Polymorphisms in Cell Cycle Regulatory Genes, Urinary Arsenic Profile and Urothelial Carcinoma

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Abstract

Introduction: Polymorphisms in p53, p21 and CCND1 could regulate the progression of the cell cycle and might increase the susceptibility to arsenic-related cancer risk. The goal of our study was to evaluate the roles of cell cycle regulatory gene polymorphisms in the carcinogenesis of arsenic-related urothelial carcinoma (UC).

Methods: A hospital-based case-controlled study was conducted to explore the relationships between the urinary arsenic profile, 8-hydroxydeoxyguanine (8-OHdG) levels, p53 codon 72, p21 codon 31 and CCND1 G870A polymorphisms and UC risk. The urinary arsenic profile was determined using high-performance liquid chromatography (HPLC) and hydride generator-atomic absorption spectrometry (HG-AAS). 8-OHdG levels were measured by high-sensitivity enzyme-linked immunosorbent assay (ELISA) kits. Genotyping was conducted using polymerase chain reaction-restriction fragment length polymerase (PCR-RFLP).

Results: Subjects carrying the p21 Arg/Arg genotype had an increased UC risk (age and gender adjusted OR=1.53; 95% CI, 1.02-2.29). However, there was no association of p53 or CCND1 polymorphisms with UC risk. Significant effects were observed in terms of a combination of the three gene polymorphisms and a cumulative exposure of cigarette smoking, along with the urinary arsenic species indices on the UC risk. The higher total arsenic concentration, MMA% groups and lower DMA% groups, that possessed greater gene variant numbers, had a higher UC risk and revealed significant dose-response relationships. However, effects of urinary 8-OHdG levels combined with three gene polymorphisms did not seem to be important for UC risk.

Conclusions: The results showed that the variant genotype of p21 might be a predictor of arsenic-related UC risk.

Key words: Urothelial carcinoma; 8-hydroxydeoxyguanine; urinary arsenic profile, cell cycle, p53, p21, CCND1, polymorphism
Introduction

Bladder cancer is the most common malignancy of the urinary tract and the ninth most common cancer in Taiwan. It was estimated that 1,858 new cases would be diagnosed and 634 deaths would occur in Taiwan in 2003 (Department of Health, the Executive Yuan, 2007). Tobacco smoking, the most important risk factor for bladder cancer, accounted for ~50% of all cases. In our previous studies (Pu et al., 2007) we found that there was an approximately two-fold higher risk of urothelial carcinoma (UC) risk in smokers than in non-smokers. Furthermore, smoking and the urinary arsenic profile were found to be related factors affecting the UC risk.

Arsenic is recognized as a potent human carcinogen. Epidemiological studies of the carcinogenicity of arsenic have been demonstrated dose-response relationships between arsenic exposure and cancers of the skin, lung, bladder, liver and kidney among arsenic-exposed populations (Bates et al., 1992; Tapio et al., 2006; Yoshida et al., 2004). The carcinogenic mechanism underlying the effects of arsenic remain unclear although putative carcinogenic modes of action have recently been proposed, implicating chromosome abnormalities, oxidative stress, altered growth factors and DNA repair as well as cell proliferation (Kitchin, 2001). Recently, we have shown that subjects who had an unfavorable urinary arsenic profile had an increased UC risk, even at low exposure levels (Pu et al., 2007). In addition, urinary arsenic was found to be associated with oxidative DNA damage by using measurements of 8-OHdG levels (Chung et al., 2008). It is therefore worthwhile to examine the mechanisms underlying the ability of inorganic arsenic to induce UC.

It is a well known that arsenic can cause DNA damage, as well as inhibiting the DNA repair pathway and this may cause genomic instability (Gonsebatt et al., 1992; Hartwig et al., 2003). The tumor suppressor protein p53 plays a major role in the control of cell growth and maintenance of genomic stability (De et al., 2006). The codon 72 Arg/Pro polymorphism of p53 that changes an arginine (Arg) to a proline (Pro), due to a G to C transversion was the first to be described (Sun et al., 1999). Missense mutations of the p53 gene marks changes in the structure of the p53 protein and were found to be among the most common genetic alterations in human disease or that led to cancer susceptibility (Furihata et al., 2002). Several studies on breast and bladder cancer have examined the relationship between p53 codon 72 polymorphism
and various malignancies, although with conflicting results (Kuroda et al., 2003; Papadakis et al., 2000; Sjalander et al., 1996; Soulitzis et al., 2002).

