

# Role of *N*-acetyltransferase polymorphisms in hepatitis B related hepatocellular carcinoma: impact of smoking on risk

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## Abstract

**Background**—Persistent infection with hepatitis B virus (HBV) causes chronic phasic necroinflammation and regenerative proliferation in the liver. The sustained hepatocellular proliferation may render chronic HBV carriers more susceptible to the effects of environmental carcinogens. Aromatic amines are potential hepatocarcinogens in humans. *N*-acetyltransferase (NAT) is involved in the metabolic activation and detoxification of these compounds.

**Aims**—To investigate if genetic polymorphisms in *N*-acetylation are related to hepatocellular carcinoma (HCC) among chronic HBV carriers.

**Methods**—Genotyping of *NAT1* and *NAT2* was performed using polymerase chain reaction-restriction fragment length polymorphism on peripheral leucocyte DNA from 151 incident cases of HCC and 211 controls. All subjects were male, and were chronic HBV surface antigen carriers.

**Results**—A significant association between *NAT2* genetic polymorphism and HCC was observed among chronic HBV carriers who were smokers but not among those who were non-smokers. For smoking HBV carriers, the odds ratios of developing HCC for those heterozygous and homozygous for the *NAT2*\*4 functional allele compared with those without any copies of the functional allele (reference group) were 2.67 (95% confidence interval 1.15–6.22) and 2.58 (95% confidence interval 1.04–6.43), respectively. The interaction between cigarette smoking and the presence of the *NAT2*\*4 allele just failed to reach statistical significance ( $p=0.06$ ). No association between *NAT1* genotype and HCC was evident overall or within the smoking stratified subgroups.

**Conclusions**—Our results suggest that *NAT2* activity may be particularly critical in smoking related hepatocarcinogenesis among chronic HBV carriers. Our data also indirectly support a role for tobacco smoke derived aromatic amines in the aetiology of HCC.

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**Keywords:** genetic polymorphism; hepatocellular carcinoma; *N*-acetyltransferase; smoking

Chronic infection with hepatitis B virus (HBV) is the major cause of at least 80% of hepatocel-

lular carcinoma (HCC) throughout the world.<sup>1</sup> However, only about one fifth of chronic HBV carriers are expected to develop HCC in their lifetime.<sup>2</sup> In addition to HBV, many other environmental risk factors, including hepatitis C virus, aflatoxin exposure, alcohol and tobacco use, and decreased intake of certain antioxidant micronutrients have been implicated in the aetiology of HCC.<sup>3–12</sup> Our molecular epidemiology studies designed to investigate gene environment interactions in hepatocarcinogenesis started in 1988 when there was little awareness of the genetic basis of HCC. To date, we have identified several polymorphic genes associated with susceptibility to the effects of exposure to certain environmental risk factors on HCC development among chronic HBV carriers.<sup>5–9, 12</sup> Most of these genes encode for xenobiotic metabolising enzymes (cytochrome P450 2E1 and 1A1, and glutathione S-transferases M1 and T1).<sup>5–9</sup>

*N*-acetyltransferase (NAT) is involved in both activation and detoxification of carcinogenic aromatic amines (including arylamines and heterocyclic amines).<sup>13–15</sup> These carcinogens are ubiquitous in the environment, present in tobacco smoke, and formed in the cooking of meats and other sources of animal protein.<sup>16–18</sup> Humans possess two independently regulated and kinetically distinct NAT isoenzymes, namely *NAT1* and *NAT2*. Several allelic variants of *NAT1* and *NAT2*, which cause variations in acetylation capacity, have been detected.<sup>14, 19, 20</sup> Epidemiological studies have shown that both slow and rapid acetylation genotypes of *NAT2* are susceptibility factors for different cancers.<sup>21–27</sup> The most consistent findings in previous studies were the association of *NAT2* genetic polymorphism with bladder and colorectal cancer. Genotypes for slow acetylation were associated with an increased risk of bladder cancer but decreased risk of colorectal cancer.<sup>21–25</sup> Several studies have also examined the role of the *NAT1* genotypic variants in the predisposition to cancer. The *NAT1* rapid genotype containing a *NAT1*\*10 allele was a significant risk factor for bladder and colorectal cancer.<sup>21, 22, 24, 25</sup> Some studies demonstrated a gene-gene interaction between *NAT1* and *NAT2*.<sup>21, 24, 25</sup>

