The role of Id-1 in chemosensitivity and epirubicin-induced apoptosis in bladder cancer cells

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Abstract. Recurrence and progression are the major problems in the treatment of bladder cancer. Increased expression of Id-1, a basic helix-loop-helix transcription factor, has recently been shown in several types of advanced cancer. Some studies have provided evidence to suggest that Id-1 can be considered a potential therapeutic target. The objective of this study was to investigate the role of Id-1 in the chemosensitivity of bladder cancer cells, and the effect of Id-1 on chemotherapeutic drug-induced apoptosis in bladder cancer cells. We compared the different sensitivity to epirubicin in RT112 and MGH-U1 cell lines with different Id-1 expression. Then, we transfected different vectors into RT112 and MGH-U1 respectively, and generated the stable Id-1 up-regulation and down-regulation transfectants. The results of cell viability assay showed up-regulation of Id-1 in RT112 leading to increased sensitivity in response to epirubicin, and down-regulation of Id-1 increased cellular sensitivity to epirubicin. Furthermore, the analysis of apoptosis related protein revealed that up-regulation of Id-1 suppressed epirubicin-induced apoptosis and down-regulation of Id-1 leading to increased epirubicin-induced apoptosis. Wound closure assay showed up-regulation of Id-1 leading to improved migration abilities of bladder cancer cells under chemotherapy. Our results suggest that up-regulation of Id-1 in bladder cancer cells lead to increased cell viability in response to epirubicin by its improved anti-apoptotic role, and down-regulation of Id-1 increases cellular sensitivity to epirubicin by increased anticancer drug-induced apoptosis.

Introduction

Approximately 68,810 new cases of bladder cancer, including 51,230 men and 17,580 women, and 14,100 deaths attributable to this disease were expected in the USA in 2008, making it the fourth most common cancer in American men and the tenth most common in women (1). The treatment of bladder cancer is guided by the stage of disease. Physicians usually pay attention to the histology, grade and depth of invasion. These factors are indicators of the probability of recurrence and progression to a more advanced stage. Despite advances in surgical technique, medical oncology and radiation therapy, the high rates of recurrence and progression remain the main problem in the management of bladder cancer (2-6). Therefore, new therapeutic strategies are needed to improve the survival of bladder cancer patients.

Advances in molecular and cell biology have led to identification of several biological markers in bladder cancer, which can be used as therapeutic targets. Id-1 (inhibitor of differentiation or DNA binding), belongs to the basic helix-loop-helix (HLH) transcription factor family which lacks a basic domain for DNA binding, therefore, it acts as a dominant inhibitor of the basic HLH transcription factors by forming heterodimers (7). Id-1 has been suggested as a potential oncogene and up-regulation of Id-1 has been found in >20 types of human cancer, including breast (8), esophagus (9), cervical (10), lung (11), renal (12) and prostate cancers (13). The research on bladder cancer also showed that up-regulation of Id-1 was associated with increased EGFR expression, clinical staging and the invasion ability of bladder cancer cells (14). Aberrant overexpression levels of Id-1 are associated with aggressive and metastatic abilities of cancer cells, and increased Id-1 expression levels are associated with advanced tumor stage, as well as poor prognosis, suggesting Id-1 might be an important regulator not only in tumorigenesis but also in the progression of human cancer (10-13).

The molecular mechanisms responsible for the role of Id-1 are not clear, but some possible mechanisms have been revealed. Overexpression of Id-1 in normal cells is able to promote cell proliferation through the inactivation of tumor suppressor pathways such as p16INK4a/RB (15). In addition, ectopic Id-1 expression induced cell proliferation and progression from androgen-sensitive stage to androgen-independent stage, which was associated with activation of the MAPK and NFκB pathways (16,17). We have found that up-regulation of Id-1 in cancer cells promoted chromosomal instability through modification of APC/C activity during mitosis (18).

