APPENDIX B1. Daily intake of selected foods or nutrients by district in Japan.

District number	Prefecture number[a]	Fat (g)[b]	Animal protein (g)[b]	Salt (g)[b]	Fish (g)[b]	Vitamin D (mg)[c]
1	01	56.7	44.2	12.7	50.1	11.4
2	02,03,04,05,06,07	57.1	41.5	13.5	49.6	10.9
3	11,12,13,14	59.5	42.3	12.5	40.7	8.5
4	08,09,10,19,20	55.5	39.9	13.6	40.4	10.2
5	15,16,17,18	56.8	39.5	12.8	45.2	8.4
6	21,22,23,24	55.9	39.8	12.1	40.1	7.8
7	26,27,28	57.5	42.6	11.8	39.3	8.4
8	25,29,30	57.5	43.6	13.4	42.9	9.5
9	31,32,33,34,35	56.6	41.8	12.5	48.0	7.3
10	36,37,38,39	55.6	42.4	12.3	44.0	7.7
11	40,41,42,44	54.7	39.5	11.6	42.6	6.2
12	43,45,46,47	53.5	40.8	13.0	55.8	6.5

[a] Refer to the first column of Appendix A1.
[b] The National Nutrition Survey in Japan 1990.
[c] The National Nutrition Survey in Japan 2000.

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Influence of alcohol consumption and gene polymorphisms of *ADH2* and *ALDH2* on hepatocellular carcinoma in a Japanese population

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Although alcohol intake as well as hepatitis viruses has been associated with hepatocellular carcinoma (HCC), gene–alcohol interactions on HCC risk remain to be elucidated. We conducted a case-control study to examine whether polymorphisms of alcohol dehydrogenase 2 (*ADH2*) and aldehyde dehydrogenase 2 (*ALDH2*) modified the HCC risk depending on the amount of alcohol intake. *ADH2* and *ALDH2* genotyping was performed by a duplex polymerase chain reaction with confronting two-pair primers in 209 newly diagnosed HCC cases and 2 different controls [275 hospital controls and 381 patients with chronic liver disease (CLD)]. Multiple logistic regression analyses revealed that heavy drinkers consuming ≥3 "go"s/day of sake (69 g of ethanol/day) showed an increased risk of HCC based on comparison of HCC cases with hospital controls [adjusted odds ratio (OR) = 13.5; 95% confidence interval (CI) 3.3–54.3] or CLD patients (adjusted OR = 7.0; 95% CI 2.5–19.2), whereas the overall risk was not elevated among light to moderate drinkers consuming <3 "go"s/day. Interestingly, light to moderate drinking was associated with an increased risk among those with *ALDH2*1/*2* (adjusted OR = 4.5 or 2.0), but not among those with *ALDH2*1/*1* (adjusted OR = 0.8 or 1.0; *p* interaction = 0.03 or 0.13). However, this gene–alcohol interaction was not observed for heavy drinking. Among light to moderate drinkers, people with the combination of *ALDH2*1/*2* and *ADH2*2/*2* revealed the highest risk of HCC. These findings indicate that the *ALDH2* polymorphism may modify HCC risk among light to moderate drinkers.
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Key words: alcohol; ADH2; ALDH2; polymorphisms; hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is one of the most common cancers in Japan. Although the major causative factor of HCC in this country is chronic infection with hepatotropic viruses, especially hepatitis C virus (HCV),[1,2] alcohol intake is also likely to contribute to the development of HCC. Many epidemiological surveys have demonstrated that chronic alcohol intake modifies the risk of HCC in patients with chronic viral hepatitis[3–7] and also is a risk factor independent of chronic viral infection.[8–11]

Although the exact mechanism of alcohol-associated hepatocarcinogenesis is still incompletely understood, heavy alcohol intake is known to cause hepatocellular injury that can lead to enhanced fibrogenesis and finally cirrhosis, the latter being *per se* associated with an increased risk of developing HCC. One of the other proposed mechanisms for ethanol-related carcinogenesis is the action of acetaldehyde. Ethanol is eliminated from the body by its oxidation, first to acetaldehyde and then to acetate. These reactions are mainly catalyzed by alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). There is ample evidence for the carcinogenicity of acetaldehyde, the first metabolite of ethanol, in experimental animals.[12,13] ALDH2, the low-K_m mitochondrial ALDH, is a key enzyme in the elimination of acetaldehyde and has a polymorphism (Glu487Lys), which is prevalent in about 50% of East Asians but has not been found in Caucasians or Africans.[14] The mutant *ALDH2*2* allele encodes a catalytically inactive subunit,[15] and the molecules containing 1 or 2 *ALDH2*2* allele subunits are considered to be inactive.[16] When this enzyme is inactive, the body fails to metabolize acetaldehyde rapidly, leading to excessive accumulation of acetaldehyde.

