Aldehyde dehydrogenase 2 (ALDH2) and alcohol dehydrogenase 1B (ADH1B) polymorphisms exacerbate bladder cancer risk associated with alcohol drinking: gene-environment interaction

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Aldehyde dehydrogenase 2 (*ALDH2*) and alcohol dehydrogenase 1B (*ADH1B*) polymorphisms exacerbate bladder cancer risk associated with alcohol drinking: Gene-environment interaction.

Running head: Bladder cancer risk and SNPs in *ALDH2*/*ADH1B*

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Abstract

Although a range of chemical exposures (cigarette smoking and occupational exposure) are recognized risk factors for the development of bladder cancer (BCa), many epidemiological studies have demonstrated that alcohol drinking is not associated with BCa risk. Aldehyde dehydrogenase 2 (*ALDH2*; rs671, Glu504Lys) and alcohol dehydrogenase 1B (*ADH1B*; rs1229984, His47Arg) polymorphisms impact the accumulation of acetaldehyde, resulting in an increased risk of various cancers. To date, however, no studies evaluating the association between BCa risk and alcohol drinking have considered these polymorphisms. Here, we conducted a matched case-control study to investigate whether *ALDH2* and *ADH1B* polymorphisms influence BCa risk associated with alcohol drinking. Cases were 74 BCa patients and controls were 740 first-visit outpatients without cancer at Aichi Cancer Center Hospital between January 2001 and December 2005. Odds ratio (OR), 95% confidence interval (CI) and gene-environment interaction were assessed by conditional logistic regression analysis with adjustment for potential confounders. Results showed that *ALDH2* Glu/Lys was associated with a significantly increased risk of BCa compared with Glu/Glu (OR 2.03, 95% CI 1.14-3.62, *P*=0.017). In contrast, *ALDH2* Glu/Lys showed no increase in risk among the stratum of never drinkers compared with Glu/Glu, indicating a

gene-environment interaction. *ADH1B* His/Arg had an OR of 1.98 (1.20-3.24, *P*=0.007) compared with His/His. *ADH1B* Arg+ showed a similar OR and 95% CI. Individuals with *ALDH2* Glu/Lys and *ADH1B* Arg+ had the highest risk of BCa compared with *ALDH2* Glu/Glu and *ADH1B* His/His [OR 4.00 (1.81-8.87), *P*=0.001].

Summary

Those with *ALDH2* Glu/Lys genotype and the *ADH1B* Arg allele were associated with an increased risk of bladder cancer. Gene-environment interaction between *ALDH2* Glu/Lys and alcohol drinking might indicate that acetaldehyde contributes to a development of bladder cancer.

Keywords: bladder cancer, ALDH2, polymorphism, carcinogenesis, alcohol consumption

Abbreviations: BCa, bladder cancer; ALDH2, aldehyde dehydrogenase 2; ADH1B, alcohol dehydrogenase 1B; HERPACC, Hospital-based Epidemiologic Research Program at Aichi Cancer Center; PY, pack-years; OR, odds ratio; CI, confidence interval; ACCH, Aichi Cancer Center Hospital

Introduction

Bladder cancer (BCa) is the 9th most common cancer, accounting for approximately 165 000 deaths worldwide in 2012 [1]. Among risk factors of BCa identified in epidemiological studies, smoking is recognized as the most important [2-4], followed by occupational exposure to carcinogens (e.g. aromatic amines, polycyclic aromatic hydrocarbons and chlorinated hydrocarbons) [4]. These findings clearly link BCa with environmental chemical exposures.

Acetaldehyde, a metabolite of ethanol, is assessed by the International Agency for Research on Cancer (IARC) as a chemical carcinogen in various types of cancers [5]. Acetaldehyde is considered to form adducts with the genome and with proteins involved in the maintenance of genomic stability, leading to DNA mutation and carcinogenic effects [6,7]. In humans, epidemiological studies have suggested that acetaldehyde modulated by functionally proven polymorphisms in genes encoding alcohol-metabolizing enzymes, such as aldehyde dehydrogenase 2 (*ALDH2*) (rs671, Glu504Lys) [8-11] or alcohol dehydrogenase 1B (*ADH1B*) (rs1229984, His47Arg) [8,9], contributes to an increased risk of esophageal cancer [12-15], head and neck cancer [16,17] and gastric cancer [18,19]. However, no studies of the association between BCa risk and alcohol drinking have taken these polymorphisms into

consideration. Although there has been no consistent evidence that alcohol consumption is a risk factor of BCa [5,20], consideration of these polymorphisms would likely allow the impact of acetaldehyde to be evaluated with greater precision.

