

## Hepatitis B and C Viruses Infection, Lifestyle and Genetic Polymorphisms as Risk Factors for Hepatocellular Carcinoma in Haimen, China

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A case-control study was carried out to investigate the impact of factors including virus infection, aflatoxin B1, microcystins, smoking/drinking and dietary habits as well as genetic polymorphisms of aldehyde dehydrogenase 2 (*ALDH2*) and cytochrome P4502E1 (*CYP2E1*), on susceptibility to hepatocellular carcinoma (HCC) in Haimen, China. A total of 248 patients with HCC and 248 sex-, age- and residence-matched population-based controls were recruited into the study. Virus infection, and *ALDH2* and *CYP2E1* gene polymorphisms were assessed in 134 paired cases and controls. By univariate analysis, hepatitis B virus (HBV) infection (odds ratio [OR]=9.75; 95% confidence interval [CI]=4.71–20.2), history of intravenous injection (OR=1.50; 95%CI=1.02–2.22), average income (OR=0.63; 95%CI=0.43–0.92), frequent intake of foods rich in protein, e.g., egg (OR=0.6; 95%CI=0.42–0.87), chicken (OR=0.53; 95%CI=0.35–0.79), pork (OR=0.67; 95%CI=0.46–0.98) and fresh fish (OR=0.58; 95%CI=0.39–0.87) significantly differed between cases and controls. However, peanut intake (OR=0.66; 95%CI=0.43–1.01), source of drinking water, including tap (OR=1.33; 95%CI=0.81–2.20), deep well (OR=0.94; 95%CI=0.56–1.55), shallow well (OR=0.85; 95%CI=0.55–1.30), river (OR=0.95; 95%CI=0.65–1.38), ditch (OR=1.09; 95%CI=0.76–1.55) and pond water (OR=1.0; 95%CI=0.14–7.10) were not significantly associated with risk. Univariate analysis also indicated that the *I-I* genotype of *ALDH2* (OR=1.38; 95%CI=0.86–2.23) as well as the Pst1- and Rsa1-digested *c1/c1* genotype of *CYP2E1* (OR=1.36; 95%CI=0.81–2.28), was slightly more frequent in the case group. On multivariate analysis, HBV infection (OR=13.9; 95%CI=5.78–33.6) and history of intravenous injection (OR=2.72; 95%CI=1.24–6.00) were still associated with significantly increased risk of HCC, while frequent intake of fresh fish (OR=0.32; 95%CI=0.12–0.86) decreased this risk. These findings suggest that whereas peanut intake, water sources as well as genetic polymorphisms in *ALDH2* and *CYP2E1* do not significantly correlate with the risk of HCC, HBV infection is a main risk factor, and dietary items rich in protein, especially fresh fish, might protect against the risk of HCC in Haimen, China.

Key words: Genetic polymorphisms — Haimen — HBV/HCV — Hepatocellular carcinoma — Lifestyle

Primary liver cancer, largely hepatocellular carcinoma (HCC), is one of the most common fatal neoplasms in China. A previous study showed the main risk factor for Chinese HCC to be chronic hepatitis B virus (HBV) infection.<sup>1)</sup> Hepatitis C virus (HCV)<sup>2,3)</sup> and lifestyle,<sup>4)</sup> as well as many natural toxins have also been implicated. Among the latter, aflatoxin (AF) is the most well-known, and has been

documented to be a hepatic carcinogen in various animals, including primates.<sup>5)</sup> However, no close relationship has been observed between dietary exposure level to AFB1 and the incidence of HCC in Chinese endemic areas.<sup>6–8)</sup> In Eastern China, including Haimen, another suspected important risk factor is microcystins, a contaminant of river, pond and ditch water, but the results are also controversial.<sup>1,6,9)</sup> Among lifestyle habits, a moderately increased risk with alcohol drinking and cigarette smoking, very prevalent in Chinese adult males, was observed

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in earlier studies,<sup>4, 10, 11)</sup> but there are few Chinese studies which have evaluated the risk of HCC with regard to alcohol drinking and cigarette smoking, taking into account polymorphisms in related genes.