The ADH holoenzyme can exist as α, β and γ subunits, each of which is encoded by a distant locus, i.e, ADH1, ADH2 and ADH3, respectively. *ADH2* has a polymorphism (Arg47His), and its mutant *ADH2*2* allele exists in more than 90% of East Asians, but in fewer than 20% of either Caucasians or Africans.[15] *In vitro* studies have shown that the *ADH2*2* allele encodes a superreactive subunit of ADH2, and that the superreactive ADH2*2 homodimer has about a 40 times higher V_{max} than the less-active ADH2*1/*1 form of ADH2.[17] Unexpectedly, investigators have found no correlation between the *ADH2* genotype and peak blood acetaldehyde concentration after drinking alcohol,[18] but alcohol-induced flushing has been reported to be less intensive in East Asians with *ADH2*1/*1*.[19]

Previous case-control studies[20,21] showed consistently positive associations of the *ALDH2*1/*2* genotype with the risk for esophageal cancer in East Asian heavy drinkers, and this enzyme-related vulnerability may extend to light to moderate drinkers. Several studies[20,22] also suggest similar associations with the risk of head and neck cancer in Japanese moderate to heavy drinkers. Although some researchers studied the possible associations between these polymorphisms and HCC,[23–26] the results have been inconsistent. Moreover, since most studies were based on a small sample size, the evaluation of the gene–alcohol interaction according to the amount of alcohol intake was difficult.

In this context, the aims of this case-control study were (*i*) to evaluate the dose-response pattern (e.g., presence or absence of threshold dose) between alcohol intake and HCC risk, and (*ii*) to examine whether *ALDH2* and *ADH2* polymorphisms modify the HCC risk, depending on the amount of alcohol intake. Since most HCC patients in Japan have chronic liver disease (CLD) as the background, we recruited 2 different controls (hospital controls and patients with CLD without HCC).

Material and methods

Study subjects

HCC cases. Patients with HCC were eligible (*i*) if the disease was diagnosed for the first time between January 2001 and March

Abbreviations: ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; CI, confidence interval; CLD, chronic liver disease; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; OR, odds ratio.

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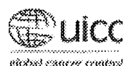
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2004, (*ii*) if they were 40–79 years old at the time of identification and (*iii*) if they were Japanese and residents of Saga Prefecture. Eligible patients were identified among those who were admitted or outpatients of Saga Medical School Hospital (from April 2001 to March 2004) or Saga Prefectural Hospital (from October 2001 to March 2004), the 2 main hospitals in Saga City. A total of 226 eligible patients were located, but 17 refused to participate, leaving 209 cases (92%) included in this study. The diagnosis of HCC was confirmed by tumor biopsy (n = 59), angiography (n = 123) or findings in ultrasonography and either enhanced computed tomography or enhanced magnetic resonance imaging (n = 27). Of the 209 cases (173 with hepatitis C alone, 13 with hepatitis B alone, 6 with both and 17 with other origins), 167 cases (80%) had preexisting liver cirrhosis, 31 cases (15%) had chronic hepatitis, 10 cases (5%) had normal serum alanine aminotransferase (40 U/l or less) but with either hepatitis C or hepatitis B and 1 case had no definite preexisting liver disease. Interferon had been administered to 25 cases (12%), with 2 virologic responses (i.e., clearance of hepatitis virus in blood) and 23 nonresponses.

Hospital controls. These controls were recruited from among first time visitors at the general outpatient clinic of Saga Medical School Hospital between May 2001 and April 2003. Such outpatients who met the selection criteria of (*ii*) and (*iii*) and had no evidence of HCC were regarded as eligible. Research nurses asked consecutive outpatients to participate in this study, unless they had any severe condition such as high fever or had no time for interviews because of practical reasons (e.g., those who immediately had to proceed to examinations). When multiple candidates were found and could not be contacted at the same time, the following order of priority by sex and age was considered: (*a*) men aged 50–79 years, (*b*) women aged 60–79 years, (*c*) men aged 40–49 years and (*d*) women aged 40–59 years; this order was determined by the sex and age distribution of deaths from liver cancer in Saga Prefecture in 1998. Of 379 eligible outpatients contacted, 275 (73%) agreed to participate. The 275 hospital controls were finally diagnosed as follows: digestive diseases (n = 50), cardiopulmonary diseases (n = 36), orthopedic diseases (n = 25), urinary tract diseases (n = 18), sensory organ diseases (n = 13), skin diseases (n = 13), malignant tumors (n = 7), other diseases (n = 28), undiagnosed symptoms (n = 49) and normal (n = 36).

CLD patients without HCC. A total of 381 patients with CLD but without HCC (298 patients with chronic hepatitis and 83 patients with liver cirrhosis) who were out- or inpatients of the 2 hospitals between September 2001 and March 2004 were enrolled as another control group. These patients satisfied the eligibility criteria of (*ii*) and (*iii*) for HCC cases. Patients with special types of CLD (primary and secondary biliary cirrhosis, autoimmune hepatitis and liver disease because of parasitosis, congestive heart failure or metabolic disorders) were excluded. Of 397 eligible patients contacted, 381 (96%) agreed to participate. Those CLD patients that were examined by ultrasonography or enhanced computed tomography did not have detectable tumor in the liver. Of the 298 patients with chronic hepatitis (266 with hepatitis C alone, 20 with hepatitis B alone, 3 with both and 9 with other origins), 191 (64%) had undergone diagnostic liver biopsy, showing chronic persistent hepatitis (n = 40) or chronic active hepatitis (n = 151) [before interferon therapy (n = 160) or without it (n = 31)]. Among the remaining 107 patients without available histology, most of whom (n = 99) had not received interferon, 56 (52%) had normal serum alanine aminotransferase levels at investigation. Interferon had been administered to 148 patients or was currently being used in 20 patients, which resulted in 34 virologic responses, 110 nonresponses, and 24 pending outcomes. The diagnosis of cirrhosis for the 83 patients (56 with hepatitis C alone, 10 with hepatitis B alone, 2 with both and 15 with other origins) was based mostly on evident clinical signs (e.g. esophageal varix) along with imaging and laboratory data (n = 71) and partly on histology (n = 11) and laparoscopy (n = 1).

