N1-guanyl-1,7-diaminoheptane sensitizes bladder cancer cells to doxorubicin by preventing epithelial–mesenchymal transition through inhibition of eukaryotic translation initiation factor 5A2 activation

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Drug resistance greatly reduces the efficacy of doxorubicin-based chemotherapy in bladder cancer treatment; however, the underlying mechanisms are poorly understood. We aimed to investigate whether N1-guanyl-1,7-diaminoheptane (GC7), which inhibits eukaryotic translation initiation factor 5A2 (eIF5A2) activation, exerts synergistic cytotoxicity with doxorubicin in bladder cancer, and whether eIF5A2 is involved in chemoresistance to doxorubicin-based bladder cancer treatment. BIU-87, J82, and UM-UC-3 bladder cancer cells were transfected with eIF5A2 siRNA or negative control siRNA before incubation with doxorubicin alone or doxorubicin plus GC7 for 48 h. Doxorubicin cytotoxicity was enhanced by GC7 in BIU-87, J82, and UM-UC-3 cells. It significantly inhibited activity of eIF5A2, suppressed doxorubicin-induced epithelial–mesenchymal transition in BIU-87 cells, and promoted mesenchymal–epithelial transition in J82 and UM-UC-3 cells. Knockdown of eIF5A2 sensitized bladder cancer cells to doxorubicin, prevented doxorubicin-induced EMT in BIU-87 cells, and encouraged mesenchymal–epithelial transition in J82 and UM-UC-3 cells. Combination therapy with GC7 may enhance the therapeutic efficacy of doxorubicin in bladder cancer by inhibiting eIF5A2 activation and preventing epithelial–mesenchymal transition.

B ladder urothelial cancer is a tumor type whose high malignancy is the major reason for the high morbidity and mortality worldwide. It has been shown that cystectomy or transurethral bladder resection is the most effective treatment and is often used in combination with chemotherapy drugs to prevent recurrence and to eradicate bladder cancer. However, chemoresistance to traditional chemotherapy agents largely compromises the effects of therapy, and a mechanism facilitating bladder tumor chemoresistance remains to be elucidated before it can be exploited. Doxorubicin is a widely used chemotherapy drug in bladder cancer treatment, especially in intravesical chemotherapy; however, the increased resistance capacity of bladder cancer and intolerable side complications are a serious obstruction to its extensive application in the treatment of bladder cancer. Several recent studies have revealed that the combination of novel molecular agents and traditional chemotherapy drugs such as doxorubicin is very helpful for enhancing the chemotherapy effect and prolonging the survival of bladder cancer patients. Tumorigenesis in the bladder is a multistep process that is believed to be regulated by several aberrantly expressed genes that cause alterations of morphological and molecular features. Initiation of EMT in bladder cancer cells has been recognized as a key procedure promoting the malignant properties of bladder cancer. The term “EMT” refers to the complicated progression in which cancer cells lose epithelial properties and barrier functions mediated by cell-to-cell adherence and gain mesenchymal morphology with capacity for metastasis. Emerging evidence suggests that EMT in bladder cancer is a response for the acquisition of invasive and chemoresistance properties. There are two bladder cancer subtypes, superficial and muscle-invasive. The latter shows more mesenchymal phenotype with greater capacity for chemoresistance and is accountable as the main cause of death in bladder cancer. Recent research has revealed that there is enrichment of mesenchymal-related gene expression in muscle-invasive bladder cancer, such as Twist-1, snail, ZEB1, and ZEB2. Along with these observations, overexpression of...
vimentin and downregulation of β-catenin or plakoglobin were proven to be closely related with poor response to chemotherapy and consequent shorter disease-free survival. These reports indicate that EMT may serve as an important factor in inducing the poor effect of chemotherapy in bladder cancer. Consistent with this hypothesis, other research groups have detected doxorubicin-induced EMT progress in many human tumor types that resulted in chemoresistance. Thus, it is of great importance to investigate whether doxorubicin-treated bladder cancer cells undergo EMT in consideration of the oncogenic potential of EMT.

Chromosomal aberrations are one of the most frequent events during the progress of cancer. This includes the amplification of 3q, which has been detected in bladder, ovarian, and ovarian cancer.

