Monoclonal antibody Zt/g4 targeting RON receptor tyrosine kinase enhances chemosensitivity of bladder cancer cells to Epirubicin by promoting G1/S arrest and apoptosis

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Abstract. Epirubicin (EPI) is one of the most used intravesical chemotherapy agents after transurethral resection to non-muscle invasive bladder tumors (NMIBC) to prevent cancer recurrence and progression. However, even after resection of bladder tumors and intravesical chemotherapy, half of them will recur and progress. RON is a membrane tyrosine kinase receptor usually overexpressed in bladder cancer cells and associated with poor pathological features. This study aims to investigate the effects of anti-RON monoclonal antibody Zt/g4 on the chemosensitivity of bladder cells to EPI. After Zt/g4 treatment, cell cytotoxicity was significantly increased and cell invasion was markedly suppressed in EPI-treated bladder cancer cells. Further investigation indicated that combing Zt/g4 with EPI promoted cell G1/S-phase arrest and apoptosis, which are the potential mechanisms that RON signaling inhibition enhances chemosensitivity of EPI. Thus, combing antibody-based RON targeted therapy enhances the therapeutic effects of intravesical chemotherapy, which provides new strategy for further improvement of NMIBC patient outcomes.

Introduction

Bladder cancer is a major cause of morbidity and mortality worldwide, and in the United States alone, 76,960 newly diagnosed cases and 16,390 deaths are estimated in 2015 (1). Approximately 70% of patients with bladder cancer are non-muscle-invasive (NMIBC) at diagnosis (2). NMIBC is characterized by significant rates of recurrence and progression. The range of recurrence is 50-80%, and progression 10-45%, depending on disease risks (based upon grade, stage, and tumor size) (3). Transurethral resection of bladder tumor (TURBT) combined with intravesical chemotherapy is the primary method for treatment of NMIBC (4). The aim of intravesical chemotherapy is to decrease the possibility of tumor recurrence and progression. At present, Epirubicin (EPI), a derivative of doxorubicin, is one of the most used intravesical chemotherapy agents to treat NMIBC (5). Comparing with TURBT alone, EPI instillation after TURBT decreased nearly half of recurrence and progression of NMIBC (6). Although intravesical EPI chemotherapy has improved the clinical outcome of patients with NMIBC, efforts to potentiate drug action and enhance chemosensitivity should be investigated for further improvement of patient outcomes (7).

Recepteur d'origine Nantais (RON) belongs to the MET proto-oncogene family (8). The expression of RON is highly altered in many primary cancer samples including colon, breast and bladder cancer, and has prognostic value in predicting patient survival and clinical outcome (9-11). Aberrant RON activation, featured by overexpression of RON protein (12-14), isoform generation (15-17), and persistent activation of downstream signaling pathways (18), has been found in various types of cancers. Those aberrations contribute to tumorigenic phenotype, malignant progression and chemoresistance (9,11,19). Due to the importance of RON in cancer pathogenesis, targeting RON signal pathway has therapeutic potential. Currently, various approaches including therapeutic monoclonal antibodies (mAb), siRNA and small molecule inhibitors (SMI) have been evaluated to inhibit RON signaling (20-22). Results from these studies demonstrate that inhibition of RON signaling contributes to reduced cell growth, diminished cell invasiveness, and impaired tumor metastasis. Combining RON signaling inhibition and chemotherapy agents were also under investigation in treating with various cancers. In colon cancer, 5-Fu in combination with RON specific mAb Zt/f2 has been showed to markedly improve treatment effects (20), suggesting

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that inhibiting RON pathway may enhance chemosensitivity of chemotherapy drugs.

In bladder cancers, RON is overexpressed in more than 35% of samples (11,14). RON expression has been documented in RT4, TCCSUP, UB09 and other bladder cancer cell lines (11). Overexpression of RON was associated with poor clinical outcome (11,23). Furthermore, MSP, the only known ligand of RON, was also detected in human urine samples (11). These findings suggested that RON plays a role in bladder cancer tumorigenesis and invasion. Evidence has indicated that RON-specific mAbs such as Zt/g4 and Zt/f2 rapidly induce RON internalization by cancer cells, which diminish RON signal transduction and enhance cytotoxic drug delivery and sensitivity (20,24-27). Thus, RON-specific mAbs are potentially effective approach to RON signal inhibition and enhancement of chemosensitivity.

In the present study, we selected a mouse mAb Zt/g4 highly specific to the RON extracellular domain to induce RON internalization and subsequent RON signal pathway inhibition. EPI was used as chemotherapeutical agent to determine the effects of RON on chemosensitivity in bladder cancer cells. This study provides new strategy to reduce NMIBC recurrence and progression.