The study protocol was approved by the ethical committees of Saga Medical School and Saga Prefectural Hospital, and written informed consent to the use of their blood and clinical information for this study was obtained from all subjects.

Interviews

Research nurses interviewed all subjects in person using a uniform questionnaire that requested demographic data and histories of alcohol and tobacco use as well as other relevant factors. Queries regarding alcohol use first ascertained current drinking status (never, former or current drinking) and, for former drinkers, the time of quitting alcohol. Then, former and current drinkers were asked to provide their usual frequency and amount of alcohol use, both at home and outside, during the last 1–2 years and at 10 years prior to the interview. The amount of alcohol use was reported in the "sake" (Japanese rice wine) equivalent of "go"s; one "go" (180 ml or 1 traditional glass) of sake which contains 23 g of ethanol and nearly corresponds to one "go" of "shochu" (distilled alcoholic beverage made in Japan) diluted with hot water (shochu:water = 6:4), 633 ml (1 large bottle) of beer, 70 ml (double) of whisky and 240 ml (2 glasses) of wine. Based on the above information, we estimated the daily intake of alcohol in "go"s at either time point for each subject. For data analysis, we defined "never drinkers" as those who had never drunk or had drunk less than once per week and/or for less than 1 year, "past drinkers" as those who quit alcohol use 1 or more years prior to the interview, and "current drinkers" as the others. In addition, the questionnaire elicited information on whether study subjects had a "history of heavy drinking", which was defined as having imbibed 3 "go"s or more almost every day (5–6 times per week or more) for 10 or more years.

A closed-end question queried about smoking habit (never, former or current smokers), with subsequent inquiries to former and current smokers about the number of cigarettes smoked per day and the duration of smoking in years, as well as the time of quitting smoking for past smokers. "Ever smokers" were defined as individuals who had smoked almost every day for 1 or more years and "never smokers" as the others. Among "ever smokers", we defined "former smokers" as those who stopped smoking 1 or more years prior to the interview and "current smokers" as the others.

Laboratory tests and genotyping ALDH2 *and* ADH2 *polymorphisms*

From all subjects, about 5–7 ml of venous blood was drawn, and plasma and buffy coat were separated and stored at −80°C until tested. All plasma samples were tested for hepatitis B surface antigen (HBsAg) by chemiluminescent immunoassay (CLIA; Dainabot, Tokyo, Japan) and for antibodies to HCV (HCVAb) by 2nd-generation enzyme immunoassay (Abbott HCV EIA II; Dainabot, Tokyo), at an external laboratory (SRL, Tokyo).

DNA was extracted from buffy coat preparations by using a commercial kit (QIAmp DNA Blood Mini Kit; QIAGEN, Tokyo). The polymorphisms of *ALDH2* Glu487Lys and *ADH2* Arg47His were genotyped by a duplex polymerase chain reaction (PCR) with confronting two-pair primers (PCR-CTPP), according to Tamakoshi *et al.*[27] Briefly, genomic DNA (10–150 ng) was amplified in a volume of 25 μl with 0.18 mM dNTPs, 12.5 pmol of each primer, 0.5 units of AmpliTaq Gold (Perkin-Elmer Corp., Foster City, CA) and 2.5 μl of 10× PCR buffer including 15 mM $MgCl_2$. The following 8 primers were used for each reaction: F1 (5′-TGC TAT GAT GTG TTT GGA GCC- 3′), R1 (5′-CCC ACA CTC ACA GTT TTC ACT TC-3′), F2 (5′-GGG CTG CAG GCA TAC ACT A-3′) and R2 (5′-GGC TCC GAG CCA CCA-3′) for *ALDH2* polymorphism, and F3 (5′-GGG CTT TAG ACT GAA TAA CCT TGG-3′), R3 (5′-AAC CAC GTG GTC ATC TGT GC-3′), F4 (5′-GGT GGC TGT AGG AAT CTG TCA-3′) and R4 (5′-AGG GAA AGA GGA AAC TCC TGA A-3′) for *ADH2* polymorphism. The PCR was conducted as follows: a 10-min initial denature at 95°C, 30 cycles for 1 min at 95°C, 1 min at 63°C and 1 min at 72°C, and a 5 min final extension at 72°C. PCR products were subjected to electrophoresis in 3% agarose gels and were visualized with