We hypothesized that there was an association between BCa risk and alcohol-metabolizing enzymes. Here, we investigated whether *ALDH2* and *ADH1B* polymorphisms influence BCa risk associated with alcohol drinking in a Japanese population.

Materials and methods

Subjects

The subjects were selected from the database of the second version of the Hospital-based Epidemiologic Research Program at Aichi Cancer Center (HERPACC). Details of the framework have been described elsewhere [21,22]. In brief, all outpatients aged 20-79 years on their first visit to Aichi Cancer Center Hospital (ACCH) were requested to provide information on lifestyle factors as well as a 7 ml blood sample. Before their first examination, patients were asked about their lifestyle when healthy or before their current symptoms developed. Responses were systematically collected and checked by trained interviewers. Completed responses were obtained from 96.7% of 29 538 enrolled subjects, of whom 50.7%

provided a blood sample [22]. Questionnaire data were loaded into the HERPACC database and periodically linked with the hospital cancer registry system to update cancer incidence. Written informed consent was obtained from all participants and the study was approved by the ethics committee of ACCH.

A total of 134 patients with no prior history of cancer were histologically diagnosed with BCa between January 2001 and December 2005 at ACCH. We excluded 60 patients who lacked blood samples or had insufficient information on alcohol drinking and smoking status, leaving 74 BCa patients included as cases. Controls were ACCH first-visit outpatients during the same period who were confirmed to be free of any cancer. Controls were randomly selected and matched for age (±3 years) and sex to cases with 1:10 case-control ratio (n=740). This matching ratio of 1:10 aimed to maintain subjects in stratified analysis in conditional logistic regression.

Genotyping of ALDH2 and ADH1B

DNA of each subject was extracted from the buffy coat fraction with a DNA Blood Mini Kit (Qiagen). Genotyping of *ALDH2* (rs671) and *ADH1B* (rs1229984) was carried out using TaqMan Assays with the Applied Biosystems 7500 Fast system (Foster City, CA). Primer

sequences for each polymorphism are listed in Supplementary Table I. Our laboratory routinely tests for Hardy-Weinberg equilibrium to assess the quality of genotyping.

Assessment of alcohol intake and smoking exposure

Environmental factors including drinking and smoking were obtained from a self-administered questionnaire. Drinking status was divided into the three categories of never, former and current. Former drinkers were defined as those who had quit drinking for more than one year. Alcohol consumption of each beverage type (Japanese sake, beer, shochu, whiskey and wine) was estimated as the average number of drinks per day, which was converted into a Japanese sake (rice wine) equivalent. One drink equates to one ‘go’ (180ml) of Japanese sake, which contains 23g of ethanol, equal to one large bottle (633ml) of beer, two shots (57ml) of whiskey or two and a half glasses of wine (200ml). One drink of ‘shochu’ (distilled spirit), which contains 25% ethanol, was rated as 108 ml. Total alcohol consumption was determined as the total sum of pure alcohol consumption (gram per day) of Japanese sake, beer, shochu, whiskey and wine among ever drinkers. Based on alcohol consumption, we then categorized subjects into the three categories of never, moderate and heavy drinkers. Heavy drinkers were defined as those who consumed alcohol on five or more days per week at 46g

or more ethanol equivalent per occasion, and moderate drinkers as those other than heavy drinkers.

We also asked about smoking status, number of cigarettes per day, age at which smoking was started, as well as period of smoking abstinence among former smokers. Smoking status was categorized into the three categories of never, former and current. Former smokers were defined as those who had quit smoking for more than one year. Cumulative smoking exposure was evaluated as pack-years (PY), the number of packs of cigarettes smoked per day multiplied by the number of years of smoking.