Aromatic amines are primarily metabolised in the liver.<sup>15, 28, 29</sup> Previous studies have shown

**Abbreviations used in this paper:** HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; NAT, *N*-acetyltransferase; OR, odds ratio; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism.

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that both rodent and human liver are capable of transforming heterocyclic amines to mutagenic species.<sup>15 30-33</sup> Certain heterocyclic amines are powerful hepatocarcinogens in rodents or monkeys.<sup>30-34</sup> Chronic administration of 4-aminobiphenyl, a well established human bladder carcinogen present in tobacco smoke, induces HCC in mice.<sup>35</sup> Exposure to 4-aminobiphenyl was recently suggested to play a role in the development of human HCC, as shown by the finding of greater adduct levels of 4-aminobiphenyl-DNA in liver tissues from HCC patients than in those with metastatic liver tumours or intrahepatic stones.<sup>36</sup> To date, only one study has examined the association between *NAT2* genetic polymorphism and HCC. In that study, *NAT2* slow acetylators were found to have an approximate twofold increase in the risk of developing HCC.<sup>37</sup> No studies have tested the hypothesis that genetic variation in the *NAT1* gene might be associated with the risk of HCC. It is not known whether these polymorphic NAT isoenzymes influence susceptibility to the risk of smoking related HCC. To further explore the association between NAT polymorphisms and HCC, we investigated the presence of the allelic variants of *NAT1* and *NAT2* in 151 HBV surface antigen (HBsAg) positive HCC patients and 211 HBsAg positive controls. We restricted our analysis to chronic HBV carriers because the majority of HCC patients in Taiwan are among these carriers.<sup>1 3</sup> Thus this study provides an unusual insight into the role of NAT polymorphisms in the transition from chronic HBV carrier state to HCC.

## Materials and methods

### STUDY SUBJECTS

The study was restricted to men because the interaction between cigarette smoking, NAT polymorphisms, and the risk of cancer has been reported previously<sup>21 23 26</sup> and the prevalence of smoking is 10 times higher in men than in women in Taiwan. Eligible cases were HCC patients diagnosed on the basis of histological findings or an elevated serum  $\alpha$  fetoprotein level ( $\geq 400$  ng/ml) combined with at least one positive image on angiography, sonography, and/or computed tomography. They were recruited through two sources. A total of 81 eligible cases were identified from a cohort study of 4841 male HBsAg carriers. The cohort characteristics and method of cancer follow up have been described elsewhere.<sup>5-9 11 12</sup>

Briefly, between August 1988 and June 1992, all male HBsAg carriers aged 30 years or older who attended the specific clinic at the liver unit of Chang-Gung Memorial Hospital for early detection of HCC and the Government Employee Central Clinics for annual health examinations were recruited. After giving written informed consent, in-person interviews were conducted by trained research assistants according to a structured questionnaire which included items of socio-demographic characteristics, lifetime habits of alcohol and tobacco use, as well as personal and family histories of major chronic diseases. Blood samples, including white blood cells,

serum, and plasma, were also obtained and frozen at  $-70^{\circ}\text{C}$  until subsequent analysis. Participants were monitored for incident cancer by follow up examination (including conventional liver function test and ultrasonography) every 6-12 months and confirmation from medical records and by searches of computer files of national death certification and cancer registry systems. Data on change in habits of cigarette smoking and alcohol consumption as well as personal histories of major chronic diseases were prospectively collected at follow up examinations. After each follow up examination, approximately 70% of the surviving HBsAg carriers continued to return for examination. By 31 December 1997, each cohort member had been followed up for an average of 6.7 years. A total of 95 incident cases of HCC were identified. Excluded from analysis were eight cases with no available DNA samples and six cases with no available data on *NAT2* genotype due to unsatisfactory genetic analysis. Hence a total of 81 cases were included in the study.