TABLE I – SELECTED CHARACTERISTIC OF STUDY SUBJECTS

Factor	HCC cases ($n = 209$)	Hospital controls ($n = 275$)	CLD patients ($n = 381$)	$p_{difference}$ [1,2]	$p_{difference}$ [1,3]
Male (%)	67.5	65.5	53.8	<0.01	<0.01
Age (median)	69	61	61	<0.01	<0.01
Age category (%)					
40–49	2.9	15.3	19.2		
50–59	13.4	30.9	24.4		
60–69	36.4	31.3	35.7		
70–79	47.4	22.6	20.7	<0.01	<0.01
HBsAg-positive (%)	9.1	2.2	9.2	<0.01	0.97
HCVAb-positive (%)	85.7	7.6	85.8	<0.01	0.95
Drinking habit (%)					
Male	($n = 141$)	($n = 180$)	($n = 205$)		
Never drinker	17.7	40.6	28.3		
Former drinker	29.1	8.9	25.9		
Current drinker	53.2	50.6	45.9	<0.01	<0.01
Female	($n = 68$)	($n = 95$)	($n = 176$)		
Never drinker	77.9	87.4	78.4		
Former drinker	13.2	1.1	11.9		
Current drinker	8.8	11.6	9.7	0.01	0.95
Smoking habit (%)					
Male	($n = 141$)	($n = 180$)	($n = 205$)		
Never smoker	17.0	27.8	26.3		
Former smoker	36.2	37.2	37.1		
Current smoker	46.8	35.0	36.6	0.03	0.07
Female	($n = 68$)	($n = 95$)	($n = 176$)		
Never smoker	89.7	92.6	85.2		
Former smoker	5.9	3.2	8.5		
Current smoker	4.4	4.2	6.3	0.70	0.66

[1] $p_{difference}$ values were calculated by χ^2 tests (for proportions) or Wilcoxon's rank sum tests (for age).–[2] Comparisons were made between HCC cases and hospital controls.–[3] Comparisons were made between HCC cases and CLD patients.

TABLE II – DISTRIBUTION OF STUDY SUBJECTS AND ADJUSTED OR WITH 95% CI OF HCC ACCORDING TO ALCOHOL INTAKE

	HCC cases *vs.* hospital controls			HCC cases *vs.* CLD patients		
	No. cases/controls	Adjusted OR[1]	95% CI	No. cases/controls	Adjusted OR[1]	95% CI
Total	209/275			209/381		
Drinking habit						
Never drinker	78/156	1.0	Reference	78/196	1.0	Reference
Former drinker	50/17	5.3	1.6–18.6	50/74	1.3	0.7–2.2
Current drinker	81/102	2.9	1.2–7.4	81/111	1.8	1.0–3.0
LRT[2]	$p = 0.01$ ($\chi^2 = 9.32$, DF=2)			$p = 0.12$ ($\chi^2 = 4.19$, DF = 2)		
Alcohol intake ("go"s/day) during last 1–2 years						
0	124/173	1.0	Reference	124/264	1.0	Reference
>0 to 0.9	33/33	3.4	1.1–10.1	33/56	1.2	0.7–2.2
1.0–1.9	20/33	0.8	0.2–2.9	20/30	1.0	0.5–2.1
2.0–2.9	15/25	0.6	0.2–2.4	15/19	1.8	0.8–4.4
3.0–3.9	8/7	10.2	1.7–60.5	8/5	5.0	1.3–19.2
>4.0	9/4	18.0	3.0–107.9	9/7	9.4	2.5–35.4
LRT[2]	$p < 0.01$ ($\chi^2 = 20.8$, DF = 5)			$p < 0.01$ ($\chi^2 = 16.9$, DF = 5)		
Alcohol intake ("go"s/day) 10 years before						
0	98/171	1.0	Reference	98/226	1.0	Reference
>0 to 0.9	22/24	4.1	1.1–15.2	22/43	1.2	0.6–2.3
1.0–1.9	25/26	1.6	0.5–5.8	25/42	1.3	0.7–2.6
2.0–2.9	20/33	0.8	0.2–2.8	20/33	1.1	0.5–2.3
3.0–3.9	19/13	8.7	2.2–34.4	19/13	2.8	1.1–6.8
>4.0	25/8	19.5	4.7–81.7	25/24	3.3	1.5–7.1
LRT[2]	$p < 0.01$ ($\chi^2 = 27.6$, DF = 5)			$p < 0.01$ ($\chi^2 = 12.3$, DF = 5)		
History of heavy drinking[3]						
No	160/252	1.0	Reference	160/342	1.0	Reference
Yes	49/23	9.2	3.4–25.2	49/39	2.4	1.4–4.1

[1] Adjusted for sex, age category (40–49, 50–59, 60–69 and 70–79 years), smoking habit (never, former, and current smokers), HBsAg and HCVAb.–[2] Likelihood ratio test.–[3] A "history of heavy drinking" was defined as having imbibed ≥3 "go"s almost every day for ≥10 years.

ethidium bromide staining. The primer pairs produced allele specific bands as follows: F1 and R1 for *ALDH2*1* (487Glu allele, 119 base pair [bp]), F2 and R2 for *ALDH2*2* (487Lys allele, 98 bp), F3 and R3 for *ADH2*1* (47Arg allele, 219 bp) and F4 and R4 for *ADH2*2* (47His allele, 280 bp).

To validate the results based on PCR-CTPP, parts of DNA samples were genotyped by using the restriction fragment polymorphism methods with restriction enzymes of Ksp632I (for *ALDH2* Glu487Lys polymorphism) and MaeIII (for *ADH2* Arg47His polymorphism), as previously described.[28,29] The results obtained from these methods turned out to be identical to those by PCR-CTPP.