Cell proliferation depends on an ordered and tightly regulated process known as the cell cycle, which is regulated by complexes composed of regulatory cyclins and catalytic cyclin-dependent kinases (CDKs) (Hernandez-Zavala et al., 2005). p21, also known as CIP1/WAF1, is a cyclin kinase inhibitor that acts as a negative regulator of the cell cycle by suppressing the activity of CDKs (Cai et al., 1998) and acts as a downstream mediator of p53 tumor suppression (Xiong et al., 1993). The p21 gene has a p53 transcriptional regulatory motif, and cells lacking functional p53 express very low levels of p21, suggesting that p53 directly regulates p21 expression (Gumerlock et al., 1997). Cyclin D1, an intracellular cell-cycle regulatory protein with checkpoint function, plays an important role in the transition from the G1 to S phase of the cell cycle and can promote cell proliferation, or induce growth arrest and apoptosis, depending on the cellular context (Ceschi et al., 2005; Yu et al., 2003). Several polymorphisms in the p21 and CCND1 gene loci have been described. A polymorphism in the p21 codon 31 has been found which produces a C-to-A change, causing a substitution from serine (Ser) to arginine (Arg) (Mousses et al., 1995). This polymorphism probably encodes for a DNA-binding zinc-finger domain, causing functional changes to the p21 protein (Ressiniotis et al., 2005). Gene alterations of p21 might be able interrupt the p53-mediated pathway of cell cycle arrest and increase the susceptibility for cancer (Mousses et al., 1995). A series of epidemiological studies found that p21 codon 31 polymorphism was associated with increased risks of lung, cervical, breast, esophageal, and nasopharyngeal cancers (Keshava et al., 2002; Roh et al., 2001; Tsai et al., 2002; Sjalander et al., 1996), although conflicting findings have been reported (Su et al., 2003; Sun et al., 1995). The gene encoding cyclin D1, CCND1, has a common G870A polymorphism at codon 242 in exon 4 that increases the frequency of alternate splicing, leading to an altered protein (Betticher et al., 1995). Several association studies show the CCND1 870AA genotype to be at an increased risk for several malignancies, including for example in the upper aero-digestive tract, larynx and colon (Izzo et al., 2003; Yu et al., 2003).

To our knowledge, no study has simultaneously examined the effect of three polymorphisms in cell cycle regulatory genes on UC risk. Furthermore, the hypothesis
that arsenic-related UC risk is associated polymorphisms of cell cycle regulatory genes under higher oxidative stress was tested.

**Materials and Methods**

**Study participants.** We conducted a hospital-based case-controlled study. The Study design has been described previously (Chung et al., 2008). Briefly, from September 2002 to April 2006, we recruited 170 UC cases and 402 healthy control participants from the Medical Center that includes National Taiwan University Hospital and Taipei Municipal Wan Fang Hospital. All UC cases were diagnosed by histological confirmation. Healthy controls were frequency matched to UC cases in terms of age, ± 5 years, as well as gender and had no prior history of cancer. All study participants mostly came from Taipei city (>80%) and drank tap water. The average arsenic concentration of tap water is 0.7 µg/L (ranging from undetectable to 4.0 µg/L), according to the Taipei Water Department of the Taipei City Government. Informed consent forms were provided by all participants before a questionnaire interview and collection of biological specimens. The Research Ethics Committee of the National Taiwan University Hospital, Taipei, Taiwan, approved the study and it was consistent with the World Medical Association Declaration of Helsinki.

**Questionnaire interview and biological specimen collection.** Trained interviewers collected information through a face-to-face interview based on a structured questionnaire. The context of the questionnaire included demographics and socioeconomic characteristics, lifestyle-related risk factors for UC risk, such as cigarette smoking and alcohol, tea and coffee consumption, also hair dye use, as well as personal and family histories of disease. Spot urine samples were collected at the time of recruitment and immediately transferred to a -20°C freezer until required for urinary arsenic species and 8-OHdG levels analyses. At the same time, blood specimens were collected and frozen at -80°C for DNA extraction.