Materials and methods

Cell lines and cell culture. The human 5637, T24, RT4, J82, UMUC and BIU87 bladder cancer cell lines were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were cultured at 37˚C in a humidified atmosphere of 5% CO_2 in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine and 100 U/ml penicillin. The medium was replaced every 3 days.

Main reagents. EPI was purchased from the Hisun Pharmaceutical Co., Ltd. (Zhejiang, China). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies (Rockville, MD, USA). Mouse mAb Zt/g4 specific to RON sema domain and rabbit antibody (R5029, specific to the RON C-terminal peptide) were kindly supplied by Professor Yao (Laboratory of Cancer Biology and Therapeutics, First Affiliated Hospital, Zhejiang University School of Medicine). Rabbit anti-Bcl-2, anti-Bax, anti-Erk1/2, anti-AKT, anti-caspase-3, anti-cyclin D1, anti-CDK4, anti-CDK6 and anti-p27 antibodies were from Cellular Signaling Technology (Danvers, MA, USA). FBS, RPMI-1640, L-glutamine and penicillin were purchased from Life Technologies Inc. (Carlsbad, CA, USA).

Western blotting. Western blot analysis was performed to measure the expression levels of various proteins in cells. Each sample equivalent of 100 μg total proteins were electrophoresed in 8% SDS-PAGE and blotted on a nitrocellulose membrane (Millipore Inc., Billerica, MA, USA). Blots were blocked at room temperature for 2 h in 1X Tris-buffered saline (TBS) buffer, and then incubated with primary antibodies specific to Ron, Bcl-2, Bax and β-actin overnight at 4˚C, respectively. After three washes for 3x10 min in TBST, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit immunoglobulin G at room temperature for 1 h. Immunoreactive proteins were visualized by enhanced chemiluminescent reagents (Thermo Scientific, Rockford, IL, USA). The optical density was quantified by VersaDoc Imaging system (Bio-Rad, Hercules, CA, USA).

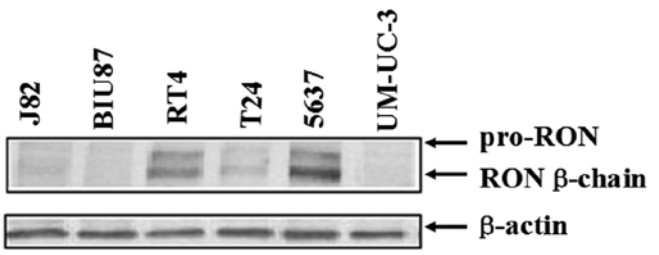

Figure 1. Expression of RON receptor proteins in various bladder cancer cells. Three bladder cancer cell lines including 5637, T24 and RT4 cell lines expressed a mature form of RON chain (150 kDa) and a precursor RON (180 kDa), while the three bladder cancer cell lines J82, BIU87, UM-UC-3 had no RON expression.

Transwell invasion assay. The cells were seeded at a concentration ratio of $5x10^4$ cells/chamber in serum-free RPMI-1640 and placed on the 8 μm pore-size upper chamber (Corning Incorporated, Corning, NY, USA). The lower chambers contained RPMI-1640 culture media with 10% FBS. After 24 h of incubation, non-invading cells on the top chamber were removed by using a cotton swab, and cells that penetrated to the lower surface were fixed with 800 μl methanol for 30 min, stained with 0.5% crystal violet solution for 2 h, washed with 1X PBS and counted under a microscope. Six fields were randomly selected from each sample, with triplicates.

Cell viability assay. Sensitivity of cells to EPI was assayed with the CCK-8 kit. Briefly, cells were seeded in 96-well plates with a density of $1x10^4$/well and incubated for 24 h at 37˚C and then treated with EPI (0.2, 1. 2, 3 and 6 μg/ml), Zt/g4 (8 μg/ml) combined with EPI for 48 h. The proportion of live cells of the two groups was analyzed by CCK-8 kit according to the manufacturer's instructions. Finally, the absorbance (OD) value of each well was measured by a microplate reader at the wavelength of 450 nm. The experiment was carried out in triplicate. Dose-dependent response curve was plotted and the half maximal inhibitory concentration (IC_{50}) was determined by fitting the concentration response curves with the sigmaplot software.

Cell cycle analysis. Cell cycle status was detected by flow cytometry and analyzed by Flowjo software. Briefly, after exposed to Zt/g4, EPI or both for 24 h, cells ($2x10^5$-10^6) were harvested, fixed with 75% ethanol overnight at -20˚C. The fixed cells were incubated in darkness at 37˚C with 1 mg/ml RNase A (Sigma-Aldrich, St. Louis, MO, USA) for 30 min and with 50 μg/ml propidium iodide (PI) (Sigma-Aldrich) for 30 min. The cells were analyzed by flow cytometry (FACScan, Becton-Dickinson, Franklin Lakes, NJ, USA).

