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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ORIGINAL PAPER

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Cytochrome P450 (CYP) 1A2, sulfotransferase (SULT) 1A1, and N-acetyltransferase (NAT) 2 polymorphisms and susceptibility to urothelial cancer

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Abstract *Purpose*: Arylamines are suspected to be the primary causative agent of urothelial cancer in tobacco smoke. In the human liver, arylamines are N-hydroxylated by a cytochrome P450 (CYP)1A2-catalyzed reaction, which produces a substrate for O-esterification that can be catalyzed by N-acetyltransferases (NAT) or sulfotransferases (SULT). Recently, several polymorphisms of CYP1A2, SULT1A1, and NAT2 that affect their activities have been reported. *Methods*: In this study, 306 Japanese patients with urothelial transitional cell carcinoma and 306 healthy controls were compared for frequencies of CYP1A2, SULT1A1, and NAT2 genotypes. *Results*: The frequencies of NAT2 intermediate or slow acetylator genotype were significantly higher in the urothelial cancer patients than in the healthy control subjects [odds ratio (OR) = 1.49, 95% confidence interval (95% CI) 1.06–2.09, OR = 3.23, 95% CI 1.72–6.08, respectively]. Stratifying by amount of smoking, among subjects who consumed >33.5 pack-years and carried the SULT1A1 *1/*1 or NAT2 slow acetylator genotype, the OR was 1.73 (95% CI 1.01–2.97) whereas it was 7.31 (95% CI 1.90–28.05) in non-smokers who carried the homozygous wild genotype, respectively. The relationships between CYP1A2, SULT1A1, and NAT2 polymorphisms and clinical findings including tumor differentiation, stage, and recurrence rate were analyzed. Only associations between NAT2 genotype and pathological findings were admitted, and the higher OR of NAT2 intermediate and slow acetylator genotype was more likely to present to a low-grade tumor (G1) among heavy-smokers. *Conclusions*: Our results suggest that SULT1A1 *1/*1 and NAT2 slow acetylator genotypes might modulate the effect of carcinogenic arylamines contained in tobacco smoke, and that the modulation of NAT2 intermediate and slow acetylator genotype has a tendency to present a higher risk for highly differentiated tumors among heavy-smokers.

Keywords CYP1A2 · SULT1A1 · NAT2 · Polymorphism · Urothelial cancer

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Introduction

It has been recognized that tobacco smoke is the main cause of human urothelial cancer (Silverman et al. 1992), and carcinogenic arylamines, including 4-aminobiphenyl (ABP), in tobacco smoke may represent one of the leading causes of urothelial cancer (Bartsch et al. 1993; Cohen et al. 2000). Studies of molecular epidemiology have suggested that the excess of urothelial cancer in smokers could be attributed to arylamines (Bartsch et al. 1990; Vineis et al. 1996). Arylamines are not carcinogenic in the parent form, but require metabolic activation to reactive electrophiles in order to exert their carcinogenic effects (Grant et al. 1997; Guengerich 1992). These can proceed via a two-step pathway involving cytochrome P450 (CYP) 1A2-catalyzed N-hydroxylation (Crofts et al. 1998; Williams et al. 1998) followed by an O-esterification step catalyzed by N-acetyltransferase(s) (NATs) and/or sulfotransferase(s) (SULTs) (Grant et al. 1997; Lewis et al. 1998; Yamazoe et al. 1999). As a consequence of these reactions, in urothelial cancers, the arylnitrenium ions generated from N-hydroxylamines are believed to be the ultimate reactive intermediates responsible for carcinogenic activity (Kadlubar et al. 1977).