Statistical analysis

χ^2 tests were used for unadjusted comparisons based on frequency. The Wilcoxon's rank sum test was conducted to com-

TABLE III – DISTRIBUTION OF *ALDH2* AND *ADH2* GENOTYPES AMONG STUDY SUBJECTS

Genotype	HCC cases (n = 209)		Hospital controls (n = 275)		CLD patients (n = 381)		$p_{difference}$[1,2]	$p_{difference}$[1,3]
	No.	%	No.	%	No.	%		
ALDH2								
*1/*1	117	56.0	146	53.1	218	57.2		
*1/*2	77	36.8	107	38.9	138	36.0		
*2/*2	15	7.2	22	8.0	26	6.8	0.81	0.96
ADH2								
*1/*1	12	5.7	13	4.7	16	4.2		
*1/*2	73	34.9	103	37.5	137	36.0		
*2/*2	124	59.3	159	57.8	228	59.8	0.73	0.70

[1]$p_{difference}$ values were calculated by χ^2 tests.–[2]Comparisons were made between HCC cases and hospital controls.–[3]Comparisons were made between HCC cases and CLD patients.

TABLE IV – ADJUSTED OR[1] (AND 95% CI) OF HCC ACCORDING TO *ALDH2* GENOTYPE AND ALCOHOL INTAKE

	HCC cases *vs.* hospital controls				$p_{interaction}$	HCC cases *vs.* CLD patients				$p_{interaction}$
	*1/*1		*1/*2			*1/*1		*1/*2		
Alcohol intake ("go"s/day) during last 1–2 years										
0	1.0 (reference)	[61/73]	0.7 (0.3–1.9)	[48/78]		1.0 (reference)	[61/131]	0.9 (0.5–1.5)	[48/107]	
<3	0.8 (0.3–2.2)	[41/64]	3.4 (0.9–12.2)	[27/27]	0.03[2]	1.0 (0.5–1.8)	[41/73]	1.8 (0.8–3.8)	[27/26]	0.13[2]
≥3	13.2 (2.8–61.0)	[15/9]	8.4 (0.3–249.4)	[2/2]	0.93[2]	8.4 (2.6–27.3)	[15/9]	1.7 (0.2–11.7)	[2/4]	0.18[2]
LRT[3]	$p < 0.01$ (χ^2 = 19.9, DF = 5)				0.08[4]	$p < 0.01$ (χ^2 = 18.1, DF = 5)				0.08[4]
Alcohol intake ("go"s/day) 10 years before										
0	1.0 (reference)	[45/69]	0.8 (0.3–2.2)	[38/80]		1.0 (reference)	[45/105]	0.8 (0.5–1.5)	[38/95]	
<3	1.1 (0.3–3.5)	[39/87]	4.5 (1.0–19.1)	[28/25]	0.05[2]	0.9 (0.5–1.8)	[39/58]	1.8 (0.8–3.8)	[28/31]	0.07[2]
≥3	9.6 (2.4–38.9)	[33/19]	44.6 (4.5–437.7)	[11/2]	0.15[2]	3.3 (1.4–7.5)	[33/26]	2.0 (0.7–5.9)	[11/11]	0.65[2]
LRT[3]	$p < 0.01$ (χ^2 = 28.8, DF = 5)				0.09[4]	$p < 0.01$ (χ^2 = 15.6, DF = 5)				0.12[4]
History of heavy drinking[5]										
No	1.0 (reference)	[76/127]	1.8 (0.8–4.0)	[69/103]		1.0 (reference)	[76/186]	1.1 (0.7–1.7)	[69/130]	
Yes	12.1 (3.8–38.2)	[41/19]	10.8 (1.2–99.3)	[8/4]	0.57[2]	2.5 (1.3–4.8)	[41/32]	2.3 (0.7–7.6)	[8/7]	0.75[2]
LRT[3]	$p < 0.01$ (χ^2 = 22.2, DF = 3)					$p = 0.02$ (χ^2 = 10.2, DF = 3)				

Values in square brackets indicate no. of cases/controls.
[1]Adjusted for sex, age category (40–49, 50–59, 60–69 and 70–79 years), smoking habit (never, former and current smokers), HBsAg and HCVAb.–[2]p value calculated by the Wald test for each interaction term (DF = 1).–[3]Likelihood ratio test.–[4]p value calculated by the likelihood ratio test for a set of interaction terms (DF = 2).–[5]A "history of heavy drinking" was defined as having imbibed ≥3 "go"s almost every day for ≥10 years.

pare the distribution of age. Unconditional logistic regression models were used to estimate the odds ratios (ORs) of HCC and their 95% confidence intervals (CIs) for alcohol consumption and *ALDH2* and *ADH2* genotypes by using dummy variables, with adjustment for potential confounders including sex, age category (40–49, 50–59, 60–69, and 70–79 years), smoking habit (never, former and current smokers), and HBsAg and anti-HCV status. The gene–alcohol interaction on HCC risk was evaluated by including in the model the product terms of variables of interest (i.e., genotype and alcohol use), as well as main effects and covariates. Likelihood ratio tests were used to examine the overall statistical significance of a set of dummy variables for alcohol and genotype and a set of interaction terms. The statistical significance of each interaction term was also evaluated by the Wald test. Because habitual female drinkers were too few to conduct separate analyses, men and women were combined, and sex was always controlled in the multiple logistic regression analysis. All of the statistical analyses were performed with the SAS/PC statistical package (SAS Institute, Cary, NC). The Hardy–Weinberg equilibrium test was done with the Stata statistical package (Stata Corp., College Station, TX). All reported p values are two-sided.

Results

Table I shows selected characteristics of study subjects. As compared with hospital controls, HCC cases showed significantly higher prevalences of older subjects ($p < 0.01$), HBsAg positives ($p < 0.01$), HCVAb positives ($p < 0.01$), male ever drinkers ($p < 0.01$) and male current smokers ($p < 0.01$). As compared to CLD patients, HCC cases revealed significantly greater proportions of males ($p < 0.01$), older subjects ($P < 0.01$), male ever drinkers ($p = 0.02$) and male current smokers ($p = 0.02$).