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Id-1 has also been reported to be a potential therapeutic target. Overexpression of Id-1 in prostate cancer cells provided protection against TNF-α-induced apoptosis (17). Inactivation of Id-1 by si-Id-1 resulted in a decrease in proliferation ability of prostate cancer cells after taxol treatment and the si-Id-1 induced sensitization to taxol was associated with activation of c-Jun N-terminal kinase (JNK) related apoptotic pathway (19). In nasopharyngeal carcinoma (NPC) cells, exogenous Id-1 expression led to resistance to taxol induced apoptosis (20). Previously, using clinical samples, we found that Id-1 expression was much higher in advanced bladder cancer, and its expression was associated with increased clinical staging. The *in vitro* research also showed up-regulation of Id-1 was associated with increased invasion ability of bladder cancer cells (14). Therefore, it raised a hypothesis that up-regulation of Id-1 protein in bladder cancer cells, may provide a survival advantage against chemotherapeutic drug-induced apoptosis, and inactivation of Id-1 may be a potential target to improve the efficiency of chemotherapeutic drugs. To test this hypothesis, we transfected the pBabe-Id-1 expression retroviral vector and retroviral vectors containing an Id-1-specific small interfering RNA oligonucleotides (si-Id-1) into two bladder cancer cell lines, respectively, and examined whether regulation of Id-1 could affect the sensitivity to epirubicin. Our results suggest that up-regulation of Id-1 in bladder cancer cells leads to increased cell viability in response to epirubicin by its improved anti-apoptotic role, and down-regulation of Id-1 can increase cellular sensitivity to epirubicin by increased anticancer drug-induced apoptosis.

Materials and methods

Chemotherapeutic drug. Clinical preparation of epirubicin (Pfizer, USA) was dissolved and diluted in phosphate-buffered saline (PBS). The stock solution was further diluted again in culture medium to obtain the desired concentrations.

Cell lines and culture conditions. Two human bladder urothelial cancer cel1 lines, RT112 and MGH-U1 were cultured. MGH-U1, is an epithelium-like cell line with an aggressive and undifferentiated phenotype. Its exact origin is uncertain, although characterization studies suggest that it is a sub-line of the T24 cell line, which was initially derived from a primary, grade 3 bladder tumor from an 81-year-old female Caucasian in 1970. RT112 is an epithelioid cell derived from a grade 2 transitional cell carcinoma (TCC) from a previously untreated female patient of unknown age in 1973. The cells were maintained in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 5% (v/v) fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 U/ml) at 37°C, 5% CO_2. To study the expression of Id-1 and the effect of chemotherapy, the cells were cultured in serum-free medium for at least 48 h before harvest and treatment. Cells were split and harvested using trypsin (Sigma), and drug exposures were performed in the cell incubator.

Generation of stable Id-1 expressing transfectants. The pBabe-Id-1 expression retroviral vector and its corresponding vector control were used for the generation of stable trans-

fectants in RT112 cells. Details on the vectors and transfection procedures have been described previously (21). The transfectant clones were selected and maintained in puromycin (2 μg/ml).

Construction of Si-RNA vectors and generation of stable si-Id-1 transfectants. The Id-1 Si-RNA vector was generated using the GeneSuppressor System kit according to the manufacturer's instructions (Imgenex, San Diego, CA, USA). Details on the vectors and transfection procedures have been described previously (14). Resulting vectors were transfected into the 293 packaging cell line using the Fugene 6 reagent. Retroviruses were collected 48 h later, mixed with polybrene (8 μg/ml), and then incubated with MGHU1 cells. Positive si-Id-1 clones were selected in neomycin (600 μg/ml), and a pool of stable transfectants was isolated after ~6 days of drug selection.

3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cell viability was measured using MTT proliferation assay kit, and the experimental procedures were described by the manufacturer (Boeringher, MO, USA). Briefly, 1500-3000 cells were seeded in 96-well plates and cultured for 24 h, and then the culture medium was changed with serum free medium for 48 h. Four concentrations of epirubicin were added, respectively. Cell viability was examined 24 h after treatment. Before testing, 20 μl of MTT labeling reagent (5 mg/ml MTT in PBS) was added and the cells were incubated for 4 h at 37°C. All culture medium and MTT was then removed from the wells before 200 ml dimethyl sulphoxide (DMSO) was added to each well. The plate was further incubated for 15 min at 37°C to dissolve the formazan crystals. The optical density was measured at a wavelength of 570 nm on a Labsystem multiscan microplate reader (Merck Eurolab, Dietikon, Switzerland). The MTT assay result is the optical density of the specimen recorded by spectro-photometer. This reflects residual viable cell biomass. The data recorded represent the OD ratio between the treated and untreated cells for different concentrations tested.

