An Association of UDP-Glucuronosyltransferase 2B7 C₈₀₂T (His₂₆₈Tyr)
Polymorphism with Bladder Cancer in Benzidine-Exposed Workers in China

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UDP-Glucuronyltransferase 2B7 (UGT2B7) is involved in benzidine metabolism, as demonstrated by in vitro experiments with liver slices. To evaluate the possible association of UGT2B7 gene polymorphism with bladder cancer risk for benzidine-exposed subjects, diagnosed bladder cancer cases (n = 36) who were members of a cohort of benzidine-exposed workers in the Chinese dyestuff industry were investigated. UGT2B7 polymorphism at locus C₈₀₂T (His₂₆₈Tyr) was detected using a PCR-RFLP based procedure. Nondiseased cohort members (156 men, 95 women) were taken as work-related control, and unexposed healthy individuals (113 men, 105 women) were taken as community control. The data showed that the polymorphism at locus UGT2B7 C₈₀₂T in a general Chinese population significantly differs from that in a Caucasian population (p = 0.00018), displaying a distinctly lower frequency of T/T genotypes (9.2 vs. 25.3%), while no significant difference to a Japanese population could be detected (p = 0.17). A higher prevalence of T/T genotype carriers was found in the cancer cases, compared with unexposed healthy controls (25 vs. 9%, odds ratio [OR] 3.30, 95% confidence interval [95% CI] 1.37–7.98, p = 0.006). A higher presentation of T allele carriers in the patients group was also confirmed (46 vs. 33%, OR 1.73, 95% CI 1.05–2.87, p = 0.03). A higher portion of the T/T genotype was also observed in bladder cancer patients compared with nondiseased members of the same benzidine-exposed cohort, although some of them displayed different degrees of cellular alterations in their exfoliated urothelial cells. This study points for the first time to an association between a homozygous mutant genotype of human UDP-glucuronosyltransferase 2B7 catalyzing the biotransformation of benzidine and an elevated bladder cancer risk for formerly benzidine-exposed workers of the dyestuff industry.

International Agency for Research of Cancer (IARC) has listed benzidine as a proven human bladder carcinogen in Group I of chemical carcinogens (IARC, 1987). Therefore, a benzidine-exposed population should be regarded as a high-risk group for bladder cancer. A research cohort of dyestuff industry employees who were exposed to benzidine in their past occupational careers was established in the 1980s. It was found in our own previous epidemiological investigation on the same cohort that standardized incidence ratio (SIR) of bladder cancer reached the level of 35 for the entire cohort, and to even higher levels (up to 75) for highly exposed working areas (Lin et al., 2001; Ma et al., 2003; You et al., 1983).

Various xenobiotic-metabolizing enzymes are involved in the metabolism of benzidine. The modulation of bladder cancer risk by polymorphic enzymes has been investigated among the cohort members for GSTM1, T1, and P1 (Lin et al., 2001; Ma et al., 2003), and the aromatic hydrocarbon receptor gene (Zhang et al., 2002). Current data of NAT2 genotyping at seven major polymorphic loci in the same cohort did not display an overtly increased bladder cancer risk for individuals with NAT2 slow acetylation genotypes (Ma et al., 2004). This finding is in line with the only other study on Chinese benzidine workers with bladder cancer in which three mutant alleles and the wild-type allele in the NAT2 gene and the NAT2 phenotype were investigated (Hayes et al., 1993). In contrast to former benzidine-exposed workers of Caucasian origin (Cartwright et al., 1982; Golk et al., 1996, 1997, 2002), there seems to be no association between elevated risk for bladder cancer and NAT2 acetylation status in exposed Chinese workers. Thus, the assumption that different mechanisms could play a role in the individual susceptibility to bladder cancer related to exposure to aromatic amines in various races or ethnic groups was proposed (Golk et al., 2002).
UDP-glucuronosyltransferases (UGTs; EC 2.4.1.17), a type of microsomal enzymes, catalyze the transformation of hydrophobic substrates to more water-soluble glucuronides to facilitate renal or biliary excretion. Target substrates for UGTs cover a wide range of compounds with divergent chemical structures, including dietary by-products, endogenous metabolites, therapeutic drugs, and occupational and environmental pollutants (Desai et al., 2003).

