Involvement of Epigenetics and EMT-Related miRNA in Arsenic-Induced Neoplastic Transformation and Their Potential Clinical Use

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Abstract

Exposure to toxicants leads to cumulative molecular changes that overtime increase a subject's risk of developing urothelial carcinoma. To assess the impact of arsenic exposure at a time progressive manner, we developed and characterized a cell culture model and tested a panel of miRNAs in urine samples from arsenic-exposed subjects, urothelial carcinoma patients, and controls. To prepare an in vitro model, we chronically exposed an immortalized normal human bladder cell line (HUC1) to arsenic. Growth of the HUC1 cells was increased in a time-dependent manner after arsenic treatment and cellular morphology was changed. In a soft agar assay, colonies were observed only in arsenic-treated cells, and the number of colonies gradually increased with longer periods of treatment. Similarly, invaded cells in an invasion assay were observed only in arsenic-treated cells. Withdrawal of arsenic treatment for 2.5 months did not reverse the tumorigenic properties of arsenic-treated cells. Western blot analysis demonstrated decreased PTEN and increased AKT and mTOR in arsenic-treated HUC1 cells. Levels of miR-200a, miR-200b, and miR-200c were downregulated in arsenic-exposed HUC1 cells by quantitative RT-PCR. Furthermore, in human urine, miR-200c and miR-205 were inversely associated with arsenic exposure (P=0.005 and 0.009, respectively). Expression of miR-205 discriminated cancer cases from controls with high sensitivity and specificity (AUC=0.845). Our study suggests that exposure to arsenic rapidly induces a multifaceted differentiation program and miR-205 has potential to be used as a marker of arsenic exposure as well as a maker of early urothelial carcinoma detection. Cancer Prev Res; 8(3); 208–21. ©2015 AACR.

Introduction

Arsenic-induced carcinogenesis emerged as an international environmental health issue in the late 1960s when arsenic-contaminated drinking water was found to cause cancer. Epidemiologic studies have demonstrated the pleiotropic nature of arsenic toxicity in humans at exposure levels that are relevant to environmental health hazards frequently experienced by human populations throughout the world (1). Previous studies suggest that arsenic causes the promotion and progression of several diseases, including cancer and noncancer illnesses (2–5). In addition, individual genetic variation, nutrition factors, and other environmental factors (such as smoking) appear to contribute to the severity of toxicities induced by arsenic (2).

On the basis of epidemiologic evidence from southwestern Taiwan, arsenic in drinking water was associated with the development of multiple cancers, including urothelial carcinoma of the bladder, in a dose-dependent manner (6). Recent studies from both Chile and northeastern Taiwan provide further support for the association between arsenic exposure in drinking supplies and increased incidence of cancers (7). As urine excretion is the primary route for arsenic elimination, the bladder epithelium may thus be exposed to higher concentrations of arsenic due to the bioconcentration of urine by the kidneys. In addition to inorganic arsenic (arsenite and arsenate), the human bladder is exposed to mono- and dimethylated arsenic metabolites systematically or urinary filtrate. Accumulated evidence, therefore, suggests that the bladder epithelium may be one of the primary targets of arsenic-induced carcinogenesis.

The etiology of urothelial carcinoma is not fully understood at this time. Large-scale epidemiologic studies have found no association between urothelial carcinoma incidence within first-degree relatives and have therefore argued strongly against a germline genetic mechanism (8). On the other hand, several environmental risk factors, such as tobacco-related carcinogens, arsenic, aromatic amines, polycyclic aromatic or halogenated hydrocarbons, and ionizing radiation, have been linked to urothelial carcinoma incidence (9). Recently, arsenic exposure...
to humans has become a significant public health concern. Consequently, an in-depth study is needed to make public health policy decisions and to identify underlying pathogenic molecular events related to arsenic-induced carcinogenesis. Although there is extensive epidemiologic evidence of increased risk for the development of urothelial carcinoma associated with arsenic exposure (10), the mechanisms by which arsenic participates in tumorigenesis are not well understood. To gain mechanistic insights, in vitro and in vivo models can be used. Arsenic-induced cancer animal models have been difficult to develop due to significant species-specific differences in arsenic metabolism. Thus suitable in vitro human-originated models that replicate arsenic exposure in humans are needed to investigate arsenic carcinogenesis (10). In vitro models of human origin need to be extensively characterized and tested to ensure adequate representation of the effects seen in humans chronically exposed to arsenic. Although the lack of a fully differentiated urothelium presents a limitation, an in vitro system provides an easily handled model to work suitable for identification of progressive genetic and epigenetic changes. Here, we report the establishment of an arsenic-exposed in vitro urothelial carcinoma carcinogenesis model. We further characterize critical cell signaling pathways (such as the NOTCH pathway and the PI3K–AKT pathway) and miRNAs related to epithelial–mesenchymal transition (EMT). Understanding these biological effects of arsenic at the molecular level will facilitate the identification of appropriate noninvasive markers of arsenic exposure and assess promising drugs for prevention and therapeutic strategies for urothelial carcinoma.

Materials and Methods

Cell lines and reagents

Normal human urothelial cell line HUC1 [Simian Virus 40 (SV40) immortalized normal human urinary tract epithelial cells] was obtained from the American Type Culture Collection (ATCC). HUC1 cells were cultured in F12K medium (Mediatech) supplemented with 10% fetal bovine serum (FBS; Mediatech) and 1% penicillin–streptomycin solution (Mediatech) under a 5% CO2 atmosphere at 95% relative humidity. Arsenic trioxide (As2O3) and DMSO were obtained from Sigma-Aldrich and DMSO was used as a control. HUC1 cells were exposed to varying concentrations of As2O3 to determine the lethal concentration in 50% of the cells (LC50) over 72 hours. The LC50 for As2O3 in HUC1 cells was determined to be 1 μmol/L. Thus, 1 μmol/L was selected for chronic testing, which was non-toxic to cells. HUC1 cells were cultured in a 25-cm flask in F12K complete medium (F12K) with or without 1 μmol/L As2O3. Medium and arsenic was changed every 2 days. Cells were subcultured as necessary and frozen down each month for future studies. To determine the arsenic withdrawal effect, we cultured the 8- and 10-month arsenic-treated HUC1 cells without arsenic for 2.5 months and performed the MTT, soft agar, and invasion assay.

