RESEARCH COMMUNICATION

HBV/HCV Infection, Alcohol, Tobacco and Genetic Polymorphisms for Hepatocellular Carcinoma in Nagoya, Japan

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Abstract

A population-based case-control study was carried out to investigate risk factors for hepatocellular carcinoma (HCC) in Nagoya, Japan, including hepatitis virus infections, drinking and smoking habits and genetic polymorphisms in aldehyde dehydrogenase2 (ALDH2) and cytochrome P4502E1 (CYP2E1). A total of 84 patients with HCC and 84 sex, age and residence pair-matched controls were recruited for this study. By univariate analysis, hepatitis B virus (HBV) (OR=5.14; 95%CI=2.29-11.6) and hepatitis C virus (HCV)(OR=32.00; 95%CI=7.83-130.7) infections, having a history of blood transfusion (OR=5.25; 95%CI=1.80-15.29), and habitual smoking (OR=2.36; 95%CI=1.17-4.78) were significantly linked to cases; by multivariate analysis, HCV infection (OR=23.5; 95%CI=5.07-108.9) and habitual smoking (OR=5.41; 95%CI=1.10-26.70) were still associated with a significantly increased risk. The c1/c1 genotype of CYP2E1 (odds ratio [OR]= 0.45; 95% confidence interval [CI]=0.21-0.99), detected by PstI and RsaI digestion was significantly more prevalent in the control group, while 1-1 genotype of ALDH2 (OR=1.24; 95%CI=0.70-2.20) did not demonstrate variation. There were no statistically significant interactions between habitual smoking/drinking and genetic polymorphisms of ALDH2/P4502E1 with reference to HCC development. These findings suggest that viruses, especially HCV infection, and habitual smoking are major independent risk factors, while genetic polymorphisms of ALDH2 and CYP2E1 have only limited contribution to the risk of HCC in Nagoya, Japan.

Asian Pacific J Cancer Prev, **1**, 239-245

Key Words: HCC - gene polymorphisms - lifestyle

Introduction

Primary liver cancer, largely hepatocellular carcinoma (HCC), is one of the most common fatal neoplasms in Eastern and Southeastern Asian countries, including Japan (Parkin et al., 1999). During the past few decades in Japan, the age-adjusted mortality rate of HCC has increased (Research Group for Population-based Cancer Registration in Japan., 1998) to became the third leading cancer death in males and fourth in females in 1995(Health and Welfare Statistics Association, Japan., 1997). Based on previous studies, the principal factors are hepatitis B virus (HBV) and hepatitis C virus (HCV) infections (Tanaka et al., 1991; Yuki et al., 1992; Kato et al.,1994; Takeshita et al., 2000).

Although the vast majority of Japanese HCC cases are thus caused by chronic viral infections, other host and environmental factors may also be implicated. Obviously, it would be helpful for predicting individual HCC risk, if genetic factor or personal susceptibility and an interaction of environmental exposures and host genetic factors could be elucidated. Among numerous personal exposures, drinking and smoking habits are of critical importance. Moderately increased risk for HCC with regard to habitual alcohol drinking and cigarette smoking have already been reported previous for many countries, including Japan(Hirayama et al.,1989; Chen et al., 1991; Tanaka et al.,1991; Tzonou et al.,1991; Yu et al., 1991; Mohamed et al.,1992; et al., 1997; Mori et al., 2000). The molecular mechanism linking

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biological or chemical carcinogens to the pathogenesis of HCC is still unclear, probably involves activation and detoxication of both endogenous and exogenous agents. Therefor, enzymes responsible for metabolism of xenobiotics may play a pivotal role. It is well-known that genes encoding metabolic enzymes are polymorphically expressed in humans(Wolf et al., 1994). Thus, one speculation is that interindividual susceptibility to HCC might partly depend on genetically determined variation in the ability to metabolize xenobiotics and chemical carcinogens. For example, there is interindividual variation of aldehyde dehydrogenase2 (ALDH2) activity, an important gene in the metabolism of alcohol. The P450 2E1(CYP2E1) gene, which encodes enzymes catalyzing not only the conversion of alcohol to acet aldehyde, but also the activation of an array of carcinogens such as N-nitrosamines in cigarette smoke (Bartsh et al., 1984; Hong et al., 1985; Wrighton et al., 1987; Yoo et al., 1988; Guengerich et al., 1991) might also be important in this context. Attempts to establish an association between HCC susceptibility and CYP2E1 genetic variations have been made, but the results were controversial (Koop et al., 1992; Yu et al., 1995).

