

## Differential Expression of Molecular Markers in Arsenic- and Non-arsenic-related Urothelial Cancer

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**Abstract.** *Background:* Little is known about the mechanisms of arsenic-related urothelial cancer (AsUC). The aim of this study was to reveal the differential expression of molecular markers between AsUC and non-arsenic-related UC (non-AsUC). *Materials and Methods:* Tissues of AsUC (n=33), non-AsUC (n=20) and normal bladder urothelia from patients with benign diseases (n=4) were examined for multiple selected molecular markers responsible for various cellular functions, including glutathione, GST- $\pi$ , Bcl-2, p53 and c-Fos. *Results:* The mean cellular glutathione content of normal mucosal samples ( $33.4 \pm 7.2$   $\mu$ M/mg protein) was significantly higher than either non-AsUC ( $22.8 \pm 1.8$ ,  $p=0.04$ ) or AsUC ( $16.4 \pm 1.6$ ,  $p=0.002$ ). The glutathione content of non-AsUC was higher than that of AsUC ( $p=0.012$ ). The expressions of Bcl-2 and c-Fos in AsUC were significantly higher than those in non-AsUC ( $p=0.004$  and  $p=0.02$ , respectively). *Conclusion:* The carcinogenic pathway for AsUC is different, in part, from that of non-AsUC. Cellular glutathione contents may be down-regulated during urothelial carcinogenesis. Bcl-2 and c-Fos may play important roles in arsenic-mediated carcinogenesis of the urothelium.

It is well known that chronic arseniasis is considered responsible for the endemicity of urothelial cancer (UC) in southwest Taiwan (1). UC may arise from the renal pelvis, ureter, urinary bladder and urethra. Long-term efforts have been devoted to exploring the epidemiology

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and pathophysiology of these arsenic-related UCs (AsUC) (2). Previous reports from other countries, such as Chile, America and Argentina, also indicated that the contamination of drinking water with arsenic was strongly associated with the occurrence of UCs in those areas (3-5). The molecular mechanisms underlying the arsenic-related carcinogenesis pathway remain, however, largely unknown. Most data were obtained from artificial models of either *in vitro* or animal studies (6).

Only a few reports in the literature have addressed the cellular or molecular changes secondary to arsenic exposure in the UC model. Shibata *et al.* reported that 62% of AsUC had p53 gene mutations (7). Warner *et al.* demonstrated that there were increased micronuclei in exfoliated bladder cells of individuals who chronically ingested arsenic-contaminated water in Nevada, USA (8). Smith *et al.* showed that, after reducing the intake of arsenic-contaminated water, exfoliated bladder cell micronuclei could be significantly decreased and this could serve as a potential biomarker for arsenic genotoxicity (9). Byrd *et al.* revealed that inorganic arseniasis was associated with a deregulated cell cycle control (10). However, more detailed and in-depth studies on arsenic-mediated carcinogenesis are still needed.

This is one of the few studies that has sought to reveal the differential expressions of molecular markers between AsUC and non-AsUC. Tissues of AsUC, non-AsUC and normal-appearing bladder mucosa from non-cancer patients were examined and compared for the expressions of multiple selected molecular factors that may be involved in arsenic-related carcinogenesis of the urothelium.

### Materials and Methods

**Tissue samples.** UC tumor samples and normal-appearing bladder mucosa were used to examine selected cellular and molecular

factors reported to be involved in the carcinogenesis of the urothelium. All UCs were confirmed by histopathology. The samples contained snap-frozen tissues (at  $-135^{\circ}\text{C}$ ) and paraffin-embedded archival tissues. Tissue collection was approved by the institutional review board of the National Taiwan University Hospital, Taiwan, R.O.C.

