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Vitamin D_3 sensitizes resistant human bladder cancer cells to cisplatin by regulating Sirtuin 1 gene expression

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Abstract. – OBJECTIVE: Cisplatin is a standard chemotherapeutic agent for advanced bladder cancer, but its efficacy is limited due to drug resistance. Vitamin D_3 may reverse cancer multidrug resistance, but the potential molecular mechanisms are still only partially known. The purpose of this study was to explore the mechanism by which vitamin D_3 reverses cisplatin resistance in bladder cancer to improve therapeutic efficacy and ameliorate the prognosis of cisplatin-resistant bladder cancer.

PATIENTS AND METHODS: The levels of vitamin D_3 and sirtuin 1 protein were detected in cisplatin-resistant bladder cancer patients and cisplatin-sensitive patients. The cisplatin-resistant bladder cancer cell lines T24/DDP and UMUC3R were used as cell experimental models, and the migration, apoptosis, mitochondrial reactive oxygen species accumulation and autophagy of cells were assessed in the present study.

RESULTS: Vitamin D_3 levels were decreased, and sirtuin 1 protein levels were increased in cisplatin-resistant bladder cancer patients compared with cisplatin-sensitive bladder cancer patients. Vitamin D_3 treatment markedly repressed sirtuin 1 expression, and overexpression of the sirtuin 1 gene led to mitochondrial reactive oxygen species generation, promoted the initiation of autophagosome formation and enhanced autophagic flux. Cisplatin treatment in the presence of vitamin D_3 inhibited cell invasion and migration and induced apoptosis and enhancing the sirtuin 1 gene abolished the effect of vitamin D_3 by regulating mitochondrial reactive oxygen species accumulation and autophagosome formation.

CONCLUSIONS: These data support a mechanism wherein the sirtuin 1 gene plays a crucial role in vitamin D_3 reversing cisplatin resistance in bladder cancer and may provide useful preventive and therapeutic applications in the future.

Key Words:
Bladder cancer, Vitamin D_3, Cisplatin, Drug resistance, Sirtuin 1.

Abbreviations
Urothelial carcinoma (UC); Cisplatin (DDP); Vitamin D_3 (VitD); Epidermal growth factor receptor (EGFR); Reactive oxygen species (ROS); Sirtuin 1 (SIRT1); Dulbecco's modified Eagle's medium (DMEM); Fetal bovine serum (FBS); Radioimmunoprecipitation assay (RIPA); Sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); Polyvinylidene fluoride (PVDF); 4'-6-diamidino-2-phenylindole (DAPI); Omium tetroxide (OsO4); Standard deviation (SD).

Introduction

Bladder cancer, referred to as urothelial carcinoma (UC), is the second most common genitourinary cancer associated with high morbidity and mortality[1,2]. Cisplatin (DDP) is a standard chemotherapeutic agent for advanced bladder cancer that interferes with DNA repair mechanisms, induces DNA damage, and subsequently causes cell apoptosis[3,4]. Despite the promising anticancer effects of DDP in bladder cancer patients, drug resistance, low response rates and toxic effects limit the clinical application of chemotherapeutic agents[5]. DDP-induced DNA damage is an important therapeutic mechanism for inhibiting proliferation and inducing apoptosis in cancer cells. However, undesirable side effects after the application of DDP were found in patients, including bone marrow suppression, neurotoxicity and decreased immunity to infections, as well as acquired drug resistance[6,7]. Accumulating evidence has demonstrated

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that vitamin D_3 (VitD) can enhance the antitumor activity induced by chemotherapeutic agents such as gemcitabine, cisplatin, and doxorubicin[8,9]. Several studies have been interlinked with the synergistic or additive antitumor effectiveness of VitD supplementation and reversal of drug resistance, and the reversal effect of VitD on erlotinib resistance was also observed in a non-small cell lung cancer cell line (HCC827R1 cells) with epidermal growth factor receptor (EGFR) mutation[10]. However, the molecular mechanisms by which vitamin D reverses multidrug resistance in cancer are still not well understood.