Apoptosis analysis. After Zt/g4, EPI or both for 48 h, the cells were collected and washed twice with cold PBS, followed by resuspension in binding buffer at the density of $1x10^6$ cells/ml. 100 μl ($1x10^5$ cells) of the solution was removed and stained

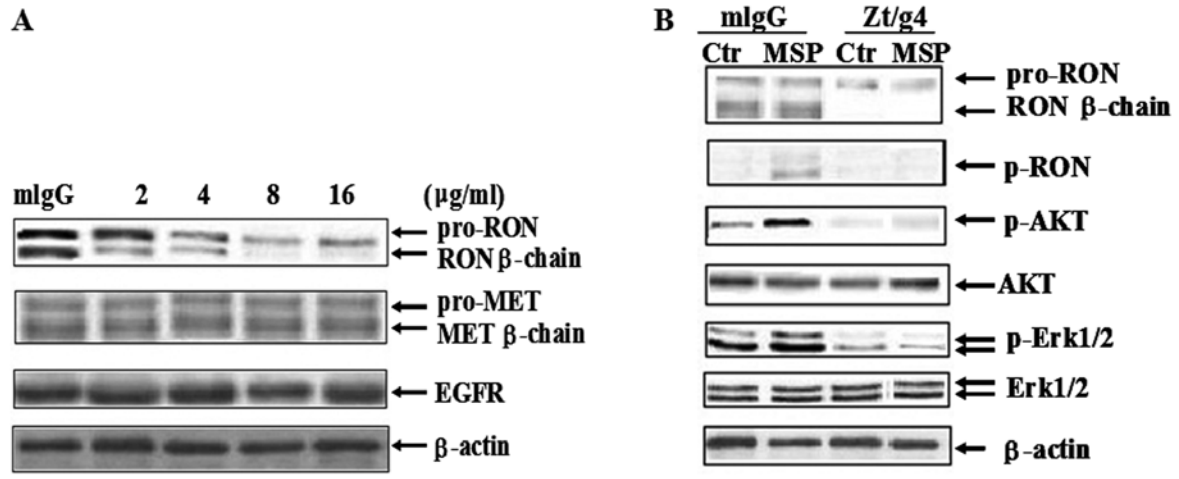

Figure 2. Concentration-dependent downregulation of RON expression after Zt/g4 treatment. (A) Dose-dependent downregulation by Zt/g4 of RON expression. RON expression levels in 5637 cells treated with Zt/g4 at 0, 2, 4, 8 and 16 μg/ml for 24 h, respectively. The levels of RON, MET and EGFR were detected by western blot analysis. (B) Zt/g4 inhibited MSP induced RON activation and downstream signal transduction. Zt/g4 or mIgG (8 μg/ml) was added to culture medium for 48 h, then cells were harvested and stimulated by MSP for 30 min, phospho-RON, Erk1/2 and AKT were detected by western blotting.

with 5 μl Annexin V-FITC and PI (BD Biosciences) for 15 min in the dark at room temperature. Then a total of 400 dilution buffer was added to each tube and cell apoptosis was analysed by flow cytometry (FACScan, Becton-Dickinson). The percentage of apoptotic cells with Annexin V^+/PI^+ was evaluated. Each group was measured three times.

Statistical analysis. Statistical analyses were performed using SPSS software (version 18; SPSS Inc., Chicago, IL, USA). Data are presented as the means ± standard deviation (SD). Statistical significance between two groups was evaluated by Student's t-test. Differences between multiple groups were performed by one-way analysis of variance. A difference was considered significant at $P<0.05$.

Results

Expression of RON receptors in bladder cancer cell lines. The expression of RON was detected by western blotting in seven bladder cancer cell lines (Fig. 1). In this panel, 5637, T24 and RT4 cell lines had a high level of RON expression, while the expression levels of RON in other three cancer cell lines were barely found. As the 5637 cells expressed a relatively higher level of RON than those in T24 and RT4 cells, we chose 5637 cells for future studies.