In total, tumor tissues of 33 AsUCs and 25 non-AsUCs, as well as 4 normal-appearing bladder mucosal samples from non-cancer patients, were studied. The tissues were harvested in such a way that neoplastic parts were separated from adjacent normal parts. All 33 patients with AsUC had lived or had been living in the arseniasis endemic area in southwest Taiwan, where people had drunk the arsenic-contaminated artesian well water for at least 10 years (2).

**Glutathione (GSH) contents.** GSH is the major antioxidant in cells and functions as a free radical scavenger, which may detoxify toxins or chemotherapeutic agents. Thus, the intracellular GSH content may be altered in urothelial carcinogenesis. Tissue extracts were prepared from frozen tissues by a standard extraction protocol. Briefly, about a  $0.5\text{-cm}^3$  tumor chip was used. The total protein was extracted by solubilizing with Laemmli buffer (PIERCE Life Science Co., Rockford, IL, USA). The protein contents of tissue extracts were determined using a commercial BCA kit (PIERCE Life Science Co.). The cells ( $3 \times 10^6$ ) were resuspended in  $0.5\text{ ml}$  5% metaphosphoric acid (Sigma Chemical Co., St. Louis, MO, USA) on ice for 20 min and were then lysed by 3 cycles of freeze-and-thaw. The supernatant was collected by centrifugation. The cellular GSH content was examined using the GSH-400 kit (Oxis International, Portland, OR, USA). Briefly,  $50\text{ ml}$  supernatant were incubated with  $0.4\text{ ml}$  reaction buffer ( $200\text{ mM}$  potassium phosphate,  $0.2\text{ mM}$  diethylene-triamine pentaacetic acid and  $0.025\%$  lubrol, pH 7.8). Then  $25\text{ ml}$  chromogenic reagent in  $0.2\text{ N}$  HCl and  $25\text{ ml}$  30% NaOH were sequentially added and mixed thoroughly. The mixture was reacted in the dark at room temperature for at least 10 min. The colorimetric density of the target chromogen was determined by a spectrophotometer (DU640i; Beckman, Fullerton, CA, USA) at  $400\text{ nm}$ .

**Western blotting.** The protein extracts ( $50\text{ mg}$ ) were separated on 10% SDS-PAGES and transferred to microporous polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After blocking with the TBST buffer ( $10\text{ mM}$  Tris-base; pH=7.5,  $100\text{ mM}$  NaCl,  $0.1\%$  Tween 20) plus 1% bovine serum albumin, the membranes were incubated with human specific anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Bcl-2 (Santa Cruz Biotechnology), anti-c-Fos (Santa Cruz Biotechnology) and anti-GST- $\pi$  (Signet Laboratories Inc., Dedham, MA, USA) antibodies at  $4^{\circ}\text{C}$  for 12 h. The membranes were then washed 3 times with the TBST buffer (20 min each) and incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit immunoglobulin (Santa Cruz Biotechnology) for 1 h at room temperature, respectively. After 3 washes, these proteins were detected by the Western Blotting Luminol Reagent (Santa Cruz Biotechnology). The protein band intensities were digitalized and quantified using the Imagemaster VDS version 3.0 software (ImagemasterVDS; Amersham Pharmacia). The band intensities of proteins were normalized to that of  $\beta$ -actin, which was run in parallel blots.

**Statistical methods.** The GSH levels between the 2 groups were compared with the Student's *t*-test. Three separate experiments

