The Journal of Toxicological Sciences (J. Toxicol. Sci.)
Vol.34, No.1, 89-97, 2009

Original Article

Polymorphisms in the promoter region of the human class II alcohol dehydrogenase (*ADH4*) gene affect both transcriptional activity and ethanol metabolism in Japanese subjects

Yukiko Kimura[1], Fusae T. Nishimura[1], Shuntaro Abe[2], Tatsushige Fukunaga[3], Hideji Tanii[1] and Kiyofumi Saijoh[1]

[1]Department of Hygiene, Kanazawa University Graduate School of Medical Science, 13-1 Takara-machi, Kanazawa 920-8640, Japan
[2]Department of Forensic Medicine, The Jikei University School of Medicine, 3-25-8 Nishi-shinbashi, Minato-ku, Tokyo 105-8461, Japan
[3]Tokyo Medical Examiner's Office, Tokyo Metropolitan Government, 4-21-18 Otsuka, Bunkyo-ku, Tokyo 122-0012, Japan

(Received October 29, 2008; Accepted November 21, 2008)

ABSTRACT — Class II alcohol dehydrogenase (π-ADH), encoded by alcohol dehydrogenase (*ADH4*), is considered to contribute to ethanol (EtOH) oxidation in the liver at high concentration. Four single nucleotide polymorphisms (SNPs) were found in the promoter region of this gene. Analysis of genotype distribution in 102 unrelated Japanese subjects revealed that four loci were in strong linkage disequilibrium and could be classified into three haplotypes. The effects of these polymorphisms on transcriptional activity were investigated in HepG2 cells. Transcriptional activity was significantly higher in cells with the -136A allele than in those with the -136C allele. To investigate whether this difference in transcriptional activity caused a difference in EtOH elimination, previous data on blood EtOH changes after 0.4 g/kg body weight alcohol ingestion were analyzed. When analyzed based on aldehyde dehydrogenase-2 gene (*ALDH2*) ^{487}Glu/Lys genotype, the significantly lower level of EtOH at peak in subjects with -136C/A and -136A/A genotype compared with subjects with -136C/C genotype indicated that -136 bp was a suggestive locus for differences in EtOH oxidation. This effect was observed only in subjects with *ALDH2* ^{487}Glu/Glu. These results suggested that the SNP at -136bp in the *ADH4* promoter had an effect on transcriptional regulation, and that the higher activity of the -136A allele compared with the -136C allele caused a lower level of blood EtOH after alcohol ingestion; that is, individuals with the -136A allele may consume more EtOH and might have a higher risk for development of alcohol dependence than those without the -136A allele.

Key words: Alcohol dehydrogenase-4, Polymorphism, Transcriptional activity, HepG2, Ethanol metabolism

INTRODUCTION

Human alcohol dehydrogenases (ADHs) are encoded by seven *ADH* genes (*ADH1A*, *1B*, *1C*, *4, 5, 6*, and *7*) and classified into five classes according to their similarities in amino acid sequences and kinetic properties (Edenberg, 2007). The class I ADHs (α, β, and γ subunit encoded by *ADH1A*, *1B*, and *1C*, respectively), in general, possess low *K*m (0.05-4.0 mM excluding mutant *1B* *3) to oxidize ethanol (EtOH) and account for most of the EtOH oxidizing capacity in the liver (Edenberg, 2007). Class II ADH (π subunit encoded by *ADH4* (von Bahr-Lindstrom *et al*., 1991)) activity is also found mainly in the liver (Ditlow *et al*., 1984) and its mRNA level is highest in the liver (Estonius *et al*., 1996). This subunit is also considered to contribute to EtOH metabolism in liver due to its abundance and kinetic properties. Namely, its *K*m value to oxidize EtOH is 34

Correspondence: Kiyofumi Saijoh (E-mail: saijohk@med.kanazawa-u.ac.jp)

mM (Bosron *et al*., 1979). In post-mortem samples, it has been demonstrated that this subunit significantly contributes to EtOH oxidation in liver at high EtOH concentration, and that this contribution increases as the concentration of EtOH increases (Lee *et al*., 2006).

Recent advances in single nucleotide polymorphism (SNP) analysis of these *ADH* genes have revealed the existence of many suggestive functional SNPs not only in the coding region, such as *1B* *3 as described above, but also in the 5' flanking region. Several case-control studies have reported an association between alcohol dependence (AD) and these genetic variations. Independent linkage studies on several populations have indicated strong evidence for linkage between a locus on chromosome 4q containing *ADH* gene cluster and AD (Martin *et al*., 1985; Reich *et al*., 1998; Reich, 1996; Williams *et al*., 1999; Long *et al*., 1998). By comprehensive analysis testing the association between AD and 110 SNPs throughout the seven *ADH* genes, the most consistent evidence for an association has been observed for 12 SNPs in and around the *ADH4* gene, while associations with SNPs in *ADH1A* and *ADH1B* were weaker, and there was no significant association with variations in *ADH1C* (Edenberg *et al*., 2006). Furthermore, a haplotype carrying both -136C (rs1800759) and -220A (rs1800761) alleles in the promoter region of *ADH4* was associated with the risk for AD in European Brazilians and African Brazilians (Guindalini *et al*., 2005). A case-control study in an American population that examined the association of seven *ADH4* SNPs with AD and drug dependence indicated that the C-136A (rs1800759) polymorphism in the promoter region and an exon 9 (rs1042364) polymorphism are associated with risk of AD (Luo *et al*., 2006).