To improve the precision of the estimation of relative risk in the stratified analysis of smoking, a sample (70 cases) of patients with newly diagnosed HCC who were admitted between October 1997 and May 1998 to the Chang-Gung Memorial Hospital (one of the sources of the study subjects in our cohort study) and the National Taiwan University Hospital, a teaching hospital that provides a major portion of the medical care in northern Taiwan, were also enrolled as cases. These patients represented approximately 20% of the male HBsAg positive incident cases seen in the two hospitals during that period. They were recruited on a voluntary basis with informed consent. Questionnaire data were obtained within two weeks after diagnosis by a trained nurse interviewer. A sample of blood (10-20 ml) was collected under standardised conditions from each subject.

Controls were recruited from the same cohort. A total of 211 HBsAg carrier controls were randomly selected from cohort subjects who were not affected by HCC throughout the follow up period and had sufficient DNA samples for genotyping of *NAT1* and *NAT2*. They were frequency matched to case patients based on ethnic group and birth year (10 year intervals).

### LABORATORY ANALYSES

HBsAg status was determined using a radioimmunoassay (Abbott Laboratories, Chicago, Illinois, USA). Genomic DNAs were isolated from peripheral leucocytes. The method described by Bell and colleagues<sup>25</sup> was used with some modifications to detect the four common variants (*NAT1\*3*, *NAT1\*4*, *NAT1\*10*, and *NAT1\*11*) in the 3' region of *NAT1* near the polyadenylation signal. Briefly, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was performed to distinguish between *NAT1\*4*, *NAT1\*11*, and a group of alleles consisting of *NAT1\*10* and *NAT1\*3*, as described previously.<sup>25</sup> To differentiate between the *NAT1\*10* and *NAT1\*3*

Table 1 Sociodemographic characteristics of hepatocellular carcinoma cases and controls

Variables	Group	Cases		Controls		p Value
		n	(%)	n	(%)	
Birth year	≤1930	33	(21.9)	64	(30.3)	
	1931–1940	52	(34.4)	74	(35.1)	
	1941–1950	48	(31.8)	58	(27.5)	
	≥1951	18	(11.9)	15	(7.1)	
Age at recruitment (y)	30–39	16	(10.6)	23	(10.9)	0.160
	40–49	38	(25.2)	65	(30.8)	
	50–59	52	(34.4)	62	(29.4)	
	≥60	45	(29.8)	61	(28.9)	
	Missing	3		0		
Ethnic group	Fukien Taiwanese	96	(63.6)	117	(55.5)	0.630
	Hakka Taiwanese	17	(11.2)	23	(10.9)	
	Mainland Chinese	38	(25.2)	71	(33.6)	
Education	Primary school and below	48	(32.4)	39	(18.5)	0.212
	Junior high school	27	(18.3)	26	(12.3)	
	Senior high school and above	73	(49.3)	146	(69.2)	
	Missing	3		0		
Cigarette smoking	No	75	(49.7)	130	(61.6)	0.024
	Yes	76	(50.3)	81	(38.4)	
Alcohol consumption	No	101	(66.9)	162	(76.8)	0.037
	Yes	50	(33.1)	49	(23.2)	

alleles, genomic DNA was then amplified by PCR in the presence of 10 pmol of each of the primers 5'-CAGCTCACCAGTTATCAACTGACGACCTAT-3' (position 1349–1378) and 5' - ATAACCACAGGCCATCTTTAAAATA CAAAT-3' (position 1528–1558), buffer (10 mmol/l Tris hydrochloride, pH 8.3; 50 mmol/l potassium chloride; and 1.5 mmol/l magnesium chloride), 2.5 mmol/l of each deoxynucleoside triphosphate, and 1 U of amplification *Thermus aquaticus* DNA in a total volume of 50 µl. The reverse primer contains mismatched bases (underlined) which create a new *SspI* restriction site when amplifying the *NAT1\*3* allele. A portion (10 µl) of the PCR product was subjected to restriction digest with *SspI* (New England Biolabs, Beverly, Massachusetts, USA) overnight at 37°C. The digestion products were electrophoresed in 3% agarose gel and visualised by ethidium bromide staining. The presence of the *NAT1\*3* allele was indicated by bands of 180 and 30 base pairs, whereas no digestion of the