An analysis of metastasis-related genes revealed that DHPS, an enzyme catalyzing hypusination, contributes to the progress of tumor malignance and poor prognosis. To date, the eukaryotic translation initiation factor 5A family (eIF5A and eIF5A2) is the only known substrate of DHPS. Post-translational modifications of eIF5A2 catalyzed by DHPS are necessary for maturity of eIF5A2. Interestingly, eIF5A2, first identified as an oncogene in ovarian cancer, is located on 3q26. The facts imply that aberrant eIF5A2 expression is a response to the malignant behavior of cancer cells. DHPS inhibitors have also been examined for antitumor effects in many solid tumors. Hence, it is important to exploit the underlying mechanism that eIF5A2 exerts and predict the oncogenic pathway, the benefit of which may be improvement of the prognosis for patients with bladder cancer.

More recently, eIF5A2 was found to have great impact on EMT progression in many cancer types, including HCC (17), liver,(16) and colorectal carcinoma, through different downstream molecular pathways; however, the relationship between eIF5A2 and EMT in bladder cancer cells has not been investigated. Herein, we examined the antitumor effect of doxorubicin-based treatment combined with GC7 in bladder cancer cells. We also investigated the underlying mechanisms of this combined therapy, and found that inactivation of eIF5A2 induced by GC7 was associated with suppression of doxorubicin-induced EMT in bladder cancer cells.

Materials and Methods

Cell culture and reagents. Human bladder cancer cell lines BIU-87, J82, and UM-UC-3 were purchased from the ATCC (Manassas, VA, USA). The cell lines were cultured in DMEM (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were maintained at 37°C in 5% CO2-95% air. Doxorubicin and GC7 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyamine spermidine and spermine were purchased from Sigma-Aldrich. The eIF5A2 and Twist-1 siRNA and negative control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell viability assay and EdU incorporation assay. Bladder cancer cells or siRNA-transfected bladder cancer cells were seeded onto 96-well plates at 3000 cells/well. The medium was replaced with the corresponding serum-free medium for 24 h to synchronize the cell cycle, then serum-free medium was replaced with complete medium containing the drugs at the indicated concentrations for 48 h. Then 10 µL/well CCK8 solution (Dojindo, Kumamoto, Japan) was added, the plates incubated for 3 h, and absorbance was measured at 450 nm using an MRX II microplate reader (Dynex, Chantilly, VA, USA). Cell viability was calculated as a percentage of untreated control. Measurement of inhibitive rate of cell proliferation was carried out using a Click-IT Edu Imaging Kit (Invitrogen, Carlsbad, CA, USA) following the procedure previously described.

Transfection of siRNA. Cells were transfected with eIF5A2 siRNA, Twist-1 siRNA, or negative control siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. The transfection medium (Opti-MEM; Gibco) was replaced with complete medium 12 h after transfection, and the cells were incubated for the indicated times. The effects of transfection of siRNA (scrambled siRNA) on cell viability and cell phenotype transition were tested by CCK8 and Western blot analyses (Fig. S1).

Western blot analysis. Bladder cancer cells were collected and lysed in 50 µL cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) containing protease inhibitors (Sigma-Aldrich). The protein concentration was quantified using a BCA Protein Kit (Thermo Fisher Scientific, Rockford, IL, USA). The cell lysates were separated by 10% SDS-PAGE, and the proteins were transferred to PVDF membranes (Millipore, Billerica, MA, USA), blocked with TBS/T containing 5% BSA, and then incubated with primary antibodies against E-cadherin, vimentin, Twist-1, Zeb-1, snail, or eIF5A2 (Abcam, Cambridge, MA, USA) at 4°C overnight. The membranes were washed three times with TBS/T and then incubated with the appropriate HRP-conjugated secondary antibodies for 1 h at room temperature. The protein bands were detected by chemiluminescence (GE Healthcare, Piscataway, NJ, USA) and visualized by autoradiography (Kodak, Rochester, NY, USA).