An *in vitro* transcriptional assay tested the effects of three polymorphic variants of the *ADH4* gene, T-253A (rs1800760), G-220A (rs1800761), and C-136A (rs1800759), revealing that haplotypes with A at -136 bp have higher activity than those with C at -136 bp (Edenberg *et al*., 1999). In addition to these three SNPs, the C-361G (rs4140388) polymorphism is also found in the *ADH4* promoter region (Iida *et al*., 2002). However, whether this polymorphism affects *ADH4* gene expression and its linkage status among other SNPs have not been investigated. Therefore, we analyzed the linkage status of these four loci and their haplotype frequencies in 102 Japanese subjects, and the differences among the effects of these haplotypes and each genotype on transcriptional activity as revealed by a luciferase reporter assay.

Based on the kinetic properties of the π subunit, as mentioned above, it is expected that ADH4 may contribute to EtOH oxidation in liver, even after moderate amounts of alcohol intake, because the EtOH concentration during absorption is higher in the liver than in circulating peripheral blood (Sawai and Takahashi, 1990). Therefore, we also analyzed the effects of the *ADH4* polymorphisms on EtOH metabolism in humans using our previously collected data of blood EtOH and acetaldehyde (AcH) changes after a moderate dose of alcohol ingestion (0.4 g/kg body weight) (Nishimura *et al*., 2006).

MATERIALS AND METHODS

Genotyping

To analyze the linkage status of the four SNPs in the *ADH4* promoter region, C-361G (rs4140388), T-263A (rs1800760), A-220G (rs1800761) and C-136A (rs1800759) in our Japanese population, genomic DNA was collected from 102 unrelated Japanese subjects (23.0 ± 3.8 (mean ± S.D.) years old, male: n = 99, female: n = 3). All subjects recruited for this study received information on the scientific purpose of this study and provided written informed consent. This study was approved by the ethical committee of our institution.

The promoter region of *ADH4*, from -433 to -14 bp, was amplified from genomic DNA isolated from oral buccal cells using primers designed for cloning into a reporter vector: forward primer, 5'-GTAGGGTACCACTAAATATGCAAGG-3' and reverse primer, 5'-TTTGGCTAGCTGTGTTGGAAGTTTC-3' (the underline denotes introduced *Kpn*I (5'-end) and *Nhe*I (3'-end) cloning sites). PCR-amplified fragments were sequenced using an ABI PRISM BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 310 Genetic Analyzer (Perkin Elmer, Norwalk, CT, USA).

Accordance with Hardy-Weinberg equilibrium was confirmed by chi-squared test to assess any discrepancies between the observed and expected genotype frequencies based on the observed allele frequencies. The *D'* value for each linkage disequilibrium pair and haplotype frequencies were calculated using the program Haploview (http://www.broad.mit.edu/mpg/haploview) (Barrett *et al*., 2005).

The genotype of *ALDH2* exon 12 was analyzed by the PCR-restriction fragment length polymorphism method as described previously (Harada and Zhang, 1993).

Reporter assay

Amplified fragments of the *ADH4* promoter region were cloned into pGL4.10, a luciferase reporter vector (Promega, Madison, WI, USA) introducing the *Kpn*I and the *Nhe*I cloning sites with forward and reverse

primers, respectively, as described above. The luciferase reporter constructs containing the three different haplotypes of *ADH4* promoter found in Japanese individuals of the present study were represented as Hap#1 (CTAC), Hap#2 (GTGA), and Hap#3 (GAGC). These constructs of *ADH4* promoter were digested at the native *Sac*I site at -240 bp and the 3' *Nhe*I cloning site and recombined to generate single base exchanged haplotypes, Hap#4 (GTGC), Hap#5 (CTGA), and Hap#6 (GAGA). All plasmid sequences were confirmed by DNA sequencing.

The human hepatoma HepG2 cell line was obtained from Health Science Research Resources Bank (Osaka, Japan) and grown in Dulbecco's modified Eagle's medium containing high glucose levels (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma). Transient transfection of reporter constructs was performed with Tfx-20 (Promega). The day before transfection, the cells were plated at 5×10^4 cells per well in 24-well plates. The cells were incubated with Tfx-20 (ratio of Tfx-20 to DNA was 2:1), 0.735 μg of pGL4 reporter construct, and 15 ng of phRL-TK (Promega) co-transfected as an internal control, for 1 hr in serum-free medium, and serum-containing medium was added to the cells at the end of the incubation period. The cells were then incubated for 48 hr and harvested for measurement of luciferase activity.

Firefly and *Renilla* luciferase activity were measured using by Dual-Luciferase Reporter Assay System (Promega). Cells were rinsed with phosphate buffered saline (PBS) twice and lysed in 100 μl of passive lysis buffer. Luciferase activity was expressed as firefly luciferase activity normalized to *Renilla* luciferase activity in each well. Data are expressed as the means ± SEM of the results of at least three independent experiments performed in triplicate. The significance of differences in promoter activity was evaluated by one-way analysis of variance (ANOVA) followed by post-hoc analysis (Fisher's PLSD).

