Overexpression of aldo-keto reductase 1C2 as a high-risk factor in bladder cancer

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Abstract. Intravesical adjuvant chemotherapy and neoadjuvant chemotherapy has been respectively administered for superficial transitional cell carcinoma (TCC) of urinary bladder and advanced TCC for years. However, the therapeutic efficacy is limited. Recently, overexpression of aldo-keto reductase (AKR) in lung, esophageal, uterine cervical and ovarian cancers was shown to be closely associated with disease progression and drug resistance. In this study, we used immunohistochemistry to determine AKR expression in pathological specimens of 347 patients with urinary bladder cancer (UBC). Some of these patients were from areas with a high risk of black foot disease (BFD), a disease that is closely associated with arsenic contamination of drinking water. The presence of AKR was confirmed by immunoblotting, matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF) and reverse transcription-polymerase chain reaction (RT-PCR). AKR isotype was determined by cDNA sequencing. Our results showed overexpression of AKR1C2 in 226 (65.1%) patients. BFD areas had a higher frequency of patients expressing AKR1C2 in UBC. Among AKR1C2-positive UBC, 148 (65.5%) were invasive, 70 (31.0%) were non-invasive and 8 (3.5%) were carcinoma *in situ* (CIS). These data indicated that AKR1C2 expression could be significantly associated with cancer invasiveness ($p<0.001$) and disease progression. Because BFD has been closely related to arsenic ingestion, our results suggested that continual intake of arsenic in drinking water might provoke AKR1C2 expression that could in turn induce drug resistance in UBC, and AKR1C2 could be a tumor marker for UBC.

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Introduction

Except for uterine cervical cancer (UCC), which is the second most common cancer in women worldwide and the third most widespread cancer among Taiwanese women [~46 cases/10^5 women-year (2003)], cancer incidence of the genitourinary system is actually quite low in Taiwan. Bladder cancer is the most prevalent urinary carcinoma [~2.79 cases/10^5 person-year (2003)] (1). However, in southwest Taiwan, particularly in coastal areas of the two specific counties that surround a mushy lagoon, the incidence rate of urinary bladder cancer (UBC) is 2- to 8-fold [12.8-23.5 cases/ 10^5 person-year (1981-1985); 6.06-9.43 cases/10^5 person-year (1992-2001)] higher than in other areas of Taiwan, as was the incidence rate of skin, hepatocellular, and lung cancers (2-5). In northeast Taiwan, following the rapid establishment of an oil refinery as well as heavy industrial well-drilling for excessive artesian water supply in the last two decades, the incidence rate of UBC increased approximately 3- to 20-fold [0.5-2.08 cases/10^5 person-year (1981-1985); 6.11-9.87 cases/ 10^5 person-year (1995-2001)]. Interestingly, in these two areas, black foot disease (BFD) is also endemically prevalent (6). BFD is a peculiar syndrome of severe peripheral vascular disorders that are closely associated with a high concentration of arsenic in artesian water (7).

Results of epidemiological studies from several different countries show that exposure to trivalent and pentavalent arsenic, which occurs primarily through either contaminated drinking water or occupational contact, can indeed increase the incidence rate of urinary bladder cancer and peripheral vascular disorders (3,6,8). Although arsenate is less toxic than arsenite, cigarette smoking and phenacetin abuse could aggravate the carcinogenic effect of arsenics (9). Shibata *et al* (10) suggested that the higher risk of UBC in these specific areas might result from increased genetic alteration of p53, which could then affect cell growth, and inflammation-associated gene expression (10-13).

Interestingly, the use of anti-inflammatory drug, piroxicam, which has been shown to induce remission of UBC in animal models (14), supported the concept that inflammation could indeed play a significant role in carcinogenesis and disease progression of UBC (15,16). The detailed mechanism of how anti-inflammation reduces the risk of bladder cancer development remains to be elucidated. It is clear though that cyclooxygenases, which convert arachidonic acid to prostanoids (15,16), and some aldo-keto reductases, which contain prostaglandin (PG) F synthase activity (17,18) for catalyzing interconversion of prostanoids, are targets of anti-inflammatory drugs.

