Urinary 8-Hydroxydeoxyguanine Levels in Urothelial Carcinoma Patients with Low Urinary Arsenic Concentrations

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Abstract

Arsenic is a well-documented human carcinogen and is known to cause oxidative stress in cultured cells and animals. In order to investigate the effect of low concentrations of arsenic exposure in producing oxidative stress, a hospital-based case-control study was conducted to evaluate the relationship between the levels of urinary 8-hydroxydeoxyquanine (8-OHdG) and the arsenic profile in urothelial carcinoma (UC) patients. Urinary 8-OHdG was measured by using high-sensitivity enzyme-linked immunosorbent assay (ELISA) kits. The urinary species of inorganic arsenic and their metabolites were analyzed by high-performance liquid chromatography (HPLC) and hydride generator-atomic absorption spectrometry (HG-AAS). **This study showed that the higher PMI and lower SMI values of 4.26 ± 0.78 and 11.57 ± 3.00 for UC patients than for healthy controls of 2.70 ± 0.46 and 21.00 ± 2.30 ($p = 0.07$ and $p < 0.01$), respectively. Urinary 8-OHdG levels correlated with urinary total arsenic concentrations ($r = 0.53$, $p < 0.01$). There were no significantly differences of 8-OHdG levels between UC patients as well as healthy controls after adjusting for age, gender and creatinine concentrations.** Multiple linear regression analyses revealed that high urinary 8-OHdG levels were associated with increased monomethylarsonic acid, dimethylarsenate, inorganic arsenite, and total arsenic concentrations as well as the primary methylation index even after adjusting for age, gender, and **creatinine concentrations**. The results suggest that **even no evident arsenic exposure, urinary arsenic is** associated with oxidative DNA damage.

Key words: Urothelial Carcinoma; 8-Hydroxydeoxyguanine; Urinary arsenic profile

Introduction

The occurrence of chronic arsenic poisoning is a worldwide public health problem, and the current maximum contaminant level of arsenic for safe drinking water is still being discussed. Arsenic is a naturally occurring element, ubiquitous in the environment in both organic and inorganic forms. **Inorganic arsenic is commonly found in groundwater, surface waters, and only a very small percentage of arsenic found in many foods, such as rice, grains, and fish (Brown and Ross, 2002). In addition, humans also experience occupational exposure (Brown and Ross, 2002). Since 1987, t**he International Agency for Research on Cancer (IARC) documented that arsenic in drinking water is carcinogenic to humans **(IARC, 1987).** Many epidemiological studies have reported that long-term exposure to inorganic arsenic is associated with increased risks of skin, liver, lung, and bladder cancers and several non-cancerous diseases (**Tapio and Grosche, 2006**; Tseng, 2002; Yoshida et al., 2004). The carcinogenic mechanism of arsenic is still unclear but arsenic-induced oxidative DNA damage has recently been proposed (Pi et al., 2002; Liu et al., 2003; Huang et al., 2004).

Results from in vitro studies demonstrated a role of various arsenic species for directly or indirectly generating oxidative stress. Reactive oxygen species (ROS) can be formed during arsenic methylation or by stimulating the NADP(H) oxidase p22phox subunit which causes oxidative DNA damage (Lynn et al., 2000; Nishikawa et al., 2002; Wei et al., 2002). The presence of arsenic-induced oxidative damage is also evident from some epidemiological studies. A study from Inner Mongolia reported that elevated serum lipid peroxide levels and a decreased non-protein sulfhydryl concentration in a high-arsenic exposure group were directly correlated with blood levels of inorganic arsenic and its methylated metabolites (Huang et al., 2004). And it has been shown that a strong inverse correlation was evident among serum nitrite/nitrate levels and blood inorganic arsenic, **MMA and DMA** (Pi et al., 2000). In Taiwan, Wu et al. found that the arsenic concentration in whole blood showed a positive association with the levels of reactive oxidants in plasma and an inverse relationship with the level of plasma antioxidant capacity (Wu et al., 2001). Recent reports have provided evidence that arsenic can cause cell damage, chromosome instability, cell proliferation, and alter telomerase activity and apoptosis.