Measurement of eIF5A2 activity. Formation of hypusinated eIF5A2 catalyzed by DHPS, which cleaves spermidine and transfers its 4-aminobutyly moiety to lysine residue of eIF5A2 to form hypusine residue, is essential for eIF5A2 maturation. Counting the radioactivity of [H]-labelled spermidine incorporated into bladder cancer cells was used to measure the activity of eIF5A2. In brief, bladder cancer cells were incubated in the presence of [1, 8-3H]-spermidine (10 Ci/µL; Perkin-Elmer/NEN, Boston, MA, USA) for 48 h. Harvested cells were precipitated in 10% trichloroacetic acid containing 1 mM unlabeled spermidine and spermine and washed repeatedly until no radioactivity was detectable. The trichloroacetic acid precipitate was used for SDS-PAGE and the radioactivity of hypusinated eIF5A2 was detected by fluorography after SDS-PAGE.

Immunofluorescence. Bladder cancer cells were seeded into 48-well plates at 6000 cells/well and treated as described for the cell viability assays. After treatment for the indicated times, the cells were fixed with 4% formaldehyde for 15 min, washed with PBS, treated with 5% BSA for 30 min at room temperature, and incubated with mouse anti-human vimentin or anti-human E-cadherin primary antibodies (Cell Signaling Technology) at 4°C overnight. The cells were incubated with goat anti-mouse FITC-conjugated secondary antibody (Abcam) at 4°C for 2 h, incubated with DAPI (Sigma-Aldrich) for 2 min at room temperature, washed twice with PBS, and observed using an inverted fluorescence microscope (Olympus, Tokyo, Japan).

Statistical analysis. Experimental data are presented as the mean ± SD. Statistical analysis was carried out using prism 5 (GraphPad, San Diego, CA, USA). The effects of combined treatment were compared using two-way ANOVA, followed by Bonferroni’s post-hoc test. Analyses for two groups comparing were carried out using Student’s t-tests. A P-value <0.05 was considered statistically significant.
Results

Low concentrations of GC7 have little effect on bladder cell viability. Activation of eIF5A2 is specifically inhibited by GC7 through inhibiting the hypusination of eIF5A2 by DHPS. However, the cytotoxicity of GC7 towards bladder cells is rarely reported. To determine the GC7 concentration appropriate for coadministration with doxorubicin, we tested the effect of a series of GC7 concentrations on bladder cell viability using the CCK8 assay. Between 0 and 50 μM, GC7 exerted little cytotoxicity in bladder cancer cells; however, higher concentrations of GC7 (e.g., 100 μM) significantly inhibited the viability of the three cell lines (Fig. 1a–c). Although 50 μM GC7 exhibited little cytotoxicity on bladder cancer cells, rare hypusinated eIF5A2 (mature form) was detected in the presence of 50 μM GC7 (Fig. 1d–f) after incubation with [1, 8-^3H]-spermidine. Western blot analyses revealed GC7 did not exert any effects on the expression levels of eIF5A2 (Fig. 1d–f) in bladder cancer cells. Therefore, 50 μM GC7, which exerted a low toxicity but effectively inhibited eIF5A2 activation, was used for further cotreatments with doxorubicin.

Cytotoxicity of doxorubicin was enhanced by GC7 in bladder cancer cells. To assess the synergistic cytotoxic effect of doxorubicin plus GC7, we used the CCK8 assay to measure cell viability and EdU incorporation assay to test the inhibition of toxicity but effectively inhibited eIF5A2 activation, was used for further cotreatments with doxorubicin.

Determination of the effect of N1-guanyl-1,7-diaminoheptane (GC7) on cytotoxicity and inhibition of eukaryotic translation initiation factor 5A2 (eIF5A2) activity in bladder cancer cells. BIU-87 (a), J82 (b), and UM-UC-3 (c) cells were incubated with different concentrations of GC7 for 48 h. The CCK8 values of the treated bladder cancer cells were normalized to the control group (Ctrl), which was incubated without GC7.

**P < 0.01; ***P < 0.001. Effects of GC7 (50 μM) on hypusine formation of eIF5A2 in BIU-87 (d), J82 (e), and UM-UC-3 (f) cells after incubation in the presence of 3H-labeled spermidine were measured by fluorography after SDS-PAGE separation. Western blot analyses showed eIF5A2 steady state protein expression.

Fig. 1. Determination of the effect of N1-guanyl-1,7-diaminoheptane (GC7) on cytotoxicity and inhibition of eukaryotic translation initiation factor 5A2 (eIF5A2) activity in bladder cancer cells. BIU-87 (a), J82 (b), and UM-UC-3 (c) cells were incubated with different concentrations of GC7 for 48 h. The CCK8 values of the treated bladder cancer cells were normalized to the control group (Ctrl), which was incubated without GC7.