Compared with nondrug-resistant cancer cells, the reactive oxygen species (ROS) level of drug-resistant cancer cells is significantly increased[11]. Therefore, drug-resistant cancer cells may be more susceptible to changes in ROS levels. Studies have found that excessive ROS generation can destroy intracellular homeostasis, lead to oxidative stress and mitochondrial dysfunction, and induce the formation of autophagosomes[12,13]. Sirtuin 1 (SIRT1) is a type III histone deacetylase that is associated with a higher tumour grade and poor long-term survival and is involved in tumour progression, apoptosis, autophagy, DNA repair, and drug resistance[14]. We will focus on the effect of the *SIRT1* gene on ROS production and autophagy levels in the regulation of VitD on the progression of tumour drug resistance, hoping that our study could provide new insights as a therapeutic target in the future.

Patients and Methods

Participants and Sampling

This study was performed in line with the principles of the Declaration of Helsinki. The research protocol was approved by the Medical Ethics Committee of Hongqi Hospital Affiliated to Mudanjiang Medical University (Data: 2017. 10. 06/No. 2017HQYY003-033), and written informed consent was obtained from each participant. Blood and tissue samples were obtained from 60 bladder cancer patients who were enrolled in this experiment from January 2018 to September 2021. Thirty patients had DDP-resistant bladder cancer (defined as tumour recurrence after R0 excision during DDP-based therapy). Thirty patients had DDP-sensitive bladder cancer (defined as no tumour recurrence during DDP-based therapy).

Cell Culture

The T24 and UMUC3 human bladder cancer cell lines were obtained from the Chinese Academy of Sciences Culture Collection. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Life Technologies, Carlsbad, CA, USA) and 1% antibiotics (100 units/ml of penicillin/streptomycin, Thermo Fischer Scientific, Waltham, MA, USA) at 37°C in a humidified incubator under 5% CO_2. The DDP-resistant cell lines were T24/DDP and UMUC3R cells in this study, which were established by the DDP concentration gradient method. To maintain the DDP-resistance phenotype, cells were stimulated with DDP at a final concentration of 5 μM.

SIRT1 Plasmid Construction and Transfection

The *SIRT1* cDNAs were subcloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). The forward primer was 5'-CAG TGG CTG GAA CAG TGA GA-3', and the reverse primer was 5'-AGC GCC ATG GAA AAT GTA AC-3′. The reconstituted pc-SIRT1 plasmid and empty plasmid (pc-NC) were transfected into human bladder cancer cells, and 2.5 μg pc-SIRT1 plasmid or 2.5 μg pc-NC was mixed with 8 μL of Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) and incubated with bladder cancer cells for 6 h.

Western Blot Analysis

Bladder cancer tissues and cells were harvested and lysed in radioimmunoprecipitation assay (RIPA) buffer. The proteins were separated via SDS-PAGE and then transferred to PVDF membrane. The membrane was blocked with 5% skim milk for 2 h and then incubated with primary monoclonal antibodies overnight at 4°C. The PVDF-membrane were washed and then probed with HRP-conjugated secondary antibodies for 2 h. The band intensities were quantified by using the Enhanced Chemiluminescence Western Detection System (Cell Signaling Technology, Danvers, MA, USA).

Immunohistochemistry

The bladder cancer tissues were fixed with 10% buffered formalin, and then embedded in paraffin. Sections of 4 μm thickness, the slices were incubated with 4% H_2O_2 for 20 min and then overnight with primary antibodies against SIRT1 at 4°C, followed by incubation with secondary

antibodies for 30 min at 37°C. The slides were visualized with DAB (Sigma-Aldrich, St. Louis, MO, USA) to produce a brown color and images were captured using inverted fluorescence microscope (Olympus, Tokyo, Japan).

Immunofluorescence Analysis

For autophagosome and autophagic flux examination, cells were fixed with 4% paraformaldehyde, blocked in a 1% BSA solution for 60 min, and then stained with 100 μL 4'-6-diamidino-2-phenylindole (DAPI) (C1002: 1 μg/mL, Beyotime, Shanghai, China). Cells were photographed with a confocal laser scanning microscope (Olympus, Tokyo, Japan). The number of LC3B puncta per cell in GFP-positive or mCherry-GFP-positive cells was calculated as reported previously[15].

Transmission Electron Microscopy (TEM)

The cells were fixed in a 2.5% paraformaldehyde-glutaraldehyde mixture (Sigma-Aldrich, St. Louis, MO, USA) overnight at 4°C and then postfixed with 1% osmium tetroxide (OsO4) for 1 h at 4°C. The pellets were dehydrated using graded ethanol and saturated in acetone and Durcupan (Fluka, Buchs, Switzerland). The cells were cut into 70~80 nm ultrathin sections and then stained with uranyl acetate and lead citrate. The ultrastructure of the cells was viewed via TEM (Hitachi, Tokyo, Japan).