Our present purpose was to reconfirm the association between HBV/HCV infections and HCC, identify whether alcohol drinking and cigarette smoking were independent risk factors of HCC, and determine whether gene polymorphisms of ALDH2 and CYP2E1, are related to the susceptibility of HCC. Further, with our population-based case-control study design, it was also possible to explore whether there were interactions between polymorphisms of ALDH2/CYP2E1 genes and corresponding metabolic substrates associated with drinking or smoking habit.

Materials and Methods

Study population:

Cases included eighty-four consecutive patients (64 men, 20 women; mean age, 62.8 years, ranging from 46 to 79 years) who were admitted into Nagoya City University Hospital and its affiliated hospitals from June to July 1994. Their diagnoses were confirmed by liver function tests, Éø-fetoprotein levels, ultrasonography, computed tomography scans and/or angiography. For every patient who received a surgical operation a histological diagnosis was available.

Eighty-four sex- and age (±2 years) matched controls, selected from the same resident community as the cases, were recruited as controls. They had no subjective symptoms related to hepatic diseases, no elevation of serum alanine aminotransferase (s-ALT; normal<45IU/L), or Éø-fetoprotein levels (<5ng/mL) and no detectable tumor like lesions in the liver by ultrasonography.

Epidemiological investigation:

An interviewer-administered questionnaire on demography, clinical conditions and possible risk factors, including smoking/drinking habits and history of hepatitis and jaundice, and a history of surgery as well as blood transfusion, was given to both HCC subjects and controls. Demographic details included age, residence, and place of birth, education, occupation (present and past), marital status, and number of children.

Laboratory methods:

1.Virus infection

One hundred and sixty eight serum samples from the case and the control groups were tested for serological viral markers. Hepatitis B surface antigen (HBsAg) was tested by a particle agglutination test (PA test; Fuji Rebio, Tokyo, Japan). Anti-hepatitis B core antibody (anti-HBc) was tested by an enzyme immunoassay (Imx; Dainabot, Tokyo, Japan). Hepatitis C virus antibody (anti-HCV) was detected with a second-generation PHA kit (Dainabot).

2.Genomic polymorphisms of ALDH2 and CYP2E1

DNA was extracted from 100É l of white blood cell rich plasma. For determination of ALDH2 genotypes, exon 12 of the ALDH2 gene was amplified by 35 cycles of polymerase chain reaction (PCR) under the following conditions: 1 min at 94o C for denaturation, 10s at 52 o C for primer annealing, and 30s at 72 o C for primer extension as previously reported (Takeshita et al., 1994). One primer (5'-CCACACTCACAGTTTTCTCTT) contained the substitution of thymine for adenine at the underlined position to create a Ksp632I recognition site in the typical allele. PCR products were digested with 2-3 units of Ksp632I, then separated in 3% agarose gels.

For detection of CYP2E1 polymorphisms, the protocol was 30 cycles of PCR amplification using Sp1 (5'-TTCATTCTGTCTTCTAACTGG) and ASP1 (5'CCAGTCGAGTCTACATTGTCA) primers were performed under the following conditions: 1 min at 95o C for denaturation, 1 min at 55 o C for primer annealing, and 1 min at 72 o C for primer extension. PCR products were subsequently digested with Rsal and Pstl restriction enzymes, and subjected to electrophoresis in 2% agarose gels.

Statistical analysis:

Crude odds ratios (OR), estimates of relative risk, linking possible risk factors to HCC, were calculated by univariate analysis. To identify variables that were independently associated with HCC, multivariate analyses were performed using conditional logistic regression methods. All p values resulted from two-sided statistical tests. The FREQ and PHREG procedures in SAS software package were employed for the statistical analysis (SAS Institute Inc.,1990).

Results

Table 1 shows the comparative results from univariate and multivariate analyses on main risk factors for HCC.