The adjusted ORs of HCC for alcohol intake are shown in Table II. Former drinkers showed an increased OR based on comparison of HCC cases with hospital controls (OR = 5.3), but not with CLD patients (OR = 1.3). Current drinkers experienced a significantly elevated risk regardless of the control groups (OR = 2.9 or 1.8). For alcohol intake during the last 1–2 years and 10 years before interviews, the overall tendency showed significantly increased risks among only heavy drinkers consuming 3 "go"s/day or more, although an exceptional risk excess was observed among light drinkers consuming less than 1 "go"/day by comparing HCC cases with hospital controls. The history of heavy drinking was also significantly positively associated with HCC risk (OR = 9.2 or 2.4).

Table III presents the distributions of *ALDH2* and *ADH2* genotypes among study subjects. No difference was seen in the distribution of either genotype between HCC cases and hospital controls (p = 0.81 for *ALDH2* and 0.73 for *ADH2*) or between HCC cases and CLD patients (p = 0.96 and 0.70). Each study group was in the Hardy-Weinberg equilibrium for both genotypes (*ALDH2*: p = 0.64 for HCC cases, 0.70 for hospital controls and 0.48 for CLD patients; *ADH2*: p = 0.77 for HCC cases, 0.47 for hospital controls, and 0.42 for CLD patients).

We studied the interaction between *ALDH2* polymorphism and alcohol intake (Table IV). Subjects with *ALDH2**2/*2* were excluded from the analysis, as there was no drinker with the geno-

TABLE V – ADJUSTED OR[1] (AND 95% CI) OF HCC ACCORDING TO *ADH2* GENOTYPE AND ALCOHOL INTAKE

	HCC cases *vs.* hospital controls		$p_{interaction}$	HCC cases *vs.* CLD patients		$p_{interaction}$
	*1/*1 + *1/*2	*2/*2		*1/*1 + *1/*2	*2/*2	
Alcohol intake ("go"s/day) during last 1–2 years						
0	1.0 (reference) [55/78]	0.5 (0.2–1.3) [69/95]		1.0 (reference) [55/100]	0.7 (0.4–1.1) [69/164]	
<3	1.5 (0.4–5.4) [23/32]	0.8 (0.3–2.4) [45/59]	0.98[2]	0.8 (0.4–1.6) [23/47]	1.2 (0.6–2.2) [45/58]	0.07[2]
≥3	4.4 (0.7–28.7) [7/6]	21.4 (3.5–129.3) [10/5]	0.07[2]	5.1 (1.2–20.8) [7/6]	5.9 (1.6–21.5) [10/6]	0.54[2]
LRT[3]	$p < 0.01$ ($\chi^2 = 19.5$, DF = 5)		0.17[4]	$p < 0.01$ ($\chi^2 = 19.1$, DF = 5)		0.18[4]
Alcohol intake ("go"s/day) 10 years before						
0	1.0 (reference) [45/78]	0.8 (0.3–2.2) [53/93]		1.0 (reference) [45/80]	0.6 (0.3–1.0) [53/146]	
<3	3.3 (0.8–13.7) [21/28]	1.1 (0.4–3.6) [46/55]	0.32[2]	0.6 (0.3–1.3) [21/56]	1.1 (0.6–2.1) [46/62]	0.01[2]
≥3	12.7 (2.8–57.6) [19/10]	12.8 (2.8–58.4) [25/11]	0.83[2]	2.5 (1.0–6.4) [19/17]	2.0 (0.9–4.7) [25/20]	0.58[2]
LRT[3]	$p < 0.01$ ($\chi^2 = 24.9$, DF = 5)		0.52[4]	$p < 0.01$ ($\chi^2 = 19.4$, DF = 5)		0.03[4]
History of heavy drinking[5]						
No	1.0 (reference) [63/107]	0.7 (0.3–1.5) [97/145]		1.0 (reference) [63/138]	0.9 (0.6–1.4) [97/204]	
Yes	11.1 (2.8–44.4) [22/9]	5.4 (1.5–20.0) [27/14]	0.70[2]	3.1 (1.3–7.2) [22/15]	1.8 (0.9–3.8) [27/24]	0.44[2]
LRT[3]	$p < 0.01$ ($\chi^2 = 20.8$, DF = 3)			$p = 0.01$ ($\chi^2 = 10.5$, DF = 3)		

Values in square brackets indicate no. of cases/controls.
[1]Adjusted for sex, age category (40–49, 50–59, 60–69 and 70–79 years), smoking habit (never, former and current smokers), HBsAg and HCVAb.–[2]p value calculated by the Wald test for each interaction term (DF = 1).–[3]Likelihood ratio test.–[4]p value calculated by the likelihood ratio test for a set of interaction terms (DF = 2).–[5]A "history of heavy drinking" was defined as having imbibed ≥3 "go"s almost every day for ≥10 years.