A possible molecular mechanism linking cigarette smoking and alcohol drinking to the pathogenesis of HCC involves activation and detoxification of chemical carcinogens. In these processes, various enzymes play important roles. For example, it is well known that there is interindividual variation in terms of the activity of aldehyde dehydrogenase 2 (*ALDH2*), which is responsible for metabolism of alcohol. Recent research has further suggested that polymorphisms of the *P450 2E1 (CYP2E1)* gene, which encodes an enzyme catalyzing the metabolism of N-nitrosamines and alcohol, might be associated with susceptibility to HCC.<sup>12)</sup>

In the present study, conducted in Haimen, an area with a high rate of HBV infection, it was hypothesized that AFB1 and microcystin exposure, together with lifestyle factors including alcohol drinking, cigarette smoking and dietary habits, contribute to the risk of HCC. Our secondary hypothesis was that genetic polymorphisms of *ALDH2* and *CYP2E1* might influence susceptibility.

**MATERIALS AND METHODS**

**Study population** Cases included 248 HCC patients who had resided in the Haimen area more than 10 years, and had been diagnosed and confirmed in Haimen People’s Hospital. Diagnosis was obtained by a combination of  $\alpha$ -fetoprotein level, ultrasonography, computed tomography, liver function tests, and/or angiography. The recruitment of cases lasted from June 1995 to February 1997. Most patients had been operated or biopsied, so histological diagnoses were available. There were 207 (83.5%) male and 41 (16.5%) female cases, of whom 87% were aged more than 40 years (range from 25 to 79 years).

Two hundred and forty-eight sex- and age ( $\pm 2$  years)-matched subjects, with the same resident community for at least 10 years as the cases, were randomly recruited as controls with the help of Haimen City Health and Anti-epidemic Station during the same time. None had any subjective symptoms related to hepatic diseases, with no elevation of serum alanine aminotransferase (normal <45 IU/liter) or  $\alpha$ -fetoprotein levels (<5 ng/ml), and also no detectable tumor-like lesions in the liver by ultrasonography. Informed consent was obtained from all study subjects. The Human Resource Committee of Shanghai Medical University approved all procedures of this study.

The general characteristics of the cases and controls are shown in Table I. Average annual income was slightly higher in the control group. Other demographic characteristics were comparable.

**Epidemiological investigation** An interviewer-adminis-

Table I. Demographic Characteristics of Hepatocellular Carcinoma (HCC) Cases and Population-based Sex-age-matched Controls in Haimen, China

Characteristics	Cases (N=248) %	Controls (N=248) %	P
Sex			—
Male	83.5	83.5	
Female	16.5	16.5	
Age (years)			
<40	12.9	11.7	
≥40	87.1	88.3	
Annual income (yuan per capita) <sup>a)</sup>			0.012
<1500	38.7	27.4	
1500–2400	28.2	28.2	
>2400	33.1	44.4	
Education			0.114
Elementary or less	66.1	59.3	
Higher than elementary	33.9	40.7	
Occupation			0.575
Peasant	59.2	61.7	
Others	40.8	38.3	

a) Estimated in Chinese yuan.

tered questionnaire on demography, clinical conditions and possible risk factors was given to both HCC cases and controls. Demographic details included age, residence, place of birth, education, job (present and past) and income, hobbies, marital status, number of children, and number of people sharing the household. Possible risk factors under consideration included: smoking/drinking habits, dietary habits including intake of peanuts, source of drinking water, categorized into tap, deep and shallow well, river, ditch and pond, as well as history of hepatitis, blood or blood-product transfusion, intravenous (i.v.) injection and family history of chronic liver disease.

**Laboratory methods**

**Virus infection:** Two hundred and sixty-eight serum samples from cases and controls were tested for serological viral markers. Hepatitis B surface antigen (HBsAg) was tested by means of a particle agglutination test (PA test; Fuji Rebio, Tokyo). Anti-hepatitis B core antibody (anti-HBc) was tested by means of an enzyme immunoassay (Imx, Dainabot, Tokyo). Hepatitis C virus antibody (anti-HCV) was detected with a second-generation PHA kit (Dainabot).