Table 2 NAT1 and NAT2 genotype frequencies in hepatocellular carcinoma cases and controls

Genotype	Cases		Controls		Odds ratio	95% CI
	n	(%)	n	(%)		
<b>NAT2 genotype</b>						
NAT2*5/*6	3	(2.0)	3	(1.4)	1.0	Reference
NAT2*5/*7	3	(2.0)	3	(1.4)		
NAT2*6/*6	8	(5.3)	16	(7.6)		
NAT2*6/*7	8	(5.3)	24	(11.4)		
NAT2*7/*7	5	(3.3)	9	(4.2)		
Sum of "0" NAT2*4	27	(17.9)	55	(26.0)		
NAT2*4/*5	5	(3.3)	10	(4.7)		
NAT2*4/*6	45	(29.8)	51	(24.2)		
NAT2*4/*7	26	(17.2)	28	(13.3)		
Sum of "one" NAT2*4	76	(50.3)	89	(42.2)		
NAT2*4/*4	48	(31.8)	67	(31.8)	1.74	1.00–3.02
<b>NAT1 genotype</b>						
NAT1*4/*4	35	(23.2)	50	(23.7)	1.0	Reference
NAT1*4/*3	7	(4.6)	4	(1.9)		
NAT1*4/*11	0	(0.0)	1	(0.5)		
NAT1*3/*3	5	(3.3)	0	(0.0)		
NAT1*3/*11	2	(1.3)	3	(1.4)		
Sum of "0" NAT1*10	49	(32.4)	58	(27.5)		
NAT1*4/*10	70	(46.4)	103	(48.8)		
NAT1*3/*10	4	(2.6)	3	(1.4)		
NAT1*10/*11	0	(0.0)	2	(1.0)		
Sum of "one" NAT1*10	74	(49.0)	108	(51.2)		
NAT1*10/*10	28	(18.6)	45	(21.3)	0.81	0.50–1.31

*NAT1\*10* allele occurred. For 81 study subjects, we confirmed PCR-RFLP genotypes by dye deoxy terminator cycle sequencing of the polymorphic region.

The *NAT1\*10* allele contains a shift in the mRNA polyadenylation signal, whereas the remaining alleles (*NAT1\*4*, *NAT1\*3*, and *NAT1\*11*) contain the consensus signal.<sup>25</sup> There was evidence suggesting that the presence of the *NAT1\*10* allele was associated with increased enzymatic activity.<sup>20</sup> In the present analysis on *NAT1* genotype and HCC, we thus grouped individuals on the basis of the number of copies of *NAT1\*10*.

PCR-RFLP methods were used to determine the five most common functional and low activity *NAT2* alleles, as described previously.<sup>38</sup> The alleles detected were *NAT2\*4* (wild-type or functional allele) and the low activity alleles *NAT2\*5* (M1: *KpnI* polymorphism, C481T), *NAT2\*6* (M2: *TaqI* polymorphism, G590A), *NAT2\*7* (M3: *BamHI* polymorphism, G857A), and *NAT2\*14* (M4: *MspI* polymorphism, G191A). *NAT2* genotype was categorised according to the identified number of copies of the *NAT2\*4* allele, as described in a previous study, which demonstrated that individuals can be segregated into slow, intermediate, and rapid acetylator phenotypes according to the presence of 0, 1, or 2 copies of the *NAT2\*4* allele.<sup>37</sup> All assays were conducted and interpreted blind to case control status.