Western blotting. Experiments of Western blotting were carried out as previously described (21). In brief, whole-cell lysate was prepared by re-suspending cell pellets in lysis buffer, and protein concentrations were measured using the protein assay kit (Bio-Rad, Hercules, CA, USA). Protein suspension (10-30 μg) was then loaded onto sodium dodecylsulfate-polyacrylamide gel electrophoresis for electrophoresis and then transferred to a polyvinylidene difluoride membrane (Amersham, Piscataway, NJ, USA). The membrane was then incubated with primary antibodies for 1-2 h at room temperature against Id-1 (Santa Cruz Biotechnology, CA, USA), caspase-3, cleaved caspase-3, PARP (Cell Signaling Technology, MA, USA), Cleaved caspase-3, Bcl-2, Bax and ß-actin (Santa Cruz Bio-technology), respectively. After washing with Tris-buffered saline Tween-20 (TBS-T), the membrane was incubated with a secondary antibody against rabbit, mouse or goat IgG and the signals were visualized using the ECL Western blotting system (Amersham, Piscataway, NJ, USA). The relative
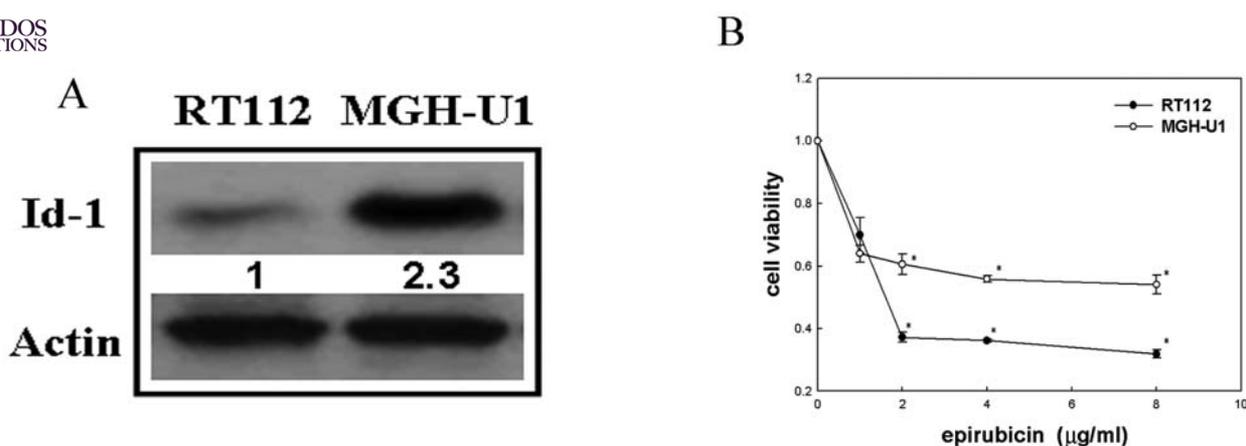
Figure 1. Correlation of Id-1 expression and cellular sensitivity to epirubicin in different bladder cancer cell lines. (A) Western blot analysis of Id-1 in two bladder cancer cell lines, RT112 and MGH-U1. Note that the expression of Id-1 in MGH-U1 is much higher than that in RT112. The relative density of Id-1 protein expression was 2.3-fold higher in MGH-U1 cells as compared to RT112 cells. (B) Cell viability of RT112 and MGH-U1, after exposure to six doses of epirubicin for 24 h, examined by MTT assay. Note that the cell viability of RT112 is lower than MGH-U1 ($^*p<0.05$, analyzed by two-tailed Student's t-test). It suggested that RT112 with relatively low Id-1 expression was more sensitive to epirubicin compared to MGH-U1.

expression levels were generated by comparing the density measured by densitometry to that of parentals and indicated underneath each band. Results represent four independent experiments.

