HIF-1α/MDR1 pathway confers chemoresistance to cisplatin in bladder cancer

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Received August 16, 2015; Accepted December 21, 2015

DOI: 10.3892/or.2015.4536

Abstract. Bladder cancer (BCa) is the 9th most common malignant tumor and the 13th leading cause of death due to cancer. The development of surgery and target drugs bring new challenges for the traditional concept for BCa therapy, and chemotherapy is still the final option for many BCa patients, and cisplatin-containing regimen the most effective one. However, the ubiquitous application of cisplatin-containing regimen in BCa results in the cisplatin-resistance, in addition, the cisplatin-resistant BCa manifests enhanced malignant behavior, the mechanism of which is unclear. In the present study, we used BCa cell lines to to clarify this point. BCa cell lines T24/J82 were pretreated with cisplatin >3 months to construct stable cisplatin-resistant cell lines (tagged $T24^{Cis-R}$ and $J82^{Cis-R}$), which manifested as enhanced capacity of proliferation and malignant behavior *in vivo* and *in vitro*, accompanied by cisplatin, and even doxorubicin resistance. The following mechanism dissection revealed that prolonged treatment time of T24/J82 cells led to elevated expression of HIF-1α, which targeted the increased expression of MDR1 on the one hand, and contributed to BCa cell proliferation, migration/invasion on the other hand. Finally, IHC staining of human BCa tissue supported our conclusion that the expression of HIF-1α and MDR1 was higher in chemoresistant tissue vs. chemosensitive tissue. Our results provided a new view of HIF-1α in chemotherapy.

Introduction

Worldwide, bladder cancer (BCa) is the 9th most common malignant tumor and the 13th leading cause of death due to cancer (1). Clinicopathologically, BCa is divided into superficial and invasive types (2-4). The instillation of chemodrug or Bacillus Calmette-Guérin (BCG) plus transurethral resection of BCa (TUR-bt) procedure is considered to be the most effective treatment for T1 and T2a superficial BCa (5). For Ta, Tis superficial BCa and invasive BCa, surgery (including radical cystectomy) accompanied by cisplatin-based chemotherapy is recommended and proved to be effective for promoting the progress-free survival (PFS) of patients (6-9). However, in instillation of chemodrug for superficial BCa or systematic chemotherapy for invasive BCa, chemoresistance is the vital obstacle, leading to treatment failure, whereas, the mechanism of how chemoresistance develops is still unclear (7).

The ABC superfamily is the most abundant transmembrane protein family encoded in the human genome, which plays important roles in pumping xenobiotics and anti-neoplastic drugs (e.g. chemodrugs) out of cells against a concentration gradient to maintenance the balance of the microenvironment, thus resulting in a low drug concentration in the cells and leading to the failure of chemotherapy (10). To date, 49 members of ABC superfamily has been discovered and divided into 7 subfamilies according to their structure (ABCA to ABCG) (11), among of them, 'ABCB1' (MDR1/P-gp) is regarded as the main one due to its special role in chemoresistance. MDR1 is a 170 kDa protein and consists of two nucleotide-binding domains (NBDs) and two transmembrane domains (TMDs), which localize in apical membrane of kidney, placenta, liver, adrenal glands, intestine and blood-brain barrier cells (12). Overexpression of MDR1 has been associated with various types of cancers, such as acute myeloid leukemia, childhood tumors, breast cancers and hematological malignancies, which can be regulated by tumor-related signaling, such as PI3K/Akt signaling (13-16). Widely, chemodrug is reported to be one of the key inducers of MDR1 (17-20).

In the present study, cisplatin-resistant BCa cell lines were generated to study the mechanism of chemoresistance in BCa, and further signaling pathway dissections demonstrated that HIF-1α→MDR1 pathway played critical role in the development of resistance to cisplatin in BCa, providing an avenue for BCa chemotherapeutics.

Materials and methods

Cell culture. Human BCa cell lines T24, and J82 were obtained from American Type Culture Collection (ATCC; Manassas,

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Key words: bladder cancer, cisplatin, MDR1, HIF-1α, malignancy

VA, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented by 10% fetal bovine serum (FBS), (Invitrogen, Carlsbad, CA, USA). Cells were cultured in an atmosphere with 5% CO_2 at 37˚C (incubators, Thermo Scientific, Germany).

In order to get cisplatin-resistant cell lines, the parental T24 and J82 cells were supplemented by 20 μM cisplatin. Medium was refreshed every two days to remove the dead cells and washed thrice using sterile phosphate-buffered saline (PBS) (pH 7.2). The cisplatin-treatment for parental T24/J82 cells for >3 months and MTT was used to verify the cisplatin sensitivity in the end of treatment (the cisplatin-resistant cell was tagged as $T24^{Cis-R}$ and $J82^{Cis-R}$).

Western blotting. Pretreated cells were harvested at 80% confluency and washed with cold PBS three times. Total cellular protein lysates were prepared with RIPA buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% NP40 and 0.5% sodium deoxycholate] containing proteinase inhibitors [1% inhibitor cocktail and 1 mM PMSF, both from Sigma (St. Louis, MO, USA)]. Protein (30 μg) was separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were blocked at room temperature for 1 h with 5% skim milk in Tris-buffered saline (pH 7.6, TBS). Primary antibodies were applied at different dilutions (GAPDH, 1:15,000; HIF-1α, 1:300; MDR1, 1:400) in 5% skim milk in TBS at 4˚C overnight, followed by TBST (with Tween-20) washes. Membranes were incubated with fluorescent secondary antibodies coupled to the first antibody at room temperature in the dark for 1 h, followed by TBST washes and signaling detection using Odyssey detection system (both from Licor, Rockford, IL, USA). MG-132 (Sigma-Aldrich, USA) was used to inhibit the proteasome-dependent degradation when necessary (10 μM, 4 h before the protein harvest). GAPDH was used as loading control (for total cell fraction).