Alcohol metabolism analysis

The effects of *ADH4* promoter polymorphisms were re-analyzed in our previous data set of 58 subjects (22.2 ± 2.7 (mean ± S.D.) years-old, 64.6 ± 3.4 kg) (Nishimura *et al*., 2006). All subjects provided written informed consent and the experiment was approved by the ethical committee of our institution. As reported previously, subjects were instructed to abstain from food for 3 hr and from drinking water for 1 hr before the experiment, and not to imbibe alcohol on the day before the experimental day. Subjects ingested 5% (w/v) orange-flavored white liquor six times, with a 4-min interval, producing a total amount of 0.4 g of EtOH per kg body weight. Peripheral blood was collected from a catheter placed in the antecubital vein at 30-min intervals, and immediately deproteinized by 0.6 N perchloric acid in saline for EtOH and AcH measurement. Blood EtOH and AcH concentrations were measured by headspace gas chromatography (Eriksson *et al*., 1982). Values are presented as means ± SEM. The significance of differences in peak blood EtOH and AcH levels among subject groups based on each genotype of the *ADH4* promoter was evaluated by unpaired t-test. The significance of differences in the changes in blood EtOH levels between subject groups based on *ADH4* haplotype was evaluated by one-way ANOVA followed by post-hoc analysis (Fisher's PLSD) and by a repeated measures two-way ANOVA (haplotype × time).

RESULTS

The genotype distribution and allele frequencies of the four SNPs in *ADH4* promoter region were analyzed in 102 unrelated Japanese subjects (Table 1). A chi-square test revealed that the observed genotype frequencies did not deviate significantly from those predicted, indicating that the allelic distribution in each of the four loci was in Hardy-Weinberg equilibrium. Minor allele frequencies of the three SNPs excluding T-253A were similar to those in an Asian population in the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/): C-361G G = 0.275, A-220G G = 0.262, and C-136A A = 0.262. Although the allele frequency of -253T in the Asian population is 1.00 in the database, individuals with -253A were observed in our samples with an allele frequency of 0.088. The two loci at -361 bp and -220 bp seemed to appear in pairs (C_A and G_G) because individuals in genotype groups based on C-361G were completely identical to those in groups based on A-220G genotype. All four *ADH4* polymorphisms were in strong linkage disequilibrium ($D' = 1$). The three haplotypes were detected by the program Haploview (Table 2). The CTAC haplotype (Hap#1) was the most common, the GTGA (Hap#2) and the GAGC (Hap#3) were rare haplotypes, while the other haplotypes were not detected in this Japanese population.

The effects of polymorphisms on transcriptional regulation were evaluated using a luciferase reporter vector containing different haplotypes of the *ADH4* promoter (Fig. 1). Three constructs, pGL-Hap#1 (CTAC), pGL-Hap#2 (GTGA) and pGL-Hap#3 (GAGC), correspond to the haplotypes found in individuals in this study. The luciferase activity of pGL-Hap#2, which is a unique haplotype with A at -136 bp among the native haplotypes, was

Table 1. Genotype and allele frequencies of the four SNPs in the *ADH4* promoter region and values for the Hardy-Weinberg equilibrium test in Japanese population

Variation[a]	dbSNP ID	Observed number (n = 102)			Allele frequency	χ^2	p
C-361G	rs4140388	C/C = 57	C/G = 38	G/G = 7	C = 0.745 G = 0.255	0.038	0.846
T-253A	rs1800760	T/T = 85	T/A = 16	A/A = 1	T = 0.912 A = 0.088	0.064	0.800
A-220G	rs1800761	A/A = 57	A/G = 38	G/G = 7	A = 0.745 G = 0.255	0.038	0.846
C-136A	rs1800759	C/C = 72	C/A = 26	A/A = 4	C = 0.833 A = 0.167	0.692	0.406

[a] Number means position from the translation start ATG codon.
χ^2: chi-square distribution; p: p-value for Hardy-Weinberg equilibrium test (goodness-of-fit)

Table 2. Haplotype frequency of the *ADH4* gene in the Japanese population

Haplotype (5' to 3')	Frequency
Hap#1 (CTAC)	0.745
Hap#2 (GTGA)	0.167
Hap#3 (GAGC)	0.088
Total	1.000

greater than those of both pGL-Hap#1 and pGL-Hap#3 haplotypes (2.48- and 2.17-fold, respectively). To further assess which locus affects transcriptional activity, recombinant constructs with single base exchanges, pGL-Hap#4 (GTGC), pGL-Hap#5 (CTGA) and pGL-Hap#6 (GAGA) were generated and compared with the native haplotypes. The luciferase activity of the recombinant construct pGL-Hap#4 was slightly lower than that of the native constructs pGL-Hap#1 and pGL-Hap#3, being 0.90- and 0.78-fold, respectively, and significantly lower than that of pGL-Hap#2 (0.36-fold). Both pGL-Hap#5 and pGL-Hap#6 had significantly higher activity than pGL-Hap#1, pGL-Hap#3, and pGL-Hap#4. The ratios of luciferase activity of pGL-Hap#5 and pGL-Hap#6 to that of native pGL-Hap#1 were 3.06-fold and 3.07-fold, respectively, while compared with the native pGL-Hap#3 they were 2.68- and 2.69-fold, respectively. Significant differences of transcriptional activity among three constructs with -136A allele (namely, Hap#2, 5 and 6) were not detected and all these constructs had significantly higher activity in comparison with -136C constructs, Hap#1 and 4. Although it is not always statistically significant, the activity of Hap#3 was also lower than that of -136A allele constructs. This showed that the luciferase activity of constructs with a -136A genotype had significantly higher luciferase activity than those with a -136C genotype.