Cellular viability assay (MTT assay)

We performed the MTT assay at 2, 4, 6, 8, and 10 months of arsenic-treated and mock-treated cells. Cell proliferation was measured by the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) proliferation assay kit from the ATCC according to the manufacturer’s instructions and as described previously (12, 13).

Immunoblotting analysis

Urothelial carcinoma tumors comprise a heterogeneous group with respect to both histopathology and clinical behavior. Alterations of different molecular pathways have been proposed and the MAPK–PI3K–AKT pathway has been reported to play a principal role in urothelial carcinoma carcinogenesis (14). Deregulation of genes included in this pathway has been reported in both non–muscle-invasive and muscle-invasive urothelial carcinoma; and we recently reported that several PI3K–AKT pathway genes have been altered in HUC1 cells after exposure to cigarette smoke (CS; ref. 12). As arsenic is a constituent of CS, we tested the expression of PI3K–AKT pathway genes (PI3K, AKT, and mTOR) in our arsenic-treated and untreated cell lines. Briefly, arsenic-treated and untreated (6, 8, 10 months) cells were lysed on ice for 30 minutes in RIPA (radioimmunoprecipitation assay buffer) buffer [150 mmol/L NaCl, 100 mmol/L Tris (pH 8.0), 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 5 mmol/L EDTA, and 10 mmol/L NaF], supplemented with 1 mmol/L phenylmethylsulfonylfluoride and protease inhibitor mixture (Sigma-Aldrich). After centrifugation at 12,000 rpm for 15 minutes, the supernatant was harvested as the total cellular protein extract. The protein concentration was determined using the Lowry protein assay (Bio-Rad Laboratories). Equal amounts of protein (40 μg each) were mixed with Laemmli sample buffer (62.5 mmol/L Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1 mol/L DTT, and 0.01% bromophenol blue), run on 4% to 12% NuPAGE and electroblotted onto nitrocellulose membrane (Bio-Rad Laboratories). The membrane was blocked with phosphate-buffered saline (PBS) supplemented with 0.1% Tween 20 and 5% nonfat milk or 5% bovine serum albumin (BSA) for 1 hour at room temperature, and probed with primary antibody overnight at 4°C followed by horseradish peroxidase (HRP)–conjugated appropriate secondary antibody for 1 hour at room temperature followed by enhanced chemiluminescence detection (Santa Cruz Biotechnology). All the immunoblotting experiments were performed at least two times. Antibodies for p16, phosphor and total ERK1/2, phosphor and total EGFR, phosphor and total PI3K, HRP-conjugated anti-rabbit IgG, and antimouse IgG were purchased from Santa Cruz Biotechnology.

Soft agar assay

Soft agar plates were made with Agar Select (Invitrogen) at 0.8% (bottom layer) and 0.3% (top layer). Five thousand arsenic-treated and untreated cells (6 months, 8 months, and 10 months, 8 months treated and 2.5 months arsenic withdrawal, 10 months treated and 2.5 months arsenic withdrawal) were counted and seeded in the top layer of agar mixed with F12K medium.
supplement with 10% FBS and 1% penicillin–streptomycin solution in 6-well plates and incubated in 37°C. Complete medium was added every 2 days (500 μL/well). The cells were allowed to grow for 2 weeks and colonies were photographed and counted using a light microscope. Each experiment was performed in triplicate wells and repeated three times.

Invasion assay

Invasion assay was performed using individual inserts coated with Matrigel (cat no. 354480; BD Biosciences) as directed by the manufacturer. Briefly, 1 × 10⁶ arsenic-treated and untreated (6 months, 8 months and 10 months, 8 months treated and 2.5 months arsenic withdrawal, 10 months treated, and 2.5 months arsenic withdrawal) cells were plated in triplicates in serum-free media. Cells were allowed to attach and grow for 48 hours. Invaded cells were fixed using 100% methanol and stained with hematoxylin and eosin (H&E). Invaded cells were counted under a microscope in 10 randomly selected fields (magnification, ×100) per well and averaged. To normalize for cell invasion differences, each cell line was also grown on an uncoated insert. Number of invaded cells was divided by the number of cell counted on the uncoated inserts.

DNA extraction

Total genomic DNA was extracted by digestion with 50 μg/mL proteinase K (Boehringer) in the presence of 1% SDS at 48°C overnight, followed by phenol/chloroform extraction and ethanol precipitation. Genomic DNA was eluted in low-salt Tris-EDTA (10 mM) buffer and stored at −20°C.

RNA and miRNA extraction

Total RNA was extracted after cell lysis with Qiazol (Qiagen) followed by phenol extraction and ethanol precipitation. Total RNA was eluted in diethyl pyrocarbonate (DEPC)–treated water and stored at −80°C. microRNA (miRNA) extraction was performed using the MiVana miRNA Isolation Kit, (Ambion; cat no. AM1560) according to the manufacturer’s instructions.