Recently, by using differential display and microarray, we have identified dihydrodiol dehydrogenase (DDH), in particular aldo-keto reductase 1C1 (AKR1C1), which was overexpressed in non-small cell lung cancer (NSCLC) (18). DDH is a member of the aldo-keto reductase superfamily that mediates oxidation of trans-dihydrodiols to the corresponding catechols (19). In human liver, four isoforms (AKR1C1-AKR1C4) have been identified with monomeric mass of 36 kDa (20). In addition to detoxification, AKRs exhibit PGF synthase activity by converting PGD_2 into 9α, 11ß-PGF_2 (17,21). Detection of AKR overexpression in transitional cancer cells would provide an alternative link between chronic inflammation and carcinogenesis of bladder and, possibly, the disease manifestation of urinary bladder cancer.

In this study, we used an immunohistochemical method to determine AKR1C2 expression in UBC specimens from various areas in Taiwan that had different prevalence of UBC and BFD. Expression of AKR1C2 was confirmed by two-dimensional (2-D) immunoblotting analysis, matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) and reverse transcription-polymerase chain reaction (RT-PCR). Correlations between living area of patients with UBC, AKR1C2 expression, tumor invasiveness and disease progression were evaluated statistically.

Patients and methods

Patients and tissue samples. From January 1992 to December 2001, 347 pathological samples were collected from patients who were diagnosed with urinary bladder cancer. Tumor staging and grading were determined in accordance with American Joint Committee on Cancer (AJCC) guidelines (22). All patients had undergone radical cystectomy with bilateral pelvic lymph node dissection (PLND) or transurethral bladder tumor resection. All tumors, including invasive transitional cell carcinoma (TCC), non-invasive TCC, and carcinoma *in situ* (CIS), were histologically classified as TCC. Patients with lymph node involvement were irradiated with 45-60 Gy at the afflicted areas depending upon the risk of residual disease and in patients with positive margins or local recurrence, the radiation dose was increased to 70 Gy. Characteristics of these patients and their respective living areas in Taiwan (Fig. 1) are listed in Table I. After treatment, all patients were routinely followed every 3-6 months in the respective Out-Patient Departments. Tumor recurrence and metastasis were identified when blood examination, biochemical studies, abdominal sonography, whole body bone scan and computerized tomography scans showed evidence of the disease.

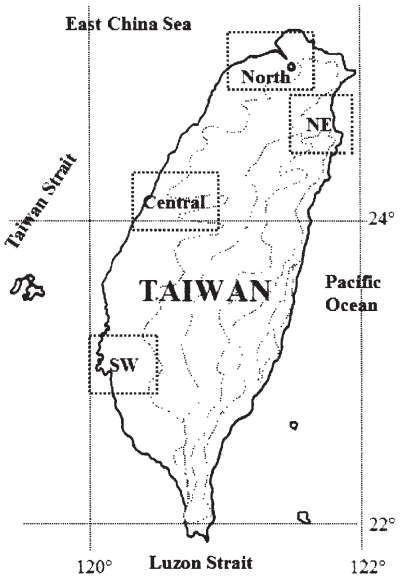

Figure 1. Sketch of four different areas in Taiwan where TCC specimens were collected. The dashed squares show the respective location. SW, southwest; NE, northeast.

Immunohistochemical staining and immunoblotting. Immunohistochemical staining was performed on paraffin-embedded tissues by an immunoperoxidase method as previously described (15,16,18). Following removal of paraffin with xylene and absolute alcohol, specimens were incubated with polyclonal antibodies specific to all AKRs or monoclonal antibodies specific to AKR1C2 (Cashmere Scientific Company, Taipei, Taiwan). Slides were treated with biotin-labeled goat anti-mouse immunoglobulin, peroxidase-conjugated streptavidin and then developed in 3-amino-9-ethylcarbazole. The crimson precipitates were identified as positive staining. The non-tumor counterpart of the bladder tissue was served as a negative control, and a section of liver tissue was used as a positive control for each run of immunostaining. The same antibodies were used for immunoblotting, of which the procedure has been described previously (15,16,18). Briefly, proteins were separated in a 10% polyacrylamide gel with 4.5% stacking gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane. The membrane was then probed with AKR1C2 specific antibodies. The signal was amplified by biotin-labeled goat anti-mouse IgG, and peroxidase-conjugated streptavidin. AKR1C2 protein was visualized by exposing the membrane to an X-Omat film (Eastman Kodak, Rochester, NY, USA) with enhanced chemiluminescent reagent (NEN, Boston, MA, USA).