Enzyme polymorphisms that have been previously associated with arylamine metabolic pathways include

CYP1A2 and NAT2 (Kadlubar 1994). Accordingly, recent NAT2 genotype studies show associations with urinary bladder cancer that are highest for particular NAT2 alleles associated with slow acetylator phenotypes, consistent with earlier phenotypic studies (Cartwright et al. 1982). Some meta-analyses of case-control studies that investigated associations between NAT2 and bladder cancer were reported and a weak interaction between smoking and NAT2 slow acetylation was observed (Green et al. 2000; Johns and Houlston 2000; Marcus et al. 2000a; Marcus et al. 2000b). Evidence linking elevated CYP1A2 activity to increased bladder cancer risk has been reported (Kaderlik and Kadlubar 1995; Lee et al. 1994). Several polymorphisms of CYP1A2 have been reported (Chida et al. 1999; Nakajima et al. 1999; Sachse et al. 1999; Chevalier et al. 2001), and a single nucleotide change from guanine (CYP1A2*1A) to adenine (CYP1A2*1C) at position −2964 in the 5'-flanking region, that caused a significant decrease of CYP1A2 activity, was reported by Nakajima et al (Nakajima et al. 1999). Therefore, categorization based on a combination of both NAT2 and CYP1A2 might be a more discerning biomarker for identification of high-risk individuals for urothelial cancer. Moreover, molecular dosimetry studies indicate that the slow NAT2/rapid CYP1A2 individual has the highest level of ABP-hemoglobin adducts and, conversely, the lowest level of ABP-hemoglobin adducts, as observed in individuals who are rapid NAT2/slow CYP1A2 (Bartsch et al. 1993; Kaderlik and Kadlubar 1995). Thus, those individuals who are rapid for CYP1A2 and slow for hepatic NAT2 might be at a higher risk for arylamine-induced urothelial cancer compared with those who are slow for CYP1A2 and rapid for NAT2 (Kloth et al. 1994).

Recently, single amino acid substitutions (^{213}Arg/His and ^{213}Met/Val) in polymorphic human phenol-sulfating phenol sulfotransferase were found (Raftogianis et al. 1997; Raftogianis et al. 1999). In a Japanese population, allele frequencies of ^{213}Arg (SULT1A1*1) and ^{213}His (SULT1A1*2) were 83.2% and 16.8%, respectively, but the ^{213}Val allele (SULT1A1*3) was not found (Ozawa et al. 1999). The SULT1A1*2 allele was associated with reduced sulfotransferase activity and thermostability in platelets (Raftogianis et al. 1997; Ozawa et al. 1998).

Given the role of CYP1A2, SULT1A1, and NAT2 in the metabolism of arylamines, in this paper we hypothesized that the rapid genotype of CYP1A2 and the slow genotypes of SULT1A1 or NAT2 might cause an increased risk of urothelial cancer among smokers. We previously reported the association of SULT1A1 and NAT2 genotypes with urothelial cancer in a preliminary case-control study, and the metabolic activation of 4-aminobiphenyl by recombinant STLT1A1*1 and SULT1A1*2 (Ozawa et al. 2002). We also found that low activity alleles of NAT2 were overall high-risk alleles (OR = 2.11, 95% CI 1.08–4.26), and recombinant SULT1A1*1 enzyme showed a tendency of catalyzing higher in vitro 3'-phosphoadenosine 5'-phosphosulfate-dependent DNA adduct formation than SULT1A1*2. In this work, we studied the CYP1A2, SULT1A1, and NAT2 genotypes with urothelial cancer in relation to smoking status among larger study populations. Additionally, we assessed CYP1A2, SULT1A1, and NAT2 genotypes in relation to clinical findings associated with the outcome. These include tumor differentiation, stage, and recurrence. We have demonstrated associations between SULT1A1, NAT2 genotype, and urothelial cancer susceptibility that are consistent with the smoking amount-related association with the SULT1A1*1/*1 genotype and NAT2 slow acetylator genotype. As clinical parameters, NAT2 intermediate and slow acetylator genotype tends to be more frequent among heavy-smokers with well-differentiated tumors, compared with moderately and poorly differentiated tumors.