Babu and coworkers reported that N-glucuronidation represents a major pathway of benzidine metabolism in the human liver (Babu et al., 1994, 1995). The main UGT isoforms involved in benzidine metabolism are UGT1A9 > UGT1A4 > UGT2B7 > UGT1A6 > UGT1A1 (Zenser et al., 2002).

UGT2B7 is a major UGT2B isoform in humans (Coffman et al., 1998; Radominska-Pandya et al., 2001). Typical substrates of UGT2B7 are endogenous substances such as hydroxyl metabolites of steroid hormones and bile acids (Jin et al., 1993) and xenobiotics including drugs like morphine, codeine, and other opioid derivatives (Coffman et al., 1998; Court et al., 2003), and carboxylic acid compounds used as nonsteroidal anti-inflammatory drugs (NSAIDs) like ketoprofen, ibuprofen, diclofenac, and naproxen, the lipid reducer gemfibrozil or the antiepileptic valproic acid (Sakaguchi et al., 2004), and a wide range of hydroxylated benzo(a)pyrene and 2-acetylaminofluorene derivatives (Jin et al., 1993). The involvement of UGT2B7 in the metabolism of benzidine or its metabolites in the human liver was demonstrated by a study with cDNA-expressed glucuronosyltransferase (Ciotti et al., 1999) and later by an investigation with liver slices as well (Zenser et al., 2002).

UGT genes have been classified into different families and subfamilies based on their evolutionary divergence. All human UGTs known so far are classified into UGT1A and 2B subfamilies (Mackenzie et al., 1997). Unlike the UGT1A subfamily, in which all isozymes are derived from a single gene locus on chromosome 2q37, the isozymes in the subfamily 2B are encoded by separate genes clustered on chromosome 4q13 (Riedy et al., 2000) and exhibit differences in amino acid sequences throughout the entire polypeptide chain.

In general, studies addressing the impact of the UGT genotype on predisposition to cancer are scarce (Wallig, 2004). To our knowledge, no study on the impact of UGT genotype on bladder cancer risk in exposed individuals has been published. Nevertheless, some papers dealing with in vitro studies in this field are available (Zenser et al., 2002). Thus, we investigated the impact of UGT2B7 gene polymorphism in 36 male bladder cancer cases from a cohort of occupationally benzidine-exposed workers.

**MATERIALS AND METHODS**

**Subjects.** All people included in this study are of ethnic Han origin (Chinese majority that represents 93% of the nation’s population). The study was approved by the Ethics Committee of the Shanghai Municipal Center for Disease Prevention and Control (CDC), and each participant provided written informed consent beforehand.

In total, 36 histologically confirmed bladder cancer cases (all male) from a cohort of former benzidine-exposed workers were included in this study. Each tumor was classified as papillary transitional cell carcinoma. All subjects were employees or former employees of the Chinese dyestuff industry. Synthesis of benzidine was first introduced in China in 1946 as a part of the benzidine-based dye production process. Benzidine had been widely used for 30 years before it was finally forbidden for all industrial purposes by the Chinese government in 1976. A research cohort of former benzidine-exposed workers in the dye industry was established in several Chinese cities in the 1980s. A follow-up study and regular medical surveillance has been performed since then.

Additionally, 251 members, including 156 males, of the benzidine-exposed cohort described before were taken as work-related control. All the subjects in this subgroup have not been diagnosed with bladder cancer or any other malignancy, although exfoliated urothelial cells of some subjects revealed different grades of cytological abnormalities according to Papanicolaou and Marshall (Papanicolaou and Marshall, 1945).

Unrelated healthy people who were permanent residents of Shanghai urban districts (n = 218, including 113 men and 105 women) were taken as community control. All individuals in this group had never been diagnosed for any kind of malignancies, cardiovascular diseases, mental disorders, or any other serious health problems at the time of survey. They were not matched for any other criteria to the case subjects or the exposed controls.

Seventeen bladder cancer cases and 73 nondiseased controls from the benzidine cohort were current and former smokers, whereas 17 bladder cancer cases and 149 nondiseased controls from the benzidine cohort were nonsmokers. Smoking habits are not known for two bladder cancer cases, 30 nondiseased controls from the benzidine cohort, and for the population controls.