2-D gel electrophoresis and protein analysis by MALDI-TOF MS. Total cell lysate was first separated by a tube gel of isoelectric focusing (IEF) system (Bio-Rad Laboratories, Inc., Hercules, CA). The pH range was from 3.0 to 10. At the end of IEF, gels were extracted from the tubes, and equilibrated with SDS buffer before SDS gel electrophoresis. One gel was processed for immunoblotting as described above, and one gel was stained with Coomasie blue. The protein spot on Coomassie-stained gel that corresponded to an immunoblot-positive spot was extracted from the gel for further identity

Table I. Characteristics of patients with urinary bladder cancer in the four different locations of Taiwan.

Category	No. of patients	Location in Taiwan				p-value
		Northeast	Southwest	Central	North	
Age						0.026[c]
≥50	262	17	92	97	56	
<50	85	6	16	37	26	
Ratio	3.08	2.83	5.75[e]	2.62	2.15	
Gender						<0.001[c]
Male	257	14	69	105	69	
Female	90	9	39	29	13	
M/F ratio	2.86	1.56[e]	1.77[e]	3.62	5.31	
Type of cancer						0.078[d]
Invasive TCC[a]	193	13	73	66	41	
Non-invasive TCC	129	9	31	57	32	
CIS[b]	25	1	4	11	9	

[a]TCC, transitional cell carcinoma; [b]CIS, carcinoma *in situ*; [c]two-sided p-value determined by χ^2 test; [d]two-sided p-value determined by χ^2 test for trend; [e]with significant difference.

analysis by MALDI-TOF MS on a Voyager-DE™ pro bio-spectrometry workstation (Applied Biosystems, Milpitas, CA, USA).

Slide evaluation. In each case, non-tumor bladder tissue served as an internal negative control. Slides were evaluated by two independent pathologists without clinicopathological knowledge. A German ImmunoReactive Scoring system was adapted for this study (23). Briefly, a specimen was considered strong positive if more than 50% of cancer cells were positively stained; intermediate positive, if positive cells were between 25-50%; weak positive, if less than 25% or more than 10% were positively stained; and negative, if less than 10% were positively stained. Both strong and intermediate positive cases were classified as overexpression, and weak and negative as low expression.

RNA extraction, and reverse transcription-polymerase chain reaction (RT-PCR). RNA extraction and gene amplification have been described previously (15,16,18). Briefly, following RNA extraction, cDNA was synthesized by random primers and AMV reverse transcriptase. An aliquot of cDNA was then subjected to 35 cycles of polymerase chain reaction (PCR) using standard procedure denaturing at 94˚C for 45 sec, hybridizing at 56˚C for 30 sec, and elongating at 72˚C for 45 sec. The primer sequences for AKR1C2 were 5'-GTGTG AAGCTGAATGATGGTCA-3' (GenBank/ABO21654, nts 20-41) and 5'-TCTGATGCGCTGCTCATTGTAGCTC-3' (GenBank/ABO21654, nts 834-810). The primer sequences for AKR1C3 were 5'-TCCAGAGGTTCCAAGAAGTAAAG CTTT-3' (GenBank/BC001479.2, nts 134-160) and 5'-TGGA TAATTAGGGTGGCTAGCAAA-3' (GenBank/BC001479.2, nts 1010-987). The primer sequences for AKR1C4 were 5'-TCCAGAGGTTCCGAGGAACAGAGCT-3' (GenBank/NM001818.2, nts 111-135) and 5'-AATGGATAATCAGGA TGGTCCATA-3' (GenBank/BC001479.2, nts 989-966).

The amplified products were resolved in a 2.5% agarose gel. Specificity of the 815 base-pair AKR1C2 fragments was determined by DNA sequencing (Perkin-Elmer, Foster City, CA, USA). The nucleotide sequences were matched with database of GenBank (http://www.ncbi.nlm.nih.gov/blast) (18).

Statistical analysis. Relations between AKR1C2 expression and clinicopathological parameters were analyzed by χ^2 test. The χ^2 test for trend was used when corresponding factors (i.e., patients' living locations) exceeded two categories. Statistical significance was set at $p<0.05$. Statistical analysis was performed by GraphPad Prism4 statistical software (San Diego, CA, USA).