Statistical analysis

We applied odds ratios (ORs) and their 95% confidence intervals (CIs) estimated in multivariate conditional logistic regression models as measures of association for the risk of BCa. We evaluated the impact of *ALDH2* and *ADH1B* polymorphisms in two multivariate models: model 1 consisted of age only and model 2 consisted of age, smoking (PY in categories, PY=0, PY<30 and PY≥30) and drinking (never, moderate and heavy drinkers). Trend analysis for drinking was done using drinking as scores (0: never, 1: moderate, and 2: heavy). Interaction between drinking and *ALDH2* or *ADH1B* polymorphisms was assessed

with the model including interaction terms of combination of drinking and genotype in categories. Accordance with Hardy-Weinberg equilibrium in controls was checked using the chi-square test.

Statistical analyses were conducted using STATA statistical software version 13.1 (StataCorp LP, College Station, Texas, USA). Two-sided *P* values<0.05 were considered statistically significant.

Results

Baseline characteristics of cases and controls are shown in Table I. Age and sex were well-balanced. Age-adjusted OR in a conditional model for ever smokers relative to never smokers was 1.99 (95% CI 1.02-3.89, *P*=0.043). With respect to cumulative smoking exposure, BCa risk for ever smokers with PY≥30 was higher than that for never smokers (OR 2.73, 95% CI 1.35-5.56, *P*=0.005), and also higher for ever smokers who smoked more than 20 cigarettes per day (OR 2.33, 95% CI 1.07-5.07, *P*=0.032). The dose-response relationship for PY categories was significant (*P* for trend=0.002), and a similar tendency was observed for the number of cigarettes per day (*P* for trend=0.035). In contrast, no significant association with BCa risk was seen for alcohol drinking status, either ever/moderate/heavy

drinkers, although OR for heavy drinkers was greater than unity.

Table II shows genotype distributions of *ALDH2* and *ADH1B* polymorphisms, and their ORs and 95% CIs for BCa risk. The *ALDH2* genotype frequencies of Glu/Glu, Glu/Lys and Lys/Lys were 36.5%, 58.1% and 5.4% among cases, and 46.9%, 43.2% and 9.9% among controls, respectively. The *ADH1B* genotype frequencies of His/His, His/Arg and Arg/Arg were 44.6%, 51.4% and 4.1% among cases, and 60.5%, 35.8% and 3.7% among controls, respectively. These frequencies in controls were in accordance with Hardy-Weinberg equilibrium. For *ALDH2*, Glu/Lys had a significantly increased risk of BCa compared with Glu/Glu (fully adjusted OR 2.03, 95% CI 1.14-3.62, *P*=0.017), while no increased risk was observed with Lys/Lys (OR 0.88, 95% CI 0.26-2.99, *P*=0.834). Regarding *ADH1B*, His/Arg had an OR of 1.98 (95% CI 1.20-3.24, *P*=0.007) compared with His/His, and Arg+ also showed similar OR and 95% CI (OR 1.94, 95% CI 1.19-3.15, *P*=0.008).

As shown in Table III, *ALDH2* Glu/Lys indicated no increased risk among the stratum of never drinkers compared with Glu/Glu (OR 0.85, 95% CI 0.26-2.73, *P*=0.780). However, for moderate drinkers, Glu/Lys and Lys+ showed a significant elevation of risk (OR 3.42, 95% CI 1.50-7.83, *P*=0.004 and OR 3.31, 95% CI 1.46-7.53, *P*=0.004, respectively). For heavy drinkers, Glu/Lys showed an OR of 4.99, which was higher than that for moderate

drinkers but not with statistical significance (*P*=0.105). In addition, among *ALDH2* Lys+, a significantly elevated risk of BCa was observed with an increase in alcohol intake (*P* for trend=0.043), and interaction between *ALDH2* Glu/Lys and moderate alcohol intake was seen (*P* for interaction=0.030). In terms of *ADH1B* polymorphism, among strata of never drinkers and moderate drinkers, His/Arg and Arg+ indicated a trend for higher BCa risk. Furthermore, impact of alcohol drinking on BCa risk stratified by *ALDH2* and *ADH1B* genotypes are shown in Supplementary Table II.