**Blood sampling and DNA extraction.** Ethylenediaminetetraacetic acid (EDTA) was used as blood anticoagulant. Genomic DNA was prepared from leukocytes after lysis of erythrocytes, incubation with proteinase K, chloroform extraction, and ethanol precipitation as described previously (Shen et al., 1998).

**UGT2B7 genotyping.** The PCR-RFLP procedure described by Holthe and coworkers (Holthe et al., 2002) was used for UGT2B7 genotyping. Total PCR reaction volume was 50 μl, including 0.3 μM of each primer (Gibco-BRL Life Technologies, Grand Island, NY; sequences: 5'-GAC AAT GGG GAA AGC TGA CG-3' and 5'-GGT CAG GGT TGC AGT G3'). PCR conditions were the following: 94°C for 10 min, 35 cycles of 94°C for 0.5 min, 65°C for 0.5 min, and 72°C for 0.5 min, followed by a final extension at 72°C for 10 min.

The resulting 116 bp PCR product was digested with 2% (w/v) ethidium bromide-stained agarose, and 8.5 μl of the PCR product was digested with Bse GI (MBI Fermentas, Hanover, MD) at 55°C for 3 h. Finally, 10 mmol/l EDTA was added for reaction termination. The digestion products were separated on a 4% (w/v) Nusieve 3:1 agarose (Amresco, Solon, OH) gel and detected by ethidium bromide staining.

**Collection and classification of exfoliated cells.** Urine samples were collected from the members of the benzidine cohort at the medical clinic in Shanghai between 0900 and 1100 h. The entire urinates, which were second or third urinates of the day, were immediately spun down, fixed, subsequently stained according to Papanicolaou (Papanicolaou, 1942), and classified according to Papanicolaou and Marshall (Papanicolaou and Marshall, 1945) as described elsewhere in detail (Ma et al., 2003).

**Statistical analysis.** Chi-square test was used to determine differences in genotype frequencies between cases and controls; p values less than 0.05 were regarded as significant. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were also calculated to estimate the risk due to different genotypes. Hardy-Weinberg equilibrium was used to calculate the expected frequencies of the investigated heterozygous genotypes.
TABLE 1

<table>
<thead>
<tr>
<th>Genotypes or alleles</th>
<th>Healthy individuals (n = 218)</th>
<th>Bladder cancer cases (n = 36)</th>
<th>OR</th>
<th>95% CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotypes C/C</td>
<td>95 (44%)</td>
<td>103 (47%)</td>
<td>0.65</td>
<td>0.31–1.36</td>
<td>0.25</td>
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<tr>
<td>Genotypes C/T</td>
<td>103 (47%)</td>
<td>15 (42%)</td>
<td>0.80</td>
<td>0.39–1.63</td>
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<td>Genotypes T/T</td>
<td>20 (9%)</td>
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<td>3.30</td>
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<td>0.006</td>
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<td>Alleles C</td>
<td>293 (67%)</td>
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<td>0.58</td>
<td>0.35–0.96</td>
<td>0.03</td>
</tr>
<tr>
<td>Alleles T</td>
<td>143 (33%)</td>
<td>33 (46%)</td>
<td>1.73</td>
<td>1.05–2.87</td>
<td>0.03</td>
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RESULTS

UGT2B7 C<sub>802</sub>T Polymorphism in General Chinese Han Population

UGT2B7 C<sub>802</sub>T polymorphism in a group of healthy individuals in the city of Shanghai (n = 218) was detected as follows: homozygous wild genotype C/C: 43.6%; homozygous mutant genotype T/T: 9.2%; heterozygous genotype C/T: 47.3%. The frequencies of the genotypes among healthy individuals were within the Hardy-Weinberg equilibrium. The observed percentages (Table 1) were quite different (p = 0.0003) from those reported for Caucasian subjects in Australia (n = 91) (Bhasker et al., 2000). In the investigated Australian subjects, the homozygous mutant genotype T/T was more frequent than in Chinese (25.3% vs. 9.2%). Nevertheless, UGT polymorphism in the investigated Chinese general population did not significantly differ (p = 0.30) from that in a group of Japanese individuals (n = 84) in which the T/T genotype displayed an even lower portion (4.8% vs. 9.2%) (Bhasker et al., 2000). This should be considered when the impact of polymorphism of UGT2B7 genotypes on certain diseases such as bladder cancer is concerned. No significant gender-dependent deviation of the frequencies of UGT2B7 C<sub>802</sub>T genotypes was found in the present study (p = 0.49).