Zt/g4 induces reduction of RON expression and signal inhibition. Western blot analysis confirmed that Zt/g4 treatment for 24 h caused diminished RON expression in a dose-dependent manner in 5637 cells (Fig. 2A). When used with 2 μg/ml of Zt/g4, Zt/g4-induced reduction of RON β-chain expression was significantly identified. However, the concentration of Zt/g4 increased up to 8 μg/ml, but did not further cause RON reduction. Thus, the maximal effect induced by Zt/g4 was at the range of 8 μg/ml. We chose this concentration as the standard for further experiments. Cross-talk between RON and MET or EGFR has been found in various cancer cells (28,29). We verified whether Zt/g4-induced RON reduction affected MET or EGFR expression in 5637 cells. Result

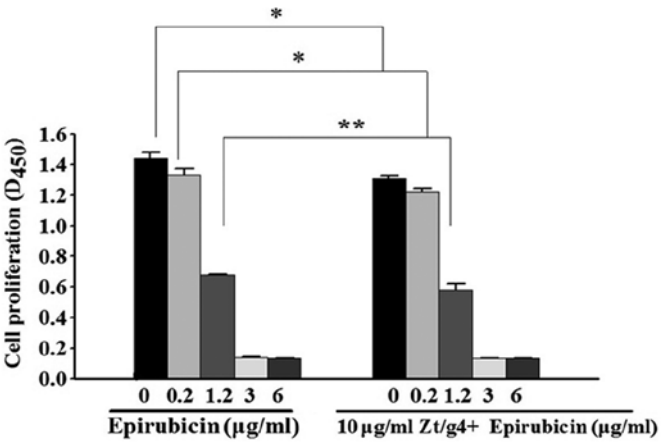

Figure 3. Effects of RON inhibition by Zt/g4 on the sensitivity to EPI in 5637 cells. CCK-8 analysis of cell growth inhibiton in EPI and EPI in combination with Zt/g4 groups. Values are presented as the means ± SD from three independent experiments. $^*P<0.05$, $^{**}P<0.01$.

showed that there were no significant differences in the total level of MET and EGFR expression in 5637 cells after Zt/g4 treatment. Taken together, these results demonstrated that Zt/g4-induced RON reduction was specific to RON and had no effect on MET or EGFR.

As MSP was detected in human urine samples (11), MSP induced RON activation and signaling transduction may be a key event in bladder tumorigenesis and invasion. To test whether Zt/g4 blocks MSP induced RON activation and downstream signaling transduction, 5 nM MSP was added to induce RON activation with or without 8 μg/ml Zt/g4 treatment (Fig. 2B). Result showed that after Zt/g4 treatment for 48 h, MSP induced RON activation was completely inhibited. Phosphorylation of downstream signal molecules such as Erk1/2 and AKT were also interrupted (Fig. 2B).

Zt/g4 enhances the chemosensitivity of EPI on 5637 cells. To determine whether inhibition of RON signaling by Zt/g4 enhance chemosensitivity of EPI in 5637 cells, a CCK-8 assay

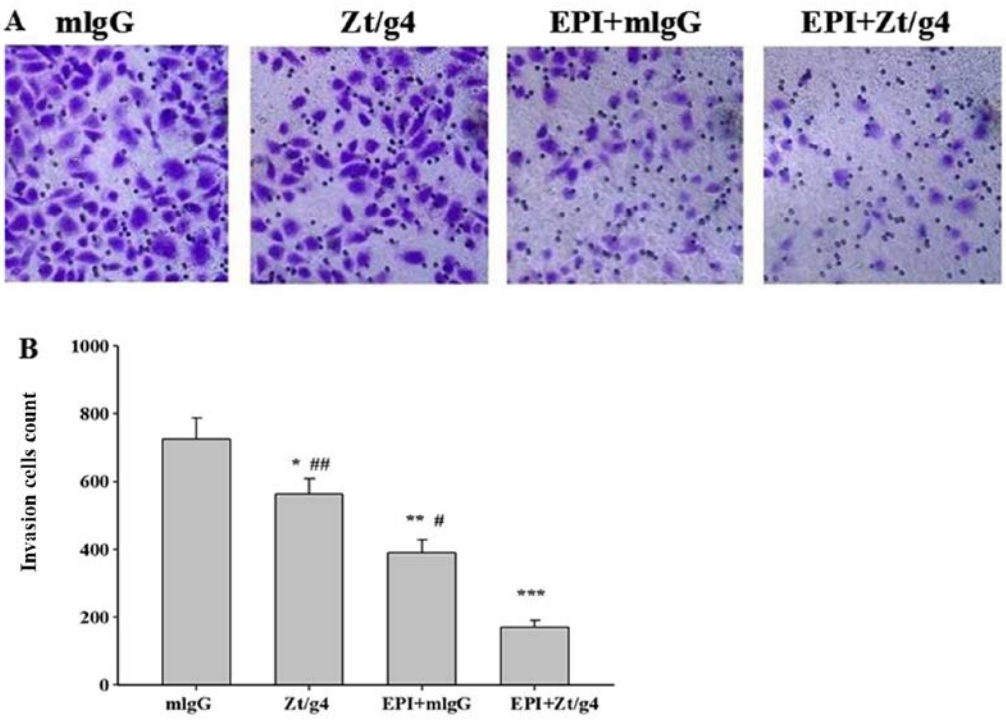

Figure 4. Effect of Zt/g4-induced cell invasion. (A) Representative staining images showing number of perforated cells in mlgG, Zt/g4 EPI+mlgG and EPI+Zt/g4 groups, respectively. (B) Quantitative data of perforated cell number in each group. *P<0.05, **P<0.01, ***P<0.001 compared to mlgG group; $^{\#}$P<0.05, $^{\#\#}$P<0.01, compared to EPI+Zt/g4 group. The data are presented as the means ± SD from three independent experiments.