Real-time reverse transcriptase PCR for quantification of miRNAs

Total RNA (20 ng), isolated from cells was reverse transcribed using TaqMan reverse transcription kit (Applied Biosystems) and RNA-specific primers provided with TaqMan microRNA assays (Applied Biosystems) in 15 μL reaction volume that contains 3 μL of RT Primer Mix, 0.15 μL of 100 mmol/L dNTPs, 1 μL of reverse transcriptase enzyme 50 U/μL, 0.19 μL of RNase inhibitor 20 U/μL, 4.16 μL of nuclease-free water, and 5 μL of RNA (20 ng). Reverse transcription (RT) reaction was carried out with annealing at 16°C for 30 minutes followed by extension at 42°C for 30 minutes. RT reaction (1.5 μL) was then used with 1-μL specific primers for each of the miR-222, miR-200a, miR-200b, miR-200c, and miR-205 (Applied Biosystems) in triplicate wells for 40-cycle PCR on a 7900HT thermocycler (Applied Biosystems). The thermal cycling parameters were as follows: 50°C for 2 minutes, 95°C for 10 minutes, followed by a third step for denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for 1 minutes repeated for 40 cycles. SDS software (Applied Biosystems) was used to determine cycle threshold (Ct) values of the fluorescence measured during PCR. Results of miRNA were normalized to miR-222 relative expression using the ΔCt method and then the tested miRNAs in the arsenic-treated periods were normalized using the ΔΔCt method to the expression levels of their untreated matching period. Selection of endogenous control miRNA for normalization is not yet established. Most prior reports used RNAU6 (RNU6B) and RNU48 and normalization. However, neither of these was expressed in all the urine supernatants and the expression levels were variable. We tested three miRNA (RNU6B, mir-222, and mir-16) in a subset of our urine supernatants from controls and cancer cases. Among these three molecules, mir-222 was found to be almost equally expressed in all the samples tested (data not shown). Therefore, we used miR-222 for normalization.

Methylation profiling of arsenic-treated and untreated HUC1 cells by NOTCH signaling pathway DNA methylation PCR array

Because of the growing interest of NOTCH signaling pathway in human carcinogenesis and reports of epigenetic alterations related to environmental exposure, we performed Notch Signaling Pathway DNA Methylation PCR Array analyses using the EpiTect Methyl II PCR System (SA Biosciences; cat no. EHSA-611ZE). The Human Notch Signaling Pathway Epitected Methyl II Signature PCR Array (SA Biosciences; cat no. EHSA-611ZE) profiles the promoter methylation status of a panel of 22 genes central to NOTCH signal transduction. The spreadsheets, gene tables, and template formulas included with the PCR array package were used to calculate relative changes in promoter methylation status of each of the gene. The method used by the EpiTect Methyl II PCR System (SA Biosciences) is based on detection of remaining input DNA after cleavage with a methylation-sensitive and/or a methylation-dependent restriction enzyme (15). These enzymes will digest unmethylated and methylated DNA, respectively. Following digestion, the remaining DNA in each individual enzyme reaction is quantified by real-time PCR using primers that flank a promoter (gene) region of interest. The relative fractions of methylated and unmethylated DNA are subsequently determined by comparing the amount in each digest with that of a mock (no enzymes added) digest using a ΔCt method. Briefly, 0.4 μg of DNA after digestion with Mo, Ms, Md, and Msd enzymes were placed in each well of a 384-well PCR array plate (SA Biosciences; cat no. EHS-611ZE) that contained a panel of primer sets for a thoroughly researched set of 22 Notch pathway genes, plus two positive/negative controls to determine assay performance. The methylation PCR reaction was performed in Applied Biosystem 7900HT sequence detector with 10-μL total volume. The amplification conditions were the following: 10 minutes at 95°C, a three-cycle step at 99°C for 30 seconds and 72°C for 1 minute and a 40 cycle step at 97°C for 15 seconds and 72°C for 1 minute. The relative quantity of methylated and unmethylated DNA in each sample in the PCR array was calculated using the following formula as instructed by the manufacturer:

\[ C_{Mo} = \frac{1}{2^{\Delta Ct(Mo)}} \quad C_{Ms} = \frac{1}{2^{\Delta Ct(Ms)}} \quad C_{Md} = \frac{1}{2^{\Delta Ct(Md)}} \quad C_{Msd} = \frac{1}{2^{\Delta Ct(Msd)}} \]

Unmethylated (U) DNA fraction: \( F_{U} = \frac{C_{Mo} + C_{Ms} + C_{Md}}{C_{Mo} + C_{Ms} + C_{Md} + C_{Msd}} \)

Hypermethylated (H) DNA fraction: \( F_{H} = \frac{C_{Mo} + C_{Ms} + C_{Md}}{C_{Mo} + C_{Ms} + C_{Md} + C_{Msd}} \)

Quantitative methylation-specific PCR for selected genes

Representative candidate genes (NFKB2 and NCSTN) with significant promoter methylation changes identified by NOTCH Signaling Pathway DNA Methylation PCR Array were chosen for
technical validation by quantitative methylation-specific PCR QMSP analysis. DNA was bisulfate-treated (Epitect Kit; Qiagen) and analyzed with QMSP. Fluorogenic PCR reactions were carried out in a reaction volume of 20 μL consisting of 600 nmol/L of each primer; 200 μmol/L probe; 0.75 U platinum Taq polymerase (Invitrogen); 200 μmol/L of each dATP, dCTP, dGTP, and dTTP; 200 nmol/L ROX dye reference (Invitrogen); 16.6 mmol/L ammonium sulfate; 67 mmol/L Trizma (Sigma-Aldrich); 6.7 mmol/L magnesium chloride; 10 mmol/L mercaptoethanol; and 0.1% DMSO. Triplicates of 3 μL of bisulfite-modified DNA solution were used in each QMSP amplification reaction. Primers and probes were designed to specifically amplify the promoters of the two genes of interest and the promoter of a reference gene, β-actin (ACTB). Primer and probe sequences and annealing temperatures are provided in Supplementary Table S1A.