Wound closure assay. Cells ($4x10^5$) were seeded into 6-well culture plates and allowed to grow to ~90-95% confluence in serum-free medium for 48 h. Similar sized wounds were introduced to monolayer cells using a sterile yellow pipette tip, and remove the medium with floating cells carefully. Pre-warmed medium (2 ml) with low dose of epirubicin (2.5 μg/ml) was added. The speed of wound closure was monitored and photographed at 0, 24, 48 and 72 h time-points using a phase-contrast microscope under x10 magnification.

Results

Correlation of Id-1 expression and cellular sensitivity to epirubicin in different bladder cancer cell lines. In order to investigate whether Id-1 expression was associated with sensitivity to chemotherapy in bladder cancer, we studied two kinds of cell lines derived from human bladder urothelial cancer. As shown in Fig. 1A, the expression of Id-1 in MGH-U1 is much higher than that in RT112. The relative density of Id-1 protein expression was 2.3-fold higher in MGH-U1 cells as compared to RT112 cells. Fig. 1B shows that the RT112 cell line was more sensitive to epirubicin compared to the MGH-U1 cell line with relatively high level of Id-1. It suggested that there may be an association between Id-1 expression and sensitivity to epirubicin in these two cell lines.

Generation of stable Id-1 expressing and si-Id-1 trans-fectants in different bladder cancer cell lines. The bladder cancer cell line RT112 has been shown to have relatively low level of Id-1. Therefore, in this study, we transfected the pBabe-Id-1 expression retroviral vector into RT112 cell line, and two stable-transfected clones were generated. As shown in Fig. 2A, increased Id-1 expression was seen in the two

clones (Id-1-C1 and Id-1-C2) compared with the vector control (Id-1-pBabe) cell lines, and the relative density of Id-1 protein expression estimated by densitometry shows the increased amplitudes are ~18.1 and 36.4%, indicating successful overexpression of the Id-1 gene. Likely, MGH-U1 has been shown to have relatively high level of Id-1. We transfected a retroviral vector containing a siRNA oligo-nucleotide targeted to the Id-1 gene into MGH-U1 cell line, and one stable transfected clone was generated. As shown in Fig. 2B, decreased Id-1 expression was found in the clone (si-Id-1) compared with the vector control (ssCON) cell line, and the decreased amplitude is ~25%, indicating successful inactivation of the Id-1 gene.

Effect of Id-1 up-regulation and down-regulation on cellular sensitivity to epirubicin. We investigated by MTT assay whether up-regulation of Id-1 could increase the sensitivity of bladder cancer cells to chemotherapy, and inversely, down-regulation of Id-1 could lead to inhibition of cell viability under the treatment of anticancer drug. As shown in Fig. 3A, the cell viability in the two Id-1 clones generated from RT112 cell line was much higher than the vector control after exposure to the same doses of epirubicin. These results indicate that increased Id-1 expression is associated with increased cell survival in response to epirubicin in bladder cancer cells. To confirm the effect of Id-1 on cellular sensitivity to chemotherapy, we performed MTT assay to investigate whether down-regulation of Id-1 had any effect on cell viability in response to epirubicin compared with the vector control. As shown in Fig. 3B, the cell viability of si-Id-1 clone was lower than that of the control, after treated with 5 doses of epirubicin. Taken together, these results indicate that up-regulation of Id-1 in bladder cancer cells leads to increased cell viability in response to epirubicin, and down-regulation of Id-1 can increase cellular sensitivity to epirubicin.