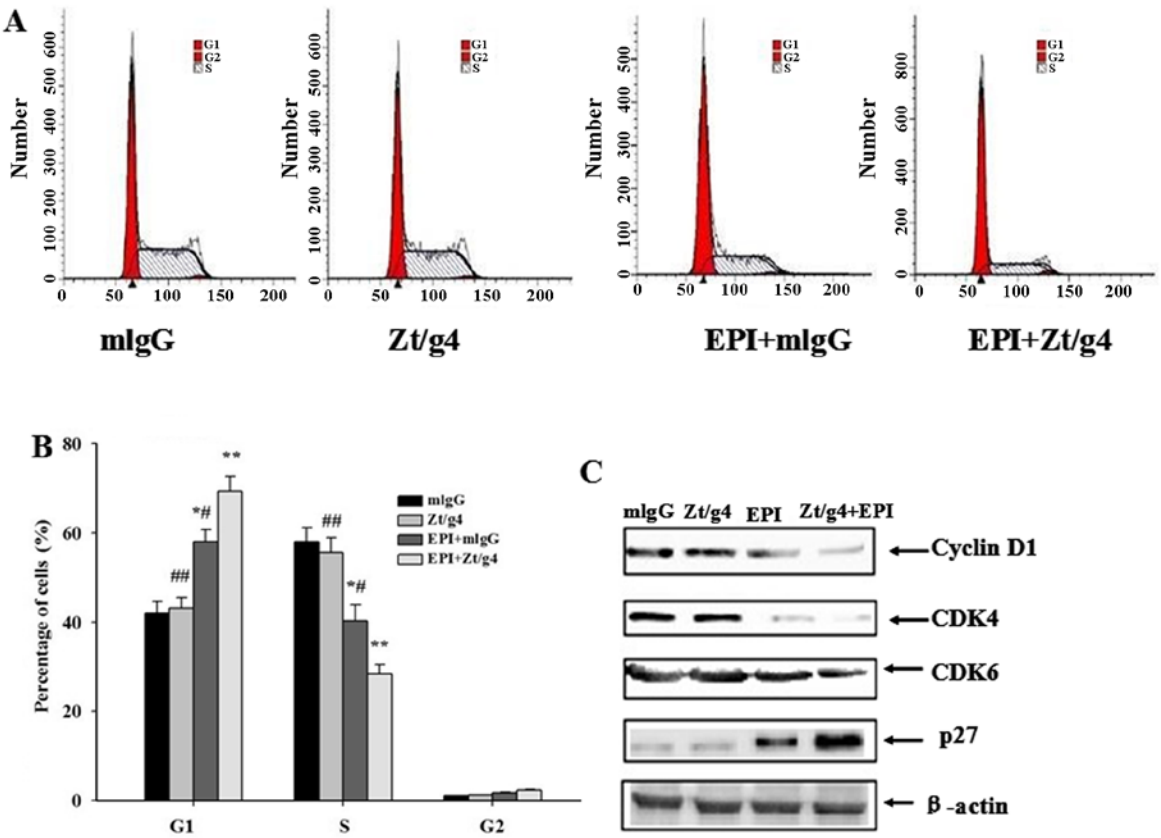

Figure 5. Effects of Zt/g4 on EPI-induced cell cycle in 5637 cells. (A) Changes in cell cycles: 5637 cells were treated at 37C with Zt/g4 alone, EPI alone or both for 24 h, collected, stained with propidium iodide, and then analyzed by flow cytometer. (B) Quantitative analysis of cell cycle distribution of 5637 cells treated with Zt/g4, EPI or both. *P<0.05, **P<0.01, ***P<0.001 compared to mlgG group; $^{\#}$P<0.05, $^{\#\#}$P<0.01, compared to EPI+Zt/g4group.Values are presented as the means ± SD from three independent experiments. (C) Representative western blot analysis showed changes in the expression of cyclins (CDK4 and CDK6), cyclin-dependent kinases (cyclin D1) and cyclin-dependent protein kinase inhibitors (p27) following Zt/g4, EPI or combined treatment in 5637 cells. Data represent the mean ± SD (n=3).