cDNA synthesis and RT-PCR
To validate the expression of differentially methylated genes due to arsenic exposure as identified by NOTCH methylation array, we performed reverse transcriptase PCR (RT-PCR) using commercially available TaqMan Expression assays (Life Technologies). Total RNA (1 μg) was converted to cDNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer’s protocol. The first-strand cDNA synthesis reaction is catalyzed by SuperScript™ II Reverse Transcriptase (RT). This enzyme has been engineered to reduce the RNase H activity that degrades mRNA during the first-strand reaction, resulting in a greater full-length cDNA synthesis and higher yields of first-strand cDNA than obtained with RNase H+ RTs. 40 ng of cDNA was used as a template in PCR reaction that contain 1 μL of each of the forward and reverse primer of the genes of interest (Applied Biosystems) in triplicate wells for 40-cycle PCR on a 7900HT thermocycler (Applied Biosystems). Thermal cycling parameters were as follows: 50°C for 2 minutes, 95°C for 10 minutes, followed by a third step for denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. SDS software (Applied Biosystems) was used to determine cycle threshold (Ct) values of the fluorescence measured during PCR. Expression level of gene of interest was normalized with GAPDH using the ΔΔCt method. RT-PCR assay information for CTBP1, ERBB2, NCSTN, NFKB2, and GAPDH was provided in Supplementary Table S1B.

Sample sources
To determine the consequences of arsenic exposure on miRNA associated with EMT, we used a human miRNA panel to test 110 Table 1. Characteristics of human urine samples tested for miRNA expression

<table>
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<th>Characteristics</th>
<th>Arsenic exposed (n = 110)</th>
<th>Arsenic unexposed (n = 67)</th>
</tr>
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<tr>
<td>Age, y</td>
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<tr>
<td>Mean</td>
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<td>59.4</td>
</tr>
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<td>Smoking history</td>
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</tr>
<tr>
<td>Never</td>
<td>Not available</td>
<td>N/A</td>
</tr>
<tr>
<td>0–20 pack years</td>
<td>Not available</td>
<td>5</td>
</tr>
<tr>
<td>&gt;20–35 pack years</td>
<td>Not available</td>
<td>1</td>
</tr>
<tr>
<td>&gt;35 pack years</td>
<td>Not available</td>
<td>5</td>
</tr>
<tr>
<td>Unknown</td>
<td>Not available</td>
<td>N/A</td>
</tr>
<tr>
<td>Water arsenic, μg/L</td>
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<tr>
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<tr>
<td>10–50</td>
<td>22</td>
<td>N/A</td>
</tr>
<tr>
<td>&gt;50</td>
<td>21</td>
<td>N/A</td>
</tr>
<tr>
<td>Unknown</td>
<td>39</td>
<td>N/A</td>
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<tr>
<td>Creatinine-adjusted urine arsenic, μg/g</td>
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<td>&lt;100</td>
<td>35</td>
<td>N/A</td>
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<tr>
<td>100–200</td>
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<td>&gt;200</td>
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<tr>
<td>≥T2</td>
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Abbreviation: N/A, not applicable.
*Included 10 unexposed samples with unknown smoking history.
Figure 1.
A, transformation of HUC1 cells with long-term arsenic exposure: a, morphologic changes: morphology differences were monitored over the entire treatment schedule, using a light microscope (×20). Mock-treated cells and arsenic-treated cells were plated on 6-cm dishes at a density of 200,000 per plate. At 6 months, arsenic-treated HUC1 started to become more rounded and had a tendency to pile on to one another. b, MTT assay: the MTT assay was performed for each month of treatment to determine whether changes in cell proliferation occurred because of arsenic treatment. (Continued on the following page.)
universe samples from subjects who were exposed to different levels of arsenic. To confirm the arsenic exposure–specific miRNA alterations, we also tested 67 urine samples collected from the Baltimore area with safe levels of arsenic using the same miRNA panel. Control patients were randomly chosen from the Johns Hopkins Urology patients with no history of genitourinary malignancy and whose urine samples were evaluated by the Cytopathology Division of the Department of Pathology. Approval for research on human subjects was obtained from The Johns Hopkins University (Baltimore, MD) Institutional Review Boards. This study qualified for exemption under the U.S. Department of Health and Human Services policy for protection of human subjects [45 CFR 46.101[b]]. A summary of arsenic-exposed and nonexposed sample information is detailed in Table 1. To assess a potential causative relationship between the four uniquely identified arsenic exposure–related miRNA alterations, we quantitatively measured concentrations of each of the four candidate miRNAs in urine samples from 32 urothelial carcinoma cases. Detailed information of urothelial carcinoma cases are shown in Table 1. Urine samples from subjects with low and high exposure to environmental arsenic were identified through the Health Effects of Arsenic Longitudinal Study (HEALS) cohort, an ongoing population-based prospective cohort study in Araihazar, Bangladesh (16). Arsenic levels in drinking water in Araihazar range from 0.1 μg/L to >100 μg/L. The cohort includes about 42,000 members, of whom 50% are exposed to arsenic >10 μg/L and 25% are exposed to >50 μg/L and approximately 10% to 12% >100 μg/L. Arsenic exposure status of subjects was determined through drinking water arsenic concentrations measured in the subject’s primary tube-well used for water consumption. Arsenic concentrations in drinking water >10 μg/L were considered exposed and concentrations <10 μg/L were considered as unexposed. This cohort has been extensively used in previous epidemiologic studies on arsenic determinants and health effects in Bangladesh (16–22). Within all studies, extensive clinicopathological information, including smoking history, was recorded.

