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Keratin 6 Expression Correlates to Areas of Squamous Differentiation in Multiple Independent Isolates of As⁺³-Induced Bladder Cancer

Ling Cao^a, Xu Dong Zhou^a, Mary Ann Sens^a, Scott H. Garrett^a, Yun Zheng^a, Jane R. Dunlevy^b, Donald A. Sens^a, and Seema Somji^{a,*}

^aDepartment of Pathology School of Medicine and Health Sciences University of North Dakota Grand Forks, ND 58202

^bDepartment of Anatomy and Cell Biology School of Medicine and Health Sciences University of North Dakota Grand Forks, ND 58202

Abstract

This laboratory has shown that arsenite (As⁺³) exposure can cause the malignant transformation of the UROtsa human urothelial cell line. This single isolate formed subcutaneous tumors with a histology similar to human urothelial cell carcinoma. The tumors also displayed areas of squamous differentiation of the urothelial cells, an infrequent, but known component of human bladder cancer. In the present study, five additional independent isolates of As⁺³-transformed urothelial cells were isolated and each were shown to produce subcutaneous urothelial cell tumors with a characteristic histology very similar to those described in the initial report. That there were underlying phenotypic differences in the 6 independent isolates was demonstrated when they were assessed for their ability to form tumors within the peritoneal cavity. It was shown that two isolates could form hundreds of small peritoneal tumor nodules, one isolate a moderate number of tumor nodules, and three isolates no or only one tumor nodule. The peritoneal tumors were also characterized for their degree of squamous differentiation of the urothelial cells and, while areas of squamous differentiation could be found, such differentiation was substantially reduced compared to subcutaneous tumors. Immunostaining for keratin 6 was tested as a potential marker for malignant urothelial cells that had undergone squamous differentiation. Keratin 6 was shown to consistently stain only cells having some evidence of squamous differentiation. Keratin 16 was shown to follow the staining pattern of keratin 6. The isolates and tumor heterotransplants were all examined for keratin 6, 16 and 17 mRNA and protein expression.

Keywords

Urothelial Cell Carcinoma; Squamous Differentiation; Bladder; Arsenite; Keratin 6; Keratin 16; Keratin 17

INTRODUCTION

Arsenic has been ranked first in priority among a listing of the top 20 hazardous substances by the Agency for Toxic Substances and Disease Registry (ATSDR) and the U.S. EPA [ATSDR, 1997]. The association between arsenic exposure and urinary bladder cancer,

*Correspondence, Reprints, and Page Proofs: Seema Somji, Ph.D. Department of Pathology School of Medicine and Health Sciences University of North Dakota 501 N. Columbia Road Grand Forks, ND 58202 Telephone: 701-777-2658 Fax: 701-777-3108 ssomji@medicine.nodak.edu.

typically urothelial carcinoma, has been observed in the same endemic areas of the world where populations with skin cancer have been identified [Cantor and Lubin, 2007; Chiou *et al.* 1995; Luster and Simeonova, 2004; Smith *et al.* 1998; Steinmaus *et al.* 2000; Tsuda *et al.* 1995]. Urothelial cell carcinoma is the fourth most common cancer in men and the fifth in women in western countries [Johansson and Cohen, 1997]. This laboratory has developed a potential model system for As⁺³-induced bladder cancer by showing that arsenite (As⁺³) can directly cause the malignant transformation of an immortalized, but non-tumorigenic, human urothelial (UROtsa) cell line [Sens *et al.* 2004]. It was also shown that these cells could form tumors when subcutaneously heterotransplanted into nude (immunocompromised) mice. The first goal of the present study was to determine the repeatability of the transformation process by isolating and characterizing additional independent As⁺³ transformed cell lines using an identical transformation protocol starting with untransformed parental UROtsa cells. Multiple independent isolates of As⁺³ transformed cell lines and their tumor heterotransplants would be a unique model to determine the degree of heterogeneity of the molecular signatures among independent isolates transformed by a single environmental toxicant.

The histology of the tumor heterotransplants produced by the original isolate of UROtsa cells malignantly transformed by As⁺³ had the classic histologic features of urothelial carcinoma. In addition to the classic urothelial cell histology, the heterotransplants also displayed prominent areas where the urothelial cells had undergone squamous differentiation. The finding that the tumor heterotransplants displayed areas of squamous differentiation is not a sign of aberrant behavior of the model since a low percentage of human urothelial cell carcinomas are known to display squamous differentiation [Frazier *et al.* 1993]. There is evidence that squamous differentiation in patients with bladder cancer is associated with a more aggressive cancer and a poor prognosis. Squamous differentiation of the urothelial cancer cells has been shown to be an unfavorable prognostic feature in patients undergoing radical cystectomy, possibly because of its association with high grade tumors [Frazier *et al.* 1993; Lopez-Meltran *et al.* 2007; Billis *et al.* 2001]. Squamous differentiation has also been reported as predictive of a poor response in patients undergoing radiation therapy [Akdas and Turkeri, 1990; Martin *et al.* 1989]. In another report, squamous differentiation was associated with a poor response to systemic chemotherapy [Logothetis *et al.* 1989]. The second goal of the present study was to determine if independent isolates of As⁺³ transformed cells would produce tumor heterotransplants with squamous differentiation of their urothelial cells. The finding that the original isolate of As⁺³ -transformed cells produced tumor heterotransplants with squamous differentiation also suggested that keratin expression might be altered in these tumors. One of the more common manifestations of chronic arsenic exposure includes hyperkeratosis and hyperpigmentation of the skin [Maloney, 1996]. An examination of keratin 6 showed that expression of this gene was increased in the As⁺³ -transformed cells and their tumor heterotransplants [Somji *et al.* 2008]. The final goal of the present study was to determine if keratin 6 expression was a marker for transformed urothelial cells that had undergone squamous differentiation and if keratin 6 might be an early biomarker to detect such differentiation.

MATERIALS AND METHODS

Cell Culture

Stock cultures of the UROtsa cell line were maintained in 75 cm² tissue culture flasks using Dulbecco's modified Eagles' medium (DMEM) containing 5% v/v fetal calf serum in a 37°C, 5% CO₂: 95% air atmosphere [Rossi *et al.* 2001]. Confluent flasks were subcultured at a 1:4 ratio using trypsin-EDTA (0.05%, 0.02%) and the cells were fed fresh growth medium every 3 days.

Arsenite-Induced Transformation of UROtsa Cells

The protocol used to malignantly transform the UROtsa cell line with As^{+3} has been detailed in an earlier report [Sens *et al.* 2004]. An identical protocol starting with the parental UROtsa cell line was used in the present study. Eight independent cultures of UROtsa cells were grown to confluency in 25 cm² cell culture flask and when confluent, each of the eight flasks were fed fresh growth media containing 1 μM As^{+3} (NaAsO_2 , Cat. No. 71287, Sigma Aldrich, St. Louis, MO). Following addition of As^{+3} , the cells were thereafter fed fresh growth media every three days that contained As^{+3} . The cultures were observed immediately before and 24 h after each feeding by light microscopy.

