple studies on *ADH* variation and its effects on transcription, enzyme activity, alcohol use and dependence and alcohol-related disease.

MATERIALS AND METHODS

Alcohol challenge study: subjects and phenotypes

Alcohol metabolism data were available for 206 pairs of twins, aged 18–34 years (mean 23), who took part in the ACTS in 1979–81 (9). Ten timed measurements of breath alcohol concentration and six of blood alcohol concentration were obtained, starting 40 min after ingestion of alcohol (0.75 $g.kg^{-1}$ body weight). Breath alcohol readings were converted to blood alcohol by a conversion factor of 2100:1; this is conservative in that it under-estimates blood alcohol concentrations. Full details are given in Martin *et al*. (9).

Blood samples and SNP assays

Some 10–20 years after completion of the ACTS, twins were recontacted to obtain blood samples for DNA extraction; at this time, we also collected blood samples from parents and/or non-twin siblings of the twins. The zygosity of ACTS twin pairs was originally determined by self-report and a limited range of serological and isoenzyme markers; extensive genotyping since the initial study has shown that some re-assignment is necessary. The revised zygosity status of participants is 91 monozygotic (MZ) twin pairs (46 female and 45 male) and 115 dizygotic (DZ) twin pairs (41 female, 35 male and 39 opposite sex).

A set of 25 polymorphic SNPs related to *ADH7* (Table 1) was typed in 817 individuals from 196 of the original 206 twin families. SNP typing was performed with the MassARRAY genotyping platform (Sequenom Inc., San Diego, CA, USA). DNA was available for 220 non-twin siblings and 215 parents, in addition to the twins, in 112 DZ and 84 MZ families. At least one parent and non-twin sibling were present in 106 families, 24 more families had at least one non-twin sibling and 37 had one or both parents; 28 families only contained twins. The extended families provided additional information for the determination of haplotype phase. The ancestry of ACTS participants, based upon reported country of origin of their four grandparents, was estimated at 87% 'Northern European' (including Australia and New Zealand), and 13% 'other', nearly all Southern European/Mediterranean (50).

Genotype quality control

Family relationships were confirmed with PREST ver. 3.02 (51) and the SNPs conformed to the expectations of Mendelian Inheritance [PEDSTATS ver. 0.4.6 (52)]. A genotyping error rate of 0.12% was estimated from 65 MZ families for which both twins were genotyped. Eleven dispersed genotyping errors or spurious point double recombinants were identified by coinheritance checks [MERLIN ver. 1.0.1 (53)]. They were confined to six SNP loci that were sporadically distributed across the *ADH7* region and not due to incorrect map locations (map order was from NCBI Build 35.1 (http://www.ncbi.nlm.nih.gov/projects/SNP/). The error rate was in the range 0.05–0.49% for each of these six SNPs. The distribution of errors by family (eight families) showed they were sporadic in this respect and they were recoded as missing. Minor allele frequencies for the 25 SNPs were obtained from three databases; Caucasians/Northern Europeans in HapMap [Public Release No. 19, The International HapMap Consortium, 2003 (54)], the CEU population (CEPH Utah Residents with Northern and Western European Ancestry), NCBI Build 35.1 (http://www.ncbi.nlm.nih.gov/projects/SNP/) and from ALFRED (http://alfred.med.yale.edu/alfred/index.asp/). The mean difference in frequency of minor alleles as genotyped in the present study and published MAFs was small (0.012 ± 0.062).

Linkage disequilibrium

Pairwise estimates of LD across the *ADH7* region were obtained from the SNP-typed data using HAPLOVIEW (ver. 3.2; http://www.broad.mit.edu.mpg/haploview/). The most likely haplotypes were identified using MERLIN 1.0.1 (53) which takes into account within-family segregation patterns and the extent of LD within the region. The definition of the haplotype block was resolved using MERLIN 1.0.1 by varying the threshold value for the genetic correlation

needed to define a haplotype block separately for the 5′and 3′ blocks using the '- -' cluster option.

SNP associations with the alcohol phenotype

Two approaches were taken to obtain the maximum information from the data on blood and breath alcohol concentrations. These involved firstly, an empirical approach based on the multiple measures of blood and breath alcohol obtained during the study and, secondly, estimation of kinetic parameters of a physiological model of alcohol absorption, distribution and metabolism.

Effect sizes for SNP alleles were estimated for each time point in the means part of the model for the multivariate normal equation using Mx (55) in a preliminary screen of the *ADH7* region. The expected sib pair covariance matrices were modeled jointly with means and were parameterized as additive and specific environmental covariances by Cholesky decomposition, taking into account the zygosity of twin pairs. Initial analysis included the effects of sex, age and the interaction of SNP effects with sex in the model for mean breath or blood alcohol levels. The interaction effects proved to be trivial and the final model only retained the effects of sex and age. Initial analysis of the association of SNP genotypes and alcohol levels showed no evidence for dominance effects.