**Genomic polymorphisms of *ALDH2/CYP2E1*:** Genotypes of *ALDH2/CYP2E1* were determined from serial numbers 1 to 134 in both cases and controls. DNA was extracted from 100  $\mu$ l of white blood cell-rich plasma. For determination of *ALDH2* genotypes, exon 12 of the *ALDH2* gene

Table II. Odds Ratios of Hepatocellular Carcinoma (HCC) for Selected Risk Factors and Dietary Items by Age, Sex and Residence Matched Case-control Study Conducted in Haimen, China

Variables	Cases (n/n)	Controls (n/n)	Crude odds ratio (95% CI)	HBV-adjusted odds ratio (95% CI)
HBsAg (positive vs. negative)	91/43	21/113	9.75 (4.71–20.2)**	— — —
HBV-HCV (positive vs. negative)	7/127	8/126	0.86 (0.29–2.55)	0.77 (0.19–3.18)
History of blood transfusion (ever vs. never)	22/223	14/231	1.46 (0.72–2.96)	1.26 (0.36–4.44)
History of intravenous injection (ever vs. never)	146/101	125/122	1.50 (1.02–2.22)*	1.95 (1.00–3.86) <sup>§</sup>
Average income (≥2400 yuan vs. <2400 yuan)	85/163	110/138	0.63 (0.43–0.92)*	0.49 (0.21–1.12)
Alcohol (ever vs. never)	106/141	97/151	1.20 (0.82–1.75)	1.38 (0.68–2.81)
Smoking (ever vs. never)	143/105	142/106	1.02 (0.68–1.55)	0.69 (0.28–1.66)
Fruit (≥2 times/mo. vs. <2 times/mo.)	135/113	118/130	1.40 (0.94–2.06)	0.73 (0.37–1.46)
Yellow vegetables (≥2 times/mo. vs. <2 times/mo.)	92/156	103/145	0.65 (0.37–1.13)	1.35 (0.55–3.31)
Pickled vegetables (≥3 times/we. vs. <3 times/we.)	114/134	121/127	0.88 (0.60–1.28)	1.23 (0.62–2.46)
Peanut (≥3 times/we. vs. <3 times/we.)	71/177	89/159	0.66 (0.43–1.01)	0.66 (0.32–1.36)
Preference for tea (yes vs. no)	33/212	36/205	0.91 (0.55–1.49)	1.10 (0.46–2.66)
Coffee (≥2 times/mo. vs. <2 times/mo.)	7/241	7/241	1.00 (0.35–2.85)	1.08 (0.19–6.23)
Milk (≥2 times/mo. vs. <2 times/mo.)	11/237	8/240	1.43 (0.54–3.75)	0.69 (0.15–3.09)
Egg (≥3 times/we. vs. <3 times/we.)	137/111	167/81	0.60 (0.42–0.87)**	0.58 (0.31–1.10)
Chicken (≥2 times/mo. vs. <2 times/mo.)	77/171	109/139	0.53 (0.35–0.79)**	0.67 (0.31–1.44)
Beef (≥2 times/mo. vs. <2 times/mo.)	12/236	21/227	0.55 (0.26–1.15)	0.60 (0.20–1.85)
Pork (≥3 times/we. vs. <3 times/we.)	102/146	124/124	0.67 (0.46–0.98)*	0.63 (0.32–1.24)
Duck (≥2 times/mo. vs. <2 times/mo.)	31/217	33/215	0.93 (0.55–1.57)	0.78 (0.29–2.08)
Salted fish (≥2 times/mo. vs. <2 times/mo.)	38/210	55/193	0.59 (0.35–0.97)*	0.61 (0.26–1.43)
Fresh fish (≥3 times/we. vs. <3 times/we.)	108/140	135/113	0.58 (0.39–0.87)**	0.31 (0.13–0.71)*
Tap water <sup>a)</sup> (ever vs. never)	107/140	97/148	1.33 (0.81–2.20)	0.93 (0.43–1.98)
Deep well water <sup>a)</sup> (ever vs. never)	119/128	120/126	0.94 (0.56–1.55)	0.80 (0.38–1.67)
Shallow well water <sup>a)</sup> (ever vs. never)	178/70	183/63	0.85 (0.55–1.30)	0.71 (0.30–1.69)
River water <sup>a)</sup> (ever vs. never)	94/154	97/149	0.95 (0.65–1.38)	0.96 (0.48–1.93)
Ditch water <sup>a)</sup> (ever vs. never)	151/97	144/102	1.09 (0.76–1.55)	1.08 (0.55–2.11)
Pond water <sup>a)</sup> (ever vs. never)	2/246	2/242	1.00 (0.14–7.10)	2.00 (0.18–22.1)