Real-time PCR. Cellular total RNA was isolated using TRIzol reagent (Invitrogen) and quantitated by absorbance at 260 nm. RNA (2 μg) was reverse transcribed using RevertAid™ First Strand cDNA Synthesis kit (MBI Fermentas, St. Leon-Rot, Germany) according to the manufacturer's protocol. For real-time PCR, we used the SYBR Premix Ex Taq™ II system (Takara Biotechnology, Co., Ltd, Dalian, China) and the Bio-Rad CFX96™ Real-Time system (Bio-Rad, Hercules, CA, USA). Briefly, 12.5 μl SSYBR Premix Ex Taq™ II, 1 μl primer (F and R, respectively), 200 ng cDNA and 9.5 μl distilled and deionized water were mixed together, followed by two stage, pre-degeneration for 95˚C, 30 sec, one repeat; and PCR reaction, 95˚C 5 sec followed by 60˚C, 30 sec, 30 repeats; and the third stage as dissociation, 95˚C, 15 sec followed by 60˚C, 30 sec and another 95˚C, 15 sec. GAPDH was used as the loading control. Primers used are as follows: *MDR1* (NM_000927) F, 5'-CAG GAA CCT GTA TTG TTT GCC ACC AC-3' and R, 5'-TGC TTC TGC CCA CCA CTC AAC TG-3'; *HIF-1α* (NM_001530.3) F, 5'-TTG CTC ATC AGT TGC CAC TTC C-3' and R, 5'-AGC AAT TCA TCT GTG CTT TCA TGT C-3'; *GAPDH* (NM_002046.4) F, 5'-AAC AGC GAC ACC CAT CCT C-3' and R, 5'-CAT ACC AGG AAA TGA GCT TGA CAA-3'.

Cell viability assay. Cell viability was assessed using a tetrazolium-based assay (MTT). Pretreated cells were incubated in the absence or presence of cisplatin/doxorubicin for the indicated times, and then washed once with PBS and incubated with 0.5 mg/ml of MTT at 37˚C for 1 h. The reagent was reduced by living cells to form an insoluble blue formazan product. After incubation, cells were lysed with DMSO. Colorimetric analysis using a 96-well microplate reader was performed at a wavelength of 490 nm. The experiments were performed in triplicate.

Boyden chamber assay. Cell ability of migration/invasion was determined by the Boyden chamber assay. Chambers with pores of 8-μm diameter were obtained from Millipore (Switzerland). For migration assay, 0.2 ml FBS-free DMEM suspension with 10,000 cells was added to the upper chamber in 24-well plates, and 0.8 ml FBS-free DMEM was added to the lower chamber. After 12 h incubation, the chambers were washed with PBS (pH 7.4) three times to remove the cells in the upper chamber and fixed with 4% formalin for 15 min, then stained with crystal violet (0.01% in the ethanol) for 25 min followed by washing three times with PBS. The cells were counted using an inverted microscope, and five visions were randomly taken in the x200 magnification, and the average number of cells was analyzed. For the invasive assay, the cell suspension (10,000 cells/well) in the upper chamber contained 0.2 ml mixture of FBS-free DMEM/Matrigel at a ratio 8/1 (Matrigel; Sigma). Cells were incubated for 36 h and the rest of procedure was conducted according to the protocol of the migration assay.

RNAi and plasmid transfections. RNAi transfection: (siRNA-HIF-1α sense, CGT TGT GAG TGG TAT TAT TTT and antisense, AAT AAT ACC ACT CAC AAC GTA; siRNA-MDR1 sense, GGA AAA GAA ACC AAC UGU CdT dT and antisense, dT dTC CUU UUC UUU GGU UGA CAG) were used to silence the expression of *HIF-1α* and *MDR1* in $T24^{Cis-R}$/$J82^{Cis-R}$ cells, Lipofectamine 2000 was used according to its protocol. Forced expression of HIF-1α or MDR1 in parental T24/J82 cells completed by HA-$HIF1\alpha^{P402A/P564A}$-pc-DNA3 and pHa-MDR^{wt} plasmids, were obtained from Addgene (Addgene plasmid, #18955 and #10957, http://www.addgene.org). Lipofectamine 2000 was used to transfect plasmid into target cells, G418 was used to select HIF-1α high-expression stable clone. Both $T24^{HIF-1\alpha}$/$J82^{HIF-1\alpha}$ and $T24^{MDR1}$/$J82^{MDR1}$ were monitored using real-time PCR for their HIF-1α or MDR1 expression efficiency.