Cell Growth

Growth curves of the malignantly transformed cells were obtained using the methylthiazolotetrazolium (MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay following a 1:20 subculture of the cells [Denizot and Lang, 1986].

Growth in Soft Agar

Before testing for tumor growth in nude mice, all cultures were tested for their ability to form colonies in soft agar using a slight modification of the procedure described by San and co-workers [San *et al.* 1979; Sens *et al.* 2004]. Briefly, 60 mm diameter dishes were prepared with a 5 ml underlay of 0.5% agar in DMEM containing 5% fetal calf serum. On top of the underlayer was placed 2×10^4 cells in 1.5 ml of 0.25% agar in DMEM containing 5% fetal calf serum. The dishes were incubated at 37°C in a 5% CO_2 : 95% air atmosphere inside humidified plastic containers to prevent evaporation. Cultures were examined microscopically 24 h after plating to confirm an absence of large clumps of cells and thereafter at 7, 14 and 21 days after plating.

Subcutaneous Injection of As^{+3} -Transformed UROtsa Isolates to Determine Tumorigenicity in Nude Mice

To test for malignant transformation, the respective cultures that showed colony formation in soft agar, along with the UROtsa parent cell line, were each inoculated subcutaneously (s.c.) at a dose of 1×10^6 cells in the dorsal thoracic midline of 5 nude (NCr-nu/nu) mice. Tumor formation and growth were assessed weekly. All mice were sacrificed by 10 weeks after injection or when clinical conditions dictated euthanasia. Tumor samples were paraffin-embedded, sectioned, stained with Hemotoxylin and Eosin (H&E), and analyzed by light microscopy. In addition, tissue samples from the tumors were also taken for the preparation of total RNA and protein.

Intraperitoneal Injection of As^{+3} -Transformed UROtsa Isolates

To determine the ability of the transformed isolates to colonize internal organs of the peritoneum, the As^{+3} -transformed isolates were each injected intraperitoneally (IP) into 6 nude (NCr-nu/nu) mice. The IP injection was performed according to the online protocol of the American Association of Laboratory Animal Science learning library. A one inch 23 gauge needle was inserted in the abdominal cavity in the lower right quadrant and each mouse received 1×10^6 cells in 200 μl of phosphate-buffered saline (PBS). All the mice were euthanized at 53 days after injection when the tumors in some groups became large and observable by visual examination of the abdomen. Necropsy was performed on each mouse according to the online dissection guide published by the National Institutes of Health (<http://www3.niaid.nih.gov/labs/aboutlabs/cmb/InfectiousDiseasePathogenesis/section/mouseNecropsy/>) with minor modifications. Briefly, at necropsy the general condition of the mouse was observed with determination of the weight, length (head to anus), and abdominal girth. The thoracic cavity, cranial cavity and especially the peritoneal cavity were examined

carefully for gross tumor formation. At least 6 photographs were taken for each mouse including: an external dorsal view; external ventral view; ventral view with the skin opened; open abdominal cavity, open abdominal cavity with intestines removed; and, open abdominal cavity with the liver removed from the abdomen. All abdominal organs and associated tumor samples were formalin fixed and paraffin-embedded. Selected specimens were sectioned, stained with H&E, and analyzed by light microscopy.

Immunostaining for Keratin 6, 16 and 17 Protein in Tumor Heterotransplants

The mouse tumor heterotransplants were routinely fixed in 10% neutral-buffered formalin for 16–18 h. The tissues were then transferred to 70% ethanol and dehydrated in 100% ethanol. Dehydrated tissues were cleared in xylene, infiltrated, and embedded in paraffin. Serial sections of the tissue blocks from the subcutaneous and intraperitoneal tumor heterotransplants were cut at 3–5 μ m for use in immunohistochemical protocols. Prior to immunostaining, sections were immersed in preheated Target Retrieval Solution high pH (Cat. No. S3308; Dako, Carpinteria, CA) for keratin 17 or Target Retrieval Solution (Cat. No. S1699; Dako) for keratin 6 and 16 in a steamer for 20 min. The sections were allowed to cool for 30 min at room temperature and immersed into Tris-buffered saline with Tween 20 for 5 min. The immunostaining was performed on a Dako Autostainer Universal Staining System (Dako). The antibodies, sources, dilution and clone number used in this study are as follows: keratin 6 (Abcam; Cambridge, MA; 1:400; LHK6/LHK6B), keratin 16 (Abcam; 1:40; LL025), keratin 17 (Dako; 1:40; E3). The primary antibody was localized using the DakoCytomation ARK (Animal Research Kit; Dako, Cat. No. K3955) according to the manufacturer's instructions. This system minimizes reactivity of secondary antimouse antibody with endogenous immunoglobulin that may be present in the heterotransplant-generated specimen. Liquid diaminobenzidine (DAB) was used for visualization. Slides were counterstained with hematoxylin, rinsed in distilled water, dehydrated in graded ethanol, cleared in xylene, and coverslipped.

Real Time Analysis of Keratin 6, 16 and 17 mRNA Expression

The expression of keratin 6a, 6b, 16 and 17 was measured with real-time reverse transcription polymerase chain reaction (RT-PCR). The culture of cells and the method used from RNA extraction from the isolates and tumor heterotransplants have been described previously [Sens *et al.* 2004]. One μ g of total RNA was subjected to cDNA synthesis using the iScript cDNA synthesis kit (Cat. No. 170-8890; Bio-Rad Laboratories, Hercules CA) in a total volume of 20 μ L. The primers for keratin 16 (Cat. No. QT00065940) and keratin 17 (Cat. No. QT00001680) were purchased from QIAGEN (Valencia, CA). The primers for keratin 6a and 6b were developed using Oligo 6.0 software. The sequences of the upper and lower primer of keratin 6a along with the product sizes are as follows: sense: CTAAAGTGCCTGCTGCTA antisense: TGGGTGCTCAGATGGTATA (product size, 184 bp). The sequences of the upper and lower primer of keratin 6b along with the product sizes are as follows: sense: TCCTTTTGTAGTTCCCGTAT, antisense: TAATGGGCAGGATGGTTAG (product size, 151bp). Amplification of the cDNA was performed using the SYBR Green kit (Cat. No. 170-8880; Bio-Rad Laboratories) with 2 μ L cDNA and 0.2 μ M primers in a total volume of 20 μ L in an iCycler iQ real-time detection system (Bio-Rad Laboratories). Amplification was monitored by SYBR Green fluorescence. Cycling parameters consisted of denaturation at 95°C for 15 s, annealing at 55°C for 45 s (for keratin 6b, annealing at 54.3°C for 45 s), and extension at 72°C, which gave optimal amplification efficiency. The levels of keratin 6a, 6b and 16 were determined by serial standards. The resulting levels were normalized to the change in β -actin expression. The levels of keratin 17 mRNA were determined relative to the UROtsa cells using serial dilutions of this sample as the standard curve. The resulting relative levels were then normalized to the change in β -actin expression assessed by the same assay using the primers,

sense: CGACAACGGCTCCGGCATGT and antisense: TGCCGTGCTCGATGGGGTACT, giving a product size of 194 base pairs and with the cycling parameters of annealing/extension at 62°C for 45 s and denaturation at 95°C for 15 s.