\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; §,  $P = 0.05$ .

a) as drinking water.

was amplified by 35 cycles of polymerase chain reaction (PCR) under the following conditions: 1 min at 94°C for denaturation, 10 s at 52°C for primer annealing, and 30 s at 72°C for primer extension as previously reported.<sup>13)</sup> One primer (5'-CCA CAC TCA CAG TTT TCT CTT) featured substitution of thymine for adenine at the underlined position to create a Ksp632 recognition site in the typical allele. PCR products were digested with 2–3 units of Ksp632, then separated in 3% agarose gels.

For detection of *CPY2E1* polymorphisms, 30 cycles of PCR amplification using Sp1 (5'-TTC ATT CTG TCT TCT AAC TGG) and ASP1 (5'-CCA GTC GAG TCT ACA TTG TCA) primers were performed under the following conditions: 1 min at 95°C for denaturation, 1 min at 55°C for primer annealing, and 1 min at 72°C for primer extension. PCR products were subsequently digested with Rsa and Pst restriction enzymes, and subjected to electrophoresis in 2% agarose gels.

**Statistical analysis** Odds ratios (ORs), used to estimate

the relative risk of HCC under various types of exposure, were calculated by using conditional logistic regression models.<sup>14)</sup> All  $P$  values resulted from two-sided statistical tests. The FREQ and PHREG procedures in the SAS software package<sup>15)</sup> were used for estimating risk, and the command called “sampsi” of STATA ver. 6<sup>16)</sup> for determining statistical power.

## RESULTS

The effects of virus infection and dietary habits on the risk of HCC were assessed by univariate analyses (crude OR), and then adjusted by HBsAg status (HBV-adjusted OR), the main risk factor of HCC. These results are presented in Table II.

The risk of developing HCC was strongly associated with the presence of HBsAg (crude OR=9.75; 95% confidence interval [95% CI]=4.71–20.2). A history of i.v. injection also conferred increased risk (crude OR=1.50;

HBV-adjusted OR=1.95). However, there was no statistically significant association between HCV infection and HCC (crude OR=0.86; HBV-adjusted OR=0.77).

Alcohol drinking and cigarette smoking elevated the risk, but without statistical significance. By univariate analyses, higher average income (crude OR=0.63; 95%CI=0.43–0.92), frequent intake of egg (crude OR=0.60; 95%CI=0.42–0.87), chicken (crude OR=0.53; 95%CI=0.35–0.79) and pork (crude OR=0.67; 95%CI=0.46–0.98) produced decreased ORs with statistical significance. Frequent intake of fresh fish showed decreased risk by univariate analyses (crude OR=0.58; 95%CI=0.39–0.87) and after controlled the confounding effect of HBV (HBV-adjusted OR=0.31; 95%CI=0.13–0.71).

Frequent intake of peanuts (crude OR=0.66; HBV-adjusted OR=0.66) and source of drinking water, including tap (crude OR=1.33; HBV-adjusted OR=0.93), deep

(crude OR=0.94; HBV-adjusted OR=0.80) and shallow wells (crude OR=0.85; HBV-adjusted OR=0.71), river (crude OR=0.95; HBV-adjusted OR=0.96) and ditch (crude OR=1.09; HBV-adjusted OR=1.08) as well as pond (crude OR=1.00; HBV-adjusted OR=2.00) did not significantly differ between the cases and controls.

Table III shows the frequencies of *ALDH2* and *CYP2E1* genotypes among HCC cases and controls. The value for the *ALDH2*<sup>1</sup>/*ALDH2*<sup>1</sup> genotype was slightly higher in cases (50.0%) than in controls (43.3%), with an OR of 1.38 (95%CI=0.86–2.23). The homozygous *c2/c2* genotype detected by PstI and RsaI digestion of *CYP2E1* was 5.2% (7/134) in HCC cases and 7.5% (10/134) in controls, giving an OR compared with the other two genotypes combined of 1.36 (95%CI=0.81–2.28).