**Urinary arsenic species and 8-OHdG assessment.** Urinary arsenic species, including iAs$^{3+}$, iAs$^{5+}$, MMA$^{5+}$ and DMA$^{5+}$ were measured by high-performance liquid chromatography (HPLC), equipped with a hydride generator and atomic absorption spectrometer (HG-AAS), as previously described (Hsueh et al., 1998). Ranges for iAs$^{3+}$, DMA$^{5+}$, MMA$^{5+}$, and iAs$^{5+}$ recovery rates were from 93.8% to
102.2%, with detection limits of 0.02, 0.08, 0.05, and 0.07 µg/L, respectively. Urinary 8-OHdG measurement was analyzed with a competitive enzyme-linked immunosorbent assay (ELISA) kit in vitro (Japan Institute for the Control of Aging, Fukuroi, Japan), as described in detail elsewhere (Chung et al., 2008). The detection range of the ELISA assay was 0.5-200 ng/mL. The intra-and inter assay coefficient of variance (CV) was 9.8% and 6.7%, respectively. Chen et al. (2002) have shown that urinary arsenic species are stable for at least 6 months when preserved at -20°C (Chen et al., 2002b), the urine sample assay of arsenic species and 8-OHdG levels were therefore performed within 6 months post-collection.

**Genotyping.** Genomic DNA for analysis was extracted from blood specimens using proteinase K digestion following phenol and chloroform extraction. Genotyping for single nucleotide polymorphisms (SNPs) in p53, p21 and CCND1 was carried out for all participants by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique (Ara et al., 1990; Le et al., 2003; Li et al., 1995). Briefly, the primers 5’-TTGCCGTCCCAAGCAATGGATGA-3’ (forward) and 5’-TCTGGGAAGGGACAGAAGATGAC-3’ (backward) for p53 Arg72Pro polymorphism, 5’-GTCAGAACCGGCTGGGGATG-3’ and 5’-CTCCTCCCAACTCATCCCGG-3’ for p21 Ser31Arg polymorphism as well as 5’-AGTTTCATTCTCCAATCGCCC-3’ and 5’-TTTCCGTGGCAGTGTGC-3’ for CCND1 G870A polymorphism were used to amplify 199 bp, 272 bp and 212 bp PCR products, respectively. PCR products were obtained in a total volume of 30 µL, consisting of an 80 ng sample DNA, 10x PCR buffer, 2.5 mM dNTP, 2 µM of each primer and 2 U Taq polymerase. After initial denaturation for 5 min at 94 °C, 35 cycles were performed at 94°C, 30 sec (denaturation), at 55°C, 1 min (annealing) and at 72°C, 1 min (extension) for p53, 94°C, 40 sec, at 61 °C, 30 sec and at 72°C, 40 sec for p21, and 94°C, 30 sec, at 60°C, 30 sec and at 72°C, 30 sec for CCND1 followed by a final step for 5 min at 72°C. The amplified products were visualized by electrophoresis in a 2 % agarose gel. PCR products were digested with BstUI (>12 hrs, at 60°C) for p53, BlpI (5 hrs, at 37°C) for p21 andMspI (2 hrs, at 37°C) for CCND1. Genotypes were analyzed by electrophoresis on 3% agarose gels. For quality control, a random 5% of the samples were repeated with a concordance of 100%.

