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ORIGINAL PAPER

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Genetic polymorphisms of tobacco- and alcohol-related metabolizing enzymes and the risk of hepatocellular carcinoma

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Abstract The effect of genetic polymorphisms for glutathione S-transferase (*GST*) *M1*, *GSTT1*, *GSTP1-1* (*GSTP1*), cytochrome P450 2E1 (*CYP2E1*) and aldehyde dehydrogenase 2 (*ALDH2*) on the risk of hepatocellular carcinoma (HCC) was observed in 78 Japanese patients with HCC and 138 non-cancer hospital controls. We found a positive association between cumulative amounts of alcohol consumption (≧600,000 ml in a lifetime) and the risk of HCC (OR = 4.52, 95% CI 2.39–8.55). However, cigarette smoking was not significantly related to the risk of HCC (OR = 1.23, 95% CI 0.57–2.68). The allelic frequencies of *GSTM1*, *GSTT1*, *GSTP1*, *CYP2E1* and *ALDH2* of HCC patients were not significantly different from those of controls when odds ratios were only adjusted for age and gender except for any *2* alleles of *ALDH2* in drinkers (OR = 2.53, 95% CI 1.21–5.31). However, the frequency of any *C2*alleles of *CYP2E1* and any *2* alleles of *ALDH2* were significantly higher than those of controls (OR = 5.77, 95% CI 1.24–27.39, OR = 9.77, 95% CI 1.63–58.60) when covariates including viremia were selected by using stepwise logistic regression analysis. We conclude that habitual alcohol drinking is likely to lead to an increased risk of HCC, and any *C2* alleles of *CYP2E1* as well as any two alleles of *ALDH2* were also associated with an increased risk of HCC.

Keywords Genetic polymorphism · GSTM1 · CYP2E1 · ALDH2 · Hepatocellular carcinoma

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Introduction

Primary liver cancer usually complicates several chronic liver diseases, mainly those induced by hepatitis B virus (HBV) and hepatitis C virus (HCV) (Ruiz et al. 1992; Tsai et al. 1994). Especially, HBV and HCV prevalence was found to be associated with 95% of hepatocellular carcinoma (HCC) patients in Japan (The Liver Cancer Study Group of Japan 2000). The proportions of HBV and HCV were 16.5% and 74.8%, respectively. Not only is HCC an inevitable consequence of chronic HBV or HCV infection, but also other HCC risk factors, such as tobacco smoking, alcohol drinking and aflatoxin exposure (Ross et al. 1992), are related to susceptibility to HCC. Epidemiological studies have shown a possible correlation between ethanol abuse and the development of HCC (Chen et al. 1991; Mohamed et al. 1992). Otherwise, the possibilities of a relationship between tobacco smoking and the occurrence of HCC are controversial (Trichopoulos et al. 1987; Tsukuma et al. 1993; Kuper et al. 2000; Tanaka et al. 1992; Hadziyannis et al. 1995).

Many chemical carcinogens are also metabolically converted into active forms that have harmful effects on the liver. The metabolizing enzymes, including

glutathione S-transferases (GSTs), cytochrome P-450s (CYPs) and aldehyde dehydrogenase 2 (ALDH2), play an important role in the detoxification or activation of carcinogens. This metabolic activation depends on genetic variations, which may be responsible for individual differences. *GSTM1*, *GSTT1* and *GSTP1* may play a part in the activation and detoxification of procarcinogens in tobacco smoke (Guengerich 1991; Mannervik and Danielson 1988). Individual variations in enzyme activities have been demonstrated for several GSTs. Some of these variations are genetically linked and may affect individual cancer risk.

When drinking alcohol, some of the proposed mechanisms for ethanol-related carcinogenesis are closely linked to the action of acetoaldehyde. Approximately half of the Japanese population lacks ALDH2 activity because of a structural point mutation in the *ALDH2 gene*. This genetic polymorphism, which is seen in Asians, including Japanese, but not in Caucasians, results in catalytic deficiency of aldehyde metabolism (Harada and Zhang 1993). Besides ALDH2, the ethanol inducible CYP2E1 catalyses the oxidation of ethanol itself. In addition, CYP2E1 is of critical importance in the metabolic activation of many carcinogens, including N-nitrosamines, benzene and aniline, that are present in tobacco smoke. Therefore, previous reports have shown that *CYP2E1* might modulate the risk of HCC (Ladero et al. 1996).