Materials and methods

Subjects

The case groups were comprised of 306 patients with urothelial cancer (bladder $n = 236$, renal pelvis and ureter $n = 35$, overlap $n = 35$) (242 men, 64 women; mean age 69.4 years) in Kitakyushu city and Miyazaki city, Japan. No statistically significant differences were found between patients in Kitakyushu and Miyazaki for age, gender, and smoking habit. The patients were treated at the University of Occupational and Environmental Health Hospital ($n = 170$) and Miyazaki Medical College Hospital ($n = 136$), and had been histologically diagnosed for urothelial transitional cell carcinoma. Tumors were graded according to the criteria of the Japanese Urological Association (Japanese Urological Association and Japanese Society of Pathology 2001) and were staged according to the TNM classification system (Sobin and Wittekind 1997). Thirty-two patients (10.5%) had well-differentiated disease (G1), 138 (45.1%) had moderately differentiated (G2) and 136 (44.4%) had poorly differentiated disease (G3). We used the term 'superficial tumors' to refer to those which were limited to the mucosa (pTis, pTa) or the lamina propria (pT1); and 'invasive tumors' to refer to those which had invaded the muscle layer (pT2, pT3) or deeper (pT4). One hundred and eighty-four patients (60.1%) had superficial tumors, 117 (38.2%) had invasive tumors, and five (1.6%) had unknown stage tumors. A total of 306 controls, frequency-matched with cases for age (±5 years) and gender, were selected from the people who visited a medical institution located in Kitakyushu city for a general health check-up (242 men, 64 women, mean age 66.9 years). All study subjects completed a questionnaire administered by a trained interviewer covering medical, residential, and occupational exposures as well as smoking history. Smoking history was summarized as the total amount of cigarettes consumed during their lifetime up until the time of the interview. The amount of tobacco smoke exposure was calculated as pack-years [1 pack (20 cigarettes)/day × years of smoking]. The median value of smoking amounts among controls who had ever smoked was 33.5 pack-years. We used the term 'light-smokers' to refer to subjects who consumed ≦33.5 pack-years; and 'heavy-smokers' to refer to subjects who consumed >33.5 pack-years. All participants were given an explanation of the nature of the study, and informed consent was obtained. This study was approved by the ethics committees of the University of Occupational and Environmental Health and Miyazaki Medical College.

Genotyping

Genomic DNA was isolated from peripheral leukocytes by proteinase K digestion and phenol/chloroform extraction. The genetic polymorphism in the 5'-flanking region of CYP1A2 was determined

by polymerase chain reaction (PCR) amplification followed by digestion with DdeI and BslI using the method described previously (Nakajima et al. 1999). Genotyping of SULT1A1 for polymorphism at codon 213 (Arg/His) was performed according to the previously published methods of PCR-restriction fragment length polymorphism (RFLP) (Ozawa et al. 1999). A NAT2 high activity allele, NAT2*4, and NAT2 low activity alleles, NAT2*5B, NAT2*6A, and NAT2*7B, were determined according to a previously published PCR-RFLP method (Bell et al. 1993). A NAT2 variant allele (NAT2*14), which is very rare (less than 1% frequency) in the Japanese population, was not considered (Katoh et al. 1998). All of the genotyping was performed by laboratory personnel unaware of case-control status, and blinded quality control samples were inserted to validate genotyping identification procedures; concordance for blinded samples was 100%. None of the genetic polymorphisms departed significantly from the Hardy-Weinberg equilibrium.

Statistical analysis

Crude ORs and 95% CIs were calculated for CYP1A2, SULT1A1, and NAT2 genotypes. Odds ratios were adjusted for age, gender, and smoking status (ever and never-smokers), using multiple logistic regression analysis by SPSS Medical Pack for Windows. To examine the interaction between environmental and genetic factors, stratification analysis of urothelial cancer risk associated with CYP1A2, SULT1A1, and NAT2 genotypes was carried out for smoking amounts. All statistical tests were based on two-tailed probability. The Cochran-Armitage method was used for a trend analysis. A P value less than 0.05 (two-tail) was considered to be statistically significant.