**Urine analysis for miRNA expression**

One to 2 mL of randomly collected urine sample was centrifuged for 5 minutes at 1,500 rpm and the supernatant was used for miRNA extraction. Detailed rationales are provided in the discussion section for RNA extraction for miRNA extraction. miRNA extraction was performed using the MirVana miRNA Isolation Kit (Ambion; cat no. AM1560) according to the manufacturer’s instructions. To remove any associated debris and nucleoprotein, the supernatant sample was first lysed in a denaturing lysis solution that stabilizes RNA and inactivates RNases. The lysate is then extracted once with Acid-Phenol:Chloroform that removes most of the other cellular components, leaving a semi-pure RNA sample. By using this kit, we obtained approximately 400 ng of total RNA. A total of 20 ng RNA was reverse transcribed using TaqMan reverse transcription kit (Applied Biosystems) and RNA-specific primers provided with TaqMan microRNA assays (Applied Biosystems) in 15-μL reaction volume containing 3 μL of reverse-transcription (RT) Primer Mix, 0.15 μL of 100 mmol/L dNTPs, 1 μL of RT enzyme 50 U/μL, 0.19 μL of RNase inhibitor 20 U/μL, 4.16 μL of nuclelease-free water, and 5μL of RNA (20 ng) RT reaction was carried out with annealing at 16°C for 30 minutes followed by extension at 42°C for 30 minutes. PCR reaction was then carried out for specific miRNA in a total volume of 20 μL that contain 2 μL of the RT reaction, 10 μL of TaqMan Universal Master Mix, 7 μL water, and 1 μL of specific primers for each of the miR-16, miR-200a, miR-200b, miR-200c, and miR-205 (Applied Biosystems) in triplicate wells for 40-cycle PCR on a 7900HT thermocycler (Applied Biosystems). The thermal cycling parameters were as follows: 50°C for 2 minutes, 95°C for 10 minutes, followed by a third step for denaturation at 95°C for 15 seconds, and annealing/extension at 60°C was for 1 minute repeated for 40 cycles. SDS software (Applied Biosystems) was used to determine cycle threshold (Ct) values of the fluorescence measured during PCR. Each of the miRNA of interest was normalized to miR-222 using the ACt method.

**Statistical analysis**

The data represent mean ± SD from independent experiments done for three to five times. Continuous variables were basically analyzed by the Student t test, two-tailed. As for miRNA expression analysis in urine samples, the Mann–Whitney U test was applied to continuous variables. All statistical analyses were performed using JMP 9 software (SAS Institute). The level of statistical significance was set at P < 0.05.

**Results**

**Characteristics of HUC1 cells after chronic arsenic exposure**

The morphologic changes of cells due to chronic arsenic treatment are shown in Fig. 1A, a. We found that the growth of the HUC1 cells was increased in a time-dependent manner after arsenic treatment (Fig. 1A, b). To determine whether the increased number of cells is due to cell proliferation, we performed bromodeoxyuridine (BrdUrd) assay using arsenic untreated and treated cell lines at various time points. We
found that the increased cell numbers in arsenic-treated cells were partially related to increased cellular proliferation (data not shown). In addition, transformation analyses including soft agar and invasion assays showed that arsenic increases both size and number of colonies on soft agar, and also increases the number of invasive cells (Fig. 1A, c–d. *P < 0.05 in all 8 and 10 months arsenic-treated cells in comparison with untreated cells, Student t test). Upon withdrawal of arsenic for 2.5 months in 8- and 10-month treated HUC1 cells, no changes in morphology and tumorigenic properties of the cells were observed (Fig. 1B, a–c). In conclusion, the observed inducible and irreversible phenotypic characteristic of HUC1 cells after arsenic treatment defines a suitable in vitro model for studying the effect of arsenic exposure on the molecular alterations in urothelial carcinoma.

Alteration of expression of some key molecules including PI3K–AKT signal transduction pathway by arsenic exposure

As shown in Fig. 2A, p-AKT was highly expressed in the arsenic-treated cells compared with the untreated ones. Similarly, m-TOR and p-PI3K demonstrate dramatically increased expression in arsenic-treated cells, specifically after 8 and 10 months. To further characterize our arsenic-treated HUC1 cells, we performed Western blot analysis for several other proteins that are known to be altered in oncogenic process. As has been seen in carcinogenesis studies in other malignancies (23, 24), p-EGFR, ERK, and cyclin D3 expression were increased in arsenic-treated cells in comparison with arsenic untreated cells. In summary, in addition to the morphologic and other phenotypic alterations as shown in Fig. 1A, this cell model was also altered at molecular level due to chronic arsenic exposure.

Exposure of HUC1 cells to arsenic affects the expression of miRNAs associated with EMT and tumorigenesis

We observed morphologic differences between the arsenic-treated and untreated HUC1 cell line using a light microscope. The round shape of the arsenic-treated HUC1 cells changed to a spindle form, suggesting that EMT-like changes might have occurred (Fig. 1A, a). To confirm the induction of EMT in arsenic-treated HUC1 cells, we analyzed the expression of the miRNAs, regulating EMT (miR-200a, miR-200b, miR-200c, and miR-205). At the molecular level, we generally observed significantly reduced expression of miR-200a, miR-200b, and miR-200c in the arsenic-treated HUC1 cell line (Fig. 2B, *P < 0.05, Student t test) in comparison with arsenic untreated cell line. However, no change or slight overexpression was observed for miR-205 after arsenic treatment. Interestingly, withdrawal of arsenic from media for 2.5 months resulted in expression recovery of the dysregulated miRNAs (miR-200a, miR-200b, and miR-200c). Fig. 2B, *P < 0.05, Student t test). However, no reversal of cells morphology was observed (data not shown).

To confirm EMT association, E-cadherin and vimentin protein expression were analyzed using arsenic-treated/untreated HUC1 cells for 6 and 10 months. E-cadherin was significantly decreased in Arsenic-treated cells of both 6 and 10 months while Vimentin expression appeared in 10 months Arsenic-treated cells (Fig. 2C).