TABLE VI – ADJUSTED OR[1] (AND 95% CI) OF HCC ACCORDING TO THE COMBINATION OF *ALDH2* AND *ADH2* GENOTYPES AMONG LIGHT TO MODERATE DRINKERS CONSUMING <3 "GO"S/DAY OF SAKE

ALDH2 genotype	*ADH2* genotype	HCC cases *vs.* hospital controls	HCC cases *vs.* CLD patients
Light to moderate drinkers during last 1–2 years[2]			
*1/*1	*1/*1 + *1/*2	1.0 (reference)	1.0 (reference)
*1/*1	*2/*2	0.2 (0.03–1.7)	1.5 (0.6–3.6)
*1/*2	*1/*1 + *1/*2	1.4 (0.1–13.0)	1.7 (0.5–5.7)
*1/*2	*2/*2	3.3 (0.5–23.5)	2.8 (0.9–8.3)
Light to moderate drinkers 10 years before[3]			
*1/*1	*1/*1 + *1/*2	1.0 (reference)	1.0 (reference)
*1/*1	*2/*2	0.3 (0.05–1.4)	2.2 (0.9–5.3)
*1/*2	*1/*1 + *1/*2	1.0 (0.1–8.1)	2.5 (0.8–7.8)
*1/*2	*2/*2	1.9 (0.3–12.7)	3.9 (1.3–11.2)

[1]Adjusted for sex, age category (40–49, 50–59, 60–69 and 70–79 years), smoking habit (never, former and current smokers), HBsAg and HCVAb.–[2]Data were based on 68 HCC cases, 91 hospital controls and 99 CLD patients.–[3]Data were based on 67 HCC cases, 112 hospital controls and 89 CLD patients.

type in either study group. We noticed a possible interaction between light to moderate alcohol intake less than 3 "go"s/day during the last 1–2 years and the *ALDH2*1/*2* genotype; although the individual OR for either factor was near unity, the OR for both factors combined was elevated. In other words, light to moderate drinking, as compared with nondrinking, was associated with an increased risk among those with *ALDH2*1/*2* [adjusted ORs: 4.5 (95% CI 1.2–16.5) based on hospital controls and 2.0 (95% CI 0.9–4.3) based on CLD patients], but not among those with *ALDH2*1/*1* (adjusted ORs: 0.8 and 1.0); alternatively, the risk for *ALDH2*1/*2* relative to *ALDH2*1/*1* was elevated among light to moderate drinkers [adjusted ORs: 4.4 (95%CI 1.2–15.4) based on hospital controls and 1.8 (95%CI 0.8–3.7) based on CLD patients], but not among nondrinkers (adjusted ORs: 0.7 and 0.9). Similar interactions were observed between alcohol use less than 3 "go"s/day 10 years prior to the interview and the *ALDH2*1/*2* genotype. However, we did not observe any significant gene–alcohol interaction among heavy drinkers consuming 3 "go"s/day or more, or among those with a history of heavy drinking.

For *ADH2* polymorphism (Table V), we noticed a possible inverse interaction between light to moderate alcohol intake and the *ADH2*2/*2* genotype by comparing HCC cases with CLD patients ($p = 0.07$ or 0.01), although the CIs of relevant ORs were too wide to substantiate this finding. In addition, this tendency was not evident based on comparison of HCC cases with hospital controls.

Finally, we evaluated the combined effect of *ALDH2* and *ADH2* polymorphisms on HCC risk among light to moderate drinkers consuming less than 3 "go"s/day of sake (Table VI). Such drinkers with the combination of *ALDH2*1/*2* and *ADH2*2/*2* had the highest risk of HCC, which was about two- to four-fold higher than the risk among those with the combination of *ALDH2*1/*1* and either *ADH2*1/*1* or *ADH2*1/*2*.

Discussion

The main results of this case-control study were as follows: (*i*) for the consumption of alcohol beverages, there appeared to be a threshold for the overall association with increased risk of HCC at about daily 3 "go"s of sake (69 g of ethanol); (*ii*) in the inactive heterozygous *ALDH2* (*ALDH2*1/*2*) group, even light to moderate alcohol intake (less than 3 "go"s/day) was associated with an increased risk of HCC; and (*iii*) among light to moderate drinkers, individuals with the combination of *ALDH2*1/*2* and *ADH2*2/*2* (active homozygote) had the highest risk of HCC.

To our knowledge, this case-control study of moderate size is the largest investigation of these gene–alcohol interactions to date. The enrollment of 2 different controls and adjustment for hepatitis virus infection also represent favorable aspects. The fairly high participation rates of study subjects (73–96%) reduced the possibility of self-selection bias. However, several limitations in the present study should be considered. We did not limit the diagnosis for hospital controls to those for which there was no prior indication of a relation with alcohol use. This might have biased alcohol-related ORs towards unity, although it would not seriously damage the interpretation of the results. Recall bias could be present, yet the employment of the patient controls may have

restricted differential recall of alcohol intake. Inaccuracy of self-reported alcohol intake, particularly 10 years prior to interviews, may have led to an underestimation of relevant ORs. Because of the lack of histology for about half the CLD patients and the administration of interferon to 48% of the CLD patients, which perhaps modified liver disease activities in a short time period, we could not analyze the influence of relevant factors on the severity of liver disease (e.g. histological grading), but combined all CLD patients into a single control group.