**Statistical analysis.** The total arsenic concentration (µg/g creatinine) was determined
by as the sum of iAs$^{3+}$, iAs$^{5+}$, MMA$^{5+}$ and DMA$^{5+}$. The relative proportion of each arsenic species (InAs%, MMA% and DMA%) was calculated by dividing the concentration of each species by the total arsenic concentration. A Log$_{10}$-transformation was applied to the 8-OHdG levels with an abnormal distribution before statistical analyses being performed. Median or triple values of age, urinary arsenic species percentage, cumulative exposure of cigarette smoking and 8-OHdG levels were used as a cutoff. The Student’s t test was used to compare the differences of variables between two levels of genotypes. A one-way ANOVA followed by a Scheffe’s test was used to compare the differences between three levels of genotypes. The frequency distribution of the polymorphisms was tested in the control group to ensure a Hardy-Weinberg equilibrium. Also we performed a multivariate logistic regression analysis to estimate odds ratios (ORs) and 95% confidence intervals (CIs), to evaluate the impact of relevant variables (cumulative exposure of cigarette smoking, urinary arsenic species percentage, 8-OHdG levels and related polymorphisms) on UC risk, after adjustment for other confounders. In our previous work, we described the UC-related risk factors, including education, paternal and maternal ethnicity, alcohol drinking and pesticide usage (Pu et al., 2007), so we adjusted for these variables in the final multivariate logistic regression. All data analyses were performed out using the SAS statistical package (SAS, version 8.0, Cary, NC).

Results

Subjects who had higher educational levels had a lower risk UC than those with lower educational levels. Mainland Chinese had a significantly lower UC risk than the Fukien Taiwanese. Age, ABO blood type, marital status or use of hair dye did not affect the UC risk. Occasional alcohol drinkers had a significantly lower UC risk than non-drinkers and frequent drinkers. Pesticide users had a significantly higher UC risk than non-users in this study, as described in our previous work (Pu et al., 2007). Age, gender, urinary 8-OHdG levels and $p53$, $p21$ and $CCND1$ genotype frequency data are shown in Table 1. The urinary 8-OHdG levels in 170 UC patients were significantly higher than in the 402 controls ($p = 0.03$). After adjusting for age and gender, a significantly elevated risk of UC with increasing urinary 8-OHdG levels (per unit) was found. However, a significant dose-response relationship between urinary 8-OHdG levels and UC risk was not observed. In controls, genotype distributions of
p53 codon 72, p21 codon 31 and CCND1 G870A were fitted in Hardy–Weinberg equilibrium. For the p21 codon 31 polymorphism, the number of cases (n = 51) that were carrying 30.18% of the variant homozygote genotype (Arg/Arg) was higher than that of the controls at 22.14% (n = 89). Subjects carrying the Arg/Arg genotype had a significantly increased risk of UC (OR = 1.53; 95% CI, 1.02 - 2.29), compared to those carrying Ser/Ser or Ser/Arg genotypes, after adjustment for age and gender. However, we did not detect a significant association between the p53 or CCND1 genotype and UC risk. The percentage of UC and urinary arsenic species, cumulative exposure of cigarette smoking and 8-OHdG levels for different genotype groups are shown in Table 2. The median ± standard error of relevant variables is similar between the two genotype groups, or among three genotype groups in p53, p21 and CCND1. Subjects carrying the p53 Arg/Pro or a Pro/Pro genotype had a higher DMA% than those with the Arg/Arg genotype. In addition, subjects with the CCND1 AA genotype had higher urinary 8-OHdG levels than those with the GA genotype (p<0.05). Multivariate-adjusted ORs for the combination of the effects of the environmental exposure and p53, p21 and CCND1 polymorphisms on the UC risk are shown in Table 3. For p53 polymorphism, those who had at least one variant allele and the highest level group of cumulative exposure of cigarette smoking, total arsenic, InAs%, and MMA% and the group with lower levels of DMA%, had a higher UC risk compared to those who had no variant genotype or the group with the lower levels of cumulative exposure of cigarette smoking and total arsenic, InAs%, and MMA% and highest level group of DMA%. The similar patterns were also observed in subjects carrying the p21 Arg/Arg genotype or carrying the CCND1 GA or AA genotype. Here, we did not observe a significant combination of effects for the three gene polymorphisms and urinary 8-OHdG levels on UC risk. The gene-gene interaction and gene-gene-environment interaction is shown in Table 4. Individuals with the p53 Arg/Arg genotype, p21 Ser/Ser or Ser/Arg genotype or CCND1 GG genotype were used as reference values and individuals with one to three gene variants were compared with the respective reference group. We evaluated simultaneously the three gene variants on the UC risk and combined the effects of urinary arsenic species percentage, 8-OHdG levels and cumulative exposure of cigarette smoking. There were significant dose-response relationships among those subjects carrying greater numbers of gene variants and with higher environmental exposure on UC risk, these including cumulative exposure of cigarette smoking, total arsenic concentration, and
MMA% (trend p value < 0.05). Furthermore, those with a lower DMA% and with greater gene variants had an increased UC risk (trend p value < 0.05).