Identification of differentially methylated genes using human notch signaling pathway DNA methylation PCR array

By array analysis, we found general reduction of promoter methylation of two genes (CTBP1 and NCSTN) and induction of promoter methylation of two genes (ERBB2 and NFKB2) in arsenic-treated HUC1 cells (Supplementary Table S2). No significant promoter methylation changes were observed in 17 genes, and one gene was failed in the analysis. Technical validation of promoter methylation was performed for two representative genes (NFKB2 and NCSTN) by QMSP assay (Supplementary Fig. S1A and S1B). From the viewpoint of dichotomization (methylation positive or negative), only two samples (8MAs/2.5MUT and 10MAs) for NFKB2, and one sample (BTFC909) for NCSTN showed different results. Therefore, the consistency between array and QMSP-based assay for NFKB2 and NCSTN was 78% and 89%, respectively. These discrepancies may be due to different sensitivity of the assay. Overall, QMSP results were generally consistent with β-values obtained from Notch methylation array data.

Promoter methylation of differentially methylated genes determined by notch methylation array in arsenic-treated and untreated HUC1 cells are inversely associated with expression

To determine whether changes in promoter methylation due to arsenic exposure had any effect on gene expression, we performed qRT-PCR for all the four differentially methylated genes (CTBP1, NCSTN, ERBB2, and NFKB2) in arsenic-treated and untreated HUC1 cells. qRT-PCR showed increase of the expression levels of CTBP1 and NCSTN genes in arsenic-treated HUC1 cells, which was an expected observation of the demethylated pattern in arsenic-treated HUC1 cells (Supplementary Fig. S2A). On the contrary, NFKB2 was found to be methylated in arsenic-treated HUC1 cells and exhibited lower expression levels compared with the untreated HUC1 cell line (Supplementary Fig. S2B). As for ERBB2 gene, 8-month arsenic-treated cells showed relatively high expression, although promoter methylation was detected. It may be due to incomplete occupation of the methylated promoter region by other associated molecules for silencing of this gene. For
further confirmation of association of ERRB2 promoter methylation with expression, we tested promoter methylation of ERRB2 in BFTC 905 and BFTC 909 cell lines. These cell lines were established from arsenic-exposed bladder cancer patients. As expected, we observed promoter methylation of ERRB2 in these two cell lines, and very low expression of ERRB2 was detected in these two cell lines. In general, promoter methylation of these genes seemed to be inversely associated with gene expression. However, these findings need to be confirmed in future studies.

miRNAs in urine from arsenic-exposed population and controls

In general, low levels of urine miRNA expression were observed in the arsenic-exposed population for all four miRNA (miR-200a, miR-200b, miR-200c, and miR-205) tested (Fig. 3A–D). Interestingly, expression of miR-205 was significantly decreased in the arsenic-exposed population (P < 0.001, Mann–Whitney U test; Fig. 3D). Furthermore, we also analyzed miRNA expression patterns according to different levels of water arsenic concentration (Supplementary Fig. S3A–S3C) and creatinine-adjusted urine arsenic concentration (Supplementary Fig. S3D–S3F). Although not conclusive, our data showed that increased levels of arsenic exposure were associated with low levels of miR-200c (P = 0.005 between creatinine-adjusted urine arsenic <100 and >200 μg/g, Mann–Whitney U test) and miR-205 (P = 0.009 between creatinine-adjusted urine arsenic <100 and >200 μg/g, Mann–Whitney U test).

Validation of miRNA candidates in the urine of urothelial carcinoma patients and controls

To determine the relationship of altered miRNA due to arsenic exposure and cancer, we subsequently tested all four miRNAs in urine samples from 32 patients with urothelial carcinoma and compared the expression levels with the urine miRNA level from 177 people without cancer. Among 177 people, we also compared 67 arsenic unexposed people and 110 exposed people. Besides, we compared 46 nonsmokers with 11 smokers in 67 arsenic unexposed people. Scatter plots in Fig. 3 showed the distribution of expression levels of all the four miRNA in urine of cancer cases in different comparison groups. The diagnostic potential of urine miRNAs was evaluated by ROC curve analysis for individual miRNA and the discriminatory accuracy presented by area under the curve (AUC) values (Supplementary Fig. S4). The urine miRNAs level detected urothelial carcinoma with high sensitivity and specificity. Furthermore, we also performed ROC curve analysis for non–muscle-invasive bladder cancer (NIMBC) (Fig. 4) and the sensitivity and accuracy was similar to previous ROC that include samples of muscle-invasive and non–muscle-invasive tumors (Supplementary Fig. S4). In particular, the threshold of miR-205 (2–ΔCt = 551) in the NIMBC cohort was the same as shown in the all bladder cancer cohort. The ROC analysis support that miR-205 may be a potential early detection marker of NIMBC.

Discussion

Arsenic is a highly toxic element and a known human carcinogen involved in the etiology of various malignancies including bladder cancer. However, relatively little is known about the key mechanisms underlying arsenic-induced carcinogenicity. We developed an in vitro model for arsenic-induced transformation in human normal urothelial cells that can be used to identify the molecular events that lead to urothelial carcinogenesis and progression after exposure to arsenic. At present, there is no suitable in vitro and in vivo model to study the stepwise molecular alterations by arsenic exposure in this cancer type. Studying stepwise progression will allow developing appropriate chemopreventive strategy in a timely fashion that will also facilitate molecular marker development for risk assessment, diagnosis, and prognosis. The bladder epithelium is one of the most affected tissues, as it is exposed to the carcinogen for several hours before it is expelled. In this study, we show that chronic arsenic exposure can promote cell proliferation of normal bladder epithelial cells within 6 months. However, significant changes in invasiveness and non-adherent growth in soft agar were observed after 8 months of treatment. These findings support the notion for a strong link between arsenic exposure and the risk for urothelial carcinoma development.