Results of multivariate analysis are presented in Table IV. Factors exhibiting statistical significance on univariate analysis were selected for consideration in the multivariate logistic regression model. Positive HBsAg (OR=13.9; 95%CI=5.78–33.6) and having a history of i.v. injection (OR=2.72; 95%CI=1.24–6.00) again exhibited significantly increased ORs for HCC; in contrast, frequent consumption of fresh fish decreased the risk (OR=0.32; 95%CI=0.12–0.86) with statistical significance. A previously described method<sup>17)</sup> was used to test possible interactions of HBsAg status with lifestyle variables, as well as with *ALDH2* and *CYP2E1* genetic polymorphisms. None of these was statistically significant.

**DISCUSSION**

According to a recent investigation, the age-adjusted death rate of HCC, along with lung cancer, is still showing an increasing trend in both rural and urban areas of mainland China.<sup>18)</sup> After adjustment for the 1985 world population, the mortality rate of HCC in mainland China was 33.7 for men and 12.3 for women (per 100 000); both ranked top in the world.<sup>19)</sup> In China, the mortality rate of HCC is highest in Jiangsu province, especially the Haimen

Table III. Frequencies of *ALDH2* and *CYP2E1* Genotypes among 134 Patients with Hepatocellular Carcinoma (HCC) and 134 Sex-age-matched Controls in Haimen, China

Genotype	No. of cases (%)	No. of controls (%)	Odds ratio	Odds ratio (95%CI)
Genotype of <i>ALDH2</i> <sup>a)</sup>				
2-2	14 (10.5)	13 (9.7)	1.00	—
1-2	51 (38.1)	63 (47.0)	1.00	—
1-1	67 (50.0)	58 (43.3)	1.38	(0.86–2.23)
<i>CYP2E1</i> <sup>b)</sup>				
<i>c1/c2</i>	41 (30.6)	47 (35.1)	1.00	—
<i>c2/c2</i>	7 (5.2)	10 (7.5)	1.00	—
<i>c1/c1</i>	83 (61.9)	77 (57.4)	1.36	(0.81–2.28)

a) Two missing values in the case group were due to insufficient DNA samples.

b) Three missing values in the case group were due to insufficient DNA samples.

Table IV. Multivariate Analysis of Major Risk Factors and Dietary Items for Hepatocellular Carcinoma (HCC) in Haimen, China

Variables	Odds ratio	(95%CI)
HBsAg (positive vs. negative)	13.9	(5.78–33.6)**
History of intravenous injection (ever vs. never)	2.72	(1.24–6.00)*
Average income (≥2400 yuan vs. <2400 yuan)	0.56	(0.21–1.54)
Egg (>3 times/we. vs. <3 times/we.)	0.69	(0.33–1.45)
Chicken (≥2 times/mo. vs. <2 times/mo.)	0.86	(0.35–2.14)
Pork (≥3 times/we. vs. <3 times/we.)	1.19	(0.53–2.65)
Salted fish (≥2 times/mo. vs. <2 times/mo.)	0.70	(0.25–1.97)
Fresh fish (≥3 times/we. vs. <3 times/we.)	0.32	(0.12–0.86)*

\*, P<0.05; \*\*, P<0.01.

area,<sup>19)</sup> where the positive rate of HBsAg was 15.0% among adult males and 10.7% among adult females,<sup>20)</sup> and the population attributable risk of HBsAg according to our previous estimation was 55%.<sup>3)</sup> Some general information concerning Haimen is available on web site [www.haimen.gov.cn](http://www.haimen.gov.cn).

Our present results reconfirmed HBV infection to be a main risk factor of HCC in Haimen, in line with etiological surveys conducted in adjacent areas.<sup>1,21)</sup> Although history of blood transfusion and/or i.v. injection is not a usual risk factor of HCC,<sup>1)</sup> our results did reveal a significantly increased risk among those having a history of i.v. injection. The possible reason is that the present patients might have received more i.v. injections (for administration of medication) at the stage of chronic hepatitis.