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Doxorubicin-induced EMT can be reversed and MET promoted by GC7 in bladder cancer cells. To investigate whether GC7 could regulate doxorubicin-induced EMT, we examined morphologic changes and expressions of EMT markers in bladder cancer cells treated by GC7 with or without doxorubicin. The dramatic shape change of BIU-87 cells induced by doxorubicin was reversed by GC7, and cells sustained the epithelial phenotype during doxorubicin plus GC7 treatment (Fig. 3a).

Table 1. Statistical analyses and IC₅₀ values of doxorubicin and doxorubicin plus N1-guanyl-1,7-diaminoheptane (GC7) treatment in bladder cancer cell lines

| Bladder cancer cell line | IC₅₀ (µg/mL)† | Treatment | Concentration | Interaction | P-value |
|-------------------------|--------------|-----------|---------------|------------|---------|
|                         | Doxorubicin  | Doxorubicin+GC7 | F₁(1,60) | F₁(5,60) | F₁(5,60) | P-value |
| BIU-87                  | 0.38 (0.31–0.44) | 0.17 (0.15–0.19) | 48.29*** | 177.41*** | 4.58** | <0.010 |
| J82                     | 0.77 (0.56–0.97) | 0.18 (0.16–0.21) | 108.81*** | 123.47*** | 6.17*** | <0.001 |
| UM-UC-3                 | 0.76 (0.56–0.97) | 0.22 (0.18–0.27) | 58.84*** | 92.92*** | 2.89* | <0.050 |

†IC₅₀ value and 95% confidence interval of doxorubicin in each treatment.

**P < 0.05; ***P < 0.01; ****P < 0.001.
However, GC7 alone exerted little effect on BIU-87 cells. In line with the morphologic change, there was an increase in E-cadherin expression and decrease in vimentin expression in BIU-87 cells after coincubation with doxorubicin plus GC7, compared to doxorubicin-treated cells. Hence, these results indicated that GC7 reverses doxorubicin-induced EMT in epithelial bladder cancer cells.

Moreover, although doxorubicin alone did not affect the mesenchymal phenotype of J82 and UM-UC-3 cells, GC7 promoted development of cell–cell contacts and reversed their epithelial/mesenchymal ratio (Fig. 3a–c). These results suggested that treatment with GC7 leads to MET in mesenchymal bladder cancer cells and contributes to the sensitization of bladder cancer cells to doxorubicin. In addition, we tested the expression of eIF5A2 after incubation with GC7 or GC7 plus doxorubicin and found that GC7 did not affect expression of eIF5A2 in the presence or absence of doxorubicin, however, GC7 significantly reduced formation of mature eIF5A2 (Fig. 3b,d).

**Doxorubicin-induced EMT is required for doxorubicin resistance in bladder cancer cells.** In order to ascertain the roles of EMT in doxorubicin sensitivity in bladder cancer cells, we examined the effect of doxorubicin on Twist-1 siRNA-treated bladder cancer cells. According to morphologic changes, Twist-1 siRNA-transfected BIU87 cells sustained the epithelial phenotype during doxorubicin treatment compared to the negative control (Fig. 4a). In line with these observations, Twist-1 siRNA reversed doxorubicin-induced changes of E-cadherin and vimentin in BIU-87 cells (Fig. 4b). These results indicated that Twist-1 siRNA reversed doxorubicin-induced EMT. Next, we explored the sensitivity change to doxorubicin in Twist-1 siRNA-treated BIU-87 cells and found that Twist-1 siRNA transfection sensitized BIU-87 cells to doxorubicin.

**Table 2. Statistical analyses and IC50 values of doxorubicin treatment in Twist-1 or negative siRNA transfected bladder cancer cell lines**

| Bladder cancer cell line | IC50 (µg/mL)† | Two-way ANOVA results |
|--------------------------|---------------|-----------------------|
|                          | Negative siRNA | Twist-1 siRNA | Treatment | Concentration | Interaction | P-value |
| BIU-87                   | 0.53 (0.44–0.62) | 0.18 (0.15–0.22) | f(1,60) = 167.49*** | f(5,60) = 277.99*** | f(5,60) = 6.99*** | <0.001 |
| J82                      | 0.96 (0.78–1.14) | 0.18 (0.17–0.23) | f(1,60) = 224.82*** | f(5,60) = 189.15*** | f(5,60) = 12.10*** | <0.001 |
| UM-UC-3                  | 0.65 (0.59–0.74) | 0.21 (0.19–0.24) | f(1,60) = 131.13*** | f(5,60) = 199.56*** | f(5,60) = 5.97** | <0.010 |