Proliferative assay. 5-Bromo-2'-deoxyuridine (BrdU) incorporation assay was used to monitor the proliferative ability of tumor cells. Pretreated cells were seeded on 8-well glass (Millipore) until 50-70% confluent, and BrdU was added into the medium (3 μg/ml), followed by 4 h incubation and then rinsed with PBS for 10 min to remove residual free BrdU. Cells were then fixed with 4% paraformaldehyde for 45 min, followed by rinsing with PBS for 20 min. 0.1% Triton X-100 was used to permeabilize the cell membrane for 15 min and 2N HCl added for 25 min to unspool DNA into single strands to allow primary antibody access to the incorporated BrdU. Cells were then rinsed with PBS for 10 min and non-specific

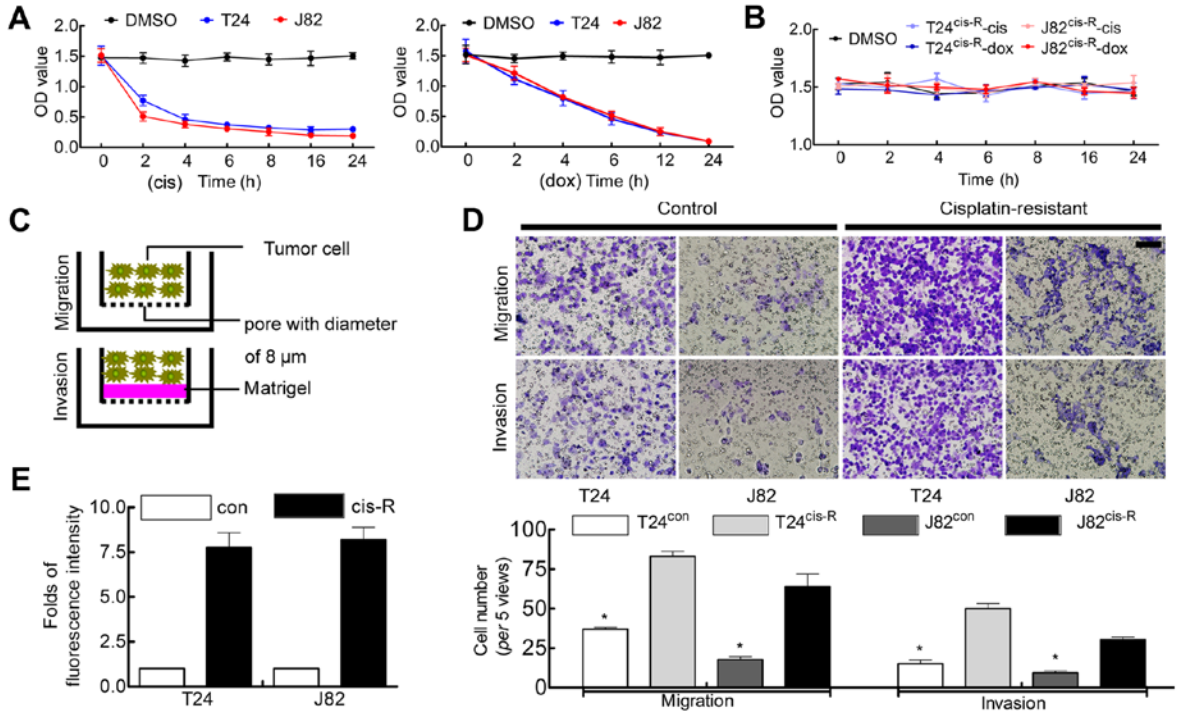

Figure 1. Prolonged time of cisplatin-treatment led to drug resistance, accompanied by enhanced ability of tumor cell migration/invasion and proliferation. (A) Bladder cancer cell lines T24 and J82 were treated with cisplatin (20 μM) and doxorubicin (40 μM), MTT showed that with time elapsing, cisplatin-sensitivity was decreased (left), but it was not so significant with doxorubicin (right). (B) MTT indicated that after 3 months treatment with cisplatin, T24 and J82 manifested non-sensitivity to both cisplatin and doxorubicin (indicated by $T24^{Cis-R}$ and $J82^{Cis-R}$). (C) Migration and invasion assay. (D) Representative figures (up) and quantification (down) of Boyden chamber assay suggesting that $T24^{Cis-R}$ and $J82^{Cis-R}$ manifested enhanced ability of migration and invasion compared with $T24^{con}$ and $J82^{con}$, magnification, x200; bar, 100 μm; *P<0.05. (E) BrdU incorporation showed enhanced ability of proliferation for $T24^{Cis-R}$ vs. $T24^{con}$ and $J82^{Cis-R}$ vs. $J82^{con}$.

epitopes were blocked by 10% BSA for 20 min. Anti-BrdU antibody (1:200) in 10% BSA was added to cells overnight at 4˚C. Cells were rinsed with PBS, followed by incubation with TRTIC-labeled secondary antibody for 1 h at room temperature, and finally rinsed with PBS to remove the free antibody. The fluorescence intensity of TRITC was monitored by SuperMicro Orifice Plate Spectrophotometer (BioTek, USA) in 547 nm.

Animal experiments. In order to demonstrate the ability of tumorigenesis, parental BCa cells, T24/J82 and chemoresistant BCa cells, $T24^{Cis-R}$/$J82^{Cis-R}$, $T24^{HIF-1\alpha}$/$J82^{HIF-1\alpha}$ and $T24^{MDR1}$/$J82^{MDR1}$ were implanted subcutaneously in both flanks of the mice. In brief, 10^6 BCa cells mixed with Matrigel (V/V=1:2) were injected into subcutaneous of the two flanks of mice, 5 weeks later, tumor mass was harvested, weighed, fixed with 4% formalin and prepared for pathological analysis.

Hematoxylin and eosin (H&E) and immunohistochemistry (IHC) staining. For H&E staining, the tissue sections were de-waxed and rehydrated routinely. The sections were stained in hematoxylin for 5 min, and washed in running tap water for 5 min. Then the sections were stained in eosin for 30 sec, dehydrated and mounted by routine methods. The representative fields were chosen for presentation in the figures.