STATISTICAL METHODS

Odds ratios (ORs) and confidence intervals (CIs) were calculated by unconditional logistic regression and adjusted for potential confounding factors. Effect modification of the relation between *NAT2* genotype and HCC by smoking was assessed using a likelihood ratio test to compare the goodness of fit of the model with the interaction term *NAT2* genotype×smoking, with the reduced model containing indicator variables of the main effects of genotype and smoking. All statistical tests were based on two tailed probability.

Results

Characteristics of HCC cases and controls are presented in table 1. No statistically significant difference in the frequency distribution of birth year, age at recruitment, or ethnic group was found between cases and controls. Cases were significantly more likely than controls to have lower educational levels, to smoke cigarettes, and to consume alcohol.

The *NAT2\*14* allele was not detected in 151 HCC cases and 211 controls. *NAT1* and *NAT2* genotype frequencies are shown in table 2. Compared with chronic HBV carriers without any copies of the *NAT2\*4* allele as the reference group, OR for HCC was 1.74 (95% CI 1.00–3.02; p=0.0498) for chronic HBV carriers heterozygous for the *NAT2\*4* allele and 1.46 (95% CI 0.81–2.64; p=0.2101) for those who were homozygous for the *NAT2\*4* allele. There was no significant case control difference in the genotype frequency distribution of *NAT1*.

Table 3 Odds ratios of hepatocellular carcinoma for *NAT1* and *NAT2* genotypes by cigarette smoking status

Genotype	Smokers				Non-smokers			
	Cases	Controls	OR	95% CI	Cases	Controls	OR	95% CI
No of <i>NAT2</i> *4								
None	11	25	1.0	Reference	16	30	1.0	Reference
One	40	34	2.67	1.15–6.22	36	55	1.23	0.59–2.57
Two	25	22	2.58	1.04–6.43	23	45	0.96	0.44–2.11
No of <i>NAT1</i> *10								
None	25	20	1.0	Reference	24	38	1.0	Reference
One	36	43	0.67	0.32–1.40	38	65	0.93	0.48–1.77
Two	15	18	0.67	0.27–1.64	13	27	0.76	0.33–1.76
<i>NAT2</i> *4/ <i>NAT1</i> *10								
–/–	4	7	1.0	Reference	3	10	1.0	Reference
–/+	7	18	0.68	0.15–3.07	13	20	2.17	0.50–9.40
+/-	21	13	2.83	0.69–11.58	21	28	2.50	0.61–10.23
+/+	44	43	1.79	0.49–6.56	38	72	1.76	0.46–6.78

Because NAT is involved in the metabolism of tobacco smoke derived aromatic amines, the risk of HCC associated with the polymorphisms of this enzyme may depend on the individual's smoking status. Table 3 presents the ORs for HCC associated with *NAT1* and *NAT2* genotypes stratified by cigarette smoking. Among non-smokers, there was essentially no difference in the *NAT2* polymorphism between cases and controls. Among smokers, the frequency of carrying at least one copy of the *NAT2*\*4 functional allele was significantly greater in cases than in controls. The OR of HCC for homozygotes of the *NAT2*\*4 functional allele (OR 2.58; 95% CI 1.04–6.43;  $p=0.0414$ ) was similar to that for the heterozygotes (OR 2.67; 95% CI 1.15–6.22;  $p=0.0223$ ). Adjustment for ethnic group, educational level, cigarette smoking, and alcohol consumption did not alter these relative risk estimates appreciably. After adjustment for multiple factors, including the main effects of smoking and the *NAT2* genotype, the interaction term between cigarette smoking and the presence of the *NAT2*\*4 allele just failed to reach statistical significance ( $p=0.06$ ).

There was no significant association between *NAT1* genotype and HCC in either cigarette smokers or non-smokers. We also investigated the association between risk of HCC and combined *NAT* genotype comparing those without either the *NAT2*\*4 or *NAT1*\*10 allele with the other subjects. Among smokers, chronic HBV carriers with the *NAT2*\*4 allele had a higher risk of HCC than those without such a factor. The ORs shown in table 3 suggest no gene-gene interaction between *NAT1* and *NAT2* in the development of HCC.