Regarding other factors, habitual smoking and drinking were associated with slightly increased risk, without statistical significance, while dietary items rich in animal protein, e.g. egg, chicken, pork and fish, seemed to be protective. However, these results should be interpreted with caution, since linkage between dietary habits and HCC might be biased by other factors. For instance information bias, due to the multistage course, with patients undergoing virus carrier, chronic hepatitis, then liver macronodular cirrhosis and eventually HCC stages, could be important. From clinical practice, we know that the course takes 15 to 20 years or even longer and during this period, many pathological changes occur, including hepatosplenomegaly, jaundice and esophageal varices, with only a few patients remaining asymptomatic. In order to relieve problems of fatigue, fullness, loss of appetite and nausea, patients may modify their dietary habits. When visiting hospital, physicians strongly recommend a sedentary life, and cessation of drinking and smoking, as well as an improved menu rich in vegetables, fruit and foods that are easily digestible. Although our interviewer requested a recall of dietary habits of at least one year before HCC diagnosis, it could not be ensured that the answers to the questionnaire were accurate. This may partially explain why frequent consumption of fruit presented an increased risk and why habitual drinking and smoking did not show statistically significant ORs for HCC in this study.

We did not estimate the risk of AFB<sub>1</sub>, considered to be an important carcinogen in Haimen.<sup>1,8)</sup> However, our previous analysis with the same study subjects revealed no significant difference between cases and controls when testing their serum for AFB<sub>1</sub>-human albumin adducts.<sup>22)</sup> The fact that since the 1980s, all families in Haimen have changed their staple food to rice from corn,<sup>1)</sup> which is a heavily AFB<sub>1</sub>-contaminated food in high epidemic areas, is important in this context. Another food item assumed to be linked with AF pollution is peanuts, but this is not a staple food item in Haimen.<sup>1)</sup>

We also did not directly estimate the risk of microcystin, another important carcinogen in the Haimen area,<sup>23,24)</sup> but this mainly exists in pond water,<sup>23)</sup> so that it can be used as a surrogate. However, since 1972, after drinking water was noted to be linked with HCC, the people have been encouraged to dig wells and discouraged from drinking pond and ditch water.<sup>6,7)</sup> This may explain why in this study, compared with those never drinking pond water, the ever-drinker did not show any increased risk for HCC.

As mentioned above, although AF exposure was closely linked with peanut intake, and microcystins with drinking water, statistical power in detecting these risks should also be considered. In the controls of this study, those consuming peanuts no less than 3 times/week accounted for 35.9%; ever-drinking river or ditch water were 39.4% and 58.5%, respectively. With these frequencies, the statistical power with 248 paired cases and controls to detect OR=2 or OR=0.5 would be 96.1% and 91.8% for peanut intake, 96.1% and 94.1% for drinking river water, and 93.4% and 96.1% for ditch water, respectively.

Our results suggested that polymorphisms of the *ALDH2* and *CYP2E1* genes do not seem to be significantly associated with HCC. This is contrary to the results of early studies conducted in Taiwan Chinese.<sup>12)</sup> We know that *CYP2E1* can be induced by ethanol and is of critical importance in the metabolism of many low-molecular-weight carcinogens including N-nitrosamines from tobacco smoke; *ALDH2* is the principal enzyme catalyzing the conversion of acetaldehyde to acetate. The polymorphism in the 5'-flanking region of *CYP2E1* gene causes a difference in transcription activity, resulting in variation in metabolic efficiency, which may to some extent explain differences in alcohol tolerance and intake behavior as well as alcohol-related diseases. However, significant interaction between habitual drinking/smoking and gene polymorphisms of *ALDH2/CYP2E1* was not detected by the present logistic regression analysis. This might suggest that smoking and alcohol are not of major importance for HCC in our population. The fact that the influence of virus infection was overwhelmingly strong might have masked weak effects of other factors.

In conclusion, the present study suggests that HBV infection is the main risk factor for HCC in Haimen, China. However, dietary items rich in protein, especially fresh fish, might reduce the susceptibility.

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