IHC staining was conducted using the Image-Pro Plus System (Olympus, Japan). Tissues were deparaffinized, rehydrated and subjected to 5-min pressure-cooking antigen retrieval, 15-min endogenous enzyme block, 60-min primary antibody incubation and 30-min DakoCytomation EnVision-HRP reagent incubation for rabbit antibodies. Signals were detected by adding substrate hydrogen peroxide using diaminobenzidine (DAB) as a chromogen followed by hematoxylin counterstaining. Negative control slices were prepared by omitting the primary antibody. Stained (brown) cells are indicated in the figures.

Statistical analysis. ANOVA test was used to analyze the statistical discrepancy in >3 groups. Student's t-test was used to detect any statistically significant difference between two groups. P-values <0.05 were considered to indicate a statistically significant result.

Results

Decreased sensitivity of BCa cells to cisplatin in prolonged treatment. Acquired drug resistance of cancer cells leads to the failure of chemotherapy (12). In BCa, cisplatin is regarded as the most effective components of classical chemoregimen, such as M-AVC regimen; however, BCa cell acquired resistance to the treatment leads to inevitable tumor progression (21). Thus, in the present study, cisplatin (20 μM) was used to treat BCa cells, in addition, doxorubicin (40 μM) was used as parallel experiment. Our results indicated that, along with the prolonged exposure to chemodrug, the slope of cell viability gradually decreased, for cisplatin (Fig. 1A), indicating the decreased sensitivity of cells induced by the chemodrug.

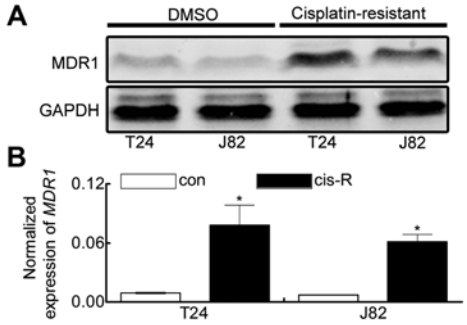

Figure 2. Prolonged time of cisplatin-treatment led to the elevated expression of MDR1. (A) Western blotting showed that comparing with DMSO, $T24^{Cis-R}$ and $J82^{Cis-R}$ gave rise to elevated expression of MDR1. (B) Real-time PCR indicates the elevated expression of *MDR1* in $T24^{Cis-R}$ and $J82^{Cis-R}$ vs. $T24^{con}$ and $J82^{con}$, $^{*}P<0.05$.

Chemoresistant BCa cells show enhanced ability of proliferation and malignant behavior. Previous experiment suggested that cisplatin sensitivity gradually decreased by the prolonged time of treatment, therefore, 20 μM of cisplatin was used to treat T24/J82 cells as described in Materials and methods. More than three months later, we observed that cisplatin-treated BCa cell lines displayed chemoresistance to cisplatin, even non-sensitivity to doxorubicin (Fig. 1B). These cell lines were tagged with $T24^{Cis-R}$ and $J82^{Cis-R}$ in the following investigation.

In order to clarify whether cisplatin-induced chemoresistance affected tumor malignancy and proliferation, Boyden chamber assay and BrdU incorporation were applied. As expected, $T24^{Cis-R}$ and $J82^{Cis-R}$ cells showed enhanced malignant behavior (Fig. 1C and D) and ability of proliferation (Fig. 1E).

Cisplatin treatment induces elevated expression of MDR1 (p-gp) in T24 and J82 cell lines. Increasing body of evidence indicates that the ABC transporter family is irreplaceable in acquired chemoresistance, and the most important one is the ABCB1 family, encoding MDR1 (P-gp) protein (22-24). Therefore we assessed whether MDR1 was involved in cisplatin-induced chemoresistance in our study. The expression of MDR1 in $T24^{Cis-R}$ and $J82^{Cis-R}$ cells were demonstrated using western blotting (Fig. 2A) and real-time PCR (Fig. 2B). Our results suggested that, comparing with control cells, $T24^{Cis-R}$ and $J82^{Cis-R}$ cells exhibited increased expression of MDR1, giving evidence that cisplatin-induced chemoresistance is possibly involved in upregulation of MDR1.

Knockdown of MDR1 in $T24^{Cis-R}$ and $J82^{Cis-R}$ cells attenuates cisplatin-induced chemoresistance. Large number of reports have pointed out the important roles of MDR1 in chemoresistance (25,26), in order to demonstrate this point, the expression of MDR1 in $T24^{Cis-R}$ and $J82^{Cis-R}$ were knocked down by RNAi, as indicated in Fig. 3A. The MTT analysis