Results

The frequencies of CYP1A2, SULT1A1, and NAT2 genotypes associated with urothelial cancer are shown in Table 1. There were no associations between CYP1A2 or SULT1A1 genotypes and urothelial cancer adjusted for age, gender, and smoking status. The NAT2 genotypes were categorized as homozygous mutant (slow), heterozygous wild-mutant (intermediate), and homozygous wild type (rapid). Compared with those who had the rapid acetylator genotype of NAT2, those who had intermediate or slow acetylator genotypes had an increased urothelial cancer risk after adjustment for age, gender, and smoking status. Those who had the intermediate or slow genotypes had an OR of 1.49 (95% CI 1.06–2.09) or 3.23 (95% CI 1.72–6.08) compared with those who had a rapid genotype, respectively, and these results provided evidence of a gene dosage effect (Cochran-Armitage test, $P=0.0001$).

In order to check the effect of the gene in combination with smoking, we calculated the OR for data that were classified by smoking status and cumulative cigarette dose (pack-years) and by gene genotypes (Table 2). Among non-smokers, there were no significant differences between CYP1A2, SULT1A1, and NAT2 wild genotypes and these variant genotypes. When the OR was investigated within the strata of cumulative cigarette dose, joint effects of tobacco smoking and SULT *1/*1 or NAT2 slow acetylator genotype were observed only among heavy-smokers. Among subjects who consumed >33.5 pack-years and carried the SULT1A1 *1/*1 or NAT2 slow acetylator genotype, the OR was 1.73 (95% CI 1.01–2.97) whereas it was 7.31 (95% CI 1.90–28.05) in non-smokers who carried the wild genotype as a reference, respectively.

The relationships between CYP1A2, SULT1A1, and NAT2 polymorphisms and pathological findings including differentiation and stage were analyzed. No statistically significant changes were observed when the CYP1A2 and SULT1A1 genotypes were examined relative to tumor differentiation or stage (data not shown). Table 3 shows the OR of developing urothelial cancer for NAT2 genotypes subdivided according to tumor differentiation and smoking status. Among non-smokers, there was no significant difference between NAT2 genotypes and tumor differentiation. Among light smokers, the OR of G1 patients with NAT2 intermediate and slow acetylator genotype was 4.16 (95% CI 0.81–21.45) compared to controls, but not significant.

Table 1 Relationship between CYP1A2, SULT1A1, and NAT2 genotypes and urothelial cancer

Genotype	Patients ($n=306$) % (n)	Controls ($n=306$) % (n)	Crude OR (95% CI)	OR[a] (95% CI)
CYP1A2 genotype				
*1A/*1A	59.8% (183)	58.5% (179)	1	1
*1A/*1C	34.0% (104)	36.9% (113)	0.90 (0.64–1.26)	0.89 (0.64–1.25)
*1C/*1C	6.2% (19)	4.6% (14)	1.33 (0.65–2.73)	1.31 (0.64–2.70)
*1A/*1C + *1C/*1C	40.2% (123)	41.5% (127)	0.95 (0.69–1.31)	0.93 (0.67–1.29)
SULT1A1 genotype				
*1/*1	77.8% (238)	79.1% (242)	1	1
*1/*2	20.3% (62)	19.6% (60)	1.05 (0.71–1.56)	1.05 (0.70–1.57)
*2/*2	2.0% (6)	1.3% (4)	1.53 (0.43–5.47)	1.74 (0.47–6.39)
*1/*2 + *2/*2	22.2% (68)	20.9% (64)	1.08 (0.74–1.59)	1.09 (0.74–1.61)
NAT2 genotype[b]				
Rapid acetylator	42.8% (131)	55.6% (170)	1	1
Intermediate acetylator	44.4% (136)	39.2% (120)	1.47 (1.05–2.06)[c]	1.49 (1.06–2.09)[c]
Slow acetylator	12.7% (39)	5.2% (16)	3.16 (1.69–5.91)[d]	3.23 (1.72–6.08)[d]
Intermediate + slow acetylator	57.2% (175)	44.4% (136)	1.67 (1.21–2.30)[e]	1.69 (1.23–2.34)[e]

[a]Odds ratios were adjusted for age, gender, and smoking status
[b]Trend analysis was carried out on the frequency of NAT2 genotype among urothelial patients and controls; $P=0.0001$
[c]$P<0.05$
[d]$P<0.001$
[e]$P<0.005$