Mitochondrial ROS Assessment

The cells (5×10^5/mL, 2.5 ml/well) were cultured in 6-well plates. Mitochondrial ROS production was detected using the MitoSOX™ Red fluorescent probe (Molecular Probes, Life Technologies, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Relative changes in MitoSOX™ Red fluorescence are shown as the fold increase over the control group.

Cell Invasion Assay

The invasion of cells was assessed using Transwell chambers (6-well plate, 8 μm pore size). One hundred microlitres of cell suspension was plated into the upper chambers, and treatment media was loaded into the bottom chambers. The membrane was fixed with 4% paraformaldehyde and then stained with crystal violet (Beyotime, Shanghai, China) for 25 min. The number of migrated cells in five random fields was calculated under an inverted microscope (Olympus, Tokyo, Japan) at a magnification of 200 ×.

Scratch Test Assay

Cell migration ability could also be measured by the scratch test. A thirty mm petri dish was scratched three straight lines with 1~2 cm intervals, and cells in log phase growth were placed in petri dishes. After 24 h of incubation, the next scratch was made with a pipette tip perpendicular to the three baselines. The dropped cells were observed for cell migration under a microscope.

Cell Apoptosis

The cells were harvested by 0.25% trypsin and resuspended with binding buffer (5.0×10^5 cells/mL), then incubated with 5 μl Annexin V and 5 μL of propidium iodide (PI) for 15 min in the dark. The cells apoptosis was calculated using flow cytometry.

Statistical Analysis

The measurement data were presented as the mean ± standard deviation (SD) at least three separate experiments. The quantitative results were analysed using SPSS version 17.0 (SPSS Inc. Chicago, IL, USA), and data comparison between the two groups were conducted by *t*-test if normally distributed, otherwise the Wilcoxon rank-sum test was used. $p < 0.05$ indicated a statistically significant difference.

Results

The Levels of VitD and SIRT1 Protein in DDP-Resistant Bladder Cancer Patients and DDP-Sensitive Patients

To clarify the relationship between VitD levels and drug resistance in bladder cancer, the serum $1,25D_3$ levels were measured in the DDP-resistant bladder cancer group (R) and the DDP-sensitive bladder cancer group (S). The results indicated that the VitD level was significantly lower in the DDP-resistant bladder cancer group than in the DDP-sensitive bladder cancer group ($t = 6.628$, $p < 0.001$, Figure 1a), and patients with DDP-resistant bladder cancer had vitamin D levels below 50 nmol/L (< 50 nmol/l is defined as vitamin D deficiency). Meanwhile, the SIRT1 protein level was detected by western blot and immunohistochemical staining, and the data showed that SIRT1 protein expression in DDP-resistant bladder cancer tissues was significantly increased compared with that in DDP-sensitive tissues (Figure 1b-c).