*NAT* plays no role in the metabolism of ethanol. As expected, we observed that the association of *NAT2* genotype and HCC was similar in both drinkers (OR 1.74 and 1.34, respectively, for heterozygotes and homozygotes of the *NAT2*\*4 allele) and non-drinkers (OR 1.64 and 1.46, respectively, for heterozygotes and homozygotes of the *NAT2*\*4 allele). Irrespective of alcohol consumption, no notable increase was found in the risk of HCC for chronic HBV carriers with the *NAT1*\*10 allele.

### Discussion

In animal models of chemically induced HCC, normal liver is mitotically inactive and rela-

tively resistant to chemical carcinogenesis. When rat or mouse hepatocytes are stimulated to divide, however, the liver becomes exquisitely sensitive to carcinogenesis.<sup>39</sup> Persistent HBV infection causes chronic phasic necroinflammation and regenerative proliferation in the liver.<sup>1</sup> The sustained hepatocellular proliferation may render chronic HBV carriers more susceptible to the effects of environmental carcinogens. We restricted our study to HBsAg carriers because the majority of HCCs in Taiwan are caused by chronic HBV infection<sup>1,3</sup> and we wished to address the possibility that *N*-acetylation polymorphisms, which have been associated with genetically based differences in carcinogen metabolism,<sup>14</sup> may modify the risk of HBV related HCC.

Both arylamines and heterocyclic amines are present in tobacco smoke.<sup>17,18</sup> Epidemiological studies have demonstrated that smoking is associated with a moderate excess risk of HCC.<sup>4-7</sup> Furthermore, exposure to a tobacco specific arylamine induces HCC in mice and has been suggested to play a role in the development of human HCC.<sup>35,36</sup> An in vitro study on liver *NAT2* enzymatic activity towards sulphamethazine (a *NAT2* specific substrate) revealed that Caucasians can be segregated into slow, intermediate, and rapid acetylator phenotypes according to the presence of 0, 1, or 2 copies of the *NAT2*\*4 functional allele.<sup>37</sup> As the *NAT2* gene codes for an enzyme involved in the metabolism of a carcinogen, increased genetic risk may be present only in those with carcinogen exposure. In this study, we found a statistically significant association between the presence of at least one copy of the *NAT2*\*4 functional allele and HCC risk among smoking HBV carriers, but there was no association between *NAT2* genotype and HCC among those who never smoked. This observation suggests that *NAT2* activity may be particularly critical in the hepatocarcinogenesis caused by tobacco smoke derived aromatic amines. Although an earlier study, which involved 100 Spanish HCC cases and 258 controls composed of men and women, reported an excess HCC risk for subjects homozygous for loss of the *NAT2*\*4 functional allele, they did not investigate the effect of *NAT2* genotype on the risk of smoking related HCC. In fact, no data regarding smoking habits in cases and

control subjects were presented in the Spanish study.<sup>37</sup>

It has been suggested that enhancement of metabolic activation of hepatocarcinogens by persistent HBV infection may be an alternative mechanism for the interactions of chemical carcinogens with chronic HBV infection in the development of HCC. In woodchucks, chronic infection with woodchuck hepatitis virus, which is genetically and biologically similar to HBV, resulted in alterations in the activities of certain enzymes involved in the activation or detoxification of hepatocarcinogens and produced enhanced activation of certain aromatic amines.<sup>40</sup> Analysis of human liver biopsy samples in a bacterial mutagenicity assay also revealed significant enhancement in the activation of an aromatic amine among HBV carriers, regardless of their histological diagnosis. However, this finding was based on a limited number of human liver tissues.<sup>41</sup> If the degree of chronic hepatitis B (duration or severity) can influence NAT biochemical activities, it may be important to consider this factor as an effect modifier in analyses on the association of HCC with the genetic polymorphisms in *NAT1* and *NAT2* because the risk of HCC increases progressively with the degree of chronic hepatitis B.<sup>42</sup>

Our finding of a significant role for the presence of the *NAT2\*4* allele in modulating smoking associated HCC risk is consistent to some extent with a recent large case control study of lung cancer for which tobacco smoke is the major aetiological factor. In the lung cancer study, the *NAT2\*4* functional allele was also higher, although not significantly, in cases than in controls, but the lung cancer risk was significantly increased only for the homozygotes of the *NAT2\*4* allele.<sup>27</sup> Based on our data, both homozygosity and heterozygosity for the *NAT2\*4* allele conferred a risk of HCC for cigarette smokers. Indeed, there was no obvious difference in the risk of smoking related HCC between the two groups.