In this study, we have made the hypothesis that alcohol abuse and/or tobacco smoking is a risk factor for the development of HCC, and we have examined the effects of the *GST*s (*M1*, *T1*, *P1-1*), *CYP2E1* and *ALDH2* polymorphism on the susceptibility of HCC among Japanese people in relation to their smoking or alcohol-drinking status.

Materials and methods

Subjects

A total of 78 HCC patients seen in the University of Occupational and Environmental Health (UOEH) Hospital in Japan from June 1997 to April 1998 were enrolled in the present study. Acid-citrate-dextrose-anti-coagulated blood was drawn from 78 patients with HCC and from 138 hospital controls with no evidence of cancer in any organ. Cases and controls were unmatched. The demographic data of both case and control groups are shown in Table 1. All study subjects completed a questionnaire administrated by a trained interviewer, covering medical, residential, occupational and smoking and drinking history. The lifetime amount of cigarette smoking was quantified by the Brinkman-Coates index, which is the product of the daily number of cigarettes smoked and years of smoking. The cumulative amount of ethanol consumption was quantified by drink-years, which was calculated by multiplying the volume of ethanol a year by the number of drinking-years. None of the subjects had had any exposure to carcinogens, heavy metals or radiation in their occupational history.

This study was approved by the ethics committee of medical care and research of the University of Occupational and Environmental Health (UOEH) under the guidelines of the Ministry of Education, Culture, Sports, Science and Technology in Japan.

Table 1 Distribution of demographic variables for cases and controls

	Variables	Cases	Controls
Gender	Males (%)	61 (78.2%)	94 (68.1%)
	Females (%)	17 (21.8%)	44 (31.9%)
Age	Mean age (±SD)	66.1±7.7	67.2±10.5
	Range	47–84	34–92
Smoking status	Non smokers (%)	20 (25.6%)	50 (36.2%)
	Smokers (%)	58 (74.4%)	88 (63.8%)
Drinking status	Non drinkers (%)	25 (32.1%)	56 (40.6%)
	Drinkers (%)	53 (67.9%)	82 (59.4%)
Viremias	Non viremias (%)	2 (2.6%)	127 (92.0%)
	HBV (%)	14 (17.9%)	1 (0.7%)
	HCV (%)	54 (69.2%)	10 (7.3%)
	HBV+HCV (%)	8 (10.3%)	0 (0%)

Genotyping

Genomic DNA was isolated from peripheral leukocytes by proteinase K digestion and phenol/chloroform extraction and ethanol precipitation. A multiplex polymerase chain reaction (PCR) method was used to detect the presence or absence of *GSTM1* and *GSTT1* (Katoh et al. 1996). The genotype of *GSTP1* (A to G substitution at nucleotide 313) was determined by PCR/RFLP according to Watson et al. (1998). The genetic polymorphism in the 5′-flanking region of *CYP2E1*was determined by PCR amplification followed by digestion with *Rsa1*, using the method described previously (Adami et al. 1992). The dominant allele (*c1*) was sensitive to *Rsa1* digestion and the *c2* allele was resistant to *Rsa1* digestion. The genotypes of *ALDH2* were identified as the homozygous genotype of a normal *ALDH2* (*1/1*), the homozygous genotype of an inactive *ALDH2* (*2/2*) and the heterozygous genotype of normal and inactive *ALDH2* (*1/2*) by the method of Harada and Zhang (1993).

Statistical analysis

Statistical analysis was performed by comparing each gene polymorphism of five metabolic enzymes in HCC patients with the hospital controls. Odds ratios and 95% confidence intervals were adjusted for age and gender by multiple logistic regression analysis with the SPSS for Windows Medical Pack (SPSS Inc., Chicago). Needing to combine heterozygous genotypes (*GSTM1/T1/P1*, *CYP2E1*ALDH2) to examine the interaction between environmental and genetic factors as well as smoking or drinking status, we carried out stratification analysis of HCC risk associated with genotypes.

Results

Table 2 demonstrates the risk of HCC by drinking, smoking habits and viremias. The age- and gender-adjusted OR of heavy drinkers, who consumed alcohol above a threshold of 600,000 ml during their lifetime, was significantly higher (OR = 5.19, 95% CI 2.53–10.64) than in non-drinkers and light drinkers who consumed alcohol under 600,000 ml during their lifetime. On the other hand, there was no tendency of increased risk in the smoker strata (OR = 1.23, 95% CI 0.57–2.68). We also confirmed a strong association between viremia and HCC (OR = 805.17, 95% CI 134.37–4,824.52).