To test whether the difference in promoter activity of *ADH4* based on haplotype has an effect on EtOH elimination, we analyzed the data on blood EtOH levels after a moderate amount of alcohol drinking that was obtained from our previous study (Nishimura *et al*., 2006). To clarify the effect of the different transcriptional activity, which was shown by our *in vitro* study, on alcohol metabolism, the subject groups were divided based on whether they were carrying the minor alleles Hap#2 and Hap#3, as listed in Table 3. The polymorphism *ALDH2* ^{487}Glu/Lys has been known to result in much higher AcH concentrations in blood, and also affect individual ability to oxidize EtOH to AcH by product inhibition (Mizoi *et al*., 1994). Therefore, a comparison between *ADH4* promoter genotypes was performed in each separate group based on *ALDH2* genotype; namely the ^{487}Glu/Glu group (n = 30) and ^{487}Glu/Lys group (n = 28). Regarding the blood EtOH levels of *ALDH2* ^{487}Glu/Glu subjects, both the main effect of the Hap#2 haplotype and interactive effect between haplotype and time were significant (haplotype: $p < 0.05$, haplotype × time: $p < 0.001$) (Fig. 2A). Blood EtOH levels at 30 and 60 min after alcohol ingestion in Hap#2+ subjects were significantly lower than in Hap#2- subjects (Fig. 2A), but were not significantly different between Hap#3 haplotype groups (Fig. 2A'). In subjects with an *ALDH2* ^{487}Glu/Lys genotype, no significant difference in blood EtOH levels between Hap#2 groups was observed (Fig. 2B). A repeated measures two-way ANOVA showed a significant interactive effect of Hap#3 haplotype and time (haplotype × time: $p < 0.05$). The blood EtOH level was lower at 30 min after ingestion but higher at 120, 150 and 180 min after ingestion in Hap#3+ individuals compared with Hap#3- subjects, although no significant differences in blood EtOH levels were detected at any time point

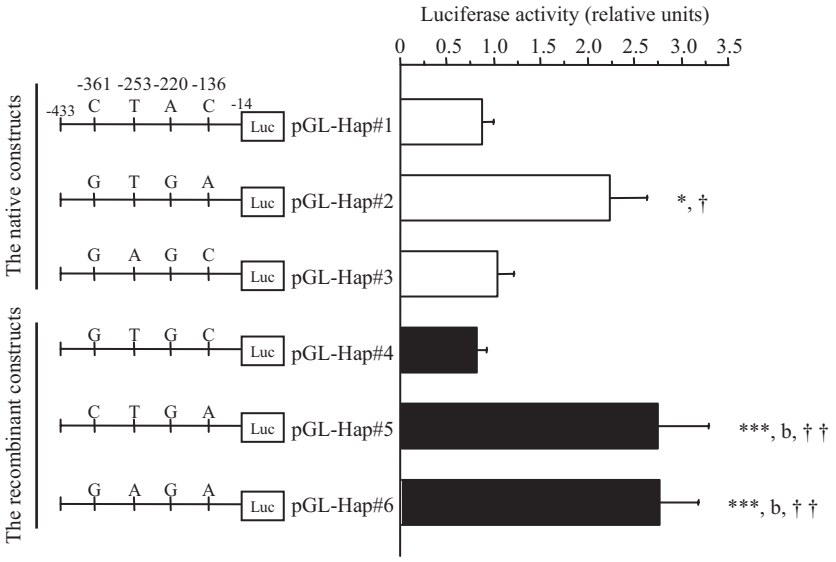

Fig. 1. Effects of polymorphisms in *ADH4* promoter region on its transcriptional activity. HepG2 cells were transfected with a luciferase reporter construct containing six different haplotypes of the *ADH4* promoter region. The *ADH4* haplotypes pGL-Hap#1 (CTAC), pGL-Hap#2 (GTGA) and pGL-Hap#3 (GAGC) were found in the population of the present study and the haplotypes pGL-Hap#4 (GTGC), pGL-Hap#5 (CTGA) and pGL-Hap#6 (GAGA) were generated in order to generate single base exchanged haplotypes. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Data are expressed as means ± SEM. The statistical significance of differences was analyzed by one-way ANOVA followed by post hoc analysis (Fisher's PLSD). * $p < 0.05$, *** $p < 0.001$ compared with cells transfected with pGL-Hap#1. b $p < 0.01$ compared with cells transfected with pGL-Hap#3. † $p < 0.05$, †† $p < 0.01$: compared with cells transfected with pGL-Hap#4.

(Fig. 2B').

The effect of each SNP on EtOH metabolism was assessed by comparison of peak EtOH and AcH levels after alcohol ingestion (Table 4). Regardless of *ALDH2* genotype, there were no significant differences in peak levels of EtOH and AcH between C-361G (A-220G) genotype groups or between T-253A genotype groups. However, in subjects with an *ALDH2* ^{487}Glu/Glu genotype, the -136C/A+A/A group displayed significantly lower EtOH levels than the -136C/C group. The peak AcH level in -136C/A+A/A subjects also tended to be lower than that in the -136C/C genotype group ($p = 0.054$). Peak EtOH and AcH levels were not significantly affected by -136C/A genotype in ^{487}Glu/Lys subjects.

DISCUSSION

Judging from the allele frequency registered in the dbSNP database and the present study genotyping 102 unrelated Japanese subjects, there is a racial difference in the allele frequencies of four SNPs in the promoter region of *ADH4*. Systematic screening of SNPs in the *ADH4* gene revealed thirteen SNPs, including four SNPs in the promoter region in a Japanese sample (Iida *et al*., 2002). The distribution status of these four loci displayed in strong linkage, and especially -361C and -220A were completely linked. Because of this strong linkage, four loci were classified into three haplotypes, that is, the expected haplotype frequencies of CTAC, GTGA and GAGC were equal to the frequencies of the -361C, -136A, -253A alleles, respectively. In this study, we demonstrated that one of these SNPs, C-136A (rs1800759), affected not only gene expression but also EtOH oxidation at peak level after alcohol ingestion.