Western Analysis of Keratin 6, 16 and 17 Protein Expression

Confluent cultures were harvested in 2% sodium dodecyl sulfate (SDS) and 50 mM Tris–HCl, pH 6.8, followed by boiling for 10 min and DNA shearing through a 23-ga needle. The protein concentration was determined using the bicinchoninic acid protein assay (Cat. No. 23225; Pierce Chemical Co., Rockford, IL) before 100 mM dithiothreitol was added to each sample. Frozen tumor heterotransplant tissue was homogenized in the buffer as mentioned above and was then treated in the same way as described for the isolates. Total cellular protein (20 µg) was separated on a 12.5% SDS-polyacrylamide gel and transferred to a hybond-P polyvinylidene difluoride membrane (Cat. No. RPN2020F; Amersham Biosciences, Piscataway, NJ). Membranes were blocked in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) and 5% (wt/vol) nonfat dry milk for 1 h at room temperature. After blocking, the membranes were probed with the keratin 6 (1:500), keratin 16 (1:500), keratin 17 (1:10000) primary antibody (Abcam) in blocking buffer overnight. After washing 3 times in TBS-T, membranes were incubated with the antimouse secondary antibody (1:2000) in antibody dilution buffer for 1 h. The blots were visualized using the Phototope-HRP Western Blot Detection System (Cat. No. 7071; Cell Signaling Technology, Beverly, MA).

Immunofluorescence Analysis of Keratin 6, 16 and 17 Protein in UROtsa Isolates

UROtsa cells were grown in 24 well plates containing 12 mm glass coverslips and processed while still at a subconfluent density. The cells were then fixed and stained as described previously [Flitney and Goldman, 2004; Khanobdee et al. 2004]. Briefly, cells to be stained for keratin 16 and 17 were fixed in ice cold 100% methanol for 3–5 min at –20°C. Cells to be stained for keratin 6 were fixed in 3.7% paraformaldehyde for 7–9 min at room temperature. Paraformaldehyde fixed cells were treated with 0.1 M NH₄Cl for 15 min to quench free aldehyde groups, followed by permeabilization with 0.1% Igepal (NP-40) for 3 × 3 min. The keratins were detected via indirect immunofluorescence using primary antibodies to cytokeratin-6, cytokeratin-16 and cytokeratin-17 from Abcam Inc, respectively. The primary antibody was diluted to a concentration of 18 µg/ml for mouse anti-keratin 6; 20 µg/ml for rabbit anti-keratin 16, and 1:50 for rabbit anti-keratin 17. Primary antibody was incubated on cells for 45–60 min at 37°C. Primary antibodies were detected using either Alexa Fluor 488 goat anti-mouse IgG (Cat. No. A11001) or Alexa Fluor 488 goat anti-rabbit (Cat. No. A11008) from Invitrogen (Carlsbad, CA). The secondary antibody was diluted to a concentration of 4.0 µg/ml and incubated on cells for 45–60 min at 37°C. Duplicate coverslips were stained with each of the keratin antibodies for all of the isolates. Controls consisted of coverslips treated only with the appropriate secondary antibody. Coverslips were mounted in ProLong Gold antifade reagent with DAPI (Invitrogen) for nuclear counter staining. Cells were observed and images captured using a Zeiss LSM 510 Meta Confocal Microscope with LSM 510 software (Carl Zeiss MicroImaging Inc.). Images were composed by capturing z-slices at a depth of 0.5 µm, stacking the z-slices together, and merging with the DAPI image of the same field so all cells in the field could be identified. Image processing and compilation was performed using Adobe Photoshop CS2.

RESULTS

Arsenite-Induced Transformation of Independent Cultures of UROtsa Cells

The results of the current protocol followed an experimental course very similar to that described previously for the malignant transformation of single cultures of UROtsa cells by As^{+3} [Sens *et al.* 2004]. Five of the eight UROtsa cell cultures originally exposed to $1\mu\text{M}$ As^{+3} were able to form colonies in soft agar and these were characterized further in this study. These resulting cultures were able to be subcultured at 1:20 ratios and multiple flasks of cells were prepared and processed for long term storage under liquid nitrogen. Combined with the individual isolates from this laboratory's previous study, 6 independent cultures of As^{+3} -transformed UROtsa cells were available for further characterization.

Cell Doubling Times, Light Level Morphology, Subcutaneous Heterotransplantation and Tumor Histology

The determination of doubling times for the As^{+3} -transformed isolates showed 4 of the 6 transformed lines to have doubling times significantly shorter than the UROtsa parental cell line and the other 2 As^{+3} -transformed isolates (Table 1). The light level morphology of the As^{+3} -transformed isolates showed that all the lines possessed an epithelial morphology (Fig 1). The cultures could be subcultured at a 1:20 ratio and formed cell monolayers with little evidence of a loss of contact inhibition of growth or the formation of multilayered foci of cells. There was no evidence of squamous differentiation such as multilayering of the cells, shedding of terminally differentiated cells into the growth medium, or the presence of keratin strands in the medium. There was no obvious correlation of light level morphology with the doubling times of the isolates.

Each isolate was inoculated s.c. at a dose of 1×10^6 cells in the dorsal thoracic midline of 5 nude mice to confirm that the cultures that were able to form colonies in soft agar were also capable of forming tumors. The mice were sacrificed 10 weeks after inoculation except for two mice, one in group UTAs#1 and one in group UTAs#3 that were sacrificed in week 8 due to displaying lethargic behavior and large tumor masses. There was tumor formation in each group of mice for each of the individual isolates of the As^{+3} -transformed isolates (Table 1). However, the parent UROtsa cell line failed to form tumors. There was some variation in the success of heterotransplantation among the isolates with tumor heterotransplants noted in 5 out of 5 mice for 3 isolates, 4 out of 5 for two isolates, and 3 of 5 for one isolate. There was no correlation of the ability of the isolates to form heterotransplants with cell doubling times or cell morphology of the tumor heterotransplants. These results demonstrated that all the As^{+3} -transformed isolates were capable of forming subcutaneous tumors in nude mice.