**P < 0.01; ***P < 0.001. †IC50 value and 95% confidence interval of doxorubicin in each treatment.**
significantly enhanced the cytotoxicity of doxorubicin (Fig. 4c, Table 2). Hence, doxorubicin-induced EMT is a major reason accounting for doxorubicin resistance. Moreover, Twist-1 siRNA promoted MET in J82 and UM-UC-3 mesenchymal bladder cancer cells (Fig. 4a,b) and sensitized them to doxorubicin treatment (Fig. 4c, Table 2). Knockdown of eIF5A2 alters doxorubicin-induced EMT in BIU-87 cells and mesenchymal phenotype of J82 and UM-UC-3 cells. Deoxyhypusine synthase is specifically inhibited by GC7 in mammalian cells, which catalyzes the post-translation modifications required to activate eIF5A2. Thus, to ascertain the role of eIF5A2 in doxorubicin-induced EMT, we used RNAi to knockdown eIF5A2 expression in bladder cancer cells. The siRNA-transfected bladder cancer cells were incubated with doxorubicin or doxorubicin plus GC7 for 48 h. The CCK8 assay and EdU incorporation assay revealed that the eIF5A2 siRNA significantly enhanced the cytotoxicity of doxorubicin in bladder cancer cells (Fig. 5, Table 3). Western blotting also revealed the upregulation of E-cadherin and downregulation of vimentin in eIF5A2 siRNA-transfected BIU-87 cells, and the contrary alteration of E-cadherin and vimentin expression in J82 and UM-UC-3 cells treated with doxorubicin for 48 h, compared to doxorubicin-treated cells transfected with the negative control siRNA (Fig. 6). We also preliminarily investigated the relationship between eIF5A2 and Twist-1 in bladder cancer cells. Knockdown of eIF5A2 decreased expression of Twist-1 in bladder cancer cells in the presence of doxorubicin (Fig. 6). Analogously, inhibition of activation of eIF5A2 by GC7 also showed similar effects (Fig. 3b). These results indicated that eIF5A2 is an important factor in the pathway involved in EMT regulations of bladder cancer cells. Twist-1, the E-cadherin repressor, may serve as an important downstream target of the eIF5A2 pathway in bladder cancer cells.

Discussion

Adjuvant or neoadjuvant chemotherapy is the most indispensable component in the treatment of bladder urothelial carcinoma, which is frequently detected at a late stage at the clinic due to a lack of early symptoms and effective diagnostic tech-
niques. In fact, chemotherapy may represent the only means of retarding the malignant progression of advanced-stage bladder cancer. However, the outcome of the doxorubicin-based treatment approach, the most frequently used chemotherapy regimen in diverse tumor types, including bladder cancer, has been reported as poor in bladder cancer patients. Thus,

Table 3. Statistical analyses and IC\textsubscript{50} values of doxorubicin and doxorubicin plus N1-guanyl-1,7-diaminoheptane (GC7) treatment in eukaryotic translation initiation factor 5A2 (eIF5A2) siRNA-transfected bladder cancer cell lines

| Bladder cancer cell line | IC\textsubscript{50} (\mu g/mL)\dagger | Treatment | Concentration | Interaction | P-value |
|--------------------------|---------------------------------|-----------|---------------|-------------|---------|
| BIU-87                   | 0.29 (0.25–0.32)                | 0.20 (0.17–0.24) | 11.39** | 2.20 | 0.074 |
| J82                      | 0.29 (0.26–0.33)                | 0.19 (0.17–0.21) | 24.79*** | 1.96 | 0.107 |
| UM-UC-3                  | 0.25 (0.21–0.28)                | 0.20 (0.18–0.22) | 4.36* | 1.43 | 0.231 |

*\(P < 0.05\); **\(P < 0.01\); ***\(P < 0.001\). \(\text{IC}_{50}\) value and 95% confidence interval of doxorubicin in each treatment.