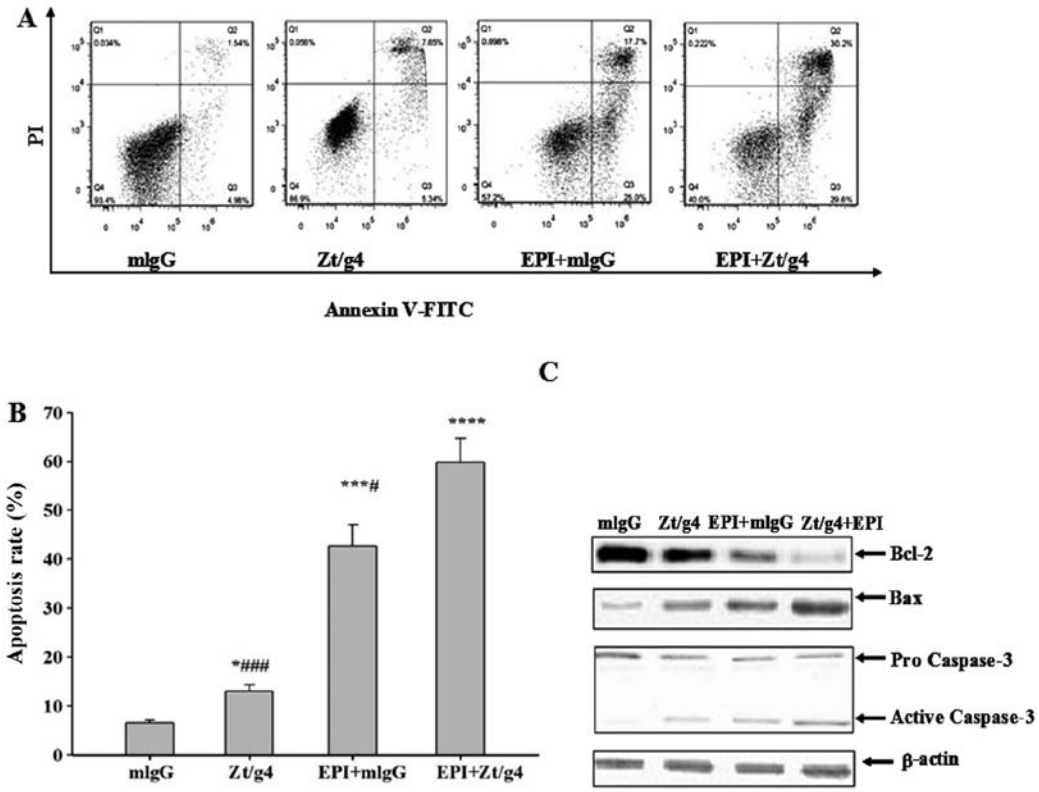

Figure 6. Effects of Zt/g4 on EPI-induced cell apoptosis in 5637 cells. Flow cytometric analysis demonstrated the apoptotic effects of Zt/g4, EPI and both treatment on 5637 cells. (A) Cells were treated with Zt/g4, EPI or both for 48 h, collected, stained with Annexin V and PI and then analyzed by flow cytometer. (B) Quantitative results obtained using Annexin V/PI staining. *P<0.05, ***P<0.01, ****P<0.0001 compared to mIgG group; $^{\#}$P<0.05, $^{\#\#\#}$P<0.001, compared to EPI+Zt/g4 group. (C) Representative western blot analysis showed changes in the expression of Bcl-2, Bax and activity-caspase-3 following Zt/g4, EPI or combined treatment in 5637 cells. Data are derived from three independent experiments and are expressed as the mean ± SD.

was carried out to measure the proliferation status of the cells. 5637 cells were treated with EPI or Zt/g4 combined with EPI at different concentrations. After 48 h, the cell viability was reduced with increasing concentrations of EPI (Fig. 3). The levels of cytotoxicity were indicated as the concentration that inhibits the response by 50% IC_{50} value. The IC_{50} values in Zt/g4 combined with EPI and EPI were (0.9±0.13, 1.2±0.09), respectively. Data showed that RON inhibition by Zt/g4 was able to enhance the sensitivity of 5637 cells to EPI at the concentrations of 0.2 and 1.2 μg/ml (P<0.05). Since the IC_{50} value in EPI treated 5637 cells was (1.2±0.09 μg/ml), the concentration of EPI intervention in subsequent experiments was determined as 1.2 μg/ml.