The subcutaneous tumor heterotransplants generated from the 5 new isolates of As^{+3} -transformed cells had a very similar histology to that described previously for UTAs#1 [Sens *et al.* 2004]. In general, the tumors were composed of infiltrating masses or nests of moderately differentiated cells with stratification of the malignant phenotype from the exterior to the central portion of the neoplastic masses (Fig 2). The peripherally located cells displayed less differentiation, with hyperchromatic nuclei, a higher nuclear to cytoplasmic ratio, and frequent mitotic profiles. All the isolates displayed a tumor histology that was similar to that expected of an invasive urothelial cell carcinoma. Also in agreement with the previous study was the finding that all the isolates contained areas of squamous differentiation of urothelial cells localized to the central or superficial cells. It was found that the degree of squamous differentiation, while constant within the tumors produced by each isolate, varied in prominence among the individual isolates (Table 1, Fig 2). The degree or prominence of squamous differentiation could be grouped into 3 patterns; mild (Fig 2A);

moderate (Fig 2D) and prominent (Fig 2 G, J). A prominent pattern of squamous differentiation was defined as a histology profile that displayed frequent keratin pearls, the presence of numerous intercellular bridges, and stratification with readily identifiable keratohyaline. Cytoplasmic vacuoles of the middle and superficial squamous layers were absent or very rarely seen in a prominent pattern of squamous differentiation. In contrast, a mild pattern of squamous differentiation was characterized by a histology that displayed no or very rare keratin pearls, intercellular bridges and stratifications with keratohyaline. In tumors with mild squamous differentiation, there were prominent cytoplasmic vacuoles or clearance in the cells of middle or superficial layer. A moderate pattern of squamous differentiation was assigned to tumors between these two patterns. Tumors with an intermediate squamous differentiation pattern displayed infrequent, but present, profiles of keratin pearls, intercellular bridges, and stratifications with keratohyaline. There were rare profiles of cytoplasmic vacuoles in the cells of the middle or superficial layer. The pattern of the squamous component did not correlate with doubling times or the success rate of tumor heterotransplantation.

Keratin 6 and 16 Staining Correlates to Areas of Squamous Differentiation in Subcutaneous Urothelial Tumors

All the subcutaneous tumors derived from the As⁺³-transformed isolates were immunostained for the expression and localization of the keratin 6 and 16 proteins. In all instances, the staining of keratin 6 and 16 was focal and localized to the cytoplasm of the cells. Microscopic examination demonstrated a conspicuous correlation of keratin 6 and 16 staining, both in localization and intensity of staining, with the prominence of squamous differentiation. An example of keratin 6 and 16 staining is illustrated for a subcutaneous tumor heterotransplant with mild squamous differentiation (Fig 2 B,C), moderate squamous differentiation (Fig 2 E, F) and two examples with prominent squamous differentiation (Fig 2 H, I, K, L). As described in the section immediately above, to the right of each panel is an H&E serial section illustrating tumor histology.

The Ability of the As⁺³-Transformed Isolates to Colonize Intraperitoneal Organs

Each isolate was inoculated at a dose of 1×10^6 cells into the peritoneal cavity of 6 nude mice to determine the ability of each isolate to colonize (seed) the organs located within the peritoneal cavity. The mice in all experimental groups were euthanized 53 days after injection, a time when visible inspection of the abdomen revealed several experimental groups to have substantial tumor growth. There was a wide variation among the respective As⁺³-transformed isolates in their ability to colonize the peritoneum (Table 2). The UTAs#1 and UTAs#3 isolates were able to form hundreds of tumor nodules within the peritoneal cavity of the nude mice. In contrast, the UTAs#4 and UTAs#6 isolates were only able to form a few tumor nodules, and the UTAs#2 and UTAs#5 isolates no tumor nodules within the peritoneal cavity (Table 2). The UTAs#1 and UTAs#3 isolates were also uniform in the ability to form large numbers of tumors within the peritoneal cavity of individual mice, with all mice in the group displaying large numbers of tumors. The UTAs#4 isolate, which produced a limited number of intraperitoneal tumors was also uniform in this ability among the entire group of 6 mice. The UTAs#6 isolate formed only one peritoneal tumor in one mouse of the experimental group. These results demonstrate that the individual As⁺³-transformed isolates varied greatly in their ability to colonize sites within the peritoneal cavity. The location of the tumors within the peritoneal cavity and the number per group of mice colonizing the sites were also determined for the As⁺³-transformed isolates that produced peritoneal tumors (Table 2).

The locations of the tumors can be grouped into several categories and an example of each location is provided in the accompanying figure (Fig 3). There are two types of tumors

associated with the peritoneum. The first is an injection site tumor that forms between the skin and the external surface of the peritoneum and is by definition a subcutaneous tumor (Fig 3 A, circled). These were not counted as a tumor of the peritoneum when analyzing tumor formation within the peritoneal cavity. The second type of peritoneal tumor (and the one listed in Table 2) is characterized by tumor nodules growing on the inside surface of the organ-facing surface of the peritoneum (Fig 3 B, C, arrows). The omentum defines tumors that grew on the greater omentum along the greater curvature of the stomach (Fig 3 C, identified with *). The pelvic cavity, superficial, defines a tumor located on the superficial part of the pelvic cavity just inferior to the intestinal loops (Fig 3 C, identified with +). The pelvic cavity, deep, defines a tumor found only following removal of the intestinal loops, is usually located in the pelvic part of the retroperitoneum, and most often attached to the posterior abdominal wall (Fig 3 D, identified with *). The liver defines a tumor located at the hepatic hilum, between the liver and the lesser curvature of the stomach. It is visualized after the liver is flipped to the cranial side (Fig 3 E, arrows), but is more apparent after the liver and stomach are removed from the peritoneum (Fig 3 F, identified with #). The spleen and pancreas defines tumors located between the greater curvature of the stomach and spleen (Fig 3 E, F, identified with *) and those that grew around the spleen. The diaphragm defines tumors when the normally thin, transparent diaphragm becomes thickened with a whitish appearance (Fig 3 G identified with * and normal diaphragmatic surface with #) or when a tumor nodule can be found on the abdominal surface of the diaphragm (Fig 3 H, identified with +). The intestine defines tumor nodules located in the mesentery (Fig 3 I) or serosal surface of intestinal tract (Fig 3 J identified with *). The kidney defines tumor nodules growing around the kidney in the retroperitoneum (Fig 3 K, identified with *). The results demonstrate that the independently generated As⁺³-transformed isolates had an appreciable variability in their ability to form tumors within the peritoneum.