These alterations may be involved in tumor progression or tumorigenesis through activation of oxidative-sensitive signaling pathways (Kamat et al., 2005; Liu et al., 2003; Zhang et al., 2003).

ROS can interact with DNA to produce damage including single- and double-stranded DNA breaks, deletions, and nucleoside modifications (Valko et al., 2006). 8-Hydroxy-2'-deoxyguanosine (8-OHdG), the oxidized form of the nucleoside 2'-deoxyguanosine present in DNA, is one of the most reliable and abundant markers of DNA damage because it reflects extremely low levels of oxidative damage (Howard et al., 1998). Previous studies demonstrated that urinary 8-OHdG levels are higher in smokers, cancer patients, chronic renal failure patients, and semiconductor workers with greater urinary arsenic and chromium exposure (Akagi et al., 2003; Hu et al., 2006; Kimura et al., 2006; Mizoue et al., 2006; Rozalski et al., 2002). In addition, it was suggested that 4 months of 4 cups/day of green tea consumption is significantly associated with decreased urinary 8-OHdG levels among heavy smokers (Hakim et al., 2004).

To investigate the relationship between urinary 8-OHdG levels and the development of arsenic-associated urothelial carcinoma (UC) among subjects who had no evident arsenic exposure history.

Materials and Methods

Study population. This was a hospital-based case-control study. Study methods have been described in detail elsewhere (Pu et al., 2007). Briefly, the study population consisted of 170 UC cases and 402 healthy control participants from September 2002 to April 2006. All cases were diagnosed UC patients with histological confirmation. Pathological verification of UC was done by routine urological practice including endoscopic biopsy or surgical resection of urinary tract tumors followed by histopathological examination by board-certified pathologists. Cytological evidence alone was not accepted as an adequate diagnosis of UC. Bladder cancer was staged into three groups: superficial (Ta, T1 and Tis), locally advanced (T2-4N0M0), and metastatic (N+ or M+). Tumor grading was based on the WHO 1999 classification system (WHO, 1999). Controls were frequency matched to UC cases in terms of age,

± 5 years, and gender. Healthy controls have no prior history of cancer. The majority of study population (>80%) lived in Taipei city, and recruited from the medical center including National Taiwan University Hospital and Taipei Municipal Wan Fang Hospital. These hospitals are located in Taipei approximately 200 to 300 kilometers away from the arsenic-contaminated areas in Taiwan. **All subjects mostly came from Taipei and drank tap water from Taipei Water Department of the Taipei City Government. And the average arsenic concentration tap water is 0.7 µg/L with ranges from non-detectable to 4.0 µg/L.** No case subjects or controls came from arsenic-contaminated areas in southwestern (Chen et al., 2003) or northeastern Taiwan (Chiou et al., 2001). The Research Ethics Committee of National Taiwan University Hospital, Taipei, Taiwan, approved the study. All patients provided informed consent forms before sample and data collection. The study was consistent with the World Medical Association Declaration of Helsinki.

Questionnaire interview and participant specimen collection. Standardized personal interviews based on a structured questionnaire were carried out by well-trained personnel. Information collected included: demographic and socioeconomic characteristics; general potential risk factors for malignancies such as lifestyle, cigarette smoking, alcohol, tea, and coffee consumption; occupational history; as well as personal and family histories of disease. Status as never, former, or current classified smoking history at the time of diagnosis. **Spot urine samples were collected from all participants and immediately transferred to -20°C freezer until further use for urinary arsenic and 8-OHdG levels analysis.**