Results

Demographic characteristics. As shown in Table I, in terms of age of detecting bladder cancer, patients who lived in the southwest area of Taiwan showed a significantly different age distribution (p=0.026), with the highest ratio being over 50 years old. The regional difference of male (M) to female (F) ratio was also significant. Compared with patients from other area, patients who lived in northeast and southwest areas of Taiwan had lower M/F ratios ($p<0.001$). The regional difference of cancer type, however, was marginal (p=0.078).

Aldo-keto reductase 1C2 phenotypes and local difference for enzyme expression. As determined by immunohistochemistry, 256 patients (68.1%) were positive for AKR1C2 expression,

Table II. Comparison of AKR1C2 expression in patients with urinary bladder cancer and patients' respective living location in Taiwan.

Type of cancer	No. of patients	AKR1C2-positive no. (%)	Living location of AKR1C2-positive patients in Taiwan				p-value
			Northeast	Southwest	Central	North	
Invasive TCC	193	148 (76.7)	11	69[b]	39	29	0.02[a]
Non-invasive TCC	129	70 (54.3)	7	16	31	16	
CIS	25	8 (32)	1	1	3	3	
Subtotal		226 (65.1)	19 (82.6)[b]	86 (79.6)[b]	73 (54.4)	48 (58.5)	<0.001[a]
Total patients no.	347		23	108	134	82	

[a]Two-sided p-value determined by χ^2 test for trend; [b]with significant difference.