Although humans are exposed to many forms of arsenic in the workplace or environment, inorganic arsenic exposure has the greatest impact on human health. In the general population, the main sources of inorganic arsenic are drinking water and food (e.g., rice and flour). As noted before, there is extensive epidemiologic evidence of increased risk for the development of urothelial carcinoma associated with arsenic in drinking water (10). Recently, Waalkes and colleagues (25–29) demonstrated multigene carcinogenesis following transplacental exposure to 0, 42.5, or 85 parts per million (ppm) arsenic during gestation days 8 to 18. Urinary bladder tumors (papilloma and carcinoma; 13% increased) were formed in the offspring of the females exposed to arsenic. The metabolism of arsenic in humans and animals is not well characterized and could be different. Therefore, our in vitro model is useful for identification of candidate stepwise molecular alterations critical for the genesis of human urothelial carcinoma.

Oxidative stress and deregulation in MAPK and NF-κB pathways have been reported in skin cancer cases related to arsenic exposure (30) and also have been recently evaluated in urothelial carcinoma cases (31, 32). By candidate gene approach, we identified deregulated expression of some genes involved in MAPK–PI3K–AKT signaling, a pathway that has been implicated even in early stages of urothelial carcinogenesis (33, 34). p-AKT, cyclin D3, and m-TOR were overexpressed in the arsenic-exposed cell lines, which is consistent with the previous findings that alterations in these molecules play a notable role in urothelial carcinoma oncogenesis (35, 36). Cyclin D3 was overexpressed in BFTC 909 cells, a cell line established from a donor who was exposed to high arsenic levels (11). The EGFR pathway has been reported to be activated in urothelial carcinoma (37). Our results showed high expression of the p-EGFR in the arsenic-treated cells compared with the untreated controls. It was previously reported that arsenic exposure disrupts cellular control over intermediates in the EGFR signaling pathway (including Src and ERK; refs. 38–41); however, a direct biochemical link between deregulated signaling and arsenic-induced transformation is yet to be fully established. Recently, it has been reported that arsenic activates EGFR pathway signaling in the lung (24), which is consistent with our findings.

EMT, a de-differentiation program that converts adherent epithelial cells into individual migratory cells, is associated with the stemness and metastatic properties of cancer cells (42). Enhanced EMT characteristics as determined by the expression level of EMT-related genes are associated with poor overall and metastasis-free survival in other patients with solid tumor (43). miRNAs—highly conserved small RNA molecules that regulate gene expression—

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Arsenic Exposure, Urothelial Carcinoma, Molecular Marker

Average $2^{\Delta C_t}$ of each miRNA expression (A: miR-200a; B: miR-200b; C: miR-200c; and D: miR-205) in urine sample was calculated and plotted after normalization with miR-222. Six groups of samples were listed. All normal urines (1: n = 177) were compared with cancer urines (6: n = 32). As subgroup analyses in normal urines, arsenic unexposed normal urines (2: n = 67) were compared with arsenic-exposed urines (3: n = 110), and nonsmoker normal urines (4: n = 46) were compared with smoker normal urines (5: n = 11). The Mann–Whitney $U$ test was performed and a $P$ value of $<0.05$ was considered as statistically significant. As for normal versus cancer, miR-200a and miR-205 expressions were significantly downregulated in cancer ($P = 0.005$ and $P < 0.001$, respectively). As for arsenic unexposed versus exposed, miR-200a and miR-205 were significantly downregulated in cancer ($P = 0.002$ and $P < 0.001$, respectively). As for smoker versus nonsmoker, miR-200c and miR-205 expression was significantly downregulated in cancer ($P = 0.044$ and $P < 0.018$, respectively). Notably, the expression of miR-205 was consistently downregulated in arsenic-exposed, smoker and cancer urines.

can act as cancer signatures, and as oncogenes or tumor suppressors depending on their main target genes (44). A recent study has shown that miRNAs are involved in regulating cancer stem cells (CSC) and EMT properties (45). For example, the miR-200 family members (miR-200a, miR-200b, miR-200c, miR-141, and miR-429), which are tumor-suppressive miRNAs, can reverse the EMT process and induce mesenchymal–epithelial transition (MET) via regulation of zinc finger E-box-binding homeobox (ZEB) 1 and ZEB2 (46). In addition, the miR-200 family is required for the maintenance of CSCs properties of breast and oral CSCs by directly targeting BMI1 (47). And loss of miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) expression has been reported in several types of advanced carcinoma, including urothelial carcinoma (48, 49). Recently, it was reported that arsenic exposure facilitates the initiation of a stem-like cell population in skin cancer oncogenesis (50–52), and EMT (53), and reduction in the levels of miR-200 family members (53). In our cell model, we observed morphologic changes characterizing EMT and we found downregulation of miR-200a, miR-200b, and miR-200c in arsenic-treated cells. These changes were associated with a decrease in E-cadherin levels, an increase in vimentin (Fig. 2C), loss of cell adhesion with subsequent tumor invasion, and alterations of novel molecules related to stemness of cells. Interestingly, we did not observe morphologic and phenotypic changes after 2.5-month withdrawal of arsenic, but did see overexpression of miR-200a, miR-200b, and miR-200c following arsenic withdrawal. Thus, to develop miRNA-based therapeutic and preventive strategies, future studies are needed to investigate the regulation of these EMT/MET-related key miRNAs and their relationship to the EMT process and CSCs in urothelial carcinoma. For example, while miR-205 was overexpressed in endometrial cancer (54) and non–small cell lung cancer (55–57), its expression was downregulated in prostate cancer (58), melanoma (59–61), and breast cancers (62, 63). The claimed tumor-suppressive functions of miR-205 in breast cancer is due to direct targeting of several oncogenes, such as VEGFA, E2F1, E2F5, PKC epsilon, and HER3 (64), as well as offsetting EMT by suppressing ZEB1 and ZEB2 (64–66). Reduced expression of miR-205 has been reported in
primary urothelial carcinoma by different groups (67–69). However, the precise mechanisms of deregulated miR-205 in the initiation and progression of urothelial carcinoma have not been elucidated. It is plausible that miR-205 plays a dual role in urothelial carcinoma; for the initiation of urothelial carcinoma, miR-205 needs to be downregulated; while after a certain stage of tumor development, it may play a role in tumor progression. Although a very small number of urine samples were tested from muscle-invasive urothelial carcinoma, the median value of miR-205 expression was 241, while it was 174 in non–muscle-invasive urothelial carcinoma.