Zt/g4 combining with EPI markedly decreases 5637 cell invasion. Tumor invasion and progression is a common event in NMIBC even after TURBT and intravesical chemotherapy. To study whether combining Zt/g4 and EPI affected cellular invasion, we further carried out Transwell assay on the 5637 cells. The cells were treated with 8 μg/ml Zt/g4 alone, 1.2 μg/ml EPI alone, EPI combined with Zt/g4, or mouse IgG in transmembrane chambers for 24 h. The results showed that the invasive cell count of Zt/g4 in combination with EPI was significantly lower than that of Zt/g4 or EPI alone (170±21 vs. 564±49 or 390±37 individually) (Fig. 4A and B), and the cell invasive capacity of the Zt/g4 treatment group (564±49) was significantly lower than the mIgG group (726±62) (mIgG was used as the control).

Cell cycle arrested at G1/S phase in 5637 cells by Zt/g4 combining with EPI treatment. EPI can often inhibit cell proliferation through induction of cell cycle arrest. To determine whether Zt/g4 intracellular delivery of EPI resulted in cell cycle changes, we incubated cells with Zt/g4, EPI or both for 24 h and then examined the DNA content using propidium iodide (PI) staining. The proportions of cells treated with Zt/g4 in each phase of the cell cycle showed no significant difference comparing with those of control group. The changes in cell-cycle profile were observed after addition of EPI combined with Zt/g4, featuring a significant reduction in S phase, an increase in G1 phase, compared to the EPI treatment alone (P<0.05) (Fig. 5A and B). These changes were present in all three 5637 cell lines tested. Moreover, we checked several key factors including cyclin D1, CDK4, CDK6 and p27 regulating the G1/S cell cycle transition. The expression of cyclin D1, CDK4, CDK6 and p27was not changed by Zt/g4 treatment in 5637 cells. However, cotreatment of cells with EPI and Zt/g4 increased the expression of p27 while downregulated cyclin D1, CDK4 and CDK6 comparing with Zt/g4 or EPI treatment alone (Fig. 5C). These results suggested that the G1/S cell cycle arrest induced by Zt/g4 combined with EPI was related with upregulation of the cell cycle inhibitory proteins and downregulation of the cyclin-dependent protein kinases (CDKs) and cyclins.

Zt/g4 combining with EPI treatment promotes apoptosis in 5637 cells. Flow cytometry (FACS) was used to further

investigate whether Zt/g4, EPI or both exerted anticancer effect on 5637 cells through inducing apoptosis. We exposed 5637 cells to Zt/g4, EPI or both as previously mentioned, and then stained them with Annexin V-PE and PI to measure apoptosis rates after incubation for 48 h. FACS analysis showed that total apoptosis rates of EPI combined with Zt/g4 were significantly increased when comparing with EPI or Zt/g4 treatment alone (Fig. 6A and B). This result indicated that Zt/g4 was able to promote the EPI-induced apoptosis in 5637 cells. To further unveil the mechanisms by which Zt/g4 enhanced EPI-induced apoptosis in 5637 cells, the cells were treated with Zt/g4, EPI or both for 48 h and subjected to western blot analysis. The bcl-2 protein expression in EPI plus Zt/g4 cells was markedly lower while Bax and active caspase-3 protein expression levels were higher than those in the Zt/g4 or EPI treatment cells (Fig. 6C). This result suggested that downregulation of RON by Zt/g4 promoted EPI induced apoptosis of 5637 cells through mitochondria-mediated apoptotic pathway.

Discussion

Bladder cancer can be divided into three categories based on its prognosis and management. The first category consists of non-muscle-invasive tumors. The second category consists of muscle invasive bladder cancer (MIBC). The third group is metastasis bladder cancer. Therapeutic aim is different to each of these categories (30). To NMIBC, the main concern is to reduce recurrences and preventing progression to a more advanced stage. TURBT followed by chemotherapy agents and immunotherapy agents are now clinically used to achieve this goal (31). However, even treated with these current approaches, half of NIMBC will recur and progress. More strategies should be investigated to further decrease the rate of NIMBC recurrence and progression.

We and others have found that RON plays an important role in the pathogenesis of bladder cancer (11,14). Although RON is recently reported to associate with the chemosensitivity in human malignancies such as breast cancer and pancreatic cancer (19,32), its role in chemotherapy of bladder cancer remains largely unknown. In this study, our results showed that Ron signaling inhibition by Zt/g4 could remarkably enhance the chemosensitivity of epirubicin (EPI) in human 5637 cells and decrease cell invasion. Possible mechanisms include promotion of cell cycle arrest and induction of Bcl-2 dependent apoptosis.