Because no controls were found to be HBsAg positive in this study, we employed anti-HBc as a surrogate for HBV infection. As anti-HBc, response to HB core antigen, is quicker and more sensitive than anti-HBs at HBV infection

Table 1. Odds Ratio s of HCC for Selected Risk Factors in Nagoya, Japan

Variables	No. of cases (%)	No. of controls (%)	Odds ratios[+] (95% CI)	Adjusted odds ratio[++] (95% CI)
Alcohol				
never	33 (39.3)	36 (42.9)	1.00	-
current+former	51 (60.7)	48 (57.1)	1.23 (0.59-2.56)	-
Smoking				
never	25 (29.8)	40 (47.6)	1.00	1.00
current+former	59 (70.2)	44 (52.4)	2.36 (1.17-4.78)*	5.41 (1.10-26.70)*
History of blood transfusion				
never	58 (60.1)	75 (89.3)	1.00	
ever	26 (30.9)	9 (10.7)	5.25 (1.80-15.29)**	3.31 (0.42-26.35)
History of surgical treatment				
never	43 (51.2)	42 (50)	1.00	-
ever	41 (48.8)	4 (50)	0.96 (0.55-1.68)	-
HBsAg				
negative	71 (85.5)	84 (100)	1.00	-
positive	12 (14.5)	0 (0)	-	-
Anti-HB_C				
negative	24 (28.6)	53 (63.1)	1.00	1.00
positive	60 (71.4)	31 (36.9)	5.14 (2.289-11.6)**	3.07 (0.62-15.12)
Anti-HCV[#]				
negative	15 (18.1)	78 (92.9)	1.00	1.00
positive	68 (81.9)	6 (7.1)	32.00 (7.833-130.7)**	23.5 (5.07-108.9)**
ALDH2				
2-2	4 (4.8)	8 (9.5)	1.00-	-
1-2	32 (38.1)	33 (39.3)	1.00	-
1-1	48 (57.1)	43 (51.2)	1.24 (0.70-2.20)	-
CYP2E1[#]				
c1/c2	25 (30.1)	17 (20.2)	1.00	1.00
c2/c2	4 (4.8)	1 (1.2)	1.00-	1.00
c1/c1	54 (65.1)	66 (78.6)	0.45 (0.21-0.99)	*0.67 (0.16-2.88)

Abbreviation: HCC: hepatocellular carcinoma.
[+]: Controlled for age and sex; *: p value <0.05; **: p value <0.01.
++:Controlled for age, sex and variables showing significant by univariate analysis.
[#]: There was one missing value in the case group due to an insufficient DNA sample.

Table 2. Combined Effect of Smoking Habit and CYP2E1 Gene Polymorphisms

	OR	95% CI
c1/c1 without smoking habit	1	
c1/c1 with smoking habit	2.07	0.6 - 7.18
c1/c2 and c2/c2 without smoking habit	0.36	0.07 - 1.95
c1/c2 and c2/c2 with smoking habit	6.22	0.67 - 57.74

Adjusted for HCV infection.

Table 3. Combined Effect of Drinking Habit and CYP2E1 Gene Polymorphisms

	OR	95% CI
c1/c1 without drinking habit	1	
c1/c1 with drinking habit	1.10	0.38 - 3.19
c1/c2 and c2/c2 without drinking habit	0.40	0.08 - 2.09
c1/c2 and c2/c2 with drinking habit	5.32	0.61 - 46.06

Adjusted for HCV infection.

Table 4. Combined Effect of Drinking Habit and ALDH2 Gene Polymorphisms

	OR	95% CI
$ALDH2^2/ALDH2^2$ and $ALDH2^2/ALDH2^1$ without drinking habit	1	
$ALDH2^2/ALDH2^2$ and $ALDH2^2/ALDH2^1$ with drinking habit	0.50	0.13 - 1.97
$ALDH2^1/ALDH2^1$ without drinking habit	1.37	0.24 - 7.69
$ALDH2^1/ALDH2^1$ with drinking habit	2.89	0.90 - 9.33

Adjusted for HCV infection

(Hoofnagle et al., 1974;1978). The OR of positive anti-HBc (5.14; 95%CI=2.29-11.6) and having a hisotry of blood transfusion (OR= 5.25; 95%CI=1.80-15.29) were very much higher tahn the corresponding referent groups. The risk of developing HCC was strongly associated with the presence of anti-HCV (OR=32.00; 95%CI=7.83-130.73).