An allelic association model was used in the preliminary analysis to test for the effect of the minor (less common) allele at each SNP as a deviation from the overall mean (effect of the common SNP allele). A profile of allelic effects against the location of each SNP in the *ADH7* region was used to identify possible sites/regions of interest. The overall variance in blood or breath alcohol levels associated with SNPs was partitioned into the average effect or consistency of allele effects for all time-points, and the heterogeneity (time-change) of allelic effects over time.

Pharmacokinetic model

Three main characteristics of the blood or breath alcohol/time curves describe the salient features of alcohol absorption and metabolism. There is an early absorptive phase when alcohol concentrations rise, a peak alcohol concentration reached when rates of absorption and elimination balance, and an elimination phase when alcohol levels fall. We have used a general approach based upon the physiological and biochemical processes of absorption and elimination that underlie BAC and BrAC curves. The model was described by Martin *et al.* (56) and used previously with the present data (9). It predicts concentration of alcohol, $C(t)$, at time t following ingestion, using Eq. (1).

$$C(t) = A_0(1 - e^{-k_1 t}) + k_2 t \quad (1)$$

A_0 represents the concentration of alcohol that would be achieved if all ingested alcohol were absorbed and evenly distributed throughout the whole body or tissue volume, k_1 the rate of absorption, and k_2 the rate of elimination. The rate of elimination is a linear term that reflects substrate saturation or independence between the rate of reaction and ethanol concentration. This has been shown to be valid with a dose of 0.67 g kg^{-1} (close to the 0.75 g kg^{-1} used in our study) and persists until alcohol levels fall to 20 mg/100 ml (57). The early stages of alcohol absorption are concentration dependent and estimated by A_0 and k_1. Maximum likelihood estimates of the three parameters A_0, k_1 and k_2 were obtained in Mx (55) by specifying Eq. (1) for the concentration of alcohol at time t after ingestion, in the time-related means vector of the multivariate normal equation.

Haplotype association

ADH7 activity variants can be expected to affect both A_0 and k_1 by way of enzyme activity in the gastric mucosa during absorption. Both A_0 and k_1 will be biologically correlated, as too are the conventional measures of alcohol pharmacokinetics: time to peak alcohol concentration and peak alcohol concentration following drinking. In the absence of genetic association (LD) or epistasis between gastric ADH7 variants and other sites affecting alcohol metabolism, any effects detected by the parameters A_0 and k_1 will not affect the rate of elimination (k_2) at high ethanol concentration.

The effects of haplotypes were estimated as deviations for A_0, k_1 and k_2 from the overall mean (defined by the most frequent haplotype pattern in the 5′ and 3′ blocks) (Table 2). Preliminary analysis did not show evidence for haplotype by sex interaction. Haplotype analysis for association was carried out separately for the 5′-haplotype block and 3′-haplotype block, defined above. We also tested for effects at SNP loci that were not in strong LD with the 5′- and 3′-haplotype blocks, where the overall reduction in log-likelihood indicated the possibility of an effect in at least one of the three estimated parameters.

Linkage and association

A joint test of linkage and association (10) allowed the estimation of the contribution of haplotypes in the *ADH7* region to the covariation in the timed series of ten BrAC and six BAC readings. In this method, the effects of the covariance between breath and blood alcohol levels are jointly estimated with the effects of the measured genotypes or haplotypes in the means part of the multivariate normal equation, by maximum likelihood in the structural equation modeling package, Mx. The chosen model followed a bivariate simplex design (10) and showed that the joint genetic covariance of BrAC and BAC levels was largely initiated before the time of the first measurements in the time series (about 40 min after completion of drinking). Similarly, the effect of linkage was only identified with this time point, and only seen for the joint covariance in BrAC and BAC levels, not the covariance specific to either BrAC or BAC levels. The contribution of the 5′ haplotypes to the covariance between blood and breath readings, due to the QTL associated with the *ADH* gene family, was estimated following the method described by Fulker *et al.* (58). The effects of the 5′ haplotypes were estimated in the means model of the bivariate simplex model jointly with the covariance between BAC and BrAC levels due to the linked QTL. The model for the 5′ haplotypes is described in the Results section. The effect of the 5′ haplotypes upon linked covariance was estimated from the increase

in the QTL covariance at the first time point in the time series that resulted from dropping the effects of the haplotypes from the joint model for linkage and association.

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