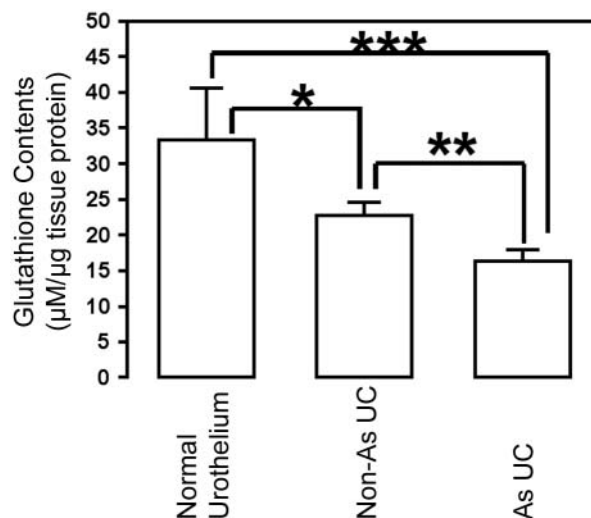


Figure 1. Cellular glutathione (GSH) contents were down-regulated in urothelial carcinogenesis. The mean GSH concentrations in both non-arsenic-related urothelial cancer (non-AsUC) ( $22.8 \pm 1.8\ \mu\text{M}/\mu\text{g}$  protein;  $p=0.04$ ) and arsenic-related urothelial cancer (AsUC) tumors ( $16.4 \pm 1.6\ \mu\text{M}/\mu\text{g}$  protein;  $p=0.002$ ) were significantly lower than normal mucosal tissues ( $33.4 \pm 7.2\ \mu\text{M}/\mu\text{g}$  protein), according to the Student's *t*-test. Of note, the GSH levels of AsUC were significantly lower than that of non-AsUC ( $p=0.012$ ).

were performed in triplicate and the data were presented as the mean  $\pm$  standard error of the means (SEM). Fisher's exact test or the Chi-square test was used to compare protein expressions determined by Western blotting analysis. All tests were two-sided, with  $p < 0.05$  being statistically significant.

## Results

The mean GSH concentrations in both non-AsUC ( $22.8 \pm 1.8\ \mu\text{M}/\mu\text{g}$  protein) and AsUC tissues ( $16.4 \pm 1.6\ \mu\text{M}/\mu\text{g}$  protein) were significantly lower than that of normal mucosal tissues ( $33.4 \pm 7.2\ \mu\text{M}/\mu\text{g}$  protein;  $p=0.04$  and  $p=0.002$ , respectively), according to the Student's *t*-test (Figure 1). In addition, the GSH levels were significantly higher in non-AsUC than in AsUC tissues ( $p=0.012$ ). These results demonstrated that the cellular GSH content may be down-regulated in urothelial carcinogenesis.

The protein levels of GST- $\pi$ , Bcl-2, p53 and c-Fos were determined by Western blotting. All AsUC expressed GST- $\pi$  and Bcl-2 proteins. A few cases of non-AsUC did not express these two proteins. The expressions of Bcl-2 and c-Fos were significantly higher in the AsUC tissues than in the non-AsUC tissues, according to Fisher's exact test ( $p=0.004$  and  $p=0.02$ , respectively) (Table I). However, there were no significant differences in GST- $\pi$  ( $p=0.18$ ) or p53 ( $p=0.29$ ) levels between the two groups.

Table I. Comparison of protein expressions of GST- $\pi$ , p53, Bcl-2 and c-Fos in two groups of tumors by Western blotting.

Molecular markers	GST- $\pi$		p53		Bcl-2		c-Fos	
	+ <sup>d</sup>	- <sup>e</sup>	+	-	+	-	+	-
AsUC <sup>a</sup> (n=33)	33	0	19	14	33	0	30	3
Non-AsUC <sup>b</sup> (n=25)	23	2	10	15	19	6	16	9
Total	56	2	29	29	52	6	46	12
<i>p</i> value <sup>c</sup>	0.18		0.29		0.004**		0.02*	

<sup>a</sup>AsUC = arsenic-related urothelial cancer.

<sup>b</sup>Non-AsUC = non-arsenic-related urothelial cancer.

<sup>c</sup>Fisher's exact test (two-sided) was used to compare protein expressions in the two groups of tumors.

<sup>d</sup>The detectable intensity of protein by Western blotting was scored as "+".

<sup>e</sup>The undetectable intensity of protein by Western blotting was scored as "-".