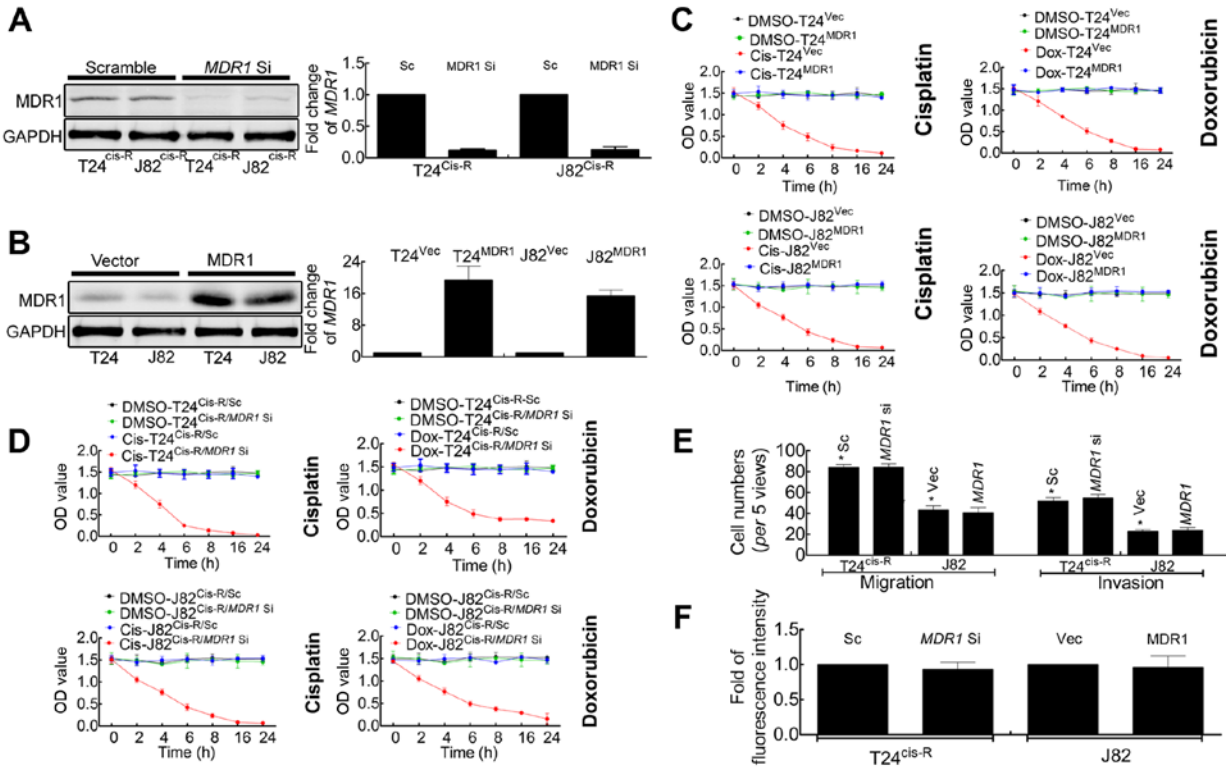

Figure 3. Alternating the expression of MDR1 in BCa cell lines led to switching of chemoresistance with non-effect on tumor migration/invasion and proliferation. (A) Western blotting (left) and real-time PCR (right) showed the efficiency of MDR1-knockdown in $T24^{Cis-R}$ and $J82^{Cis-R}$ by RNAi. (B) Western blotting (left) and real-time PCR (right) showed the efficiency of MDR1-overexpression in parental T24 and J82. (C) MTT showed that forced expression of MDR1 in parental T24 (up) and J82 (down) cells resulted in decreasing cisplatin (left) and doxorubicin (right) sensitivity. (D) MTT suggested that knockdown of the expression of MDR1 in $T24^{Cis-R}$ (up) and $J82^{Cis-R}$ (down) cells resulted in increasing cisplatin (left) and doxorubicin (right) sensitivity. (E) Quantification of Boyden chamber assay indicated that either forced expression of MDR1 in parental T24 (data not shown)/J82 or the knockdown of MDR1 in $T24^{Cis-R}$/$J82^{Cis-R}$ (data not shown) had non effect on tumor cell migration/invasion, $^{*}P<0.05$. (F) BrdU incorporation indicated that alternating the expression of MDR1 either in parental T24 (data not shown)/J82 cells or $T24^{Cis-R}$/$J82^{Cis-R}$ (data not shown) had non effect on tumor cell proliferation.