Table 2 Odds ratio of hepatocellular carcinoma by drinking, smoking and viremias

		Odds ratio (95% CI)
Drinking status	Non drinkers	1
	Drinkers	1.45 (0.81–2.60)
Alcohol consumption	1– < 200,000 ml	0.31 (0.15–0.62)
	≦200,000– < 600,000 ml	0.79 (0.40–1.57)
	≧600,000 ml	4.52 (2.39–8.55)
Smoking status	Non smokers	1
	Smokers	1.23 (0.56–2.67)
Smoking exposure (Blinkman-Coates index)	0– < 400	1.14 (0.58–2.25)
	400≦– < 800	1.09 (0.56–2.14)
	≧800	1.09 (0.56–2.15)
Viremias	Non viremias	1
	HBV or HCV	438.72 (94.69–2,032.61)

Odds ratios were adjusted for age and gender

Table 3 Relationship between *GSTM1*, *GSTT1*, *GSTP1*, *CYP2E1* and *ALDH2* genotypes and HCC

		Controls (n = 138) % (n)	Cases (n = 78) % (n)	Odds ratio (95% CI)
GSTM1	Positive genotype	50.7% (70)	61.5% (48) [a]	1
	Null genotype	49.3% (68)	38.5% (29) [a]	0.59 (0.33–1.05)
GSTT1	Positive genotype	52.2% (72)	48.7% (38) [a]	1
	Null genotype	47.8% (66)	51.3% (39) [a]	0.97 (0.57–1.76)
GSTP1	*A/A*	66.7% (92)	76.9% (60)	1
	Any *G*	33.3% (46)	23.1% (18)	0.66 (0.35–1.27)
CYP2E1	*C1/C1* homozygote	64.5% (89)	57.7% (45) [a]	1
	Any *C2*	35.5% (49)	42.3% (32) [a]	1.22 (0.68–2.17)
ALDH2	*1/1* homozygote	55.1% (76)	43.6% (34)	1
	Any *2* allele	44.9% (62)	56.4% (44)	1.54 (0.87–2.71)

Odds ratio of *GSTM1*, *GSTT1*, *GSTP1*, *CYP2E1* and *ALDH2* were adjusted for age and gender.
[a]The total number is different from the total of cases (n = 78) because it was impossible to obtain PCR products for some patients

Table 4 Odds ratio for the genotypes related to HCC by drinking or smoking status

	Non smokers	Smokers
	OR (95%CI)	OR (95%CI)
GSTM 1 null type vs. positive type	0.48 (0.16–1.48)	0.59 (0.29–1.22)
GSTT1 null type vs. positive type	1.19 (0.41–3.47)	0.93 (0.47–1.87)
GSTP1 A/A genotype vs. any *G* allele	0.50 (0.15–1.69)	0.73 (0.33–1.59)
*CYP2E1*C1/*C1* genotype vs. any *C2* allele	0.89 (0.30–2.61)	1.33 (0.65–2.73)
	Non drinkers	Drinkers
	OR (95%CI)	OR (95%CI)
CYP2E1 C1/*C1* genotype vs. any *C2* allele	2.10 (0.79–5.64)	0.85 (0.40–1.81)
ALDH2 any *2* allele vs. *1/1* genotype	0.75 (0.24–2.34)	2.53 (1.21–5.31)

ORs were adjusted for age and gender

The age- and gender-adjusted frequencies of *GSTM1*, *GSTT1*, *GSTP1*, CYP2E1 and *ALDH2* genotypes associated with HCC are shown in Table 3. There was no significant difference between controls and HCC in terms of frequency distribution of their genes. To evaluate the interaction between the genotypes, we analyzed the combination of the genes. No significant association was observed for any interaction of genes (data not shown).

Furthermore, we calculated the OR for data that was classified by smoking or drinking to evaluate the effect of the gene in combination with smoking or drinking. The summarized data and the ORs are shown in Table 4, together with the 95% confidence interval. The frequency of any *2* allele of *ALDH2* had a significant correlation with increased risk of HCC among alcohol drinkers (OR = 2.53, 95% CI 1.21–5.31). However, other genotype distributions of HCC were not significantly different from those of the controls (data not shown).