## Discussion

It has been shown that AsUC and non-AsUC may be different from each other with regard to clinicopathological parameters (11), suggesting that the carcinogenic processes of the two may also be different. In this study, it was shown that a reduction of intracellular GSH levels may be an important feature in urothelial carcinogenesis. The reasons why the GSH levels of non-AsUC were significantly higher than in AsUC, however, are unclear. The glutathione/glutathione-S-transferase (GSH/GST) system in normal and tumor tissues plays a role in the susceptibility to endogenous and/or exogenous toxic compounds. The GSH content and GST- $\pi$  activity have been analyzed in several tissues, including human bladder tumors (12). These results revealed that the reduced GSH levels of bladder carcinoma were significantly lower in patients as compared with the control group (12, 13). Interestingly, similar results were also found in this study. The low GSH concentration of the urothelia may be responsible for the vulnerability of UC to chemical carcinogenesis. Previous studies have reported overexpression of GST- $\pi$  and elevations of GSH levels in some arsenic-resistant cancer cell lines (14). The same features were also detected in AsUC cell lines in our previous report (15). There were no significant differences in the GST- $\pi$  protein levels between AsUC and non-AsUC in this study. However, some studies have suggested that it is the GST- $\pi$  activity or other isoforms of GST (such as  $\alpha$ ,  $\mu$  and  $\theta$  isoforms), not GST- $\pi$  total protein levels, that are involved in arsenic-mediated carcinogenesis (12, 16, 17). Further study is necessary to clarify the issue.

The expressions of the oncoproteins Bcl-2 and c-Fos were significantly up-regulated in AsUC compared to non-AsUC.

Hu *et al.* (18) suggested that the Bcl-2 protein level was one of the practical biomarkers for screening arseniasis-mediated skin carcinoma. In our series, all 33 cases of AsUC expressed Bcl-2, detected by Western blotting. In contrast, 6 of the 25 non-AsUC (24%) did not express the Bcl-2 protein, which appears compatible with the findings of Hu *et al.* Recent studies showed that the Bcl-2 protein was positively expressed in UC according to immunochemical staining (19, 20). However, up to 76% of non-AsUC were found to express Bcl-2 in this study. Therefore, Bcl-2 may be a sensitive marker, but not a marker with good specificity for arseniasis. AP-1, a heterodimeric transcription factor of c-Fos and c-Jun, can mediate many biological effects of tumor promoters and is an important regulator of cell growth. Arsenic has been shown to modulate the mitogen-activated protein kinase cascade in several cell systems, resulting in the activation of transcription factors, including AP-1 (21). Recently, many studies have similarly demonstrated that arsenic induction of AP-1 DNA-binding activity was accompanied by up-regulation of c-Fos and c-Jun nuclear proteins in bladder epithelial cells (22). We also observed that more AsUC than non-AsUC expressed c-Fos. Of particular relevance to the present studies was a report that c-Fos expression was a concomitant factor associated with arsenic-mediated carcinogenesis (23). However, a larger sample size is needed to validate the hypothesis that c-Fos up-regulation is closely associated with AsUC.

No statistically significant difference was found in the expression of wild-type p53 protein levels between the two groups of tumors. Tchounwou *et al.* (24) also showed that the level of p53 protein did not significantly differ between arsenic trioxide-treated and control liver carcinoma cells. On

the contrary, many studies have shown that high protein levels of mutant-type p53 were detected in AsUC (7, 18). It is, thus, possible that arsenic-mediated urothelial carcinogenesis involves p53 mutation, but not transcriptional or translational modification of the p53 protein levels. Further studies are needed to determine whether the mutant-type p53 is involved in arsenic-mediated urothelial carcinogenesis.

In summary, our results suggest that the cellular GSH content is down-regulated in urothelial carcinogenesis, especially in arsenic-related tumors. The Bcl-2 and c-Fos oncoproteins may play important roles in arsenic-mediated urothelial carcinogenesis. The carcinogenic pathway of AsUC is, at least partly, different from that of non-AsUC.

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