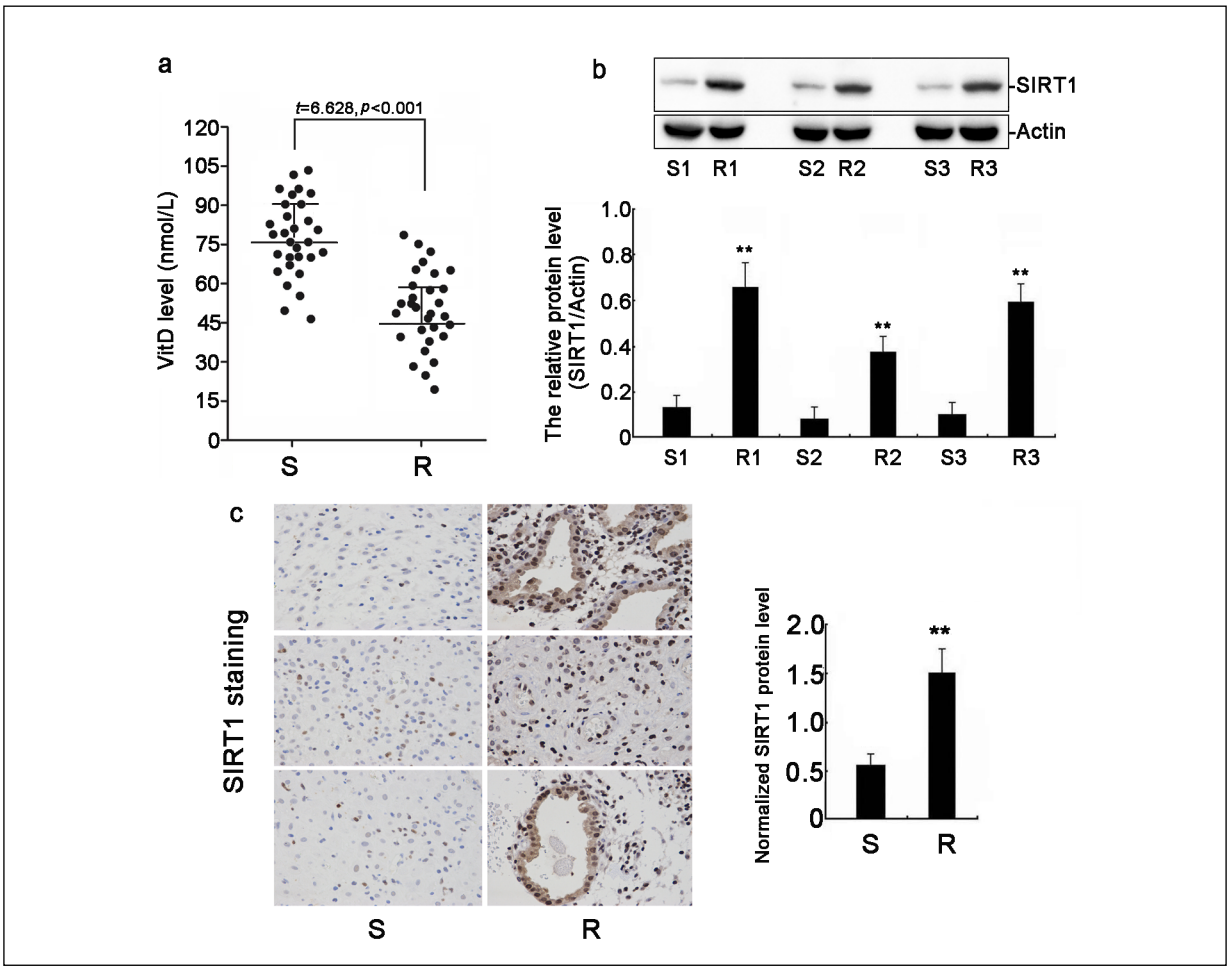

Figure 1. VitD levels and SIRT1 protein levels in DDP-resistant bladder cancer patients and DDP-sensitive patients. **(a)** Serum VitD levels were measured using chemiluminescence immunoassays from 30 patients with DDP-resistant bladder cancer (R) and 30 patients with DDP-sensitive patients (S). **(b)** SIRT1 protein levels in DDP-resistant bladder cancer tissues (R) and DDP-sensitive tissues (S) were checked using western blot analysis. Relative protein expression was normalized to actin. $n=3$ independent experiments, $p < 0.01$ *vs.* the S group. **(c)** SIRT1 protein expression in DDP-resistant bladder cancer tissues (R) and DDP-sensitive tissues (S) was observed using immunohistochemical staining (magnification, ×400), showing positive staining (brown, scale bars = 50 μm). $n = 3$, $p < 0.01$ *vs.* the S group.

Effect of the SIRT1 Gene on Mitochondrial ROS Production and Autophagy in T24 and UMUC3 Cells

First, the effect of VitD treatment on SIRT1 protein expression was observed in T24 and UMUC3 cells, as shown in Figure 2a. Treatment with 1,25-dihydroxyvitamin D_3 ($1,25D_3$, the active VitD metabolite) significantly inhibited SIRT1 protein expression, which was significantly different from that of the mock group. Second, we observed the intracellular localization of SIRT1 protein by immunofluroscence of T24 and UMUC3 cells. Our results indicated that SIRT1 protein is present in the cytoplasm of cells, consistent with a cytoplasmic role for SIRT1 protein (Figure 2b). Third, changes in mitochondrial ROS content were utilized to evaluate the alteration of mitochondrial oxidative stress in T24 and UMUC3 cells. The results indicated that transfection with pc-SIRT1 significantly induced mitochondrial ROS accumulation compared with pc-NC transfection ($p < 0.01$, Figure 2c). To further assess the role of the *SIRT1* gene in autophagy, we measured the effect of the *SIRT1* gene on autophagic flux using the mCherry-GFP-LC3B plasmid. The data indicated that the *SIRT1* gene (pc-SIRT1 group) potently enhanced autophagic flux (the rate of red puncta formation in each cell, Figure 2d). Finally, the expression of the autophagy-associated proteins Beclin 1 and ATG5 was