Table IV shows the combination of *ALDH2* and *ADH1B* polymorphisms on BCa risk. Individuals carrying *ADH1B* His/His with *ALDH2* Glu/Lys and Lys/Lys showed ORs of 2.48 (95% CI 1.09-5.67, *P*=0.031) and 1.23 (0.23-6.40, *P*=0.809), respectively, compared with *ALDH2* Glu/Glu and *ADH1B* His/His, the most prevalent genotype among study subjects. On the other hand, individuals carrying the *ADH1B* Arg allele with *ALDH2* Glu/Glu, Glu/Lys and Lys/Lys showed ORs of 2.57 (95% CI 1.15-5.75, *P*=0.022), 4.00 (1.81-8.87, *P*=0.001) and 1.48 (0.28-7.92, *P*=0.650), respectively. Those with *ALDH2* Glu/Lys and *ADH1B* Arg+ had the highest risk of BCa, although no significant gene-gene interaction was seen (*P* for interaction=0.399). A forestplot and a bar graph are shown based on information in Table II to IV (Supplementary Figure I and II).

We investigated whether *ALDH2* and *ADH1B* polymorphisms affected risks for muscle invasive, high grade and multiple tumors despite small sample size. As shown in Supplementary Table III, 57 patients had sufficient pathological information and were diagnosed with urothelial carcinoma. Those with Lys allele were more likely to have muscle invasive and high grade tumors than those with Glu/Glu, but failed to show statistical significance. *ADH1B* polymorphisms indicated no trend in pathological findings.

Discussion

This is the first study to investigate whether *ALDH2* and *ADH1B* polymorphisms influence BCa risk associated with alcohol drinking. We found that *ALDH2* and *ADH1B* functional polymorphisms were associated with BCa risk according to drinking status. Among never drinkers, no significant elevation of risk was observed with *ALDH2* Glu/Lys compared to Glu/Glu. In contrast, Glu/Lys had a significantly increased risk of BCa among drinkers, indicating gene-environment interaction between alcohol consumption and *ALDH2* Glu/Lys genotype. Regarding *ADH1B*, Arg allele carriers (slow metabolizers) had a higher risk for BCa than those with His/His (rapid metabolizers). When *ALDH2* and *ADH1B* were combined, those with *ALDH2* Glu/Lys and *ADH1B* Arg+ had the highest risk, but no clear evidence for

gene-gene interaction was observed.

The present results that *ALDH2* Glu/Lys is associated with increased risk of BCa among drinkers but not never drinkers imply that acetaldehyde accumulation related to the consumption of alcoholic beverages is involved in BCa risk. Acetaldehyde is considered a plausible candidate for having carcinogenic effects on various cancers [12-19], particularly esophageal cancers [5], and previous studies of esophageal cancer demonstrated that drinkers with the *ALDH2* Glu/Lys genotype were at elevated risk of esophageal cancer compared with other genotypes [12-15]. Our present results are consistent with these previous findings, providing further evidence that acetaldehyde also contributes to the development of BCa as a carcinogen.

Ethanol and acetaldehyde have been detected in urine as well as in blood [23,24]. Tominaga et al. reported that urinary levels of free and bound acetaldehyde in alcoholics at admission for abstinence treatment were approximately 2 times higher than in healthy volunteers, and that bound acetaldehyde levels after abstinence for 3 months among alcoholics remained significantly higher than those in healthy volunteers [23]. Furthermore, concentration of acetaldehyde in urine 30 to 300 minutes after alcohol intake was 2-6 times higher among those with *ALDH2* Glu/Lys or Lys/Lys genotype than those with Glu/Glu [24],

suggesting that individuals with *ALDH2* Glu/Lys who chronically drink alcohol in large amounts are subject to prolonged exposure to acetaldehyde in urine. These findings might explain our present finding that drinkers with *ALDH2* Glu/Lys had a higher BCa risk. The lack of any increase in risk in Lys/Lys carriers may be because they refrain from drinking due to severe adverse reactions caused by acetaldehyde, e.g. facial flushing, nausea and headache [25,26].