From a DNA Methylation PCR Array panel of 22 genes in the Notch signaling pathway, we identified four differentially methylated genes after arsenic treatment. CTBP1 and NCSTN exhibited low methylation levels in the treated cells, whereas ERBB2 and NFKB2 showed high methylation levels in the arsenic-treated cells. Expression levels determined by RT-PCR for all these four genes were generally inversely associated with methylation status (Supplementary Fig. S2A and S2B). It was previously reported by our group and others that gene-specific promoter hypomethylation and hypermethylation occur in cancers (70, 71). Furthermore, endogenous and exogenous exposures are related to promoter methylation (12, 72). For CTBP1 and NCSTN, we confirmed our array-based data by QMSP; however, we have not tested the promoter methylation status of these two genes in arsenic-exposed human samples. IRB and subjects will not allow us to obtain primary urothelial tissues directly from the bladder wall from arsenic-exposed population without any symptomatic condition. As urothelial cells directly shed into urine, we are in the process of testing methylation status of these genes in urine samples of arsenic-exposed populations and appropriate controls.

Identification of causative agents can be challenging as exposures may occur by inhalation, ingestion, and skin contact both in occupational and nonoccupational settings (73). As noted above, the urothelium is one of the most affected tissues, as it is exposed to carcinogens for several hours before they are expelled (12). Discovering new risk factors and confirming suspicions is difficult as there is typically a long latency period from time of exposure to the development of cancer and environmental carcinogens may occur together, limiting the ability to identify the risk posed by each individual exposure. Arsenic contamination in drinking water is a worldwide concern and remains a considerable cancer risk factor in many countries including Bangladesh, Taiwan, India, Mexico, China, Chile, Argentina, and the United States (74). By far, arsenic in drinking water poses the greatest threat to human health (17, 75). Recent studies revealing high levels of arsenic in food, such as poultry, rice, and apple juices, also highlight the importance of understanding arsenic’s carcinogenic mechanisms of action (76). Armed with such knowledge, effective preventive and therapeutic strategies for urothelial carcinoma can be developed.

Unique expression patterns of miRNAs are observed in individual tissues and differ between cancer and normal tissues (57, 77). Furthermore, miRNAs are deregulated by carcinogenic environmental exposure (78). Some miRNAs are overexpressed or downregulated exclusively or preferentially in certain cancer types and due to exposure to certain carcinogens (79). In our in vitro study, we found that some of EMT-related miRNAs (miR-200a, miR-200b, and miR-200c) were deregulated after arsenic treatment. Also, these in vitro data were consistent with our findings in human urine cohort. It could be due to the presence of molecularly altered mesenchymal cells in the urine of patients with urothelial carcinoma. Interestingly, miR-205 decreases in urine samples of arsenic-exposed subjects compared with unexposed controls. Notably, lower level of expression of miR-205 was observed in the urine of urothelial cancer cases in comparison with controls without any cancer. As no change or slight overexpression of miR-205 was observed in arsenic-treated HUC1 cell lines, it might be the result of the dual role of miR-205 in tumor initiation and progression, or depend on cell line–specific molecular characteristics. The urothelial carcinoma specificity together with the remarkable stability, robustness, and reproducibility of miRNAs in urine warrant a larger appropriately powered
investigation of miRNAs as biomarkers for cancer detection and risk assessment of different solid tumors including urothelial carcinoma. Previous reports also suggest that the assessment of multiple miRNA expression levels, also referred to as miRNA signatures, can accurately predict prognosis in various types of cancers (80, 81).

Urine sediment generally consists of different cell types, including renal tubular cells, lymphocytes, red blood cells, normal urothelial cells, and tumor cells. As the proportion of nontumor cells may differ between subjects, the obtained results could be influenced by varying expression of studied targets in different cell types. miRNAs in urine sediment reflect the level of intracellular expression, whereas miRNAs in supernatant are cell free and originate mainly from microvesicles secreted into extracellular space. In a subset of samples, we separated three fractions: (i) 600 µL taken directly from voided urine without any centrifugation; (ii) 600 µL after centrifugation; and (iii) urine sediment after centrifugation. For the selected miRNAs, we found essentially the same patterns of expression (data not shown). We decided to use supernatant for our study as miRNAs in supernatant arise from microvesicles from urothelial cells secreted into the urine (82).

In summary, (i) we established and characterized an urothelial cell model that will allow the scientific community to molecularly understand the stepwise carcinogenesis process of urothelial carcinoma due to arsenic exposure. Further understanding of this model at the molecular level will allow development of preventive strategies in subjects who were exposed to arsenic or cigarette smoking; (ii) we demonstrated that arsenic exposure was related to miRNA deregulation that was reported roles in EMT, an emerging area of interest for cancer researchers; (iii) our initial analysis of miR-200 family members and miR-205 indicated that deregulated miRNAs were potential biomarkers of arsenic exposure, and diagnostic markers of urothelial carcinoma in urine. However, as urothelial carcinoma is extremely heterogeneous, perhaps a cadre of miRNAs may synergistically improve the noninvasive detection of arsenic exposure and presence of urothelial carcinoma. Further studies including a larger panel of urothelial carcinoma–related miRNAs should be tested for optimal selection of markers of arsenic exposure and cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Involvement of Epigenetics and EMT-Related miRNA in Arsenic-Induced Neoplastic Transformation and Their Potential Clinical Use

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