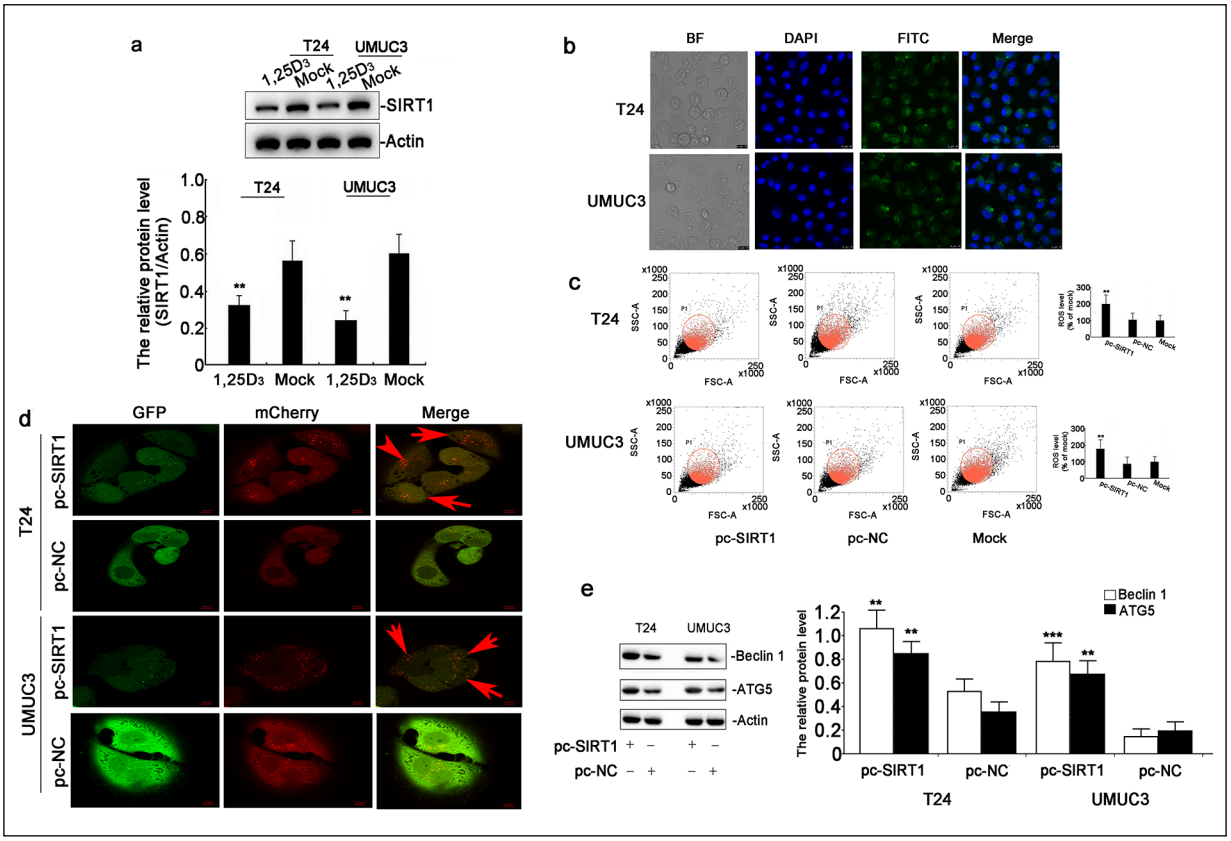

Figure 2. Effect of *the SIRT1* gene on mitochondrial ROS production and autophagy in T24 and UMUC3 cells. **(a)** T24 and UMUC3 cells were treated with 1,25D_3 (100 nmol/L) for 24 h, and SIRT1 protein levels were detected using western blot analysis. Relative protein expression was normalized to actin. $n = 3$, $p < 0.01$ *vs.* mock group. **(b)** T24 and UMUC3 cells were transfected with pc-SIRT1 (2.5 μg/ml) for 48 h, and SIRT1 protein expression was observed by laser confocal microscopy. SIRT1 protein was stained green, and the nucleus was stained with DAPI (blue) (scale bars = 10 μm). **(c)** T24 and UMUC3 cells were transfected with pc-SIRT1 (2.5 μg/ml) or pc-NC (2.5 μg/ml) for 48 h, and mitochondrial ROS levels were tested using the fluorescent probe MitoSOX™ Red. $n = 3$, $^{**}p < 0.01$ *vs.* pc-NC group. **(d)** Autophagic flux was observed using the mCherry-GFP-LC3 reporter in T24 and UMUC3 cells by laser confocal microscopy. Scale bars, 10 μm. **(e)** Western blot analysis of the autophagy-associated proteins Beclin 1 and ATG5 in T24 and UMUC3 cells (actin was used as the loading control). $n = 3$ independent experiments, $^{***}p < 0.001$, $^{**}p < 0.01$ *vs.* the pc-NC group.

determined by western blot analysis in T24 and UMUC3 cells, and the results indicated that Beclin 1 and ATG5 protein levels in the pc-SIRT1-treated group were significantly higher than those in the pc-NC group (Figure 2e). Taken together, these data suggest that the *SIRT1* gene induces mitochondrial ROS production, promotes the initiation of autophagosome formation and enhances autophagic flux in T24 and UMUC3 cells.

Effect of the SIRT1 Gene on Mitochondrial ROS Production and Autophagy in Cisplatin (DDP) Combined with VitD-Treated T24/DDP and UMUC3R Cells

As shown in Figure 3a, DDP-induced mitochondrial ROS accumulation was markedly attenuated by VitD treatment, and DDP + VitD treatment significantly decreased ROS generation relative to that of the DDP alone group ($p < 0.05$). However, overexpression of the *SIRT1* gene markedly abolished VitD-inhibited ROS generation, and DDP + VitD + pc-SIRT1 treatment significantly increased ROS accumulation, which was significantly different from DDP + VitD treatment ($p < 0.01$). In addition, electron microscopy showed that the DDP + VitD + pc-SIRT1 group and DDP alone groups showed obvious autophagosomes (red arrow), but DDP + VitD administration and DDP + VitD + pc-NC treatment abolished the adverse effects and restored normal mitochondrial morphology (Figure 3b). Consistent with these results, the protein levels of the autophagy adaptor protein p62 in