Tobacco smoke is also an established cause of bladder cancer. In contrast, several investigations on *NAT2* genotype and bladder cancer have indicated that slow acetylation, which is caused by homozygous loss of the *NAT2\*4* functional allele, is a risk factor for smokers.<sup>21-23</sup> In the liver, aromatic amines may be *N*-acetylated by NAT enzymes, rendering them less reactive. Alternatively, aromatic amines may be *N*-hydroxylated by cytochrome P450 1A2.<sup>13</sup> The *N*-hydroxy derivative can be subsequently metabolised by *O*-acetylation via the NAT enzymes to a highly reactive species that is capable of forming DNA adducts.<sup>13 14</sup> It has been hypothesised that the increased susceptibility to bladder cancer in *NAT2* slow acetylators is related to the decreased detoxification of carcinogenic aromatic amines by *N*-acetylation in the liver, so that excess hydroxylated aromatic amines reach the bladder epithelium where they can undergo a further activation step.<sup>21</sup> However, there are limited *in vivo* data on the metabolic pathway of various aromatic amines.

Differences in the carcinogenic dose-response relationship with 2-acetylaminofluorene, an aromatic amine, between liver and urinary bladder have been demonstrated in an animal model involving more than 24 000 mice. The dose-response curve for the liver was nearly linear down to the lowest amount of the carcinogen treated, while the dose-response curve for the bladder was non-linear with a threshold at low doses.<sup>43</sup> This observation raises the possibility that the liver may be more sensitive to the carcinogenic effect of aromatic amines than the bladder at low concentrations of these carcinogens. Furthermore, it has been shown that cytochrome P450 1A2 activity can be induced by some components of tobacco smoke such as polycyclic aromatic hydrocarbons.<sup>44</sup> Higher cytochrome P450 1A2 activities could theoretically shift metabolism of aromatic amines towards direct *N*-hydroxylation. Although rapid acetylators may have a greater capacity to detoxify carcinogenic aromatic amines by *N*-acetylation than slow acetylators and thus have less hydroxylated metabolites produced that are proximate carcinogens, this discrepancy may be decreased among smokers due to the increased hepatic induction of cytochrome P450 1A2. Consequently, the ability to *O*-acetylate may be the principal determinant of the production of aromatic amine DNA reactive species, so that rapid acetylators are more susceptible to smoking related hepatocarcinogenesis. Both *NAT1* and *NAT2* can perform *O*-acetylation but our data revealed no notable effect of *NAT1* rapid genotypes containing the *NAT1\*10* allele on smoking related HCC. Although *NAT1* activity is higher than *NAT2* in certain extrahepatic tissues,<sup>20</sup> *NAT2* is expressed at a higher level than *NAT1* in the liver.<sup>13</sup> Thus it is likely that *NAT2* plays a more dominant role than *NAT1* in the induction of smoking related HCC.

Carcinogenic heterocyclic amines are also present in meats cooked at high temperatures.<sup>16</sup> In a recent nested case control study of colorectal cancer, rapid acetylation genotypes, defined as carrying at least one copy of the *NAT1\*10* and/or *NAT2\*4* allele, had increased risk of red meat intake.<sup>24</sup> Certain food borne heterocyclic amines have been demonstrated to be powerful hepatocarcinogens in rodents and monkeys.<sup>30-34</sup> In this study, no effort was made to study the consumption of meat because the Chinese diet is so complex. Our failure to observe an association between *NAT2* genotype and HCC in non-smokers may be related to the lack of sufficient dietary exposure to heterocyclic amines and/or the small range of such dietary intake in the Chinese population. However, we cannot exclude the possibility that low exposure to dietary heterocyclic amines may have an effect on HCC risk among chronic HBV carriers with the *NAT2\*4* functional allele who have a long history of tobacco use, which may cause sustained elevated induction of cytochrome P450 1A2 activity. Development of biomarkers for measuring food borne heterocyclic amines is needed to clarify this issue.