Fig. 5. Cytotoxicity of doxorubicin or doxorubicin plus N1-guanyl-1,7-diaminoheptane (GC7) in eukaryotic translation initiation factor 5A2 (eIF5A2) siRNA-transfected bladder cancer cells. Knockdown of eIF5A2 reduced the synergistic effect of GC7 plus doxorubicin in BIU-87 (a), J82 (b), and UM-UC-3 (c) cells. Solid and dashed lines denote the best fit and 95% confidence intervals, respectively, of the different treatments. Bonferroni’s post-hoc test revealed no significant difference (\(P > 0.05\) for doxorubicin versus doxorubicin plus GC7). Photomicrographs and bar charts depict the 5-ethynyl-2′-deoxyuridine (EdU) staining pattern and relative EdU-positive ratio, respectively, of eIF5A2 siRNA-transfected BIU-87 (d), J82 (e), and UM-UC-3 (f) cells after 48 h of treatment with doxorubicin or doxorubicin plus GC7.
type transition, however, doxorubicin-induced upregulation of eIF5A2 initiated EMT. In mesenchymal cells, in which the expression of eIF5A2 and Twist-1 are relatively high, GC7-inhibited activation of eIF5A2 abolished its function of maintaining mesenchyme and induced MET. Hence, we showed that GC7 reversed the expression pattern of EMT markers in mesenchymal cells and induced MET in J82 and UM-UC-3 cells.

To ascertain the mechanism of action of GC7 in combination with doxorubicin, we silenced eIF5A2 in the three cell lines using RNAi. Consistent with our hypothesis, the eIF5A2 siRNA-transfected bladder cancer cells showed epithelial phenotype properties whether they were incubated with doxorubicin or not, and these cells were more sensitive to doxorubicin. In addition, eIF5A2 knockdown significantly downregulated expression of Twist-1 in doxorubicin-treated bladder cancer cells, which further confirms that eIF5A2 may function as an upstream factor in the Twist-1 EMT molecular pathways regulating EMT in bladder cancer. The EMT process is believed to be regulated by a complicated cellular signaling network. It has also been reported that doxorubicin can induce EMT in other tumor types; however, the mechanism underlying doxorubicin-induced EMT in bladder cancer cells has not been investigated. This is the first indication of the involvement of eIF5A2 in the network regulating EMT, which contributes to the acquired chemoresistance of bladder cancer cells.

Epithelial–mesenchymal transition may play a key role in bladder cancer progression and acquired chemoresistance. Accumulating evidence suggests overexpression of the multidrug resistance (MDR) gene was frequently detected in EMT cancer cells, which may be responsible for chemoresistance during chemotherapy. Consistent with these observations, our data also proved that inhibition of EMT enhanced sensitivity of bladder cancer cells to doxorubicin. However, clinical aims for the future treatment of bladder cancer are not only to increase the efficacy of chemotherapeutic agents but, more importantly, to eradicate bladder cancer cells with more aggressive properties, such as mesenchymal-phenotype bladder cancer cells which survive in stress conditions including chemotherapeutic drugs treatment. Our data revealed that combination therapy with GC7 could significantly reduce the dose of doxorubicin and simultaneously prevent doxorubicin-induced EMT in bladder cancer treatment, which may benefit to reduction of occurrence rates of the adverse side-effects induced by high doses of doxorubicin.

In summary, we showed that combined treatment with GC7 enhances the cytotoxicity of doxorubicin in bladder cancer cells through inhibiting activation of eIF5A2 and preventing doxorubicin-induced EMT. Therefore, combination therapy with GC7 may contribute to a better effect with a lower recurrence rate in doxorubicin-based chemotherapy. This study provides a new insight for improving the survival rate of bladder cancer patients in the clinic.

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Disclosure Statement

The authors have no conflict of interest.
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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig S1. Effects of transfection of negative control siRNA on cytotoxicity and phenotype transition in bladder cancer cells. The cell viability and expression pattern of epithelial–mesenchymal transition markers were tested after 48 h incubation in control (Ctrl) or negative control siRNA (siRNA) transfected bladder cancer cells. Transfection of siRNA per se did not exert any effects on the cell viability (A) (all three bladder cancer cell lines, Ctrl versus siRNA, P > 0.05) or expression levels of epithelial–mesenchymal transition markers (B).