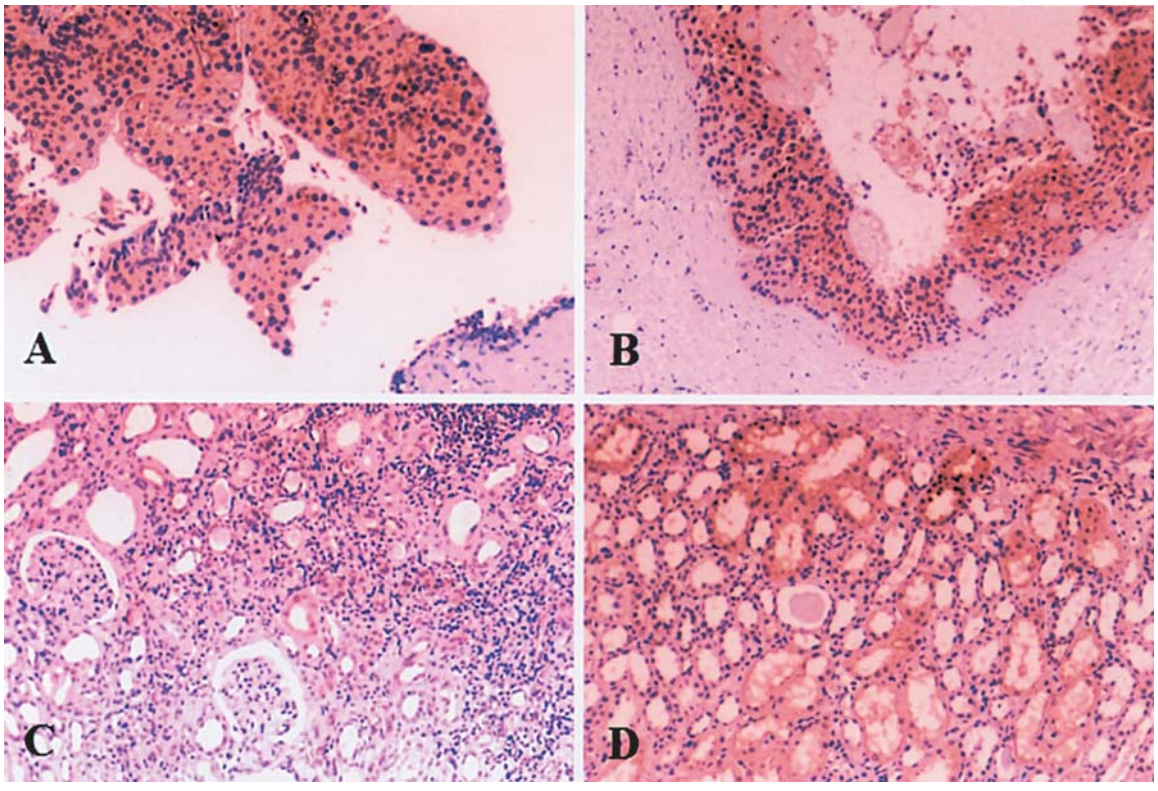

Figure 2. Immunohistochemical analysis of AKR1C2 expression in transitional cell carcinomas. (A) Expression of AKR1C2 was detected in transitional cell carcinoma (TCC) cells (crimson precipitates in cytoplasm are positive signals), but not in bladder epithelial cells. (B) Expression of AKR1C2 was detected in carcinoma *in situ* of TCC. (C) Expression of AKR1C2 in the proximal convoluted tubules, and (D) expression of AKR1C2 in the distal convoluted tubules of kidney. The slides were counterstained with hematoxylin (original magnification, x250).

and AKR1C2 staining was homogeneously distributed in the cytoplasm. The correlation between AKR1C2 expression and patients' living area was significant (Table II). Patients from northeast (82.6%) and southwest (79.6%) areas of Taiwan had significantly higher frequency (p<0.001) of expressing AKR1C2. Among all AKR1C2-positive patients, 148 (76.7%) were invasive TCC (Fig. 2A), 70 (54.3%) were non-invasive TCC and 8 (32%) were carcinoma *in situ* (CIS) (Fig. 2B). The correlation between AKR1C2 expression and type of cancer was also significant. As shown in Table II, significantly more invasive TCC was observed among AKR1C2-positive patients from the southeast area of Taiwan (p=0.02). Overall, the AKR1C2-positive frequency was higher (p<0.001) among patients from northeast (82.6%) and southwest (79.6%) areas of Taiwan. AKR1C2 was not detected in non-tumor urinary bladder epithelium (Fig. 2A) and renal corpuscles (Fig. 2C), but it was highly expressed in the proximal (Fig. 2C) and distal convoluted tubules (Fig. 2D) of kidney. Although TCC was occasionally observed amid non-tumor urinary bladder epithelium, AKR1C2-positive TCC did not increase AKR1C2

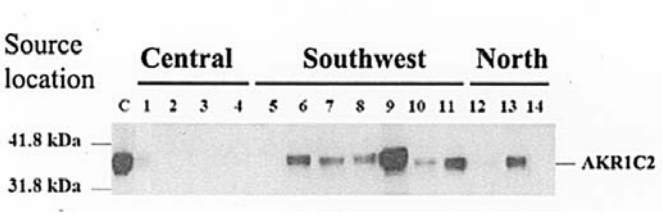

Figure 3. Overexpression of AKR1C2 was determined by immunoblotting in TCC from different locations of Taiwan. Expression of AKR1C2 was detected by monoclonal antibodies specific to AKR1C2. C, lung adenocarcinoma cell line H838 served as a control.

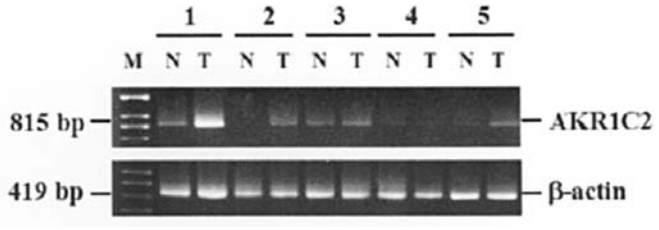

Figure 5. Overexpression of AKR1C2 was detected in most of the tumor fractions of TCC by RT-PCR. N, non-tumor lung tissue; T, tumor fraction of surgical resections. Expression of ß-actin served as an internal control of RT-PCR.