Zt/g4 has unique binding specificity to the RON extracellular domains. Recent studies have shown that Zt/g4 is highly effective in downregulation of RON expression by colon, breast and pancreatic cancer cells (26). The binding of Zt/g4 to the epitopes either on sema or IPT domains is sufficient to cause RON reduction due to RON internalization and degradation by proteasome (33). In this study, we found the effect of Zt/g4 is concentration-dependent. Significant reduction of RON was seen when using 2 μg per ml of Zt/g4 and the maximal effect was at the range of 8 μg per ml. However, further increase of Zt/g4 up to 16 μg per ml did not show additional effect (Fig. 2A). In a recent study, Li reported that the maximal rate of Zt/g4 was 10 μg per ml in colon SW620 cells (26). The maximal effect is related to the amounts of Zt/g4 that bind to RON extracellular domain. Cross-talk between RON and MET or EGFR exist on the cell membrane surface, and specifically blocking RON is under intensive investigation (28,29). In the present study, the effect of Zt/g4 was only specific to RON. It had no effect on the structurally-related MET or unrelated EGFR in Fig. 2B. Moreover, after persistent Zt/g4 treatment, MSP induced RON activation was completely inhibited also the downstream pMAPK and pAkt activation was inhibited in 5637 cells.

Cancer cell unlimited growth and invasion are the most fatal features of malignant tumors, accounting for >90% of tumor-related mortality (34). Overexpression of RON contributes to increased cell growth and invasion (35,36). The results of this study showed that Zt/g4 or EPI alone could moderately inhibit cell proliferation and invasion in 5637 cells, but when Zt/g4 was used in combination with EPI it showed significant inhibition of cell proliferation (Fig. 3)and invasion (Fig. 4). Without Zt/g4, IC_{50} of EPI to 5637 cells was 1.2±0.09 μg/ml per 10^5 cells, after combining with Zt/g4, IC_{50} of EPI to 5637 cells was markedly decreased to 0.9±0.13 μg/ml per 10^5 cells. Therefore, Zt/g4 was efficient in enhancing chemosensitivity of EPI.

It was documented recently that Zt/g4 intracellular delivery of maytansinoid could result in cell cycle changes, suggesting that cell cycle arrest might contribute to enhancement of chemosensitivity by Zt/g4 (37). Therefore, we analyzed the cell cycle distribution of EPI-treated 5637 cells. EPI alone moderately affected cell cycle distribution in the cells, but when it was combined with Zt/g4, an obvious G1/S arrest was found (Fig. 5). Thus, Zt/g4-targeted delivery of EPI affects the cell cycle in 5637 cells. The cyclin-dependent kinases (CDKs) and cyclins play a crucial role in the regulation of cell cycle progression (38,39). The G1 cyclin-CDK complex cyclin D-CDK4/6 is required for S phase entry (40). CDK inhibitors (CKIs) such as p21CIP1/WAF1 and p27KIP1 bind to cyclin-CDK complexes and render them inactive, which inhibit cell cycle progression (41). In our study, we found that cotreatment of Zt/g4 and EPI leads to G1/S arrest into 5637 cells (Fig. 5A and B), which is accompanied by the downregulation of cyclin D1, CDK4 and CDK6, whereas it increased the level of P27 (Fig. 5C).

Apoptosis is a defensive mechanism of the body against the progression and development of tumor. Several studies have suggested that RON is associated with apoptosis in various cancer cells (42,43). However, the impact of RON on apoptosis in human bladder cancer is not reported. Thus, investigating the effect of Zt/g4 on EPI-induced cell apoptosis in 5637 cells is highly desirable. Our experiments showed that EPI-treated 5637 cells exhibited an increase in Annexin V(+)/PI(-) staining, and combined with Zt/g4 treatment strengthened this effect, indicating that Zt/g4 promoted EPI induced apoptosis (Fig. 6B). The mitochondrial apoptotic pathway is mainly mediated by proteins of the Bcl-2 family such as Bcl-2 and Bax (44). Bcl-2 is an anti-apoptotic protein, which negatively regulates the activation of caspase-3 that acted as an effector of mammalian cell death pathways (45). Bax is a proapoptotic protein, and the activation of Bax can increase the mitochondrial permeability and the release of pro-apoptotic molecules such as cytochrome-c. Releasing of cytochrome c can active caspase-3 and leads finally to apoptosis (46). In this

study, combining EPI and Zt/g4 significantly decreased the protein expression levels of Bcl-2 while increased the protein expression levels of Bax and cleaved caspase-3, suggesting Zt/g4 promoted EPI-induced apoptosis via the mitochondrial-dependent pathway.

In summary, the results of this study demonstrated that inhibition of RON signaling pathway by Zt/g4 markedly improved chemosensitivity of EPI in bladder cancer cells. Both cell proliferation and invasion were effectively inhibited by combining Zt/g4 and EPI treatment. Possible mechanisms underlying this combination include induction of cell cycle arrested at G1/S and promotion of mitochondrial pathway of apoptosis. These data provide new strategies to prevent recurrence and progression, which may further improve clinical outcomes of current approaches in treatment with NMIBC.

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