Measurements of urinary arsenic species. It has been shown that urinary arsenic species are stable for at least 6 months when preserved at -20°C (Chen et al., 2002); therefore, the urine sample assay was performed within 6 months post-collection. Urinary arsenic species concentrations were determined using high-performance liquid chromatography (HPLC), linked on line a to hydride generator and atomic absorption spectrometric (HG-AAS) method (Hsueh et al., 1998). Briefly, an aliquot of 200 µL was used for separation of arsenic species by HPLC (Waters 501, Waters Associates, Milford, MA, USA), and then the levels of the individual arsenic species including **iAs^{3+}, iAs^{5+}, MMA^{5+} and DMA^{5+}** were quantified by HG-AAS. Recovery rates for iAs^{3+}, DMA^{5+}, MMA^{5+}, and iAs^{5+} ranged from 93.8% to 102.2% with detection limits of 0.02, 0.08, 0.05, and 0.07 µg/L, respectively. Freeze-dried SRM

2670 urine, which was obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) containing 480 ± 100 μg/L arsenic, was analyzed together with urine samples of subjects as a quality control. **A measurement of total arsenic independent to the analysis of arsenic species in SRM 2670** is 507 ± 17 μg/L (n = 4) was recorded.

Determination of urinary 8-OHdG levels. Urinary specimens were centrifuged at 1,500 rpm for 10 min to remove particulates. The supernatants were used for the measurement of the 8-OHdG levels using a competitive in vitro enzyme-linked immunosorbent assay (ELISA) kit (Japan Institute for the Control of Aging, Fukuroi, Japan) (Saito et al., 2000). A 50 μl urine sample and 50 μl of reconstituted primary antibody were added into each well of a 8-OHdG coated microtiter plate and incubated at 37°C for 1 h for the ELISA assay. The antibodies in the sample bound to the coated 8-OHdG were washed three times with phosphate-buffered saline. The horseradish peroxidase-conjugated secondary antibody was added to the plate, followed by incubation at 37°C for 1 h, and the unbound enzyme-labeled secondary antibody was removed and the plates again washed three times. The amount of antibody bound to the plate was determined by the development of color intensity after the addition of a substrate containing 3,3',5,5'-tetra-methyl-benzidine. The reaction was terminated by the addition of phosphoric acid, and the absorbance was measured using a computer-controlled spectrophotometric plate reader at a wavelength of 450 nm. The concentration of 8-OHdG of the urine samples was interpolated from a standard curve drawn with the assistance of logarithmic transformation. The detection range of the ELISA assay was 0.5 to 200 ng/mL. The intra-assay coefficient of variance (CV) was 9.8%, and the inter-assay CV was 6.7%.

Statistical analysis. Total arsenic concentration (μg/L) was the sum of urinary inorganic arsenic (iAs^{3+}and iAs^{5+}), and its metabolites such as MMA^{5+} and DMA^{5+}. The arsenic methylation capability was assessed by primary methylation index (PMI), defined as the ratio between the MMA^{5+} and inorganic arsenic levels, and secondary methylation index (SMI), defined as the ratio between DMA^{5+} and MMA^{5+} (Tseng et al., 2005). A decrease of PMI and/or a decrease of SMI reflected a decrease methylation capability. **Each individual arsenic and 8-OHdG measurement was $\log_{10}$-transformed to stabilize the variance and to approach to normal distribution. All tests of difference between arsenic and**

8-OHdG levels presented in the figures and tables were log_{10}-transformed. Student's t test was used to compare the differences of urinary arsenic profile and 8-OHdG levels between UC cases and healthy controls. ANOVA test was used to evaluate the differences of urinary 8-OHdG levels between more than two strata of baseline characteristics. Pearson's correlation was used to assess the relationship between urinary 8-OHdG levels and the concentrations of various arsenic species. Subsequently, we developed a multiple linear regression model to estimate the main effects of various arsenic species on urinary 8-OHdG, with adjustment for potential confounders. All data were analyzed using the SAS statistical package (SAS, version 8.0, Cary, NC). A p value of < 0.05 (two-sided) was considered significant.