Up-regulation of Id-1 suppressed epirubicin-induced apoptosis in the bladder cancer cells. As discussed
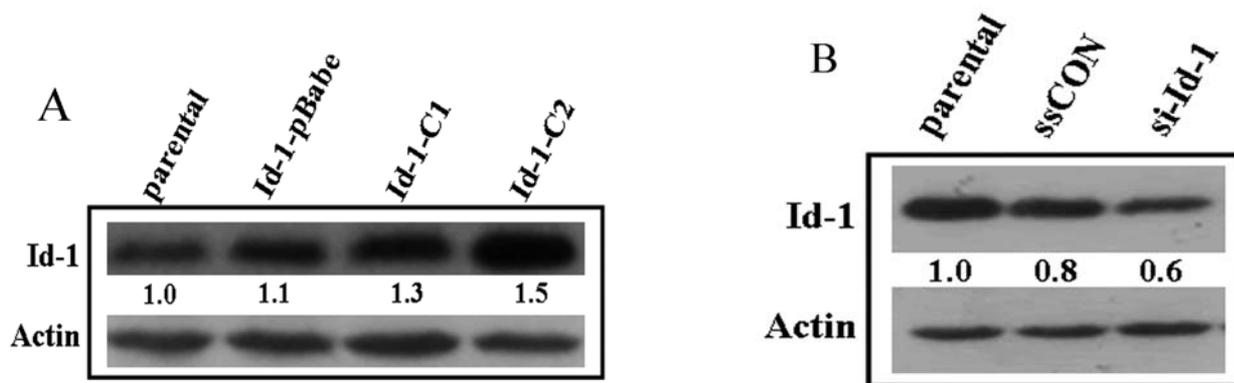
Figure 2. Generation of stable Id-1 expressing and si-Id-1 transfectants in different bladder cancer cell lines. (A) The pBabe-Id-1 expression retroviral vectors were transfected into RT112 cells, and two stable transfected clones and vector control (Id-1-pBabe) were generated. (B) The retroviral vector containing siRNA oligonucleotides targeted to the Id-1 gene were transfected into MGH-U1, and a stable transfectant (si-Id-1) and vector control cells (ssCON) were generated. Relative density of Id-1 protein expression was estimated by densitometry and indicated at the bottom of each band.
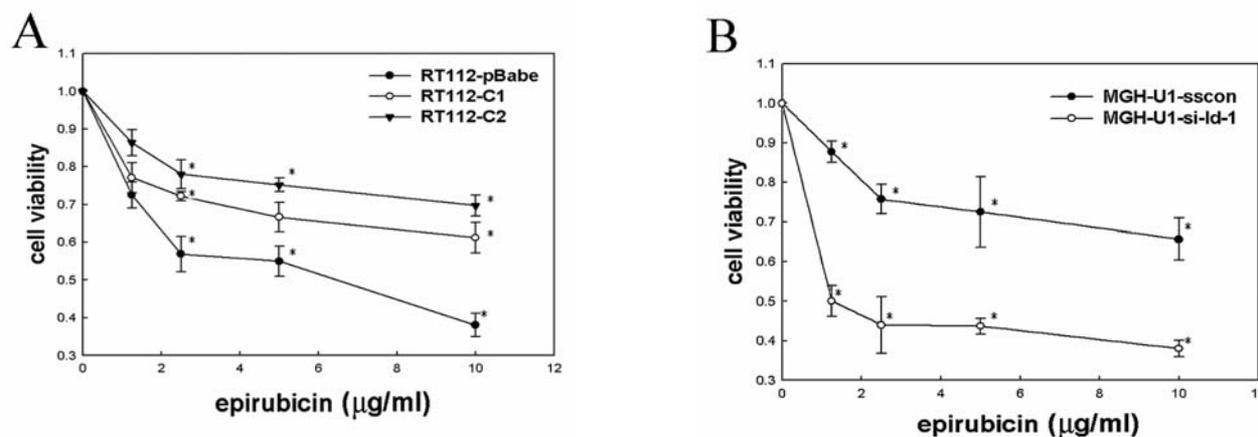

Figure 3. Effect of Id-1 up-regulation and down-regulation on cellular sensitivity to epirubicin. (A) Cell viability of RT112 pBabe, RT112 Id-1-C1 and RT112 Id-1-C2, after exposure to 5 doses of epirubicin for 24 h, examined by MTT assay. Note that the cell viability of RT112 Id-1-C1 and RT112 Id-1-C2 is higher than pBabe. (B) Cell viability of MGH-U1 ssCON and MGH-U1 si-Id-1, after exposure to 5 doses of epirubicin for 24 h, examined by MTT assay. Note that the cell viability of MGH-U1 si-Id-1 is lower compared with MGH-U1 ssCON. (*p<0.05, analyzed by two-tailed Student's t-test).