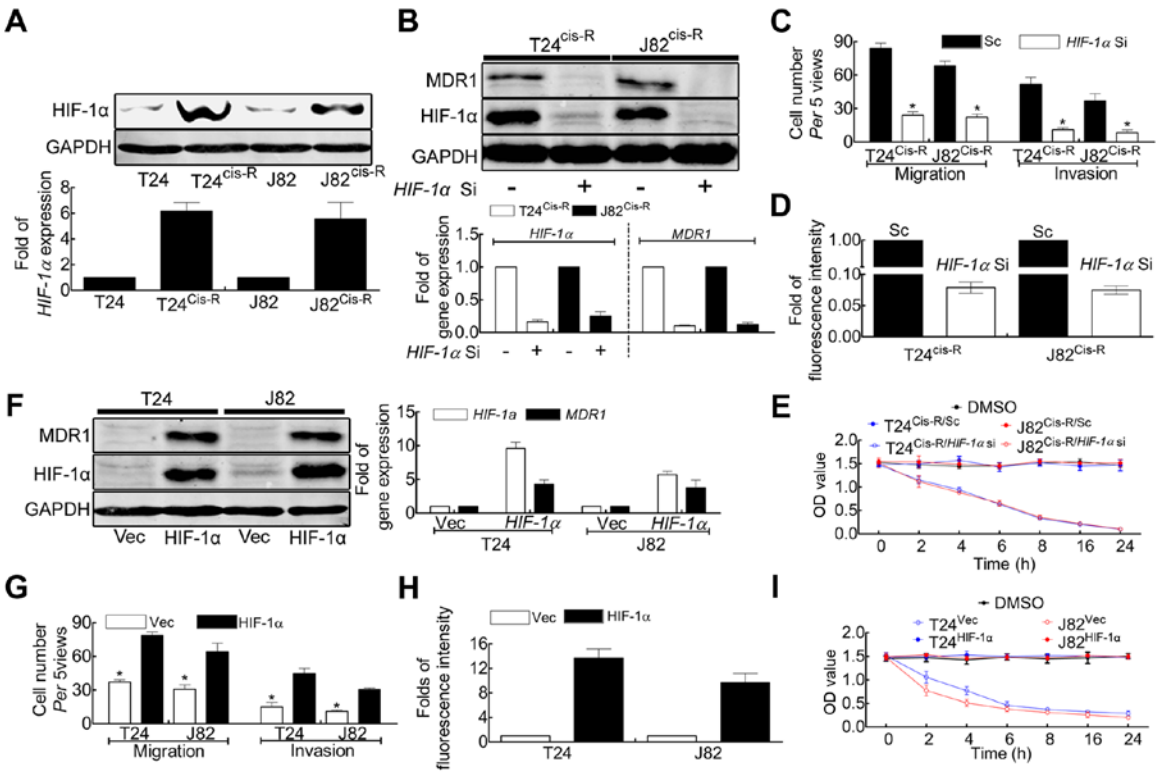

Figure 4. Prolonged time of cisplatin-treatment induces elevation of MDR1 involved in upregulating of HIF-1α. (A) Western blotting (up) and real-time PCR (down) indicated that the expression of HIF-1α was elevated in $T24^{Cis-R}$ vs. T24 and $J82^{Cis-R}$ vs. J82. (B) Western blotting (up) and real-time PCR (down) showed that knockdown of the expression of HIF-1α in $T24^{Cis-R}$ and $J82^{Cis-R}$ cells resulted in decreased expression of MDR1. (C) Quantification of Boyden chamber assay indicated that knockdown of the expression of HIF-1α in $T24^{Cis-R}$ and $J82^{Cis-R}$ cells led to the attenuated ability of cell migration and invasion, $^{*}P<0.05$. (D) BrdU incorporation indicated that knockdown of the expression of HIF-1α in $T24^{Cis-R}$ and $J82^{Cis-R}$ cells led to attenuated proliferative ability. (E) MTT showed that the knockdown the expression of HIF-1α in $T24^{Cis-R}$ and $J82^{Cis-R}$ cells resulted in increased chemodrug sensitivity. (F) Western blotting (left) and real-time PCR (right) showed that forced expression of HIF-1α in parental T24 and J82 led to elevated expression of MDR1. (G) Quantification of Boyden chamber assay indicated that forced expression of HIF-1α in T24 and J82 cells induced the enhanced ability of cell migration and invasion, $^{*}P<0.05$. (H) BrdU incorporation indicated that forced expression of HIF-1α in T24 and J82 cells led to enhanced proliferative ability. (I) MTT showed that forced expression of HIF-1α in T24 and J82 cells resulted in decreased chemodrug sensitivity.

suggested that chemodrug sensitivity significantly increased in $T24^{Cis-R/MDR1\ Si}$ and $J82^{Cis-R/MDR1\ Si}$ compared with $T24^{Cis-R/Sc}$ and $J82^{Cis-R/Sc}$, respectively (Fig. 3C).

In addition to chemoresistance, we postulated that the decreased expression of MDR1 may affect the malignancy of $T24^{Cis-R}$ and $J82^{Cis-R}$. Notably, the Boyden chamber assay (Fig. 3E) and BrdU incorporation (Fig. 3F) suggested that there was no significant difference in cell proliferation and malignant behaviors between $T24^{Cis-R/MDR1\ Si}$ vs. $T24^{Cis-R/Sc}$, or the $J82^{Cis-R/MDR1\ Si}$ vs. $J82^{Cis-R/Sc}$ (data not shown).

Forced expression of MDR1 decreases drug sensitivity in parental T24 and J82 cells. The above results suggested that knockdown of MDR1 in $T24^{Cis-R}$ and $J82^{Cis-R}$ cells led to the increased chemodrug sensitivity, but had non-effect on tumor cell malignancy, necessitating re-direction of our research. MDR1 plasmid was used to force expression of MDR1 in parental T24 and J82 cells (Fig. 3B), followed by MTT, Boyden chamber assay and BrdU incorporation to monitor the cell viability, malignancy and proliferation. Our results suggested that, consistent with the above findings, forced expression of MDR1 in parental T24 and J82 cells resulted in decreased chemodrug sensitivity (Fig. 3D) and still had non effect on tumor cell malignancy (Fig. 3E) and proliferation (Fig. 3F) (T24 data not shown).

Taken together, our results indicated that in T24 and J82 cells, cisplatin-induced chemodrug resistance was mediated by upregulation of MDR1, the process of which was proved to have non-effect on tumor cell malignancy and proliferation. However, $T24^{Cis-R}$ and $J82^{Cis-R}$ cells manifested enhanced ability of malignancy and proliferation, giving us new questions and need for further investigation.

HIF-1α is involved in cisplatin-induced upregulation of MDR1 in $T24^{Cis-R}$ and $J82^{Cis-R}$ cells. Our previous investigation proved that hypoxia-induced factor-1α (HIF-1α) played key roles in promoting BCa cell migration/invasion and proliferation (27). Yet, MDR1 was reported to be one of the target genes of HIF-1α (28), evoking us to postulate that cisplatin-induced tumor malignancy maybe medicated by HIF-1α. Expectedly, the expression of HIF-1α was strongly elevated in $T24^{Cis-R}$ and $J82^{Cis-R}$ cells comparing with parental T24 and J82 (Fig. 4A), leading us to investigate the mechanism. Thus, HIF-1α was knocked down in $T24^{Cis-R}$ and $J82^{Cis-R}$ cells (Fig. 4B), followed by Boyden chamber assay and BrdU incorporation. Our results were consistence with the postulation that knockdown of HIF-1α expression in $T24^{Cis-R}$ and $J82^{Cis-R}$ cells resulted in deceased expression of MDR1, attenuated ability of migration/invasion (Fig. 4C) and proliferation (Fig. 4D), accompanied by increased chemodrug sensitivity (Fig. 4E).