Results

A total of 572 subjects, 170 UC patients and 402 healthy controls, were included in this study. Their average age was 61.7 with a standard error of 0.6 years. The percentages of former smokers and current smokers were 25.1% and 16.5% respectively. The urinary arsenic species concentrations of all subjects are shown in Table 1. **The healthy controls age ≥ 63 years had significantly higher total arsenic, DMA^{5+} and PMI than those in controls age < 63 years. In addition, females had significantly lower concentrations of iAs^{3+} and MMA than males. UC patients had higher PMI and lower SMI than healthy controls. After adjusting for age, gender, cigarette smoking and urinary creatinine, a strong dose-response relationship was found between urinary total arsenic concentrations and the risk of UC (trend analysis $p < 0.01$) (data not shown). Subjects with urinary total arsenic ≥ 26.70 μg/L had a significantly higher risk of UC compared to those with a urinary total arsenic < 10.95 μg/L (Odds ratio (OR) = 7.11, 95% confidence interval (CI), 3.52 to 14.37) (data not shown).**

The median urinary 8-OHdG levels for all study subjects was 3.54 ng/mL (range, 0.19 to 48.27). UC subjects had a significantly lower urinary 8-OHdG level than healthy controls ($p < 0.05$) **under non-adjustment for creatinine** (Table 2). **Neither age nor gender had insignificant effects on 8-OHdG levels in both healthy controls and UC patients.** Urinary 8-OHdG levels significantly differ among different total arsenic strata. Notably, urinary 8-OHdG levels did not increase with cigarette smoking or with UC stage or grade. **After adjusting for age, gender and**

urine creatinine, log_{10}-transformed urinary 8-OHdG levels were found to be significantly associated with the log_{10}-transformed concentrations of iAs^{3+}, MMA^{5+}, DMA^{5+}, total arsenic and PMI (Pearson's correlation r = 0.33 for iAs^{3+}, p < 0.01; r = 0.36 for MMA^{5+}, p < 0.01; r = 0.56 for DMA^{5+}, p < 0.01; r = 0.53 for total arsenic, p < 0.01 and r = 0.23 for PMI, p < 0.01) as shown in Figure 1. Because the PMI value is available in those inorganic arsenic levels > 0, the subject number is 520.

Table 3 shows multiple linear regression analyses predicting the level of urinary 8-OHdG. There were significant correlations between urinary 8-OHdG levels and the concentration of most arsenic species, including iAs^{3+}, MMA^{5+}, DMA^{5+}, total arsenics, and PMI. **Neither disease state nor cigarette smoking affected urinary 8-OHdG levels after adjusting for age, gender, and urine creatinine. Our previous study found increase cancer risk associated with MMA percentage (Pu et al., 2007); however, we did not find the relationship between percentage of MMA and 8-OHdG, but 8-OHdG was significantly positively associated with DMA percentage (data not shown).** In addition, elevated urinary 8-OHdG levels were not associated with an increased UC risk (data not shown). The data were adjusted for significant risk factors of age, gender and creatinine in the multiple linear regression models. These adjustments indicated that age, gender, and arsenic species concentrations including iAs^{3+}, MMA^{5+}, DMA^{5+}, total arsenics, and PMI were significantly associated with urinary 8-OHdG levels.

Discussion

Our study evaluated the oxidative stress in UC patients and healthy controls by measuring urinary 8-OHdG levels, which was found to be correlated with the levels of individual urinary arsenic species. **Lower percentages of ever smokers was 41.6% in this study compared to 53.6% of the official statistical survey from Taiwanese age >18 years old. Hence, in our study we did not observe the effect of cigarettes smoking on oxidative stress, which was the same as Wen et al. study (Wen et al., 2005). The effects of alcohol, tea, coffee, hair dyes, and analgesic medicines were eliminated from having had any effects on urinary 8-OHdG levels, because there were no significant associations between these variables and urinary 8-OHdG levels in our study. Therefore, we might accept that urinary arsenic species were**

the main effect on evaluated 8-OHdG levels.