To assess differences in transcriptional activity of these three haplotypes, a reporter assay was performed using HepG2 cells. The *ADH4* promoter has a TATA box and a CCAAT box (von Bahr-Lindstrom *et al*., 1991). Two transcription factors, C/EBP and AP-1, which bind to several sites in the promoter regions of *ADH4* and members of the C/EBP gene family, may be involved in the transcription regulation of the *ADH4* gene (Li and Edenberg, 1998). Although the expression level of C/EBP in HepG2 cells is lower than that in the liver (Friedman *et al*., 1989), the *ADH4* promoter was active in these cells. Among the three native haplotypes, the GTGA haplotype was associated with higher transcriptional activity than the others. Subsequently, the effect of each SNP on the high activ-

Table 3. Subject classification in a drinking experiment based on haplotype of *ADH4* and genotype of *ALDH2*

Groups	Haplotype	*ALDH2* genotype	
		^{487}Glu/Glu	^{487}Glu/Lys
Hap#2+	CTAC/GTGA	8	7
	GTGA/GAGC	1	1
	GTGA/GTGA	0	1
	Total	9	9
Hap#2-	CTAC/CTAC	14	16
	CTAC/GAGC	7	3
	GAGC/GAGC	0	0
	Total	21	19
Hap#3+	CTAC/GAGC	7	3
	GTGA/GAGC	1	1
	GAGC/GAGC	0	0
	Total	8	4
Hap#3-	CTAC/CTAC	14	16
	CTAC/GTGA	8	7
	GTGA/GTGA	0	1
	Total	22	24

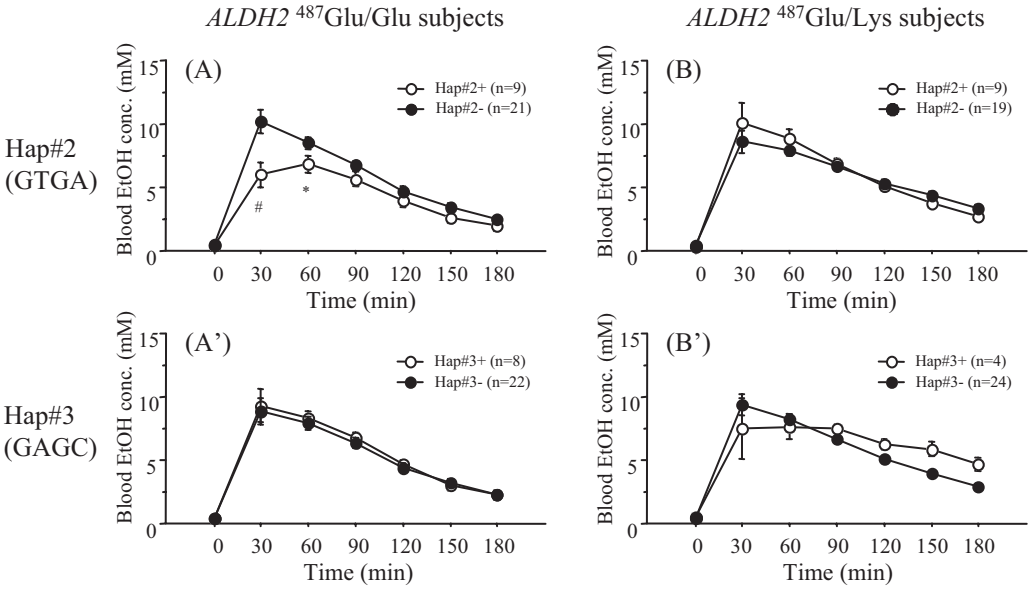

Fig. 2. The effects of *ADH4* haplotypes of Hap#2 (GTGA, upper panel) and Hap#3 (GAGC, lower panel) on blood EtOH levels after alcohol ingestion. Subjects with *ALDH2* ^{487}Glu/Glu (A and A') and *ALDH2* ^{487}Glu/Lys (B and B') genotypes took 0.4 g/kg body weight of alcohol and blood EtOH concentrations were measured at 30-min intervals. The statistical significance of differences was analyzed by one-way ANOVA followed by post hoc analysis (Fisher's PLSD) and by a repeated measures two-way ANOVA (haplotype × time). * $p < 0.05$, # $p < 0.0001$ compared between subject groups at the same time point.

ity of the GTGA haplotype was examined by comparisons among recombinant constructs. No significant difference in transcriptional activity between Hap#2 (GTGA) and Hap#5 (CTGA) haplotypes revealed a low contribution of the C-361G polymorphism to transcriptional activity. The SNP at -253 bp also seems to have a low contribution because the promoter activities of Hap#2 (GTGA) and Hap#6 (GAGA), which differ at -253 bp, were almost the same. Consistent with this result, activities of Hap#3 (GAGC) and Hap#4 (GTGC) were also almost the same. On the other hand, the activity of Hap#2 (GTGA) was 2.8 times greater than that of Hap#4 (GTGC). Similarly, the activity of Hap#6 (GAGA) was 2.7 times greater than that of Hap#3 (GAGC). These results suggested that the SNP at -136 bp was involved in the different transcriptional activation of the gene. The higher activity of promoters with -136A than those with -136C is consistent with a previous report that examined the effects of three