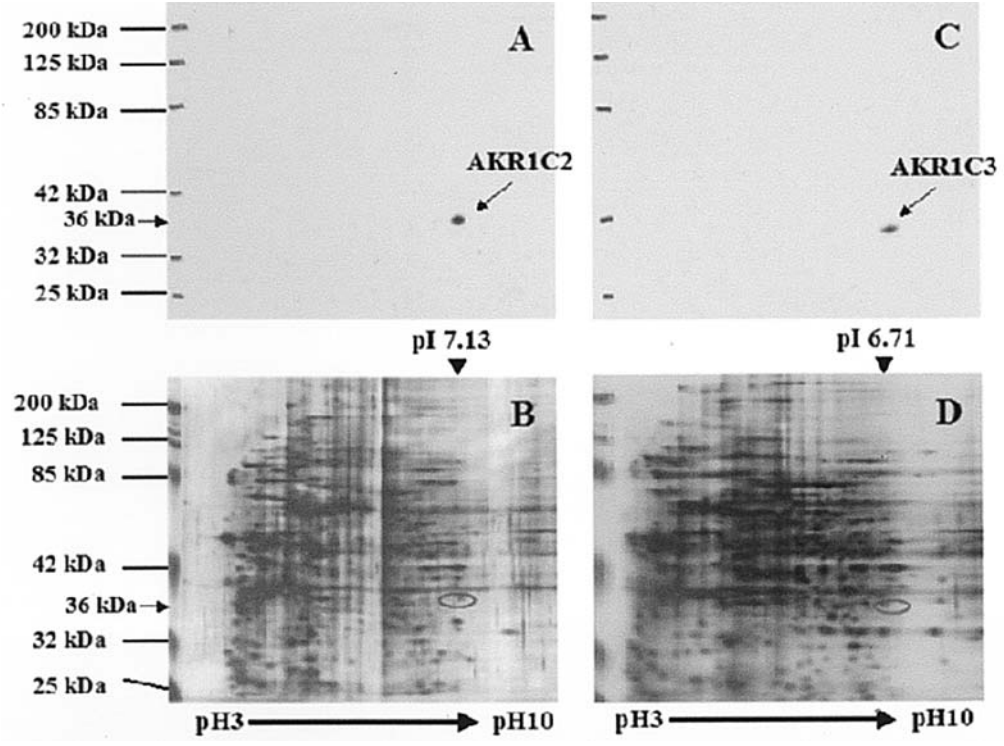

Figure 4. Overexpression of AKR1C2 was detected in TCC by 2-D gel electrophoresis and immunoblotting (A). The corresponding protein spot (pI 7.13) was identified (circled) in a silver-stained 2-D gel (B). As a positive control, AKR1C3 overexpression was detected in uterine cervical cancer cells by 2-D gel electrophoresis and immunoblotting (C). The corresponding protein spot (pI 6.71) was identified (circled) in a silver-stained 2-D gel (D).

expression in the non-tumor urinary bladder epithelium (Fig. 2B).

By immunoblot analysis, expression of AKR1C2 was detected in seven surgical specimens (Fig. 3). Interestingly, six of seven UBC specimens from the southwest area were positive for AKR1C2, while only one of three samples from the north area was positive for AKR1C2. None of the four specimens from central Taiwan was positive for AKR1C2. AKR1C2-positive samples were confirmed by 2-D immunoblotting (Fig. 4A and C) and the corresponding protein (Fig. 4B and D) spot was identified by MALDI-TOF MS. AKR1C2-positive specimens and their corresponding non-tumor fractions were further verified by RT-PCR (Fig. 5). To confirm the isotype of AKRs expressed in TCC, DNA fragments of RT-PCR products from 38 TCC specimens were further sequenced. Nucleotide sequences from seventeen samples matched to AKR1C2: GenBank|AB031084|*Homo sapiens* aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid-binding protein) (AKR1C2), identities = 99-100%. Thirteen of the 17 samples, in which AKR1C2 was detected, were from BFD-endemic areas. No mutation was detected in AKR1C2 of TCC.

Discussion

The results presented above showed that a regional preference for AKR1C2 overexpression in TCC was observed in UBC patients from northeast and southwest areas, which matched well with higher prevalent areas of BFD in Taiwan. Unlike our previous studies in lung, esophageal and ovarian cancers (18,24,25), the major isotype of dihydrodiol dehydrogenase (DDH) detected in TCC was AKR1C2 (type II 3α-hydroxysteroid dehydrogenase), which was identical to that identified in the proximal and distal convoluted tubules of kidney.