Recently, the risk of low doses arsenic has been a questioned in the US, European Union, and other countries. The European Union adopted a new drinking water standard of 10 μg/L for arsenic in 2003 while the US Environmental Protection Agency had not adopted the new standard of 10 μg/L until 2006. Some developing countries such as Bangladesh have kept their arsenic standard at 50 μg/L (Tapio and Grosche, 2006). In Taiwan, the standard of arsenic concentration in drinking water was decreased from 50 to 10 μg/L in 2000. There may be minor differences in arsenic levels between various regions in Taiwan. **However, majority of our study population (>80%) lived in Taipei city.** All subjects recruited in this study had a urinary total arsenic concentration of 20 to 40 μg/L even though they had consumed drinking water containing low arsenic concentration for many years. Besides, we found subjects who have an unfavorable urinary arsenic profile have an increased UC risk even at low exposure levels recently (Pu et al., 2007). The exact origin of any other possible environmental sources of inorganic arsenic in these subjects is unknown. These study subjects had significantly lower urinary total arsenic concentrations than the residents of the Blackfoot disease endemic area whose urinary total arsenic ranged from 60 to 90 μg/L (Tseng et al., 2005). These results showed that UC patients had a significantly increased urinary arsenic profile compared to healthy controls. The evidence for arsenic-associated bladder cancer was previously shown with animal models and human studies primarily through measuring environmental arsenic concentrations in drinking water (Chiou et al., 2001; Karagas et al., 2004; Su et al., 2006). In addition, in a study by Steinmaus et al. (2005), the mean urinary arsenic concentration was 27.8 μg/L among metabolic products measured in urine repeatedly collected over nearly 1 year from 81 individuals, while the adjusted urinary total arsenic concentrations in individuals remained constant over time (Steinmaus et al., 2005). In the following year, Steinmaus et al. studied 137 patients with bladder cancer and 163 controls from Argentina and the US. They measured the individual urinary arsenic species and found that individuals who excreted an increased proportion of the MMA species were more susceptible to arsenic-related bladder cancer (Steinmaus et al., 2006). However, two other studies have demonstrated that the association of low arsenic and UC risk only existed among smokers (Bates et al., 2004; Steinmaus et al., 2003).

Conflicting data has existed for the relationship between 8-OHdG production and age, gender, cigarette smoking, and alcohol consumption (Irie et al., 2005; Proteggente et al., 2002; Yamauchi et al., 2004). We found an age-related increase in urinary 8-OHdG levels, which supports the results of Dhawan et al. (2005). They showed that 8-OHdG levels were positively correlated with age in patients with essential hypertension (Dhawan et al., 2005). In a Japanese study based on 372 healthy workers, Irie et al. showed that males had higher urinary 8-OHdG levels than females (mean ± standard error, 4.17 ± 0.10 vs. 3.20 ± 0.20, $p < 0.01$, respectively). In addition, smokers and alcohol consumers were reported to have higher urinary 8-OHdG levels than non-smokers, and those not consuming alcohol (Irie et al., 2005; Kimura et al., 2006). However, Kimura et al. (2006) studied 248 healthy Japanese and found that the mean urinary 8-OHdG levels did not significantly differ among groups based upon ages (< 45 and ≥ 45 years), gender, cigarette smoking status, or alcohol consumption (Kimura et al., 2006). In the present study females were found to have significantly higher urinary 8-OHdG levels than males. The reason remains to be investigated. Until now, little information is available on the effects of other oxidative stress sources such as coffee and tea consumption, hair dyes, and medicines. A randomized controlled study in 2003 revealed that regular green tea consumption might protect smokers from oxidative damage and that drinking decaffeinated green tea for four months was associated with a significant decrease in urinary 8-OHdG levels (Hakim et al., 2003). The present study did not find a significant association between urinary 8-OHdG levels and UC-related risk factors such as cigarette smoking, tea and alcohol consumption, hair dyes, and clinical stage or grade. This may be related to small numbers of subjects with these risk factors.