Table 5 Logistic regression analysis output

Factor	Odds ratio	95% confidence interval
CYP2E1 C1/*C1* genotype vs. any *C2* allele	5.77	1.24–27.39
ALDH2 any *2* allele vs. *1/1* genotype	9.77	1.63–58.60

Covariates were selected by using stepwise logistic regression; variable available for selection include age, gender, drinking status, smoking status, viremia, *GSTM1*, *GSTT1*, *GSTP1*, *CYP2E1* and *ALDH2* genotypes. ORs were adjusted for age, gender, drinking status and viremia

Finally, we had a multivariate analysis including viremias; variables available for selection include age, gender, drinking status, smoking status, viremia and each genotype of five enzymes (Table 5). The frequencies of *C2* alleles of *CYP2E1* (OR = 5.77, 95% CI 1.24–27.39) and *2* alleles of *ALDH2* (OR = 9.77, 95% CI

1.63–58.60) were significantly higher than those of controls (Table 5).

Discussion

We have observed the correlation between habitual alcohol drinking and the risk of HCC for many years. Our results showed that there was a significant association between heavy alcohol drinking, which is over 600,000 ml in a lifetime, and an increase in the risk of HCC: the OR for alcohol drinkers was 4.52 (95% CI 2.39–8.55) in our HCC patients (Table 2). This relationship is in agreement with most of the many previous reports on this topic (Mohamed et al. 1992; Kuper et al. 2000). This seems to be a valid finding because alcohol has been assumed to be a promoter or growth enhancer of HCC (Adami et al. 1992).

We also examined the association between tobacco smoking and the risk of HCC. Some risk excess was observed among tobacco smokers (OR = 1.23, 95% CI 0.57–2.68) compared with non-smokers, but it was not significant.

Otherwise, the data on smoking and risk of HCC are contradictory (Trichopoulos et al. 1987; Tsukuma et al. 1993; Kuper et al. 2000; Tanaka et al. 1992; Hadziyannis et al. 1995). Our data revealed that there was likely to be no positive relationship between tobacco smoking and HCC. If tobacco smoking is one of the causes of HCC, this discrepancy could be due to some biases. The first one was that the smoking histories were excessively error prone. The second was that it was impossible to distinguish between two kinds of non-smoker. One of them had never smoked in their life, and another had quit smoking, but had a past history of smoking. The last was that the alcohol habit confounded it in the present study. We need a further examination without biases such as smoking history, alcohol and viremia.

We present data on the frequency of the *ALDH2* genotype in HCC. A significant relationship between the occurrence of certain cancers and the *ALDH2* polymorphism has been reported, particularly in alcoholics (Hori et al. 1997). Other reports also indicated that the differences of *ALDH2* genotypes has no association with HCC development (Takeshita et al. 2000). However, in a multivariate analysis including the viral factor, the frequency of any *2* allele of *ALDH2* was significantly different from controls (OR = 9.77, 95%CI 1.63–58.60). Moreover, we found evidence of a significant effect of drinking depending on the difference of the genetic polymorphism of *ALDH2*. Statistically, there was an association between any *2* allele of *ALDH2* and HCC patients in habitual drinkers (OR = 2.53, 95% CI 1.12–5.31). It is likely that alcoholic liver diseases with the *ALDH2* heterozygote (*1/2*) are more severe than those with the *ALDH2* homozygote (*1/1*) (Enomoto et al. 1991), since those with the *ALDH2* heterozygote (*1/2*) would have higher internal exposure to acetaldehyde after drinking alcohol (Takeshita et al. 1997). Ohhira et al. (1996) studied primary hepatocellular carcinoma associated with alcoholic liver disease without hepatitis virus infection. In the analysis of genetic polymorphism of *ALDH2*, all of the subjects had the *ALDH2* homozygote (*1/1* or *2/2*). Otherwise, Shibata et al. (1998) showed that ORs resulting from the *ALDH2* homozygote and some accumulated amount of alcohol intake by age 40 based on community controls were statistically significant in HCC. Although it is inconsistent which is a risk factor, the homozygote or heterozygote gene, these results might imply that individual differences of *ALDH2* genotypes change the risk of HCC by alcohol consumption.