Table 4. Effects of *ADH4* genotype on peak ethanol and acetaldehyde levels after alcohol ingestion

ADH4 subject group			n	Peak EtOH (mean ± SEM, mM)			Peak AcH (mean ± SEM, μM)		
ALDH2 ^{487}Glu/Glu	C-361G*	C/C	14	11.0 ± 1.15	t	1.66	13.9 ± 1.68	t	0.70
		C/G+G/G	16	8.77 ± 0.72	p	0.11	12.0 ± 2.07	p	0.49
	T-253A	T/T	22	9.74 ± 0.85	t	0.13	12.2 ± 1.36	t	0.89
		T/A	8	9.95 ± 1.09	p	0.90	14.9 ± 3.47	p	0.38
	C-136A	C/C	21	10.7 ± 0.85	t	–2.28	14.6 ± 1.65	t	–2.02
		C/A + A/A	9	7.58 ± 0.71	p	0.03	8.94 ± 1.78	p	0.05
ALDH2 ^{487}Glu/Lys	C-361G*	C/C	16	9.94 ± 0.76	t	–0.26	46.9 ± 10.9	t	1.08
		C/G+G/G	12	10.3 ± 0.96	p	0.80	32.7 ± 4.50	p	0.29
	T-253A	T/T	24	10.2 ± 0.64	t	–0.36	43.3 ± 7.45	t	–0.91
		T/A	4	9.54 ± 1.61	p	0.72	26.1 ± 8.91	p	0.37
	C-136A	C/C	19	9.63 ± 0.66	t	1.10	42.3 ± 9.48	t	–0.32
		C/A + A/A	9	11.0 ± 1.18	p	0.28	37.8 ± 4.84	p	0.76

EtOH: ethanol; AcH: acetaldehyde; t: t-value; p: p-value for unpaired t-test.
* Subjects in genotype groups based on C-361G are identical to those in A-220G genotype groups.

the SNPs at -253, -220, and -136 bp (Edenberg *et al*., 1999). However, the effect of this C-136A polymorphism on the level of ADH4 activity in human liver remained unknown.

Little is known about the transcription factors binding to this site or the effects of C-136A substitution on their binding affinity. Seven sites in the *ADH4* proximal promoter have been identified to bind nuclear protein from mouse liver (Li and Edenberg, 1998). However, none of four SNPs overlapped with these identified binding regions. The sequence corresponding to four loci examined in their study was C-T-A-A (from 5' to 3') (Li and Edenberg, 1998). The potential transcriptional factor binding the alternative sequence remains unclear. By a database search (http://www.gene-regulation.com/pub/programs/alibaba2/), an HNF-1β-C binding site was predicted to be located at –136C. HNF-1β-C, also known as vHNF1-C, is a variant isoform of the HNF1 family that acts as a transdominant repressor (Bach and Yaniv, 1993). The low transcription activity of promoters carrying the -136C genotype might be explained by this transcription factor acting as a negative element. Further investigation is required to identify whether this transcription factor is involved in the difference in transcriptional activity due to SNP at -136bp.

Human liver contains multiple molecular forms of ADH enzyme and plays a major role of circulating blood EtOH metabolism. ADH4 is expressed mainly in the liver, faintly in small intestine, pancreas and stomach (Estonius *et al*., 1996). Thus ADH4 enzyme may contribute to hepatic EtOH oxidation at high concentration. According to a study using human postmortem samples, the contribution of *ADH4* to total EtOH oxidation in the liver may be less than 15% (0 to 13% with an average of 7%) at 5 mM and as much as 40% (17 to 39% with an average of 27%) at 60 mM (Li *et al*., 1977). A kinetic equation study providing a model for quantitative assessment of EtOH metabolism in the liver demonstrated that *ADH4* contributed about 14% of total oxidizing capacity at 10 to 20 mM in blood (Lee *et al*., 2006); and this is within the range of peak EtOH concentrations under the condition of 0.4 g/kg body weight alcohol ingestion. Thus it was postulated that the *ADH4* enzyme takes part in EtOH oxidation during the absorption and distribution period, when the blood concentration reached its highest level, even in case of moderate amounts of alcohol intake. We previously investigated the effects of the polymorphisms *ALDH2* Glu^{487}Lys, *ALDH2* G-360A, *ADH1B* His^{48}Arg, and *CYP2E1* C-1019T on EtOH metabolism, suggesting that only the *ALDH2* ^{487}Glu/Lys genotype had a marked and significant effect on AcH oxidation (Nishimura *et al*., 2006). Thus the effects of *ADH4* genotype and haplotype were analyzed in combination with *ALDH2* ^{487}Glu/Lys genotype. As expected from the result of *in vitro* study, the high transcriptional activity of Hap#2 participated in EtOH metabolism. Blood EtOH levels were significantly lower in subjects with the Hap#2 haplotype than in subjects with other haplotypes, where-