Our results indicated that, overall, heavy alcohol consumption (more than 3 "go"s/day), but not light to moderate intake, was associated with an increased risk of HCC. This observation was in agreement with those of most previous studies on this topic.[8–11] The apparent threshold level was similar to that of 1 recent case-control study in Italy, which showed a linear increase in HCC risk with increasing alcohol intake from a level of about daily 60 g of ethanol.[8] Although the threshold level in CLD patients could be speculated as lower than in those without CLD because of possible interaction between alcohol and hepatotropic viruses (e.g., the elevation of oxidative stress observed even in moderate alcohol drinkers with HCV),[30] our data showed that the risk of HCC began to increase at about daily 3 "go"s by comparing HCC cases with CLD patients, as well as hospital controls. This result was unchanged when the analysis was restricted to HCVAb-positive subjects (data not shown).

We found elevated ORs of HCC for former drinkers and light drinkers (less than daily 1 "go") based on comparison of HCC cases with hospital controls, but not with CLD patients. The most likely reason for these results is that part of HCC cases (particularly, light to moderate drinkers) may have stopped or reduced alcohol intake due to their preexisting CLD (e.g., symptoms related to their liver disease or advice from surroundings). Similar findings were reported in other studies.[8] Alternatively, hospital controls might have unrepresentative drinking habits or a distorted distribution within the light to moderate drinking category, although we could not address this issue. For the analysis of gene–alcohol interactions, we used the combined category of light to moderate drinking less than 3 "go"s/day.

Acetaldehyde has mutagenic and carcinogenic effects leading to metaplasia, inhibition of DNA repair,[31] sister chromatid exchanges,[32] stimulation of apoptosis and enhanced cell injury associated with hyperregeneration.[12] According to IARC, there is sufficient evidence to identify acetaldehyde as a carcinogen in animals.[12] An alcohol challenge test showed that in the *ALDH2*1/*2* group after drinking a small amount of ethanol (0.1 g/kg body weight), the average peak blood acetaldehyde level was about five times higher than that in the *ALDH2 *1/*1* group after drinking a moderate amount of ethanol (0.8 g/kg body weight).[33] Thus, the lack of ALDH2 activity retards the elimination of acetaldehyde and elevates blood acetaldehyde even with a small account of alcohol intake. Despite the above biological plausibility, the results on the association between the *ALDH2* polymorphism and HCC from several epidemiologic studies[23–26] have been inconsistent.

When we examined the relation between the *ALDH2* polymorphism and disease, a complicating issue was that the polymorphism substantially influences alcohol intake, which could affect disease occurrence through various mechanisms; people with *ALDH2*2/*2* seldom drink alcohol, and those with *ALDH2*1/*2* include fewer heavy drinkers than those with *ALDH2*1/*1*. Therefore, it is indispensable to consider the amount of alcohol intake, yet there have been few epidemiologic studies of HCC addressing this issue. Only 1 case-control study[25] showed that HCC risk was slightly elevated in the *ALDH2*1/*2* relative to *ALDH2*1/*1* genotype after adjustment for age, smoking and drinking amount (OR = 1.6, 95% CI 0.8–3.2) and that this elevation was observed even in the small intake category (OR = 1.8, 95% CI 0.6–5.1). A recent case-control study[23] also demonstrated that the frequency of any *ALDH2*2* allele had a significant correlation with increased risk of HCC among habitual alcohol drinkers (OR = 2.5, 95% CI 1.2–5.3); since habitual drinkers with any *ALDH2*2* allele probably consume much less alcohol than those with *ALDH2*1/*1* and this would seemingly reduce the HCC risk, the actual risk associated with having any *ALDH2*2* allele would be even higher. In the present study, we observed about a two- to four-fold increased risk for *ALDH2*1/*2*, as compared with *ALDH2*1/*1*, among light to moderate drinkers, which partially agrees with the findings in the above case-control studies.

In the heavy drinking category (3 "go"s/day or more), we found no evidence of an elevated risk of HCC for *ALDH2*1/*2* vs. *ALDH2*1/*1*. Caution must be exercised in interpreting this data because the number of heavy drinkers with *ALDH2*1/*2* was small (Table IV). However, there exists a possibility that, among heavy drinkers, the role of the acetaldehyde-mediated pathway may be overwhelmed by those of other postulated pathways (e.g., induction of the microsomal ethanol-oxidizing system involving cytochrome P450[34,35] and immunosuppression[36]).

Although blood acetaldehyde levels after drinking mainly depend on the *ALDH2* polymorphism (Glu487Lys), the *ADH2* polymorphism (Arg47His) may also affect the blood acetaldehyde level in combination with the *ALDH2* polymorphism, possibly resulting in different profiles of HCC risk among alcohol users. Regarding the possible inverse interaction between light to moderate alcohol intake and the *ADH2*2/*2* genotype (Table V), which was observed in comparison of HCC cases with CLD patients but not with hospital controls, we currently have no plausible explanation, and chance could be responsible. Taking into account both *ALDH2* and *ADH2* genotypes, we found that the combination of *ALDH2*1/*2* and *ADH2*2/*2* revealed the highest risk of HCC among light to moderate drinkers. This result may support the involvement of the acetaldehyde-mediated pathway in hepatocarcinogenesis, at least among light to moderate alcohol users.

In conclusion, our results suggest that, although the independent role of the *ADH2* polymorphism is uncertain, the *ALDH2* polymorphism may modify HCC risk among light to moderate drinkers through the difference in acetaldehyde formation in the liver. The apparent absence of this gene–alcohol interaction for heavy drinking, which was strongly related to HCC risk, suggests the relevance of additional pathways in alcohol-related hepatocarcinogenesis, and further studies are warranted on this issue.

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