As noted above, Shibata *et al* (10) showed that the higher risk of bladder cancer in BFD-endemic areas of Taiwan was

associated with the increased mutation of *p53*, and most of the mutations identified in exons 5-8 of *p53* were nucleoside transition, a genetic alteration that was most likely caused by the excessive intracellular level of reactive oxygen species (ROS) (26). By an animal carcinogenicity study, Wei *et al* (27) further showed that feeding of male F344 rats with 50-200 mg/liter dimethylarsinic acid (DMA), a major metabolite of arsenic in most mammals, in drinking-water over 2 years significantly increased the genomic level of 8-hydroxy-2'-deoxyguanosine and frequency of urinary bladder tumor. These results corresponded well with those from a critical epidemiological survey, which showed that arsenic content in artesian water in BFD-endemic areas in Taiwan could be up to 1.8 mg/liter (7). Furthermore, recent evidence suggests that independent of direct DNA damage, arsenic may interact with thiol groups on proteins to alter their activities, which are essential for gene regulation and cell proliferation (28). The intracellular events that follow arsenic-thiol formation are not well elucidated. However, a variety of evidence indicates that this interaction is indispensable for gene activation (29) as well as for cytotoxicity to occur (30). The present study sheds further light on the effect of arsenic on AKR1C2 expression in TCC carcinogenesis and disease progression and how this may influence the drug sensitivity of urinary bladder cancer cells, in particular during intravesical adjuvant chemotherapy for superficial TCC or neoadjuvant chemotherapy for advanced disease, of which the efficacy gain was still limited (31-34).

In fact, following our findings of DDH overexpression in non-small cell lung cancer (NSCLC) by using differential display and cDNA sequencing (18), we have anticipated that DDH or other members of the AKR superfamily could play a role in drug resistance of cancer cells. Based on the highly similar chemical structures between polycyclic aromatic hydrocarbon (PAH)-derivatives and anticancer drugs, such as daunorubicin and doxorubicin, the presence of DDH may be responsible for drug inactivation, and hence increase drug resistance in tumor cells. Detection of DDH expression in spontaneous daunorubicin-resistant stomach cancer cells and ethacrynic acid-induced drug-resistant colon cancer cells further supported this speculation (35,36). By using cDNA microarray to compare gene expression profiles between parental and cisplatin-resistant ovarian cancer cells, Deng *et al* (37) also identified DDH as one of the major genes activated. Clinically, Chen *et al* (25) showed that overexpression of AKR1C3 was associated with resistance to cisplatin-based chemotherapy in patients with ovarian cancer. Results of the present study not only strengthen their observations, but also suggest that the presence of AKR1C2 may by some means fortuitously induce a common drug resistance mechanism, which is shared among a number of structurally diverse anticancer agents. Although our results were in accordance with those of Kioka *et al* (38) and Presetra *et al* (39), the effect of arsenic per se on drug resistance of TCC remains to be elucidated.

It is worth noting that although inorganic arsenicals [arsenic trioxide (As_2O_3), arsenite and arsenate] are genotoxic, As_2O_3 itself is also a chemotherapeutic agent that can selectively inhibit tumor cell growth and induce tumor cell death. The cytotoxic mechanisms were suggested via signal pathway to induce peroxide accumulation, cytochrome c-related apoptosis, and depletion of glutathione (40,41). These corresponded well with our previous data, in which expression of glutathione-S-transferase π was inversely correlated with that of AKR1C2 (24). Moreover, our results showed that AKR1C2 was frequently detected in TCC cells, in BFD-endemic areas where a high level of arsenic in drinking water might induce AKR1C2 expression. Detection of AKR1C2 in the proximal and distal convoluted tubules of kidney, but not in renal corpuscles or epithelium of urinary bladder further implicated that a common factor, i.e. arsenic and/or cigarette smoking (9), which might directly affect cell function, could be present during re-absorption or retention of excretory waste in those specific tissues.

At the present time, our results showed that expression of AKR1C2 was frequently detected in the pathologic specimens of TCC. Expression of AKR1C2, moreover, was correlated with the prevalence of BFD. Although there is not yet a clear explanation for the clinical correlation between increased AKR1C2 expression and disease progression of TCC, the cause of AKR1C2 overexpression could be a physiological response to reduce arsenic toxicity and to remove superfluous free radicals due to the immediate mitochondria damage (37,42,43). In particular, if the impact of AKR1C2 over-expression is on drug sensitivity, these observations can then serve as a focus to elucidate the mechanism by which AKR1C2 activity in TCC is regulated pathophysiologically.

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