The higher risk for *ADH1B* Arg+ might be explained as follows. First, those with *ADH1B* Arg+ are prone to drink more and to have a higher risk of alcoholism than His/His [25,27,28] because ethanol is more slowly metabolized into acetaldehyde, which induces uncomfortable symptoms. Our study also showed that drinkers with *ADH1B* Arg+ and *ALDH2* Glu/Lys tended to have higher alcohol consumption than those with *ADH1B* His/His and *ALDH2* Glu/Lys (data not shown). As a result, drinkers with *ADH1B* Arg+ are likely to have higher exposure to acetaldehyde than those with His/His. Second, *ADH1B* polymorphisms might influence acetaldehyde levels in urine. Yokoyama et al. found that ethanol and acetaldehyde levels in blood and saliva among alcoholics were significantly higher in those with *ADH1B* Arg/Arg than those with the His allele [29]. It appears likely that those with the *ADH1B* Arg allele have higher acetaldehyde levels in urine than those with

His/His, although no study has yet investigated the association between *ADH1B* polymorphisms and concentration of acetaldehyde in urine.

To our knowledge, only one study has evaluated the association between alcohol-metabolizing enzymes and BCa risk [30]. Dijk et al reported that moderate drinkers with the fast *ADH1C* (previously called *ADH3*) genotype had an approximately 3-fold higher risk of BCa than those with the slow *ADH1C* genotype [30]. This result appears discrepant with our finding that risk was higher in those with Arg+, the slow *ADH1B* genotype, than the fast *ADH1B* genotype. The discrepancy might be due to small sample size. A second explanation is that *ADH1B* polymorphisms may exert a larger impact on alcohol behaviors than *ADH1C* polymorphisms. Whitfield et al. reported that the *ADH1C* genotype had considerably less effect on the amount and frequency of alcohol intake than the *ADH1B* genotype [31]. In addition, a meta-analysis failed to identify an alcoholism risk associated with the *ADH1C* genotype in Caucasians [28]. The association between *ADH1C* polymorphism and BCa might therefore be inconsistent with that of *ADH1B* polymorphism. We did not examine *ADH1C* polymorphisms in the present study because these are relatively uncommon among Asians [10].

We believe a role of acetaldehyde in the etiology of bladder cancer is plausible. ALDH2 enzyme is expressed in various organs including bladder, whereas a majority of ADH1B enzyme is expressed in liver (the database of the human protein atlas, website: http://www.proteinatlas.org). It remains unclear whether acetaldehyde in blood is directly excreted through urinary tract or ethanol excreted in urine is converted to acetaldehyde in bladder, however, acetaldehyde has been actually detected in urine. Furthermore, Yukawa et al. reported that level of N^2-ethylidene-dG, the most abundant DNA adduct derived from acetaldehyde, was significantly higher in the esophagus of ethanol drinking and intraperitoneally-injected mice with *Aldh2*$^{-/-}$ than those with *Aldh2*$^{+/+}$ [32]. This finding suggests that acetaldehyde may induce accumulation of DNA mutations in bladder mucosa as well as in esophageal mucosa and lead to development of BCa. A meta-analysis conducted in 2011 concluded the absence of significant association between alcohol drinking and BCa risk as a whole [20]. This meta-analysis included two case-control studies among Japanese, which failed to show BCa risk associated with alcohol drinking. Lack of information on *ALDH2* and *ADH1B* polymorphisms might attenuate the association between alcohol drinking and BCa risk among Asian. Another possible explanation for observed association could be that a

substance, rather than alcohol itself, which is a substrate of *ALDH2* and *ADH1B* enzymes lead to an increased BCa risk.

Our study has several potential limitations. First, sample size of BCa patients was limited, and statistical power might therefore have been insufficient. Second, our study lacks information on occupational exposure. In Japan, however, manufacturing and handling of benzidine and 2-naphtylamine, known carcinogens leading to bladder cancer, were prohibited by the Occupational Safety and Hygiene Law in 1972 [33]. Our findings, therefore, might be less affected by occupational exposure. Third, the existence of selection bias should be considered. We selected cases and controls in a single study who participated in this study with high response rate. Controls were first-time visitors to ACCH who were confirmed to be free of any cancer, and are expected to visit the same hospital if cancer does develop in the future. It is therefore reasonable to assume that controls were selected from the same base population as cases. We previously confirmed that our control populations have similar characteristics to the general population with respect to exposures of interest, here alcohol drinking [34]. Furthermore, the genotype distribution of *ALDH2* and *ADH1B* polymorphisms in controls was similar to that in other studies conducted in Japanese populations [35,36]. These findings might warrant the internal and external validity of the present study, and