Absence of Overt Squamous Differentiation in Peritoneal Tumor Heterotransplants and Potential Identification by Keratin 6 and 16 Immunoreactivity

To determine if a difference in squamous differentiation existed between peritoneal tumors and subcutaneous tumors, the histology of tumor nodules located in the pelvic cavity were examined for evidence of squamous differentiation. This examination was performed on H&E stained tissue sections from multiple intraperitoneal tumors produced by the UTAs#1 and UTAs#3 isolates. The results of this determination showed that tumors located within the peritoneal cavity had very few, if any, areas of overt squamous differentiation that would be identified on routine H&E examination. For illustration, sections were chosen that had the most potential within the histology profile for squamous character (Fig 4 A, D). In these examples, the majority of the cells were basal-like and similar to that expected for transitional cell carcinomas without overt squamous differentiation. The histology of the peritoneal tumors routinely showed none of the individual features of squamous differentiation such as keratinization, keratin pearls, intracellular bridges and keratohyaline. Tumors from the peritoneal region did display areas composed of larger cells with more cytoplasm which could be early markers of squamous differentiation. The method used to produce peritoneal tumors also produces a subcutaneous tumor at the injection site due to the leakage of small numbers of cells into the subcutaneous space. These small subcutaneous tumors provided an internal control to assure that the reduced squamous differentiation in the peritoneal tumors was not due to the experimental variable of heterotransplantation at different times in different mice. All the subcutaneous tumors at the injection site showed squamous differentiation similar to that described previously (Table 2, Fig 2). These results show that a subcutaneous location promotes heightened squamous differentiation of the tumors produced from the As⁺³-transformed isolates.

Immunostaining the intraperitoneal tumors generated from the UTAs#1 and #3 isolates for keratin 6 and 16 was remarkable for identifying areas of the tumors that might possess early features of squamous differentiation. There were frequent microscopic fields for each peritoneal tumor that were negative or that had only weak to moderate immunoreactive for keratin 6 and that showed no evidence of squamous differentiation. In contrast, when tissue sections showed particularly strong immunoreactivity for keratin 6, these areas could then be identified as areas with cells having possible early squamous differentiation. The majority of the peritoneal tumors had at least a few sections with such areas. An example of this staining is shown in Figure 4, panels B and E for keratin 6 and in panels C and F for keratin 16; both of which are serial sections of the H&E profiles described above (Fig 4 A, D). In these profiles, areas of strong keratin 6 staining and weak to moderate keratin 16 staining, identified by arrows, correlate to areas on H&E where the cells are somewhat larger and possess a more eosinophilic or clear cytoplasm. These features would be consistent with early to mild squamous differentiation. This is in contrast to the transitional cell morphology, indicated by stars that are negative or only weakly stained for keratin 6 and 16. Overall, strong immunoreactivity for keratin 6 and was an efficient means of identifying areas of potential squamous differentiation.

Keratin 17 Immunoreactivity in Subcutaneous and Peritoneal Tumor Heterotransplants

The series of subcutaneous tumors produced from the As⁺³-transformed isolates were also immunostained for the expression of keratin 17. Expression of keratin 17 was similar among all the heterotransplants generated from each individual As⁺³-transformed isolate (data not shown). There were three patterns of keratin 17 expression in all the subcutaneous tumor heterotransplants (Fig 5 A, B). The first pattern was a consistent correlation of keratin 17 expression and areas of squamous differentiation that could be readily identified on H&E stained tissue sections. The second pattern was keratin 17 expression in areas with a transitional or basal-like morphology and no evidence of squamous differentiation. The final pattern were areas that did not express keratin 17, but had the transitional or basal-like morphology identical to that described above in the second pattern that did display keratin 17 expression. The results of keratin 17 staining on the intraperitoneal tumors generated from the UTAs#1 and #3 isolates was similar to that for the subcutaneous tumors (Fig 5 C, D). The major difference being that there were far fewer areas of squamous differentiation available on which to correlate keratin 17 expression with squamous differentiation.

Keratin 6, 16 and 17 mRNA and Protein Expression in the As⁺³-Transformed Isolates and Subcutaneous Heterotransplants

The expression of mRNA for the keratin 6a, 6b, 16 and 17 genes was determined for the As⁺³-transformed isolates and their subcutaneous heterotransplants using real time RT-PCR (Fig 6 A–H). The corresponding expression of keratin 6, 16 and 17 protein was determined by western analysis (Fig 6 I, J). Due to the high degree of sequence homology, there are currently no methods to determine the specific expression of the keratin 6a and keratin 6b proteins. It was shown that each of the As⁺³-transformed isolates expressed keratin 6a mRNA and that the expression levels varied among the isolates, with 4 of 6 isolates being elevated above the expression level of the parental cell line (Fig 6 A). The expression of keratin 6a was also present in all the tumor heterotransplants and expression varied among the heterotransplants generated from the isolates (Fig 6 B). There was no apparent correlation between the level of expression of keratin 6a mRNA between the isolates and their corresponding tumor heterotransplant. There was no expression of keratin 6b mRNA in the As⁺³-transformed isolates (Fig 6 C). In contrast, there was expression of keratin 6b mRNA in all the tumor heterotransplants generated from the As⁺³-transformed isolates (Fig 6 D). The keratin 6 protein was expressed in all the As⁺³-transformed isolates (some at very low levels) including the parental control (very low expression) and there was a weak trend

for expression to follow that of keratin 6a mRNA expression (Fig 6 I). Keratin 6 protein was also expressed in all the tumor heterotransplants at similar levels, with the exception of that from UTAs#1, which showed a high level of expression compared to the other heterotransplants. The keratin 16 mRNA was expressed in all the As⁺³-transformed isolates and the parental control (Fig 6 E). Expression was variable and significantly increased in 5 of the 6 transformed lines compared to the parental control. Keratin 16 mRNA was also expressed in all the tumor heterotransplants and, while expression was variable, there was no correlation with the level of expression in the corresponding isolates (Fig 6 F). Keratin 16 protein was expressed in all the As⁺³-transformed isolates and the corresponding heterotransplants (Fig 6 I, J). Keratin 16 protein expression in the isolates correlated to the expression level of keratin 16 mRNA (Fig 6 E, I). The expression of keratin 16 protein was similar in level among the heterotransplants generated from the As⁺³-transformed isolates and had no correlation to the level of keratin 16 mRNA (Fig 6 F, J). The keratin 17 mRNA and protein was expressed in the As⁺³-transformed isolates in levels similar to that of the parental cell line (Fig 6 G, I). The expression of keratin 16 mRNA and protein was also similar among the corresponding tumor heterotransplants (Fig 6 H, J). The levels of keratin 6, 16 and 17 mRNA and protein were not determined for tumors produced by IP injection as all the tissue was processed for microscopic analysis.

Intracellular Localization of Keratin 6, 16 and 17 in the As⁺³-Transformed Isolates

The keratin 6 protein was expressed in all of the As⁺³-transformed isolates and in the parent UROtsa cells (Figure 7, A–G). There were two distinct patterns of keratin 6 expression. In the As#1, 3, and 4 isolates keratin 6 was organized into strongly stained intermediate filaments in over 50% of the total cell population (Fig 7 B, D, E). In the As #2, 5, 6 and parental cell lines keratin 6 staining was diffusely cytoplasmic in a small number of cells and there were very few, if any, profiles of keratin 6 filamentous staining similar to that described above for the As #1, 3 and 4 isolates (Fig 7 A, C, F, G). Keratin 16 expression was also examined in each of the As⁺³-transformed isolates and the UROtsa parent. The pattern of staining was found to be very similar among all the individual isolates and a representative illustration of the As#4 isolate is shown for reference (Fig 7 H). Keratin 16 was found to be organized in strongly stained filaments and this pattern was present in over 40% of the cell population in each of the isolates. Keratin 17 staining was also found to be very similar among the As⁺³-transformed isolates and the UROtsa parent. Keratin 17 was found to be organized into strongly stained filaments in approximately 100% of the cells of all the isolates and a representative image from the As #6 isolate is shown (Figure 7 I).