Table 2 Odds ratio of developing urothelial cancer for CYP1A2, SULT1A1, and NAT2 genotypes stratified by smoking amounts

Genotype	Non-smokers			Smokers					
				≤ 33.5 pack-years			> 33.5 pack-years		
	No.[a]	Crude OR (95%CI)	OR[b] (95%CI)	No.[a]	Crude OR (95%CI)	OR[b] (95%CI)	No.[a]	Crude OR (95%CI)	OR[b] (95%CI)
All	94/98	1[c]	1[c]	83/104	0.83 (0.56–1.25)	0.88 (0.56–1.40)	129/104	1.29 (0.88–1.90)	1.42 (0.88–2.28)
CYP1A2									
*1A/*1A	60/59	1[c]	1[c]	46/60	0.75 (0.45–1.28)	0.75 (0.41–1.36)	77/60	1.26 (0.77–2.07)	1.30 (0.72–2.36)
*1A/*1C	30/34	0.87 (0.47–1.59)	0.80 (0.43–1.49)	30/40	0.74 (0.41–1.34)	0.71 (0.37–1.37)	44/39	1.11 (0.63–1.95)	1.04 (0.53–2.05)
*1C/*1C	4/5	0.79 (0.20–3.07)	0.76 (0.19–3.03)	7/4	1.72 (0.48–6.19)	1.69 (0.44–6.43)	8/5	1.57 (0.49–5.09)	1.49 (0.43–5.16)
SULT1A1									
*1/*1	71/77	1[c]	1[c]	63/87	0.79 (0.50–1.24)	0.91 (0.54–1.52)	104/78	1.45 (0.94–2.24)	1.73 (1.01–2.97)[d]
*1/*2 + *2/*2	23/21	1.19 (0.61–2.33)	1.23 (0.62–2.44)	20/17	1.28 (0.62–2.63)	1.46 (0.65–3.26)	25/26	1.04 (0.55–1.97)	1.25 (0.60–2.62)
NAT2									
Rapid acetylator	44/51	1[c]	1[c]	39/59	0.77 (0.43–1.36)	0.74 (0.39–1.41)	48/60	0.93 (0.53–1.61)	0.93 (0.48–1.79)
Intermediate acetylator	38/40	1.10 (0.60–2.01)	1.09 (0.59–2.00)	37/39	1.10 (0.60–2.01)	1.10 (0.57–2.14)	61/41	1.72 (0.98–3.03)	1.70 (0.85–3.40)
Slow acetylator	12/7	1.99 (0.72–5.49)	1.97 (0.71–5.48)	7/6	1.35 (0.42–4.33)	1.29 (0.38–4.42)	20/3	7.72 (2.15–27.72)[e]	7.31 (1.90–28.05)[e]

[a]Number of cases/number of controls
[b]Odds ratios were adjusted for age and gender
[c]Reference category
[d]$P < 0.05$
[e]$P < 0.005$

Among heavy-smokers, the OR of G1 patients who carried NAT2 intermediate and slow acetylator genotype was 6.21 (95% CI 1.26–30.59) when compared to the controls. Similarly, among heavy-smokers, the OR of G2 or G3 patients who carried NAT2 intermediate and slow acetylator genotype was 1.97 (95% CI 1.01–3.85) or 2.53 (95% CI 1.31–4.88), respectively. Table 4 shows the OR of developing urothelial cancer for NAT2 genotypes subdivided according to tumor stage and smoking status. A significant association was not found between NAT2 genotypes and tumor stage among non-smokers and light-smokers. Among heavy-smokers, the OR of superficial tumor or invasive tumor patients who carried NAT2 intermediate and slow acetylator genotype was 2.54 (95% CI 1.32–4.89) or 2.45 (95% CI 1.26–4.73) when compared to the controls. Furthermore we analyzed the interaction ORs between NAT2 genotypes and pathological findings among only case groups subdivided by smoking status (never, ever, light and heavy-smokers) (data not shown). A significant interaction of NAT2 intermediate and slow acetylator genotype was found only between G1 tumor patients and G2 tumor patients among ever-smokers ($P = 0.03$).