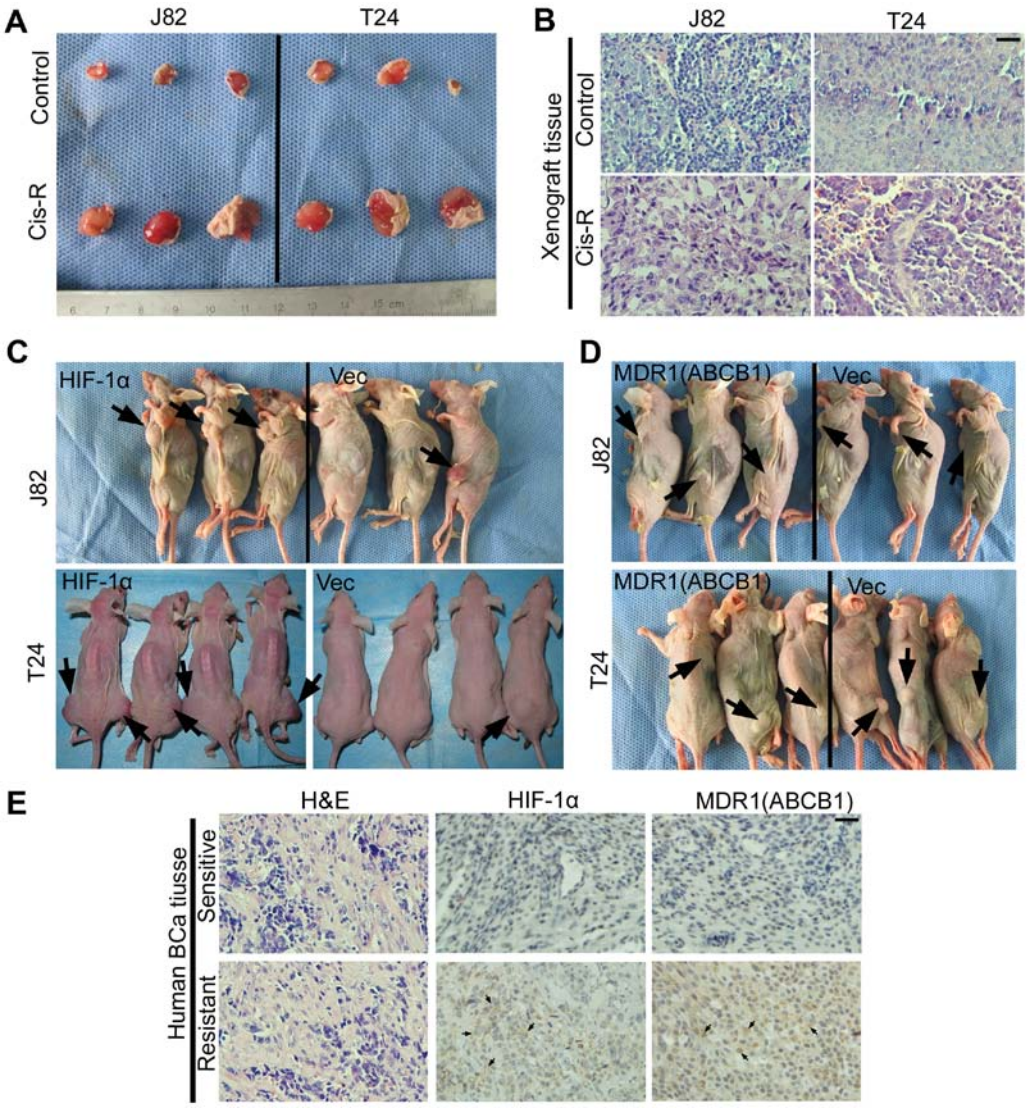

Figure 5. Validation of our conclusion *in vivo* and human BCa tissue. (A) The harvested xenograft tumor mass generated from $J82^{con}/J82^{Cis\text{-}R}$ and $T24^{con}/J82^{Cis\text{-}R}$ indicating the enhanced tumorigenetic ability of cisplatin-resistant cell lines. (B) H&E staining for validation of the xenograft tumor mass, indicating the cancerous structure, bar, 100 μm. (C) Xenograft of $T24^{HIF\text{-}1\alpha}/T24^{Vec}$ and $J82^{HIF\text{-}1\alpha}/J82^{Vec}$ in mice to monitor the discrepancy of tumorigenesis, suggesting enhanced tumorigenic ability of higher-HIF-1α-expression cell lines vs. the corresponding Vector, black arrow, the tumor mass. (D) Xenografts of T24MDR1/$T24^{Vec}$and J82MDR1/$J82^{Vec}$ in mice to monitor the discrepancy of tumorigenesis, suggesting that there is no visible difference between the enhanced tumorigenic ability of higher-MDR1-expression cell lines vs. the corresponding vector, black arrow, and the tumor mass. (E) H&E and IHC staining for the human BCa tissue, indicating the elevated expression of HIF-1α and MDR1 in chemoresistant BCa tissues vs. chemosensitive BCa tissues. Black arrow, positive cell; bar, 100 μm.