previously, Id-1 has been suggested as an anti-apoptotic factor which protects cells against apoptosis in several cell types. To test the hypothesis that Id-1 might play a positive role in protecting bladder cancer cells from programmed cell death, we studied the role of Id-1 on transfectants. Epirubicin were used to examine the association between Id-1 expression and the anticancer drug-induced apoptosis. The actual doses 0, 1 and 2 are 0, 5, 10 μg/ml, respectively. As shown in Fig. 4, after exposure to the anticancer agents, all of the cell lines showed a dose-dependent down-regulation of the Id-1 protein. Correspondingly, the expression of cleaved PARP and cleaved caspase-3, two indicators of activation of the apoptosis pathway, increased with the increasing dose, and decreased with the increasing level of Id-1. In contrast, the expression of PARP and caspase-3, two anti-apoptotic factors, decreased with the increasing dose, and increased with the increasing level of Id-1. However, the expression of the pro-apoptotic factor Bax and the anti-apoptotic factor Bcl-2, were not completely and consistently altered in

response to Id-1 expression in the treatments. The alterations of expression in apoptosis-related genes were consistent with the result of MTT assay for cell viability (Fig. 3A). These results suggest that overexpression of Id-1 is correlated with the protection of epirubicin-induced apoptosis in human bladder cancer cells in response to the anticancer drug.