indicate that selection bias is relatively small. In addition, the trial design of HERPACC requires that the self-administered questionnaires be completed before diagnosis, which may minimize the recall bias which is inherent to case-control studies. We also compared lifestyle and clinicopathological characteristics of 74 patients included in this study with those of 60 patients without any blood samples. As shown in Supplementary Table IV, no significant differences were observed between two groups, indicating lack of selection bias by blood sample availability.

We conclude that those with the *ALDH2* Glu/Lys and *ADH1B* Arg+ genotype are at increased risk of BCa. Gene-environment interaction between *ALDH2* Glu/Lys and alcohol drinking might suggest that acetaldehyde in urine contributes to the development of BCa. Measurement of *ALDH2* and *ADH1B* and targeted prevention of individuals with a high-risk genotype to reduce alcohol intake may decrease the incidence of BCa. Replication in larger studies is highly warranted.

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TABLE AND FIGURE LEGENDS

Table I. Characteristics of cases and controls

[a]Adjusted for age and sex. [b]In conditional logistic regression model. [c]For drinking categories (never, moderate, heavy drinkers). OR, odds ratio; CI, confidence interval; SD, standard deviation.

Table II. Genotype distributions of *ALDH2* and *ADH1B* polymorphisms and their impact on bladder cancer risk

[a]Model 1 adjusted for age and sex. [b]Model 2 adjusted for age, sex, smoking (pack-year; 0, <30, ≥30) and drinking (never, moderate, heavy drinkers). [c]HWE, Hardy-Weinberg Equilibrium in controls. OR, odds ratio; CI, confidence interval.

Table III. *ALDH2* and *ADH1B* polymorphisms and their impact on bladder cancer risk stratified by drinking status

[a]Adjusted for age, sex and smoking (pack-year; 0, <30, ≥30). OR, odds ratio; CI, confidence interval; Ca, case; Co, control; NE, not estimated.

Table IV. Impact of combination of *ALDH2* and *ADH1B* polymorphisms on bladder cancer risk

[a]Adjusted for age, sex, smoking (pack-year; 0, <30, ≥30) and drinking (never, moderate, heavy drinkers). OR, odds ratio; CI, confidence interval.

Supplementary Table I. Primer sequences of *ALDH2* (rs671) and *ADH1B* (rs1229984)

polymorphism by TaqMan assays

Supplementary Table II. Impact of alcohol drinking on bladder cancer risk stratified by *ALDH2* and *ADH1B* genotypes

[a]Adjusted for age, sex and smoking (pack-year; 0, <30, ≥30)

OR, odds ratio; CI, confidence interval; Ca, case; Co, control; NE, not estimated.

Supplementary Table III. Impact of *ALDH2* and *ADH1B* polymorphisms on pathological characteristics of patients diagnosed with urothelial carcinoma

We excluded patients without sufficient pathological information (n=14) and diagnosed with non-urothelial carcinoma (n=3).

[a]Muscle invasive bladder cancer was defined as T2 or more. [b]Low grade was defined as Grade1, and high grade as Grade2 and Grade3. [c]Adjusted for age, sex, smoking (pack-year; 0, <30, ≥30) and drinking (never, moderate, heavy drinkers). [d]In multivariate logistic regression model

OR, odds ratio; CI, confidence interval.

Supplementary Table IV. Lifestyle and clinicopathological characteristics of bladder cancer patients with or without blood samples

[a]For chi-square test. [b]We excluded patients without sufficient pathological information (Patients with blood samples, n=14; Patients without blood samples, n=15). [c]We included patients diagnosed with urothelial carcinoma (Patients with blood samples, n=57; Patients without blood samples, n=43).

Supplementary Figure I. Forest plot of odds ratio (OR) with 95% confidence interval (CI) for bladder cancer risk according to *ALDH2* and *ADH1B* polymorphisms

Supplementary Figure II. Impact of combination of *ALDH2* and *ADH1B* polymorphisms on bladder cancer risk