**Discussion**

To our knowledge, this study is the first to simultaneously evaluate the impact of $p53$, $p21$, and $CCND1$ cell cycle regulatory gene polymorphisms on the susceptibility to arsenic-related UC risk. A significant association between $p21$ polymorphism and UC risk (Table 1), after adjustment for age and gender (OR=1.53; 95%CI, 1.02-2.29), was clearly observable. Also individuals harboring a greater number of cell cycle regulatory gene variants had a significantly increased risk of UC, when they also had a higher cumulative exposure of cigarette smoking, total arsenic, InAs%, MMA% and decreased DMA% than those with lower levels of these variables.

Previous associations among the $p53$ codon72, $CCND1$ G870A polymorphisms and cancer risk have been reported for esophageal, lung and cervical cancers as well as sporadic adenoma (Jain et al., 2007; Lee et al., 2006; Lewis et al., 2003; Settheetham-Ishida et al., 2004; Zhang et al., 2006). However, our results indicate that $p53$ codon72 and $CCND1$ G870A polymorphisms are not associated with UC risk, which is in agreement with previous studies of bladder cancer for $p53$ codon72 polymorphism (Chen et al., 2004; Toruner et al., 2001), as well as with other studies of Cortessis et al. on bladder cancer and Yu et al. on esophageal squamous cell carcinoma for $CCND1$ G870A polymorphism (Cortessis et al., 2003; Yu et al., 2003). For each of cell cycle regulatory gene polymorphism, we found that the genotype distributions all met Hardy-Weinberg equilibrium conditions. The diverse results may be due to the difference in ethnic background leading to a variation in the allele frequency. The allele frequencies shown in the present study are more or less similar to those reported in northern and southern Taiwan (Huang et al., 2004; Wong et al., 2003; Wu et al., 2003). Our findings suggest that $p53$ codon 72 and $CCND1$ G870A polymorphisms may not influence the development of UC.

$p21$ is a cyclin-dependent kinase inhibitor resulting in cell-cycle arrest by inhibiting the G1 to S phase stage (Maddika et al., 2007) and is upregulated by wild-type tumor suppressor protein $p53$ in cell cycle. A transfection study in with human bladder cancer cells has shown that elevated $p21$ levels by adenoviral infection may be a
potent growth suppressor, especially in the WH cell line (Hall et al., 2000). Also, altered $p21$ expression, affecting the activity of CDKs, was observed among different cancers. Several immunohistochemical studies have shown the expression of $p21$ to be related to the clinical outcome and progression of cancers. Ozdemir et al. (2000) found that $p21$ over-expression was observed in about 52% of 60 patients with UC while only 39% of the tumors had a functional $p53$ loss (Ozdemir et al., 2000). It was pointed out that $p21$ expression was regulated by both $p53$-dependent and independent pathways, which is in agreement with the in vivo study of Makri et al. (1998) (Makri et al., 1998). Xie et al. (2004) indicated that a loss of $p21$ expression and a mutation of $p53$ were related to carcinogenesis in gastric carcinoma. They also showed that a loss of $p21$ expression is related to the TNM stage and invasion depth (Xie et al., 2004). Zlotta et al. (1999) revealed that a $p21$ over-expression positively correlated with $p53$ and was observed in 23 of 47 patients with superficial bladder tumors and that it was associated with shorter recurrence-free survival. These alternations of $p21$ expression might interrupt the cell cycle and be inducing apoptosis (Zlotta et al., 1999). The $p21$ gene is localized at chromosome 6p21.2 and the most abundant $p21$ polymorphism is located at codon 31, which involves a base change from AGC to AGA and amino acid changes from Serine to Arginine (el-Deiry et al., 1993; Koopmann et al., 1995; Xiong et al., 1993).