Seven single base mutations have been identified within the coding region of the *NAT2* gene. These point mutations are at nucleotide positions 191 (G→A), 282 (C→T), 341 (T→C), 481 (C→T), 590 (G→A), 803 (A→G), and 857 (G→A). Mutation 481T was found to rarely occur without 341C and/or 803G, and vice versa. The mutation at position 282 was strongly associated with the 590A mutation.<sup>27 37 45</sup> Thus in this study we analysed nucleotide substitutions only at positions 481 (*NAT2\*5*), 590 (*NAT2\*6*), 857 (*NAT2\*7*), and 191 (*NAT2\*14*). Most previous investigations of the association between *NAT2* genetic polymorphism and cancer risk were performed in Caucasian populations in which the *NAT2\*5* allele is the most frequent slow acetylator allele and the two mutant alleles, *NAT2\*5* and *NAT2\*6*, account for over 90% of the alleles associated with slow acetylation.<sup>22 23 26 27 37</sup> In Asians, the *NAT2\*5* allele is rare, but the *NAT2\*7* allele is more prevalent than in Caucasians.<sup>45</sup> Almost 85% of the slow acetylators classified according to the *NAT2* genotype in this study were carriers of only the *NAT2\*6* or *NAT2\*7* allele. The catalytic capacity to activate *N*-hydroxy aromatic amines in vitro was in the relative order of *NAT2\*4* > *NAT2\*7* > *NAT2\*6* > *NAT2\*5*.<sup>14</sup> Characterising the impact of various *NAT2* genotypic variants consisting of different alleles with differential activities on the risk of smoking related HCC may provide additional insight into the mechanism of the *NAT2* gene involvement in the tumorigenesis of HCC. Our data did not allow us to distinguish the size of the effect between different genotypic variants because of the limited number of cases and controls. However, the apparent association between the *NAT2* rapid genotype and HCC in this study indicates that moderate reduction in *NAT2* activity may appreciably reduce the risk of HCC.

In conclusion, our data support a role for *NAT2* genetic polymorphism in the aetiology of HBV related HCC. However, our results provide no support for the hypothesis of a link between *NAT1* genotype and HCC. In contrast with the abundant data in animal studies,<sup>30-35 43</sup> the evidence showing the effect of aromatic amines on human hepatocarcinogenesis is scanty.<sup>36</sup> The synergistic interaction between the presence of the *NAT2\*4* functional allele and smoking in the development of HCC observed in this study implies that exposure to tobacco derived aromatic amines that are substrates for *NAT2* may be associated with an increased risk of HCC. The frequency of the *NAT2\*4* allele is high in human populations.<sup>21-27 37</sup> The elevated HCC risk associated with the presence of the *NAT2\*4* allele for smoking HBV carriers was only modest (OR~2.6) according to our data. Using the weighted sum approach<sup>46</sup> to calculate separately the contribution of the gene in non-smokers and smokers, however, we estimate that about 30% of the HCCs occurring among male HBV carriers in this high risk area may be attributable to the genotypes containing the *NAT2\*4* functional allele. This surprisingly

large attributable fraction is a consequence of the high frequency of the at risk *NAT2\*4* functional allele and the high prevalence of smoking in the population. This demonstrates that modest genetic risks conferred by common genes, combined with environmental exposure, can determine a large proportion of a common neoplastic disease. Intervention aimed at reducing cigarette smoking and industrial/dietary exposure to aromatic amines may be important for the prevention of HCC in areas where chronic HBV infection is common.

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