From univariate analysis, alcohol drinking had also slightly increased the risk, but without statistical significance (OR=1.23; 95%CI=0.59-2.56); cigarette smoking elevated the risk for HCC with statistical significance (OR=2.36; 95%CI=1.17-4.78).

The frequency of the ALDH21/ALDH21 genotype was slightly higher in cases 57.1% (48/84) than in controls 51.2% (43/84), the odds ratio being 1.24 (95% CI=0.72-2.20). There were three genotypes of CYP2E1 resulting from digestion of PstI and RsaI: homozygote c1/c1; heterozygote c1/c2 and homozygote c2/c2. Homozygous c2/c2 of CYP2E1 was rare and accounted for only 1.2%(1/84) in controls and 4.8% (4/83) in HCC cases. Controls were more likely to be homozygous for the PstI and RsaI digested c1/c1 genotype (78.6% vs. 65.1%). The odds ratio for HCC with the c1/c1 genotype compared with two other genotypes combined was 0.45 (95% CI=0.21-0.98).

After the factors which were statistically significant by univariate analysis were put into multivariate logistic regression model, positive for anti-HCV (OR=23.5; 95%CI=5.07-108.9) and ever smoking (OR=5.41; 95%CI=1.10-26.70) increased the risk for HCC with statistical significance (right hand of Table 1).

Tables 2,3,and 4 present odds ratios for smoking and drinking habits with regard to different polymorphism statuses of ALDH2 and CYP2E1 genes. In table 2, compared to non-smokers with homogeneous c1/c1 genotype, risk for HCC increased in subjects with c1/c2 or c2/c2 genotype who had the smoking habit (OR=6.22; 95%CI=0.67-57.74). Compared with c1 homogeneous for CYP2E1 bearing subjects without drinking habit, an increased OR of 5.32 (95%CI=0.61-46.06) was also observed among drinkers with the c2 allele (Table 3). As shown in Table 4, the drinking habit and polymorphism of the ALDH2 genotype combined together gave an OR for drinkers carrying homogeneous 1/1 genotype of ALDH2 slightly elevated to 2.89 (95%CI=0.90-9.33).

Discussion

Our results suggest that HCV infection, in addition to HBV, is the principal risk factor for primary HCC in Nagoya, consistent with prior researches in Japan (Tanaka et al., 1991; Yuki et al., 1992; Kato et al., 1994; Takeshita et al.,2000). Additionally having a history of blood transfusion was also associated with significantly higher risk of HCCC by univariate analysis, but seemed not independent judged by the multivariate model. This perhaps stemmed from the fact that blood transfusion is a route of HCV infection, so that its effect on HCC would overlap other stronger factors, especially, HCV infection itself.

It was somewhat unexpected that alcohol drinking was only associated with a slightly elevated risk for HCC; while habitual smoking was another risk factor, with statistical significance by both univariate and multivariate analyses. This result should be interpreted with caution however, as many factors might bias the relation established between drinking/smoking habits and HCC. Most of Japanese HCC cases experience a multistage course, first as virus carriers, then suffering chronic active hepatitis, liver cirrhosis and

eventually HCC. From clinical practice, we also knew this course requires 30 years or longer(Hoofnagle et al., 1997). During this period, many pathological changes can occur, including hepatosplenomagaly, jaundice and esophageal varices, and only few patients have no complaints. In order to relieve symptoms, e.g. fatigue, fullness, loss of appetite and nausea, patients might automatically modify their smoking and drinking habits. When they visited hospital, physicians would always strongly recommend to stop drinking, in particular, and smoking. Although our interviewer requested a recall of drinking and smoking habits which should be at least one year before HCC diagnosis, it could not be ensured that answers to our questionnaire was free of self or physicians' modification. Therefore, the ORs for HCC with regard to both drinking and smoking habits might have been underestimated in our study.