TABLE 2

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DISCUSSION

When benzidine was incubated with liver slices from different mammalian species (dogs, rats and humans), various proportions of N-acetylated and N-glucuronidated benzidine metabolites were detected (Babu et al., 1995). Human liver slices demonstrated both extensive N-acetylation and N-glucuronidation abilities (Babu et al., 1994, 1995). Most recently, a modified concept of activating and deactivating steps for benzidine, based on the concepts of Babu et al. (1995), Zenser et al. (2002), and their own findings was published by Degen et al. (2004). N-Glucuronides of benzidine, N-acetylbenzidine, and N’-hydroxy-N-acetylbenzidine were acid labile. Since human bladder urothelial cells contain relatively high activities of prostaglandin H synthase, but only low activities of cytochrome P450 (Wise et al., 1984), the per-oxidative metabolism of N-acetylbenzidine was assessed. N’-(3’-monophospho-deoxyguanosin-8-yl)-N-acetylbenzidine (dGp-ABZ) was the major DNA adduct detected. This adduct was also the major adduct detected in bladder urothelial cells from subjects occupationally exposed to benzidine (Zenser et al., 2002).
urine samples from these workers, an inverse relationship between urine pH values and levels of free benzidine and N-acetylbenzidine was observed. A similar inverse relationship was also observed for urine pH values and levels of dGp-ABZ adducts in urothelial cells (Ciotti et al., 1999). These observations suggest that conjugation of benzidine, catalyzed by UDP-glucuronosyltransferases, and its release from the conjugate in the urinary tract due to acidic conditions might play an important role in the etiology of benzidine-induced human bladder cancer.

Although Bhasker and coworkers (2000) and Holthe and coworkers (2002) have suggested that the polymorphism of UGT2B7 at codon 268 is probably not associated with altered enzyme activities, Sawyer and coworkers reported (Sawyer et al., 2003) that homozygous mutant genotype T/T802 (Y/Y268) carriers displayed the strongest catalyzing abilities toward morphine. In the present study, benzidine-exposed bladder cancer cases providing the T/T802 genotype or just the T allele had a higher bladder cancer risk compared with healthy community controls. This finding is in line with lower portions of the T/T802 genotype or just the T allele carriers in benzidine-exposed subjects without bladder cancer but showing exfoliated urothelial cells of grade III, and higher according to Papanicolaou, compared with benzidine-exposed bladder cancer cases. These observations are pointing to an important role of UGT2B7 in the metabolism of benzidine in the Chinese population. It might be speculated whether a mutation related with increased metabolic activity may result in a higher portion of glucuronidated benzidine and, due to cleavage in the urinary bladder at certain urine pH values, in a higher amount of released benzidine.

Various UGT2B genes show a high degree of structural and sequence conservation, and the available data suggest that the UGT2B gene cluster evolutionarily resulted from recent gene duplications. Mutations might result in the overlapping of substrate specificities of UGT isoforms that have been relatively poorly defined so far. Thus, UGT isoforms other than UGT2B7 might overlap its glucuronidation activity, thereby masking an effect of the investigated polymorphism. Unlike cytochrome P450, UGT isozyme-specific inhibitors are, mostly, not available so far, and this has impaired the investigation of isozyme selectivity for glucuronidation pathways in the human.

For the first time, the present work provided evidence that carriers of homozygous mutant genotypes of UGT2B7, involved in the metabolism of benzidine, are at elevated risk for bladder cancer if they had been occupationally exposed to benzidine in the past. Possibly, genotyping of the Chinese bladder cancer cases for other UGT polymorphic genes (e.g., UGT1A9, UGT1A4, UGT1A6, UGT1A1), which have been also shown to be involved in the biotransformation of benzidine or its metabolites in human Caucasian liver (Zenser et al., 2002), might provide further understanding.

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REFERENCES