We analyzed the associations between CYP1A2, SULT1A1, and NAT2 polymorphisms and the recurrence rate of superficial tumor patients ($n = 142$), who had been treated with bladder reserving operation for the first time and been followed for more than 1 year after operations (mean follow-up, 69.8 months). However, no significant association between CYP1A2, SULT1A1, and NAT2 polymorphisms and recurrence rate of superficial tumor patients was found (data not shown).

Discussion

Recently, several polymorphisms of CYP1A2 have been reported (Chida et al. 1999; Nakajima et al. 1999; Sachse et al. 1999; Chevalier et al. 2001), and two variant alleles which affect CYP1A2 activity were reported. One variant allele was a point mutation from guanine (CYP1A2*1A) to adenine (CYP1A2*1C) at position −2964 in the gene, which caused a significant decrease in CYP1A2 activity (Nakajima et al. 1999). Another variant allele was a C → A transversion (CYP1A2*1F) in intron 1 at position 734 downstream of the first transcribed nucleotide, which has recently been associated with increased CYP1A2 inducibility (Sachse et al. 1999).

In this study, we did not find any significant association between CYP1A2*1C polymorphisms and urothelial cancer. Additional polymorphic variants could be implicated. Moreover, the relationship between genotype and phenotype may be complex and will have to be explored in detail.

Different alleles of SULT1A1 were reported as *2 (213Arg → His), *3 (223Met → Val) and *4 (37Arg → Gln) (Raftogianis et al. 1997; Raftogianis et al. 1999). In Oriental populations, the frequency of

Table 3 Relationship between NAT2 genotypes and pathological differentiation of urothelial cancer

Smoking status		NAT2 genotype (Intermediate and slow) vs rapid	
		Crude OR[a] (95%CI)	OR[a,b](95%CI)
Nonsmokers			
	Controls ($n=98$)	1	1
	Differentiation		
	G1 ($n=11$)	1.90 (0.52–6.90)	1.91 (0.53–6.98)
	G2 ($n=42$)	1.19 (0.58–2.46)	1.20 (0.58–2.49)
	G3 ($n=41$)	1.14 (0.55–2.36)	1.15 (0.55–2.40)
Light-smokers			
≤ 33.5 pack-years	Controls ($n=104$)	1	1
	Differentiation		
	G1 ($n=9$)	4.59 (0.91–23.16)	4.16 (0.81–21.45)
	G2 ($n=40$)	1.19 (0.57–2.47)	1.23 (0.59–2.56)
	G3 ($n=34$)	1.48 (0.68–3.21)	1.45 (0.67–3.16)
Heavy-smokers			
> 33.5 pack-years	Controls ($n=104$)	1	1
	Differentiation		
	G1 ($n=12$)	4.09 (1.05–15.99)[c]	6.21 (1.26–30.59)[c]
	G2 ($n=56$)	1.82 (0.94–3.51)	1.97 (1.01–3.85)[c]
	G3 ($n=61$)	2.60 (1.35–5.01)[d]	2.53 (1.31–4.88)[d]

[a]A combination of NAT2 rapid acetylator genotype and controls was used as a referent group to calculate the OR and 95%CI of urothelial patients subdivided according to tumor differentiation
[b]ORs were adjusted for age and gender
[c]$P<0.05$
[d]$P<0.005$

Table 4 Relationship between NAT2 genotypes and pathological stage of urothelial cancer

Smoking status		NAT2 genotype (Intermediate and slow) vs Rapid	
		Crude OR[a] (95% CI)	OR[a,b] (95%CI)
Nonsmokers			
	Controls ($n=98$)	1	1
	Stage		
	Superficial ($n=59$)	1.12 (0.59–2.14)	1.12 (0.59–2.15)
	Invasive ($n=32$)	1.23 (0.55–2.74)	1.28 (0.57–2.90)
Light-smokers			
≤ 33.5 pack-years	Controls ($n=104$)	1	1
	Stage		
	Superficial ($n=59$)	1.45 (0.76–2.76)	1.45 (0.76–2.78)
	Invasive ($n=24$)	1.55 (0.64–3.78)	1.55 (0.63–3.78)
Heavy-smokers			
> 33.5 pack-years	Controls ($n=104$)	1	1
	Stage		
	Superficial ($n=66$)	2.24 (1.19–4.21)[c]	2.54 (1.32–4.89)[d]
	Invasive ($n=61$)	2.42 (1.26–4.64)[d]	2.45 (1.26–4.73)[d]