as Hap#3 had no effect. Aside from the SNP at –136 bp, Hap#2 also had the SNP at –220 bp, whereas further analysis of the respective SNPs indicated that C-136A was a suggestive locus for the difference in EtOH oxidation observed between haplotypes. Namely, the peak EtOH level of -136C/A and -136A/A subjects was significantly lower than that of -136C/C subjects, and the peak AcH level also tended to be lower. Together with human and *in vitro* experiments, it was revealed that the differences in transcriptional activity caused by C-136A were involved in the differences in EtOH oxidation. It was expected that the blood EtOH level in the absorption and distribution period was kept lower, resulting in the capacity for alcohol intake being increased in individuals with the -136A allele. This hypothesis is supported by the previous case-control association study that indicated C-136A was one of the markers genetically closest to the functional risk loci for AD (Luo *et al*., 2006). Interestingly, these effects of promoter SNPs on both EtOH and AcH levels were observed only in subjects with the *ALDH2* ^{487}Glu/Glu genotype. It is generally regarded that the *ALDH2* ^{487}Lys allele, encoding an inactive subunit, is responsible for alcohol sensitivity and has a genetic protective effect against the development of AD. Because the distribution of the -136A allele differs across racial groups (the -136C allele is common in Asians, while the -136A allele is common in European-Americans and African-Americans), the *ADH4* C-136A polymorphism may in part explain the alcohol sensitivity and subsequent development of AD in East Asians, in combination with *ALDH2* genotype.

In conclusion, we demonstrated here that the polymorphisms in the *ADH4* promoter region affect EtOH oxidation after moderate amount of ingestion. Although four SNPs in the promoter were strongly linked and classified into three haplotypes, only the SNP at -136 bp had an effect on transcriptional activity. The higher activity of the -136A allele than the -136C allele caused a lower peak level of blood EtOH after alcohol ingestion, which caused a difference in EtOH metabolism during the absorption and distribution period, and might be involved in the capacity of alcohol intake, drinking behavior, and consequent development of AD.

REFERENCES

Bach, I. and Yaniv, M. (1993): More potent transcriptional activators or a transdominant inhibitor of the HNF1 homeoprotein family are generated by alternative RNA processing. EMBO. J., **12**, 4229-4242.

Barrett, J.C., Fry, B., Maller, J. and Daly, M.J. (2005): Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics., **21**, 263-265.

Bosron, W.F., Li, T.K., Dafeldecker, W.P. and Vallee, B.L. (1979): Human liver pi-alcohol dehydrogenase: kinetic and molecular properties. Biochemistry, **18**, 1101-1105.

Chou, W.Y., Stewart, M.J., Carr, L.G., Zheng, D., Stewart, T.R., Williams, A., Pinaire, J. and Crabb, D.W. (1999): An A/G polymorphism in the promoter of mitochondrial aldehyde dehydrogenase (ALDH2): effects of the sequence variant on transcription factor binding and promoter strength. Alcohol. Clin. Exp. Res., **23**, 963-968.

Ditlow, C.C., Holmquist, B., Morelock, M.M. and Vallee, B.L. (1984): Physical and enzymatic properties of a class II alcohol dehydrogenase isozyme of human liver: pi-ADH. Biochemistry, **23**, 6363-6368.

Edenberg, H.J. (2007): The genetics of alcohol metabolism: role of alcohol dehydrogenase and aldehyde dehydrogenase variants. Alcohol Res. Health, **30,** 5-13.

Edenberg, H.J., Jerome, R.E. and Li, M. (1999): Polymorphism of the human alcohol dehydrogenase 4 (ADH4) promoter affects gene expression. Pharmacogenetics, **9**, 25-30.

Edenberg, H.J., Xuei, X., Chen, H.J., Tian, H., Wetherill, L.F., Dick, D.M., Almasy, L., Bierut, L., Bucholz, K.K., Goate, A., Hesselbrock, V., Kuperman, S., Nurnberger, J., Porjesz, B., Rice, J., Schuckit, M., Tischfield, J., Begleiter, H. and Foroud, T. (2006): Association of alcohol dehydrogenase genes with alcohol dependence: a comprehensive analysis. Hum. Mol. Genet., **15**, 1539-1549.

Eriksson, C.J., Mizoi, Y. and Fukunaga, T. (1982): The determination of acetaldehyde in human blood by the perchloric acid precipitation method: the characterization and elimination of artefactual acetaldehyde formation. Anal. Biochem., **125**, 259-263.

Estonius, M., Svensson, S. and Hoog, J.O. (1996): Alcohol dehydrogenase in human tissues: localisation of transcripts coding for five classes of the enzyme. FEBS Lett., **397**, 338-342.

Friedman, A.D., Landschulz, W.H. and McKnight, S.L. (1989): CCAAT/enhancer binding protein activates the promoter of the serum albumin gene in cultured hepatoma cells. Genes Dev., **3**, 1314-1322.

Guindalini, C., Scivoletto, S., Ferreira, R.G., Breen, G., Zilberman, M., Peluso, M.A. and Zatz, M. (2005): Association of genetic variants in alcohol dehydrogenase 4 with alcohol dependence in Brazilian patients. Am. J. Psychiatry., **162**, 1005-1007.

Harada, S. and Zhang, S. (1993): New strategy for detection of ALDH2 mutant. Alcohol. Alcohol. Suppl., **1A**, 11-13.

Harada, S., Okubo, T., Nakamura, T., Fujii, C., Nomura, F., Higuchi, S. and Tsutsumi, M. (1999): A novel polymorphism (-357 G/A) of the ALDH2 gene: linkage disequilibrium and an association with alcoholism. Alcohol. Clin. Exp. Res., **23**, 958-962.

Iida, A., Saito, S., Sekine, A., Kondo, K., Mishima, C., Kitamura, Y., Harigae, S., Osawa, S. and Nakamura, Y. (2002): Thirteen single-nucleotide polymorphisms (SNPs) in the alcohol dehydrogenase 4 (ADH4) gene locus. J. Hum. Genet., **47**, 74-76.