Although arsenic is a human carcinogen, the mechanism of arsenic carcinogenesis is largely unknown. Recent advances from in vivo studies have provided strong evidence for arsenic-induced ROS generation. It has been shown that inorganic arsenic induced concentration-dependent and time-dependent superoxide generation in a human keratinocyte cell line (Shi et al., 2004). Dimethylated arsenic peroxide was produced by the reaction of trivalent dimethylated arsenic with molecular oxygen (Yamanaka et al., 2004). Therefore, **trivalent dimethylated arsenic** might be more genotoxic than inorganic arsenic. Furthermore, Wu et al. recruited 64 residents of the Lanyang Basin in northeastern Taiwan and measured their reactive oxidants and

antioxidant capacity in plasma. A positive association was found between the blood arsenic concentrations and levels of reactive oxidants and an inverse relationship was found between blood arsenic concentrations and levels of plasma antioxidant capacity (Wu et al., 2001). Oxidative stress induced by reactive oxygen species has been shown to be involved in several pathophysiological processes, such as cell proliferation, differentiation, apoptosis, and carcinogenesis. Bashir et al. demonstrated that exposure to arsenate may increase apoptosis in rat liver cells (Bashir et al., 2005). Mesencephalic cells treated with low concentrations of sodium arsenate resulted in the activation of early transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), which regulate the expression of a variety of downstream target genes, such as proinflammatory genes that are known to be involved in carcinogenesis (Felix et al., 2005). Our results showed an increase in urinary 8-OHdG levels and an increased iAs^{3+}, MMA, DMA, total arsenics, and PMI. These results are compatible with the association of urine creatinine adjusted 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) with MMA and PMI, with correlation coefficients of 0.44 and 0.40 ($p < 0.005$), respectively among semiconductor workers with arsenic exposure as suggested by Hu et al. (Hu et al., 2006). Because the workers had been exposed to arsenic, the total arsenic concentrations and urinary 8-OHdG were higher than the participants in our study. Even with low urinary total arsenic concentrations, a clear association was observed between urinary total arsenic concentrations and 8-OHdG levels.

Our study has several limitations that need to be considered when interpreting our results. In the current study, selection bias was minimized even through cases and controls recruited from two different hospitals, because these hospitals both belonged to medical centers and located in southern Taipei. Furthermore, majority of cases and controls lived in Taipei and were similar to each other in socioeconomic characteristics. The UC patients were prevalence cases and some individuals might have changed their diet habit or increased vitamins consumption to such an extent that their measured levels of urinary 8-OHdG were lower compared to those of other studies (Miyake et al., 2004; Yamauchi et al., 2004). **In addition, we only collected tap water from 37 subjects and the mean (standard error) of total arsenic level is 17.14 (0.55) μg/L. Nevertheless we did not collect the quantity of drinking water and could not explore their historical arsenic exposure. Finally, the accuracy of**

one spot evaluation of urinary arsenic and 8-OHdG may be in doubt. However, the values might be reliable under no change of life style in all subjects. Future studies should evaluate in more detail exposure to arsenic and 8-OHdG levels to elucidate the mechanisms of oxidative stress in arsenic carcinogenesis.

Conclusions

To our knowledge, this is the first study showing that urinary 8-OHdG levels are correlated with individual urinary arsenic species concentrations in a human population with low arsenic exposure. Our data provide evidence that chronic low arsenic exposure from drinking water in humans may be related to the induction of oxidative stress as indicated by the increase in urinary 8-OHdG levels. Arsenic-induced oxidative stress was associated with high levels of iAs^{3+}, MMA^{5+}, DMA^{5+}, and PMI.

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