[a]Combination of NAT2 rapid acetylator genotype and controls was used as a referent group to calculate the OR and 95%CI of urothelial patients subdivided according to tumor stage
[b]OR were adjusted for age and gender
[c]$P<0.05$.
[d]$P<0.01$

the SULT1A1*2 allele was 0.16–0.33 in previous studies (Ozawa et al. 1999; Carlini et al. 2001) and 0.11 in our controls. The variant SULT1A1*2 was associated with low SULT1A1 activity and thermostability (Ozawa et al. 1999). There are several reports which studied the association between SULT1A1 genotypes and cancer susceptibility. Two studies investigating the association between breast cancer and SULT1A1 genotype were reported. One study reported no effect of SULT1A1 genotype on the risk of breast cancer (Seth et al. 2000); however, another study reported a borderline association between SULT1A1*2/*2 genotype and breast cancer risk (OR = 1.80, 95% CI 1.0–3.2) (Zheng et al. 2001). It was also reported that the SULT1A1*1/*2 or *2/*2 genotype was associated with moderately elevated risk of lung cancer after adjusting for age, sex, and smoking status (OR = 1.41, 95% CI 1.04–1.91) (Wang et al. 2002). Furthermore, the risk was significantly higher in current smokers (OR = 1.74, 95% CI 1.08–2.29) and heavy smokers (OR = 1.45, 95% CI 1.05–2.00). These results of positive association between SULT1A1 polymorphism and cancer susceptibility suggested that

SULT enzymes are involved in numerous detoxification pathways and are responsible for metabolizing a wide range of procarcinogens to less reactive substrates. Therefore, reduced SULT1A1 activity and thermostability conferred by the polymorphism may result in inefficient metabolism and excretion of procarcinogens, which may cause DNA damage. In our study, both the urothelial cancer patients and the controls demonstrated similar frequencies of the SULT1A1*1/*2 or *2/*2 genotype. However, stratifying by amounts of smoking among subjects who consumed >33.5 pack-years and carried the SULT1A1*1/*1 genotype, the OR was significantly higher with non-smokers (OR = 1.73, 95% CI 1.01–2.97), but not among subjects who consumed >33.5 pack-years and carried the SULT1A1*1/*2 and *2/*2 genotype (OR = 1.25, 95% CI 0.60–2.62). This result is not consistent with previous results (Zheng et al. 2001; Wang et al. 2002) and the reason for this discrepancy is unknown at the present. However, a rational hypothesis can be invoked to explain this finding. SULT1A1 protein is found almost ubiquitously in human tissues (Gilissen et al. 1994), perhaps including urothelial mucosa, and particularly prevalent in the liver. Arylamines are detoxicated by SULT1A1 in the liver, and decreased SULT activity conferred by the SULT1A1 polymorphism prevents arylamines from being detoxicated. The quantity of SULT1A1 in the liver is abundant and detoxication of arylamines would not be so influenced by SULT1A1 polymorphism. After arylamines are metabolized in the liver through N-hydroxylation by CYP1A2, N-hydroxy arylamines enter the circulation and undergo renal filtration into the urothelial lumen, where they can be reabsorbed into the urothelial mucosa (Kaderlik and Kadlubar 1995). N-hydroxy arylamines are activated by NAT1 and SULT1A1, and form the arylnitrenium ions in urothelial mucosa. A polymorphism in the polyadenylation signal site of the NAT1 gene (NAT1*10 allele) that has been reported to have higher NAT1 enzyme activity in bladder tissue relative to NAT1 wild allele (NAT1*4 allele), was associated with an increased risk of urothelial cancer (Katoh et al. 1999). Similar to NAT1, the high activity SULT1A1*1 allozyme would activate N-hydroxy arylamines and form the arylnitrenium ions leading to DNA adducts, mutation, and neoplasia.