DISCUSSION

This laboratory has shown previously that As⁺³ exposure can cause the direct malignant transformation of the UROtsa human urothelial cell line. This single isolate was shown to form subcutaneous tumor heterotransplants consistent with those of a urothelial carcinoma of the bladder [Sens *et al.* 2004]. An additional interesting feature of these tumors was the presence of prominent areas of urothelial cells that had undergone squamous differentiation. As detailed in the introduction, a poor outcome has been associated with patients having urothelial carcinoma cells with squamous differentiation. Since the initial study was based on the generation of a single As⁺³-transformed urothelial cell culture, the first goal of the present study was to determine the repeatability of the transformation event. Eight independent cultures of UROtsa cells were exposed to 1.0 μ M As⁺³ following the identical protocol to that described previously by the laboratory [Sens *et al.* 2004]. The successful isolation of a new As⁺³-transformed transitional isolate was defined by its ability to form colonies in soft agar and subcutaneous tumor heterotransplants in immunocompromised mice. Employing this criteria, five new isolates were isolated that were shown to have the

ability to form colonies in soft agar and form subcutaneous tumors in nude mice. The initial characterization of the 5 isolates showed very similar phenotypic properties to the initial isolate reported using the above protocol. The morphology of the cells at the light microscopic level of examination were very similar and all showed an epithelial morphology. An important feature was that each isolate produced a subcutaneous tumor that displayed a similar tumor histology and that this histology was similar to that expected for an *in situ* human urothelial cell cancer. Furthermore, while the extent of the areas of urothelial cells showing squamous differentiation varied among each independent isolate, all displayed prominent areas of urothelial cell squamous differentiation. Thus, the 5 additional As⁺³-transformed urothelial isolates had very similar phenotypic properties to that reported previously for the initial As⁺³-transformed isolate.

An important phenotypic difference between the isolates was discovered when the 6 As⁺³-transformed isolates were assessed for the ability to establish peritoneal tumors following intraperitoneal injection of the cells. Two of the As⁺³-transformed isolates were able to form hundreds of tumors within the peritoneal cavity, one isolate a modest number of peritoneal tumors, and three isolates no or only one peritoneal tumor. This finding is potentially important since it is known from patients with bladder cancer that they tend to spread locally, requiring the ability of tumor cells to colonize the peritoneal organs following escape from the bladder. Approximately 80% of high grade transitional cell cancers are invasive. Aggressive tumors may extend only into the bladder wall; however, the more advanced stages invade the adjacent prostate and seminal vesicles in males, and the ureters and retroperitoneum in both males and females, and some produce fistulous communications to the vagina or rectum. Approximately 40% of these deeply invasive tumors metastasize to regional lymph nodes. Hematogenous dissemination, principally to the liver, lungs and bone marrow, generally occur late in bladder cancer and only with highly anaplastic tumors. As such, the current isolates may be particularly valuable in defining the genotypes underlying the differences in the ability of the individual isolates to colonize local sites outside of the bladder. It should be noted that the ability of the cells to colonize the peritoneal cavity gives no information on the initial stages involved in metastasis, which includes invasion and escape from the bladder proper. However, the isolates could provide valuable information on the ability of cells that have escaped the local tumor environment and need to re-seed and grow at distant organ sites to complete the cycle of metastasis. Thus, the phenotypic differences among the As⁺³-transformed isolates and their tumor heterotransplants should provide an excellent platform to define underlying genomic and proteomic differences that correlate with these individual phenotypes.

The ability of several of the As⁺³-transformed isolates to form peritoneal tumors also allowed a comparison of tumor histology between the peritoneal and subcutaneous transplant sites. It was one of the goals of the present study to determine if the subcutaneous environment was one that favored prominent squamous differentiation of the urothelial cells. This was especially important in the present study since human exposure to arsenic is known to cause hyperkeratosis of the skin [Steinmaus *et al.* 2000]. The results of this analysis clearly showed that peritoneal tumors displayed vastly reduced squamous differentiation compared to the subcutaneous transplant site. While areas of squamous differentiation could be found for each peritoneal tumor examined, the occurrence and degree of such differentiation was greatly reduced for the peritoneal tumors. The majority of individual tissue sections showed urothelial cells with no evidence of squamous differentiation. A fortuitous aspect of the intraperitoneal injection also provided an internal control to compare squamous differentiation between the peritoneal and subcutaneous tumors in the same mouse. There is leakage of tumor cells into the subcutaneous space during the intraperitoneal injections which results in a small subcutaneous tumor. These subcutaneous tumors displayed prominent squamous differentiation and tumor histology identical to those

described initially when each isolate was tested specifically for subcutaneous tumor growth. The results show that peritoneal tumors produced from the two As⁺³-transformed isolates have reduced squamous differentiation, but that areas of squamous differentiation of the urothelial cells can still be visualized on H&E examination by diagnostic pathologists. As detailed in the introduction there is evidence that squamous differentiation is an indication of tumors having a poor prognosis for the patient. Despite this association of squamous differentiation with a poor prognosis, there is limited or no information in the literature on the mechanism that promotes the malignant urothelial cell to undergo squamous differentiation. This is likely due to a lack of a urothelial cell model system that undergoes such differentiation either *in vitro* or *in vivo*. Thus, the present cell culture and heterotransplant model should provide a unique system to probe the mechanism underlying the ability of a malignant urothelial cell to undergo squamous differentiation.

The laboratory has shown that keratin 6 was overexpressed in subcutaneous tumor heterotransplants generated from the original As⁺³-transformed UROtsa isolate [Somji et al. 2008]. It was also shown in this report using immunohistochemistry, that a subset of archival specimens of human bladder cancer also displayed focal expression of keratin 6 protein. These findings have allowed the laboratory to hypothesize that keratin 6 might be a general marker for squamous differentiation in bladder cancer and a biomarker for bladder cancers arising from heavy metal exposure. Further evidence in support of the first hypothesis was generated in the current study. An immunohistochemical analysis of keratin 6 staining in the subcutaneous heterotransplants generated from the 6 As⁺³-transformed isolates showed a strong correlation of keratin 6 staining with areas of squamous differentiation. No areas of squamous differentiation were found on pathologic examination that were not immunoreactive for keratin 6. Similarly, there was a corresponding absence of keratin 6 immunoreactivity in areas of urothelial cells that displayed no squamous differentiation. The evidence for a direct relationship between keratin 6 immunoreactivity and squamous differentiation was reinforced further in an examination of the peritoneal tumors. In these tumors, squamous differentiation was reduced, and keratin 6 staining was identified only in regions having or suspected of having squamous differentiation on H&E examination. In fact, keratin 6 immunoreactivity allowed one to focus on, and more easily identified, areas in the peritoneal tumors that had areas of squamous differentiation. It was also noted, that keratin 16, the known type I keratin pair for keratin 6 [Moll et al. 2008], followed an identical pattern of immunoreactivity to keratin 6, but with less intense staining. Thus, there was a strong correlation of keratin 6 immunoreactivity with areas of squamous differentiation in tumors derived from As⁺³-transformed urothelial cells.