A multivariate analysis showed that an increase of risk for HCC also was found to a significant degree in the difference of *CYP2E1* genotypes (OR = 5.77, 95% CI 1.24–27.39). The rate of *CYP2E1* activity increases in the liver after alcohol induction. This means that the *c2 CYP2E1* gene increases in habitual drinkers, especially those with chronic liver disease (Ladero et al. 1996; Tsutsumi et al. 1994a, 1994b). As a result, the activation of carcinogens increases in the liver. It is possible that the CYP2E1 activity in the human liver is associated with the susceptibility of HCC. There are two different mechanisms that influence its rate of activity. One of them is the genetic functional difference between *c1* and *c2* alleles. The other depends on environmental factors, mainly ethanol or other inducers, which also frequently show a carcinogenic potential in the liver. Earlier reports have suggested the *CYP2E1* polymorphisms may play an important role in smoking-related HCC. Homozygosity for the *c1/c1* genotype significantly increased the risk of developing HCC in cigarette smokers (Yu et al. 1995). In contrast, there was no significant association between HCC risk and genotype *c1/c2* or *c2/c2* in all HCC patients (Lee et al. 1997).

In this study, the possible effects of GSTs metabolic enzymes in modulating the development of HCC were not confirmed among alcohol drinkers or tobacco smokers. Members of the GST family are important candidates for involvement in susceptibility to commonly occurring forms of cancer, because they may regulate an individual's ability to metabolize environmental carcinogens. Normal or increased GST enzyme activity or levels may protect susceptible tissues from somatic mutations in DNA by facilitating the conjugation and subsequent elimination of electrophilic carcinogens. Absent or deficient GST enzyme activity may result in poorer elimination of electrophilic carcinogens, particularly in the presence of very active electrophilic activation by phaseI enzymes. If an individual's inherited genotype at a GST locus does not permit the efficient metabolism of compounds involved in carcinogens, then that individual may be at increased cancer risk.

For example, the *GSTM1*/ *GSTT1* is polymorphic in humans. *GSTM1* has been shown to be polymorphic and is absent in 35–60% of individuals (Bell et al. 1993; Katoh et al. 1995). Similarly, *GSTT1* is also polymorphic and is absent in 10–65% of human populations

(Chenevix-Trench et al. 1995). The lack of GSTM1 activity is due to the inherited homozygous deletion of the genes, and *GSTM1* deficiency has been linked with risk for various cancers (Bell et al. 1993; Brockmoller et al. 1996; Rebbeck 1997). Less is known about the association between *GSTT1* and cancer risk, but persons with the *GSTT1* null type show reduced ability to detoxify metabolites of 1,3-butadiene (Pemble et al. 1994) and ethylene oxide (Wiencke et al. 1995). A report suggested that the *GSTT1* null type might be a risk modifier in the occurrence of colorectal cancer (Deakin et al. 1996). Also, the difference of *GSTM1/T1* polymorphisms may be subject to increased risk of urothelial cancer in tobacco smokers (Katoh et al. 1998).

The *GSTP1* is also widely expressed in normal epithelial tissue and is particularly abundant in the urinary, respiratory and digestive tracts, suggesting a possible role for *GSTP1* in the detoxification and elimination of toxic products in these tissues. GSTP1 is a major enzyme involved in the inactivation of carcinogens in cigarette smoke, such as benzo(a)pyrene diol epoxide and acrolein, as well as other cigarette smoke toxins. The gene is also suggested to be involved in the development of acquired resistance towards anti-cancer drugs. The *GG* genotype of *GSTP1* was significantly more frequent among patients with oral squamous cell carcinoma and lung cancer (Katoh et al. 1999; Ryberg et al. 1997).

Overall, the differences of genetic polymorphisms on GST enzymes have no association with the development of HCC, although alcohol drinking showed a significant association with it. The discrepancy between our results and previous reports could be explained by the following suggestions: first, there was a racial difference in the frequencies of each genotype (Kato et al. 1992); for another reason, the risk of genetic polymorphism to HCC could be overshadowed by the great etiologic role of HBV and HCV viremia in the development of HCC (Tsukuma et al. 1990; Yu et al. 1994; Donato et al. 1998). However, HBV positive patients had significantly lower GST activity than those who were HBV negative (Zhou et al. 1997). These results suggest that the risk of HCC is not only associated with *GST* polymorphism, but also GST activity.

In conclusion, we found that there was a significant association between *CYP2E1* and *ALDH2* polymorphisms with the interaction of alcohol and the risk of HCC in Japanese people.

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