From the present study, allelic frequencies of ALDH2 and the CYP2E1 5'-flanking region in our control group were comparable to those reported earlier for Japanese, Korean and Chinese (Hayashi et al., 1991; Goedde et al., 1992; Maezawa et al., 1994; Tsutsumi et al., 1994; Yu et al.,1995; Lee et al., 1997), but different from data for Western populations (Goedde et al., 1992).

Most of the acet aldehyde generated during alcohol metabolism is eliminated by ALDH2 (Borsron et al., 1986), and the mutant allele has dramatically diminished enzyme activity (Yokoyama et al., 1998), so that we expected a positive association with the risk of HCC. However, the ALDH2 polymorphism was not significantly associated with HCC in this investigation, in line with two other Japanese studies (Higuchi et al., 1995; Takeshita et al.,2000). The most probable explanation might be that alcohol consumption was low among individuals who carry one or two ALDH2*2 alleles. This is because ALDH2*2 generally serves as a strong protective factor against alcoholism by making drinking unpleasant (Higuchi et al., 1995), including facial flushing, palpitations, headache, vomiting and sweating (Lieber et al., 1994). Thus, the ALDH2*2 allele might have opposing influence: it negatively influences drinking behavior, but strengthens the carcinogenic effects of alcohol exposure on drinkers. Another possible explanation might be that the precancerous stage of liver disease, which was a long-term condition, might serve as a health warning and protect affected individuals from alcohol abuse. In this study, we failed to detect a joint effect between ALDH2 polymorphism and drinking habit; perhaps the real reason was due to a low-level relation of alcohol drinking to HCC carcinogenesis in our study.

Our results revealed a significant association between genetically determined differences in CYP2E1 and HCC risk, but the association was neither strong nor stable compared with HCV infection. By PstI or RsaI digestion, a decreased OR was observed among individuals homozygous for the CYP2E1 c1 allele. This result could be explained by previous findings. CYP2E1 is known to be involved in the metabolic activation of precarcinogens, including alcohol and cigarette smoke related acet aldehydes and N-nitrosamines (Koop et al., 1992). Besides, it also had been proved that the PstI and RsaI restriction sites are in the transcription-regulation region of CYP2E1 that is linked with gene expression (Hayashi et al., 1991); the variant form of CYP2E1 gene was expressed 10 times higher in allele c2 than in c1 (Hayashi et al., 1991). The hypothesis that there would be certain kinds of interaction between different genotypes of CYP2E1 and different smoking or drinking statuses. When c2 carrying smokers were compared with c1 homozygote non-smokers, risk for HCC increased more than 6-fold; and with c2 drinkers compared to c1 homozygous non-drinkers, 5 times elevated risk was found. However, with the adjustment for HCV infection, none of the interaction terms were statistically significant. These findings do not necessarily seem to be contradictory to our hypothesis because the sample size of this study is not big enough to detect a statistical significance of the current interactions; or the interactions might be over-shadowed by the prominent etiologic role of HCV in the carcinogenesis of HCC. However compared with virus infection, ethanol and smoking may be considered only as syncarcinogens or cocarcinogens for Japanese patients with HCV.

Therefore, it could be concluded from this study that hepatitis virus infections, HCV in particular, are the main risk factors for developing to HCC among Japanese patients with HCC. By univariate analysis, a gene polymorphism for CYP2E1, but not ALDH2 significantly correlated with HCC; there were no statistically significant interactions between drinking/smoking habit and gene polymorphisms of CYP2E1 or ALDH2 for HCC, in Nagoya, Japan.

Acknowledgement

The authors are grateful to Ms.Y. Kubo and Ms.Y.Ito for data preparation and Dr. Malcolm A. Moore for linguistic advice. This work was supported in part by Grant-in-Aid(08042010, 03670286 and 06454242) from Japanese Ministry of Education, Sports, Science and Culture. Xin-En Huang was also financially supported by scholarships from GAKUJUTSU SHINKOU Association and Daiko Scientific Foundation.

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Since he was a young boy, Tsuneo Koide has been fascinated by the culture and history of China before the modern era. At midnight, after finishing his everyday work, he often enjoys poring over some literary treatise about the splendours of China in the past.