Jeong, H.J., Hong, S.H., Park, R.K., An, N.H. and Kim, H.M. (2005): Ethanol induces the production of cytokines via the Ca^{2+}, MAP kinase, HIF-1alpha, and NF-kappaB pathway. Life Sci., **77**, 2179-2192.

Kimura, A., Nishiyori, A., Murakami, T., Tsukamoto, T., Hata, S., Osumi, T., Okamura, R., Mori, M. and Takiguchi, M. (1993): Chicken ovalbumin upstream promoter-transcription factor (COUP-TF) represses transcription from the promoter of the gene for ornithine transcarbamylase in a manner antagonistic to hepatocyte nuclear factor-4 (HNF-4). J. Biol. Chem., **268**, 11125-11133.

Lee, S.L., Chau, G.Y., Yao, C.T., Wu, C.W. and Yin, S.J. (2006): Functional assessment of human alcohol dehydrogenase family in ethanol metabolism: significance of first-pass metabolism. Alcohol. Clin. Exp. Res., **30**, 1132-1142.

Lee, Y.J., Aroor, A.R. and Shukla, S.D. (2002): Temporal activation of p42/44 mitogen-activated protein kinase and c-Jun N-terminal kinase by acetaldehyde in rat hepatocytes and its loss after chronic ethanol exposure. J. Pharmacol. Exp. Ther., **301**, 908-914.

Li, M. and Edenberg, H.J. (1998): Function of cis-acting elements in human alcohol dehydrogenase 4 (ADH4) promoter and role of C/EBP proteins in gene expression. DNA Cell Biol., **17**, 387-397.

Li, T.K., Bosron, W.F., Dafeldecker, W.P., Lange, L.G. and Vallee, B.L. (1977): Isolation of pi-alcohol dehydrogenase of human liver: is it a determinant of alcoholism? PNAS, **74**, 4378-4381.

Long, J.C., Knowler, W.C., Hanson, R.L., Robin, R.W., Urbanek, M., Moore, E., Bennett, P.H. and Goldman, D. (1998): Evidence for genetic linkage to alcohol dependence on chromosomes 4 and 11 from an autosome-wide scan in an American Indian population. Am. J. Med. Genet., **81**, 216-221.

Luo, X., Kranzler, H.R., Zuo, L., Lappalainen, J., Yang, B.Z. and Gelernter, J. (2006): ADH4 gene variation is associated with alcohol dependence and drug dependence in European Americans: results from HWD tests and case-control association studies. Neuropsychopharmacology, **31**, 1085-1095.

Martin, N.G., Perl, J., Oakeshott, J.G., Gibson, J.B., Starmer, G.A. and Wilks, A.V. (1985): A twin study of ethanol metabolism. Behav. Genet., **15**, 93-109.

Mizoi, Y., Yamamoto, K., Ueno, Y., Fukunaga, T. and Harada, S. (1994): Involvement of genetic polymorphism of alcohol and aldehyde dehydrogenases in individual variation of alcohol metabolism. Alcohol Alcohol., **29**, 707-710.

Nishimura, F.T., Kimura, Y., Abe, S., Fukunaga, T. and Saijoh, K. (2006): Effect of -361 G/A polymorphism of aldehyde dehydrogenase-2 gene on alcohol metabolism and its expression in human peripheral blood leukocytes. Nihon Arukoru Yakubutsu Igakkai Zasshi, **41**, 108-119.

Quertemont, E., Grant, K.A., Correa, M., Arizzi, M.N., Salamone, J.D., Tambour, S., Aragon, C.M., McBride, W.J., Rodd, Z.A., Goldstein, A., Zaffaroni, A., Li, T.K., Pisano, M. and Diana, M. (2005): The role of acetaldehyde in the central effects of ethanol. Alcohol. Clin. Exp. Res., **29**, 221-234.

Reich, T. (1996): A genomic survey of alcohol dependence and related phenotypes: results from the Collaborative Study on the Genetics of Alcoholism (COGA). Alcohol. Clin. Exp. Res., **20**, 133A-137A.

Reich, T., Edenberg, H.J., Goate, A., Williams, J.T., Rice, J.P., Van Eerdewegh, P., Foroud, T., Hesselbrock, V., Schuckit, M.A., Bucholz, K., Porjesz, B., Li, T.K., Conneally, P.M., Nurnberger, J.I. Jr, Tischfield, J.A., Crowe, R.R., Cloninger, C.R., Wu, W., Shears, S., Carr, K., Crose, C., Willig, C. and Begleiter, H. (1998): Genome-wide search for genes affecting the risk for alcohol dependence. Am. J. Med. Genet., **81**, 207-215.

Sawai, S. and Takahashi, S. (1990): Radiochemical and macroautoradiographic study of the mechanism of action of alcohol on the nervous system; the distribution of volatile and involatile ^{14}C in the rat injected with ^{14}C-ethanol. J. Nihon Univ. Med. Ass., **49**, 399-412.

von Bahr-Lindstrom, H., Jornvall, H. and Hoog, J.O. (1991): Cloning and characterization of the human ADH4 gene. Gene, **103**, 269-274.

Williams, J.T., Begleiter, H., Porjesz, B., Edenberg, H.J., Foroud, T., Reich, T., Goate, A., Van Eerdewegh, P., Almasy, L. and Blangero, J. (1999) : Joint multipoint linkage analysis of multivariate qualitative and quantitative traits. II. Alcoholism and event-related potentials. Am. J. Hum. Genet., **65**, 1148-1160.