Down-regulation of Id-1 leads to increased epirubicin-induced apoptosis in bladder cancer cells. To further confirm the possibility of using Id-1 as a target to increase chemosensitivity in bladder cancer cells, we utilized the si-Id-1 transfectants in MGH-U1 cells and studied the effect of Id-1 suppression on anticancer drug-induced apoptosis. As shown in Fig. 5, Western blot analysis showed that after exposure to different doses of epirubicin (the doses were same to before), the Id-1 expression between the si-Id-1 and the control cells was decreased in a dose-dependent manner. The decreased Id-1 protein expression was associated with higher levels of cleaved PARP and cleaved caspase-3, whose expression was
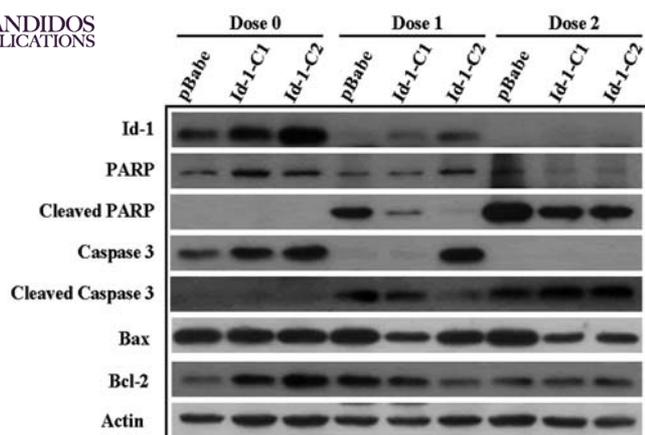
Figure 4. Effect of Id-1 up-regulation on cellular sensitivity to epirubicin. Western blot analysis of Id-1, PARP, cleaved PARP, caspase-3, cleaved caspase-3, Bax and Bcl-2 expression in response to 3 doses of epirubicin. The cells of Id-1-pBabe, Id-1C1 and Id-1-C2 were exposed with three doses of epirubicin for 24 h, respectively, and protein lysates were analyzed. Note that the expression of cleaved PARP and cleaved caspase-3 increased with the increasing dose, and decreased with the increasing level of Id-1. In addition, the expression of PARP and caspase-3 decreased with the increasing dose, and increased with the increasing level of Id-1.
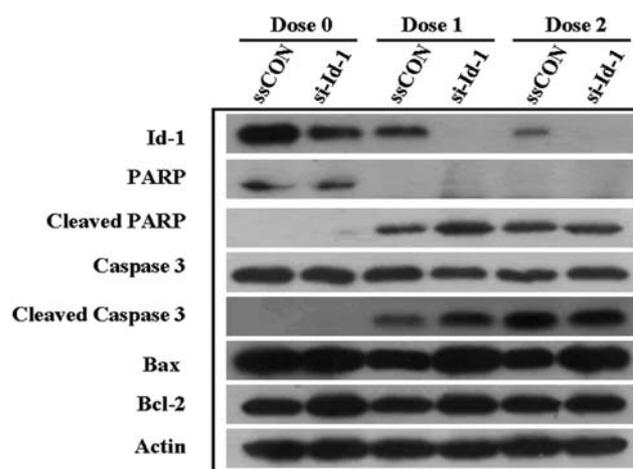
Figure 5. Effect of Id-1 down-regulation on cellular sensitivity to epirubicin. Western blot analysis of Id-1, PARP, cleaved PARP, caspase-3, cleaved caspase-3, Bax and Bcl-2 expression in response to 3 doses of epirubicin. The cells of ssCon and si-Id-1were exposed with three doses of epirubicin for 24 h, respectively, and protein lysates were analyzed. Note that the decreased Id-1 protein expression was associated with higher levels of cleaved PARP and cleaved caspase-3, whose expression was higher in the si-Id-1 cells.
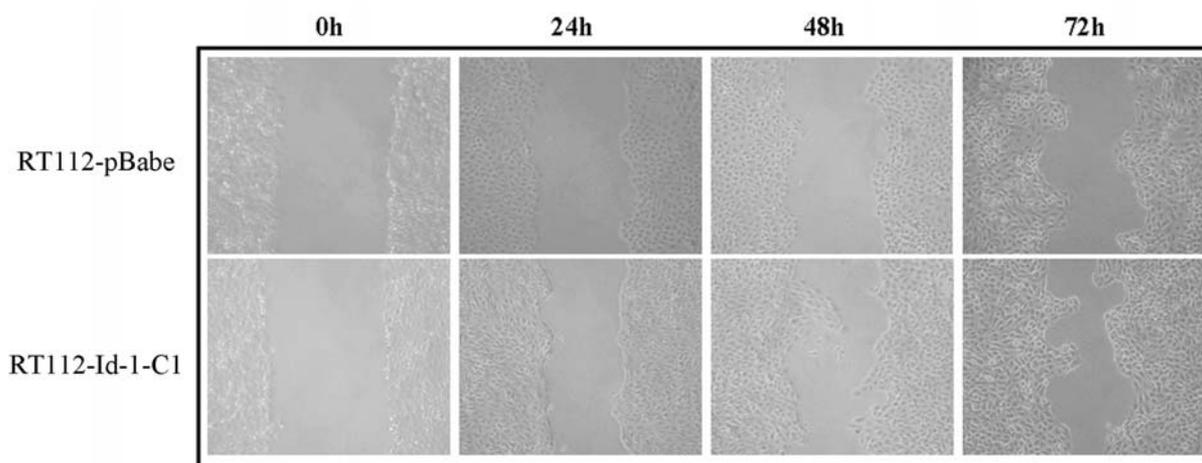

Figure 6. Effect of Id-1 up-regulation on bladder cancer cell migration treated by epirubicin. Representative results of the wound closure assay. A scraped wound was introduced on confluent monolayer RT112 cells and the speed of wound closure was monitored at 0, 24, 48 and 72 h time-points. The differential motility rate between the cells with high and low levels of Id-1. Note that up-regulation of Id-1 leads to improved migration abilities of bladder cancer cells.

higher in the si-Id-1 cells. However, the expression of PARP, caspase-3, Bax and Bcl-2 were not significantly altered. These results further support the negative role of Id-1 in anticancer drug-induced apoptosis and suggest inactivation of Id-1 might be a target to induce chemosensiti-zation to the anticancer drug.

Effect of Id-1 up-regulation on bladder cancer cell migration treated by epirubicin. To further study the association between Id-1 expression and metastatic ability of bladder cancer cells, we then tested the migration ability of the RT112 transfectans expressing high and low levels of the Id-1 protein. As shown in Fig. 6, the migration rates of Id-1-C1, treated by low dose of epirubicin, were much faster than

the control at different time points, indicating that up-regulation of Id-1 is also associated with increased cellular migration ability.