A number of studies have been conducted on urothelial cancer and the NAT2 polymorphisms. It has been suggested that NAT2 slow acetylators may be at increased risk of urothelial cancer when exposed to environmental arylamine carcinogens, due to their slower inactivation. Recently, some pooled meta-analyses of NAT2 status (phenotype and genotype) studies were reported (Green et al. 2000; Johns and Houlston 2000; Marcus et al. 2000a; Marcus et al. 2000b). Marcus et al. (2000a) reported that slow acetylators had an approximately 40% increase in risk compared with rapid acetylators (OR = 1.4, 95% CI 1.2–1.6) (22 studies, 2,496 cases, 3,340 controls). Studies conducted in Asia generated a summary OR of 2.1 (95% CI 1.2–3.8), in Europe, a summary OR of 1.4 (95% CI 1.2–1.6), and in the USA, a summary OR of 0.9 (95% CI 0.7–1.3). Vineis et al. (2001) reported a pooled analysis of NAT2 genotype-based studies in Caucasian populations (six studies, 1,530 cases, and 731 controls), and a significant association between NAT2 and bladder cancer (OR = 1.42, 95% CI 1.14–1.77). The risk of cancer was elevated in smokers and occupationally exposed subjects, with the highest risk among slow acetylators. They suggested that NAT2 was not a risk factor but modulated the effect of carcinogens contained in tobacco smoke (probably arylamines) or associated with occupational exposures. In our study, there is no significant association between NAT2 genotypes and urothelial cancer among non-smokers, but among smokers the ORs of developing urothelial cancer for NAT2 slow acetylator genotype were much higher according to smoking amounts. Our present results provide support for the findings of Vineis et al. (2001).

There are several inconsistent reports about the association between NAT2 polymorphism and pathological findings of urothelial cancer. Some have reported that NAT2 slow acetylator genotype was likely to have a higher risk for less-differentiated or advanced stage tumors (Cartwright et al. 1982; Inatomi et al. 1999; Mommsen and Aagaard 1986); however, others have reported a tendency towards a higher risk for highly-differentiated and superficial tumors (Hanssen et al. 1985). In our study, the higher OR of NAT2 intermediate and slow acetylator genotypes was more likely to present a well-differentiated tumor (G1) among heavy-smokers, although the risk of urothelial cancer for NAT2 intermediate and slow acetylator genotypes did not differ between superficial and invasive tumors. However, significant interaction ORs between NAT2 genotypes and pathological findings among the case group only were not found, with the exception of the interaction OR of NAT2 intermediate and slow acetylator genotypes between G1 tumor patients and G2 tumor patients among ever-smokers ($P = 0.03$). These non-significant interactions between NAT2 genotypes and pathological findings might be due to insufficient power, because urothelial cancer cases were subdivided into subgroups according to pathological findings and smoking status. Further investigation should clear up this issue.

Smoking is thought to be a moderate risk factor for recurrence of urothelial cancer (Aveyard et al. 2002), and 55.6% (79/142) of superficial tumor patients had recurrence in this study. We did not find any associations between CYP1A2, SULT1A1, and NAT2 polymorphisms and recurrence rate of superficial urothelial cancer patients. To our knowledge, there have been no reports on the recurrence rates of urothelial cancer patients in relation to polymorphisms of the NAT2 gene. Such associations become more difficult as, during the therapeutic process, patients are given intravesical Bacillus Calmette-Guerin, which helps to reduce the recurrence rate of superficial urothelial cancer tremendously (Morales et al. 2002).

In conclusion, our data also suggest a potentially modulating effects of the SULT1A1 polymorphism or NAT2 polymorphism on the association of smoking and urothelial cancer, and the modulation of NAT2 intermediate and slow acetylator genotypes has a tendency to present a higher risk for highly differentiated tumors among heavy-smokers.

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