To further confirm this mechanism, forced expression of HIF-1α/Vec was monitored in parental T24 and J82 cells as shown in Fig. 4F, indicating that forced expression of HIF-1α upregulated MDR1 in both protein and mRNA levels. The followed results suggested that forced expression of HIF-1α in parental T24 and J82 cells gave rise to tumor cell migration/invasion (Fig. 4G) and proliferation (Fig. 4H), accompanied by decreased chemodrug sensitivity (Fig. 4I).

Collectively, we provided evidence that HIF-1α was elevated in $T24^{Cis\text{-}R}$ and $J82^{Cis\text{-}R}$ cells, through which cisplatin induced upregulation of MDR1, leading to chemoresistance.

Demonstration the enhanced ability of tumorigenesis in vivo and validation of our conclusion in human BCa tissue. To demonstrate the enhanced ability of tumorigenesis in $T24^{Cis\text{-}R}$ and $J82^{Cis\text{-}R}$, both cell lines, in line with their parental cells, were mixed with Matrigel and injected into both flanks of the nude mice (Fig. 5A). The visible tumor mass was harvested and validated by H&E staining (Fig. 5B).

To clarify whether there is a difference in tumorigenic ability between high-HIF-1α expressing cells and parental cells, both the $T24^{Vec}/T24^{HIF\text{-}1\alpha}$ and $J82^{Vec}/J82^{HIF\text{-}1\alpha}$ cells were used. As indicated in Fig. 5C, comparing with vector, $T24^{HIF\text{-}1\alpha}/J82^{HIF\text{-}1\alpha}$ showed enhanced ability of tumorigenesis. In addition, in agreement with our mechanistic conclusion, there is no significant discrepancy of tumorigenesis between $T24^{MDR1}/J82^{MDR1}$ and $T24^{Vec}/J82^{Vec}$ (Fig. 5D).

Finally, to further confirm our conclusion in human BCa tissue, 8 chemoresistant BCa tissues and 5 chemosensitive BCa tissues were used to monitor the expression of HIF-1α and MDR1. As expected, and in line with mechanistic investigation, IHC staining for HIF-1α and MDR1 in human BCa

tissues (Fig. 5D) suggested that, comparing with chemosensitive BCa, chemoresistant BCa tissue showed higher expression of HIF-1α and MDR1.

Discussion

Despite the improvement of surgery, chemotherapy/radiotherapy is still the final regimen for invasive BCa patient or the first choice for patient who cannot be helped by surgery. Cisplatin-based chemodrug is recommended for most BCa patients due to its high efficiency, such as M-VAC regimen (3); however, due to multiple chemoresistance, treatment failure still exists, and its mechanism is unknown.

Theoretically, chemoresistance of tumor cells is divided into initial and acquired types (29). The former suggests that there is a fraction of chemoresistant cells in the tumor mass, which initiates tumorigenesis after chemotherapy; the latter emphasizes that the ability of chemoresistance is induced by chemodrugs, manifesting an inevitable result for chemotherapy. Mechanistically, chemoresistance can be ascribed to various reasons including drug inactivation, off-target, cell death inhibition, epigenetics, decreased drug uptake/increased drug efflux and EMT (30,31). The drug efflux system attracts more and more attentions for its irreplaceable roles in chemoresistance, which is mainly mediated by ABC superfamily. Consistent with other studies, our data suggested that prolonged time of cisplatin-treatment obviously led to the elevated expression of MDR1, accompanied by attenuation of cisplatin sensitivity and enhanced tumor cell malignant behavior (Figs. 1 and 5A).

In our reversal experiment, we found that forced expression of MDR1 in T24 and J82 ($T24^{MDR1}$ and $J82^{MDR1}$) resulted in decreased sensitivity to chemodrug, however, with no effect on tumor malignancy and proliferation (Figs. 3 and 5D). These results created a dilemma since in $T24^{Cis\text{-}R}$ and $J82^{Cis\text{-}R}$, high expression of MDR1 was accompanied by enhanced ability of malignancy and proliferation (Figs. 3 and 5A). In BCa cell lines, our previous investigation had proved that HIF-1α played irreplaceable roles in monitoring the tumor cell malignancy (27). Furthermore, HIF-1α was proved to target the expression of MDR1 directly (32). Previous investigations indicated that activation of HIF-1α could be induced by hypoxia (33), VHL mutation, and PHD mutation; in addition, reactive oxygen species (ROS) was regarded as the key (34). Mechanistical investigation indicated that ROS was elevated in cisplatin-treated tumor cells (35). Therefore, we postulate that in our study, elevated expression of HIF-1α was possibly induced by ROS, which was the production of cisplatin-treatment, leading to the expression of MDR1, accompanied by enhanced tumor cell malignancy (Fig. 4) and proliferation (Fig. 5C). However, more studies are needed to support our postulation. In other aspects, we found that, both cisplatin-induced MDR1 and forced elevation of MDR1 led to doxorubicin resistance, indicating a ubiquitous role in chemoresistance for MDR1.

Taken together, in the present investigation, we provide evidence that elevated expression of HIF-1α induced by cisplatin monitor the upregulation of MDR1, resulting in chemoresistance, in other aspects, elevated expression of HIF-1α initiates enhanced tumor cell malignant behavior and ability of proliferation. Thus, HIF-1α plays vital roles in cisplatin-induced chemoresistance in BCa therapeutics, which provides us another view to understand the acquired chemoresistance in BCa.

Acknowledgements

The present study was partly supported by the National Natural Science Foundation of China (NSFC; no. 81172436 to Y.S.), and Overall Innovation Projects of Scientific and Technological Resources of Shaanxi Province (No. 2013KTCL03-04 to Y.X.).

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