This polymorphism, within the DNA-binding zinc finger motif, might encode functionally distinct proteins resulting in an increasing susceptibility for cancer (Chedid et al., 1994; Huppi et al., 1994). Previous studies have demonstrated people carrying $p21$ Arg/Arg genotype had increased risk of prostate, bladder and esophageal cancers (Huang et al., 2004; Chen et al., 2002a; Wu et al., 2003). However, Hsieh et al. (2001) reported no association between $p21$ codon 31 polymorphism and endometriosis in pre-menopausal Taiwanese women (Hsieh et al., 2001). In a case-controlled study of a small subset of 140 Caucasians, with primary open angle glaucoma (POAG) and 73 healthy individuals, Ressiniotis et al. found $p21$ codon 31 polymorphism did not contribute to the risk of POAG (Ressiniotis et al., 2005). In the present study, men carried a greater frequency of the $p21$ Arg/Arg genotype than women. Also after adjusting for age and gender, subjects with the Arg/Arg genotype had a 1.53-fold increased risk of developing UC, compared with those with Ser/Ser or Ser/Arg genotypes. Further study would be warranted in order to clarify the functional
effects of p21 codon 31 polymorphisms and to explore the importance of other polymorphic sites in p21 (Bahl et al., 2000; Ralhan et al., 2000) on UC risk.

The induction of p21 results in CDK inhibition and cell cycle arrest as well as preventing the replication of damaged DNA (Ko et al., 1996). Although the correlation between enzyme activity and genotype is unclear, our data shows that subjects with the p21 Arg/Arg variant genotype had increasing UC risks with increasing cumulative exposure of cigarette smoking, total arsenic, InAs%, MMA% and decreasing DMA%. In addition, the significant dose-response relationship of the occurrence of arsenic-related UC risk is due to hereditary differences in cell cycle regulatory genes, including p53 and CCND1 polymorphisms. Although arsenic toxicity has been suggested to be dependent on the species of arsenic and various mechanisms have been proposed to explain its effects, a postulated mechanism of toxicity for could be through oxidative stress (Cohen et al., 2007). Oxidative DNA damage, induced by environmental and endogenous processes, is repaired through efficient DNA repair mechanisms, as well as through cell cycle control, to maintain DNA integrity (Hartwig et al., 2002). Impairment of these protective repair mechanisms by arsenic exposure may lead to an increased risk of cancer (Chanda et al., 2006; Hamadeh et al., 1999; Kelsey et al., 2005; Moore et al., 2003).

Experimental studies of a sodium arsenite-treated HT1197 bladder cell line have showed that iAs (III) treatment (1-10 μM) for 24 h induced a dose-dependent increase in the proportion of cells in the S-phase. The change in S-phase was accompanied by an increase in p53 protein content and a transient increase in p21 protein levels under the time-course of iAs (III) effects (10 μM). This suggests that p21 is not able to block the activity of the CDK2-cyclin E complex and is unable to arrest cells in G1 and allowing progression to the S-phase (Hernandez-Zavala et al., 2005). Previously, we demonstrated that oxidative damage as urinary 8-OHdG levels, was associated with urinary arsenic levels at low arsenic exposure (Chung et al., 2008). Also UC cases with higher urinary 8-OHdG levels had a 2.19-fold increased risk, compared with healthily controls, after adjusting age and gender. In the present study, subjects carrying the variant genotype of cell cycle genes exposed to high total arsenic or possessing a poor arsenic methylation capability may lead to a dose-response increased risk of UC. However, we did not find an interaction of 8-OHdG levels (or cumulative exposure tobacco smoking) with cell cycle gene polymorphisms with
respect to UC risk. In addition to the regulation of cell cycle progression, one other response to DNA damage is the DNA-repair process (Hartwig et al., 2002). This suggests that arsenic could directly regulate cell cycle progression for increased UC risk and arsenic-induced oxidative damage might be repaired through other DNA mechanisms. Hence, in future studies we plan to evaluate the role of DNA-repair related gene polymorphisms and arsenic methylation capability on the UC risk.

In summary, this study shows that higher urinary 8-OHdG levels may be responsible for an increased UC risk. A combination of cell cycle gene polymorphisms with arsenic methylation capability (or cumulative exposure cigarette smoking) significantly modifies the UC risk. Moreover, a variant genotype of \( p21 \) might be a predictor of arsenic-related UC risk.

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