There is a limited amount of information on the expression of K6/16 in normal and malignant cells and tissues [Moll et al. 2008]. In normal human epithelial tissues, keratin 6/16 expression has been reported in basal cells of the respiratory epithelium and in the suprabasal compartment of non-keratinizing stratified squamous epithelia [Moll et al. 2008]. In agreement, the previous study from this laboratory showed that the K6/16 pair was not expressed in normal urothelium [Somji et al. 2008]. In normal tissues, the K6/16 pair have been associated with various hyperproliferative epidermal disorders, suggesting these keratins may be molecular markers for hyperproliferative keratinocytes [Weiss et al. 1984; Moll et al. 2008]. They are not expressed in non-proliferating skin keratinocytes, but during skin wounding the K6/16 pair are rapidly induced at the wound edge before migration and regeneration begins to occur [Paladini et al. 1996]. Once healing is complete, the expression of K6/16 is down-regulated to undetectable levels. Keratin 6 has been detected in the luminal cells of the embryonic mammary gland but not the mature gland [Grimm et al. 2006]. Keratin 6 has been detected in cells of the prostate gland with high potential for proliferation and differentiation [Schmelz et al. 2006]. Keratin 6/16 expression has not been previously associated with squamous differentiation in human urothelial cell cancer or in

As⁺³ derived experimental tumors. In contrast, K6/16 have been shown to be strongly expressed in squamous cell carcinomas of different sites, preferentially in inner, maturing layers of tumor cell nests [Moll *et al.* 1982]. A low expression of keratin 6 has been found in adenocarcinomas of the uterine cervix, but it was not reported to be associated with areas of squamous differentiation but rather with metaplasia [Smedts *et al.* 1993]. An antibody that recognizes both keratin 5 and keratin 6 has been advanced as a possible adjunct in the diagnosis of breast cancer of basal character, but these studies do not indicate which keratin/s (5, 6 or both) are being expressed in the tumors [Livasy *et al.* 2007; Siziopikou and Cobleigh, 2007; Tischkowitz *et al.* 2007]. It had been suggested in early reports that keratin 5, and not keratin 6, is the keratin usually being observed in immunostaining protocols of breast tumors [Otterbach *et al.* 2000]. A unique aspect of the present study is that keratin 6/16 expression identifies only the subpopulation of malignant urothelial cells showing squamous differentiation.

The expression of keratin 6, 16 and 17 mRNA and protein were examined in the As⁺³ -transformed isolates and tumor heterotransplants. The results posed more questions for future study than immediate answers. Keratin 6 and 16 were expressed in all the As⁺³ -transformed isolates and heterotransplants. However, there was only a weak, if any, correlation between the levels of mRNA and protein expression in the individual isolates. This was also true between the isolates and the corresponding tumor heterotransplants. That this variability was not simply experimental error was suggested by the findings with keratin 17, where expression of mRNA and protein was very consistent among and between the isolates and tumor heterotransplants. This suggests that there may be a component of post-transcriptional regulation in keratin 6 and 16 expression. A particularly interesting finding was that only the keratin 6a gene was expressed in the As⁺³ -transformed cell culture isolates; where as, both the keratin 6a and keratin 6b genes were expressed in all the tumor heterotransplants. Another potentially significant finding was found during the confocal analysis of the intracellular organization of keratin 6 expression in the As⁺³ -transformed isolates. In the As#1, As#3 and As#4 isolates, keratin 6 was organized into strongly stained intermediate filaments in a majority of the cells and it was these isolates that were able to form peritoneal tumor heterotransplants. In contrast, the other isolates and the parent UROtsa cells displayed no such organization of keratin 6 and no peritoneal tumors were formed by these isolates. Keratin 16 and 17 organization was similar among all the isolates.

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Figure 1. Phase contrast light microscopy of the six As^{+3} -transformed isolates demonstrating epithelial morphology in all isolates

Panel A is UTAs#1, B) is UTAs#2, C) is UTAs#3, D) is UTAs#4, E) is UTAs#5, F) is UTAs#6.

Figure 2. H&E Histology and Keratin 6 and 16 Immunostaining for As³⁺-Transformed Tumor Heterotransplants Exhibiting Mild, Moderate and Prominent Squamous Differentiation

Panels A, B and C shows the histology (A), keratin 6 immunostaining (B), and keratin 16 immunostaining (C) of a subcutaneous tumor heterotransplant that exhibits a mild degree of squamous differentiation. Panels D, E, and F shows the histology (D), keratin 6 immunostaining (E) and keratin 16 immunostaining (F) of a subcutaneous tumor heterotransplant that exhibits a moderate degree of squamous differentiation. Panel G through L shows the histology (G, J), keratin 6 immunostaining (H, K) and keratin 16 immunostaining (I, L) of two subcutaneous tumor heterotransplants that both exhibit a prominent degree of squamous differentiation. The histology, keratin 6 immunostaining and keratin 16 immunostaining in each panel are serial sections of the respective tumor heterotransplant. Arrows illustrate representative areas of squamous differentiation while stars illustrate representative areas without squamous differentiation and a morphology similar to that seen in transitional cell carcinomas. Keratin 6 and 16 are strongly immunoreactive in areas of squamous differentiation, but negative or weak in areas of transitional cell morphology. Keratin pearls illustrated with a + were negative for the expression of keratin 6 and 16. All images represent a magnification of 200 $\times$.

Figure 3. Examples of the distribution of tumor nodules in abdomen after IP injection of As^{+3} -transformed UROtsa isolates in nude mouse

Panel A shows the injection site nodule on outside of peritoneum, B) Peritoneal (internal) tumor nodule, C) *In situ* view, peritoneal (arrows), greater omentum (*) and superficial pelvic cavity (+) tumors, D) *In situ* with intestines removed, deep pelvic cavity tumors (*), E) *In situ*, intestines removed, liver reflected upward. Liver (arrows) and splenic area (*) tumors, F) *In bloc*, liver, stomach, duodenum, spleen: Tumor at inferior liver surface (#) and splenic area (*), G) *In situ*, liver and intestines removed. Tumor deposits (*) on abdominal surface of diaphragm appear as whitish growths in contrast to normal (#) diaphragmatic surface, H) *In situ*, liver and intestines removed. Larger tumor deposits (+) and normal (#) diaphragmatic surface, I) *In situ*, reflection of large intestine cephalad, mesenteric tumors (*), J) *In situ*, intestinal and mesenteric tumors (*), K) *In situ*, intestines and liver removed. Tumor deposits in retroperitoneum and surrounding kidney (*).