Discussion

Therapy failure after intravesical and systemic chemotherapy for bladder cancer is still high. For the treatment of non-invasive bladder cancer (Ta, T1 and Tis), intravesical treatment with chemotherapy or biologic agents such as Bacillus Calmette-Guérin (BCG) is recommended as the standard approach. Patients who received 6 weekly mitomycin instillations, followed by monthly instillations for 3 years, had a 3-year recurrence rate of 13.9% (2). To improve the results

of surgery for muscle invasive bladder cancer, neoadjuvant and adjuvant chemotherapy have been explored. Prospective, randomized trials show ~6.5% survival advantage for patients who undergo cisplatin-based neoadjuvant chemotherapy prior to cystectomy (22). In cases of adjuvant systemic chemotherapy in patients in advanced stages of the disease, 5-year progression-free and overall survival rates have recently been limited to 11.3 and 15.3%, respectively (23). This relatively small benefit indicates that new treatment modalities are required.

Cases of drug resistance, and of patients not responding to chemotherapy have been associated with a failure of apoptosis in different kinds of cancer cells, including bladder cancer (24). Recent evidence has suggested that high levels of Id-1 expression in cancer cells might lead to suppression of the apoptosis pathway, resulting in promotion of cell survival and cancer progression. Therefore, as therapeutic target, Id-1 has showed its efficiency in many kinds of cancer cells. Inactivation of Id-1 in androgen-independent prostate cancer cells leads to increased sensitivity to taxol-induced apoptosis through activation of the JNK signaling pathway (19,25). In nasopharyngeal carcinoma cells, Id-1-induced Raf/MEK pathway activation is essential for its protective role against taxol-induced apoptosis (20). Id-1 may be a general negative regulator of anticancer drug-induced apoptosis and can reduce chemosensitization in many cancer cells, such as nasopharyngeal carcinoma (CNE1), cervical carcinoma (HeLa), breast cancer (MCF7), hepatocarcinoma (Huh7) and prostate cancer (PC3) (26).

The evidence presented previously has showed that Id-1 was overexpressed in human bladder cancer tissue and was associated with increased clinical staging and the invasion ability of bladder cancer cells (14). In this study, we investigated the significance of Id-1 as a potential therapeutic target in bladder cancer cell lines. As shown in Fig. 1, the different sensitivity to epirubicin in RT112 and MGH-U1 maybe the result of different expression level of Id-1 in these two cell lines. In order to exclude the interference factors in different cell lines, we transfected different vectors into RT112 and MGH-U1 respectively, and generated the stable Id-1 up-regulation and down-regulation transfectants (Fig. 2). The results of cell viability assay showed up-regulation of Id-1 in RT112 leading to increased sensitivity in response to epirubicin, and down-regulation of Id-1 increased cellular sensitivity to epirubicin (Fig. 3). Furthermore, the analysis of apoptosis-related protein revealed that up-regulation of Id-1 suppressed epirubicin-induced apoptosis and down-regulation of Id-1 leaded to increased epirubicin-induced apoptosis (Figs. 4 and 5). Finally, wound closure assay showed up-regulation of Id-1 leading to improved migration abilities of bladder cancer cells under chemotherapy (Fig. 6). Our results confirm the hypothesis that increased expression of anti-apoptotic protein Id-1 is suggested as being responsible for the resistance to chemotherapies in bladder cancer, and Id-1 supposedly promotes chemoresistance by protecting the transformed cells from entering the apoptosis pathway induced by the chemotherapeutic agent. It is consistent with our hypothesis that Id-1 could be a promising candidate for future therapy concepts and that inhibiting Id-1 expression might benefit patients with bladder cancer.

In summary, the results presented in this study show the expression of Id-1 in bladder cancer cells can affect cellular chemosensitivity. Overexpression of Id-1 can decrease the sensitivity to epirubicin and suppress epirubicin-induced apoptosis. Down-regulation of Id-1 can increase chemo-sensitivity and increased epirubicin induced apoptosis. All the results indicate a novel target in improving chemo-sensitivity in human bladder cancer. Based on our results, we are investigating how Id-1 regulates the apoptotic signal pathways in responses to a variety of anticancer drugs.

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