Figure 4. Histology and Keratin 6 and 16 Immunoreactivity of Peritoneal Tumor Heterotransplants

Panel A: H&E histology of a peritoneal tumor formed by the UTAs#1 isolate. The tumor is composed of sheets of tumor cells with cells at the periphery having a morphology similar to transitional cell carcinoma (stars). Some cells in the center are larger with an enhanced eosinophilic or clear cytoplasm (arrows). Panel B: Keratin 6 immunostaining of a serial section to that shown in Panel A. The large cells with an enhanced eosinophilic or clear cytoplasm show strong staining for keratin 6 (arrows). The smaller cells with a transitional cell morphology show absent to moderate staining for keratin 6. Panel C: Keratin 16 immunostaining of a serial section to that shown in panel A and B. Keratin 16 staining is localized to the same areas as that of keratin 6, but there is less intensity and frequency of staining of keratin 16 compared to that of keratin 6. Panel D: H&E histology of a peritoneal tumor formed by the UTAs#3 isolate. The morphology is similar to that in Panel A except there are fewer areas of cells with an enhanced eosinophilic or clear cytoplasm (arrows). Panel E: Keratin 6 immunostaining for a serial section to that shown in Panel D. The large more eosinophilic cells or those with a clear cytoplasm show strong staining for keratin 6 (arrows) while the smaller cells with a transitional cell morphology show absent to moderate staining for keratin 6. Panel F: Keratin 16 immunostaining of a serial section to that shown in panel D and E. Keratin 16 staining is localized to the same areas as that of keratin 6, but again there is less intensity and frequency of staining of keratin 16 compared to that of keratin 6. The sections were chosen to illustrate areas that displayed intense keratin 6 immunoreactivity. Magnification is 200 $\times$.

Figure 5. Immunostaining of keratin 17 in subcutaneous and intraperitoneal tumor heterotransplants

A) Immunostaining of keratin 17 in subcutaneous tumor heterotransplants. The squamous differentiated areas in the center of the tumor nests are strongly positive for keratin 17 (arrows), while the areas with urothelial or basal like morphology are negative or faintly positive (*). B) Immunostaining of keratin 17 in subcutaneous tumor heterotransplants. The peripheral basal like cells of tumor masses (arrow heads) are strongly positive for keratin 17 while the central areas are negative or very weakly positive. C) Immunostaining of keratin 17 in intraperitoneal tumor heterotransplants. The larger cells with more cytoplasm in the center of the tumor nest are positive for keratin 17 (arrows), whereas the peripheral areas with morphology resembling urothelial carcinoma (*) are negative or only faintly positive. D) Immunostaining of keratin 17 in intraperitoneal tumor heterotransplants. Areas with transitional or basal-like morphology are positive for keratin 17 (arrow heads) whereas most of the tumor composed of large tumor cells with abundant cytoplasm are negative.

Figure 6. Expression of Keratin 6, 16 and 17 mRNA and protein in UROtsa parent, As⁺₃-transformed isolates and subcutaneous tumor heterotransplants

A, C, E and G: Real time PCR analysis of keratin 6a, 6b, 16 and 17 in UROtsa parent and transformed isolates. B, D, F and H: Real time PCR analysis of keratin 6a, 6b, 16 and 17 in tumor heterotransplants. I: Western analysis of keratin 6, 16 and 17 in UROtsa parent and As⁺₃-transformed isolates. J: Western analysis of keratin 6, 16 and 17 in tumor heterotransplants. Statistical analysis consisted of ANOVA with Tukey post-hoc testing, performed by GraphPad PRISM 4. * denote a significant difference from UROtsa parental cells ($p < 0.05$). Real time PCR data is plotted as the mean $\pm$ SEM of triplicate determinations.

Figure 7. Keratin immunofluorescent staining with DAPI counter stain in arsenite-transformed isolates

Keratin 6 staining is shown for (A) UROtsa parent; (B) As #1; (C) As #2; (D) As #3; (E) As #4; (F) As #5; (G) As #6. Keratin 6 positive staining was observed in all isolates, however, keratin 6 was found to organize into strongly stained intermediate filaments in only a subset of the isolates As #1 (B), As #3 (D), and As #4 (E). Strongly stained keratin 16 intermediate filaments are shown for the As #4 isolate (H) and strongly stained keratin 17 filaments are shown for the As #6 isolate (I). Controls included staining using the secondary-antibody only and were found to be negative for all isolates and for both secondary antibody conditions (not shown). Bar = 20 μ m.

TABLE 1
Initial Characterization of Independently Generated Arsenite Transformed Cell Lines

Cell Line	Subcutaneous Transplantation	Size (cm) Median	Doubling Time (h)	Tumor Histology	Squamous Component
UT As #1	5/5	2.5	26.4 + 1.1	Urothelial Carcinoma	Moderate
UT As #2	4/5	1.2	14.1 + 0.9 ^d	Urothelial Carcinoma	Moderate
UT As #3	5/5	2.5	33.3 + 1.4	Urothelial Carcinoma	Mild
UT As #4	5/5	1.8	21.0 + 0.9 ^d	Urothelial Carcinoma	Prominent
UT As #5	3/5	1.1	16.4 + 0.7 ^d	Urothelial Carcinoma	Mild
UT As #6	4/5	1.2	21.6 + 1.6 ^d	Urothelial Carcinoma	Prominent
UROtsa Parent			33.2 + 0.8		

^dSignificantly different (P=.05) from Parent UROtsa cells and UTAS#1 and #3

Ability of Arsenite Transformed UROtsa Cells to Seed Organs Following IP Injection

TABLE 2

Cell Lines	Tumor Formation	Wt (g)/Average	# of Tumor per mouse	Seeding of tumor nodules in peritoneal cavity								Intestine	Kidney
				Peritoneum	Greatum Omentum	Pelvic cavity Superficial	Pelvic cavity, Deep	Liver	Spleen & Pancreas	Diaphragm			
UTAs#1	6/6	29.45	>100	6/6	6/6	6/6	6/6	6/6	5/6	4/6	5/6	6/6	
UTAs#2	0/6	24.29	0	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	
UTAs#3	6/6	28.85	>100	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	
UTAs#4	6/6	26.13	3-20	6/6	0/6	0/6	0/6	3/6	0/6	0/6	6/6	0/6	
UTAs#5	0/6	25.35	0	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	
UTAs#6	1/6	25.25	1	1/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	