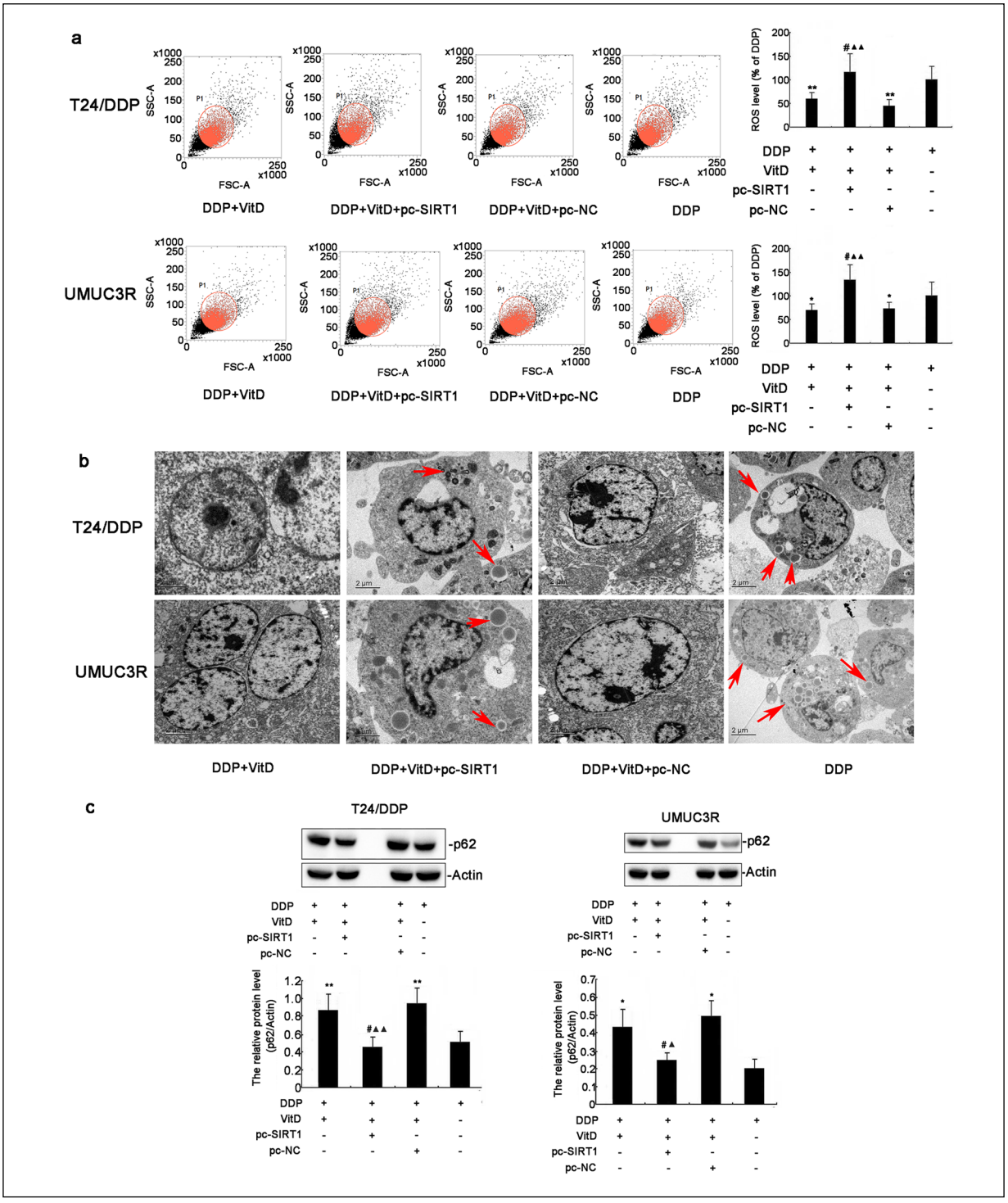

Figure 3. Effect of the SIRT1 gene on mitochondrial ROS production and autophagy in cisplatin (DDP) combined with VitD-treated T24/DDP and UMUC3R cells. T24/DDP and UMUC3R cells were separately exposed to 20 μM DDP in the absence or presence of VitD (100 nmol/L) for 24 h and then transfected with pc-SIRT1 (2.5 μg/ml) or pc-NC (2.5 μg/ml) for another 24 h. **(a)** The mitochondrial ROS level was measured using the fluorescent probe MitoSOX™ Red. $n = 3$, $^{**}p < 0.01$, $^{\#}p > 0.05$ *vs.* the DDP group; $^{\blacktriangle\blacktriangle}p < 0.01$ *vs.* the DDP+ VitD group. **(b)** Mitochondrial autophagosomes in T24/DDP and UMUC3R cells were observed by electron microscopy (5200-fold magnification), and autophagosomes are indicated by red arrows. Scale bar = 2 μm. **(c)** Western blot analysis of the autophagy-related protein p62 in T24/DDP and UMUC3R cells (actin was used as the loading control). $n = 3$, $^{**}p < 0.01$, $^{*}p < 0.05$, $^{\#}p > 0.05$ *vs.* the DDP group; $^{\blacktriangle\blacktriangle}p < 0.01$, $^{\blacktriangle}p < 0.05$ *vs.* the DDP+ VitD group.

T24/DDP and UMUC3R cells were explored. Western blot analysis showed that p62 expression after DDP + VitD treatment and DDP + VitD + pc-NC treatment was markedly higher than that after DDP treatment alone. DDP + VitD + pc-SIRT1 treatment significantly decreased p62 expression, which was significantly different from that after DDP + VitD treatment ($p < 0.05$, Figure 3c).

Effect of the SIRT1 Gene on Migration and Apoptosis in Cisplatin (DDP)- and VitD-treated T24/DDP and UMUC3R Cells

To evaluate whether VitD treatment reverses DDP resistance by a mechanism dependent on its interaction with the *SIRT1* gene, we performed Transwell, scratch test and flow cytometric analysis to assess the effect of the *SIRT1* gene on migration and apoptosis in DDP combined with VitD-treated T24/DDP and UMUC3R cells. The results indicated that DDP-treated T24/DDP and UMUC3R cells retained their invasion and migration ability (Figure 4a-b), and the amount of cell invasion and the distance of cell migration were maintained at a high level; however, DDP treatment in the presence of VitD treatment significantly decreased the number of invaded cells and reduced the migration distance compared with DDP treatment alone but enhancing *SIRT1* gene expression abolished the effect of VitD treatment. The amount of cell invasion and the distance of cell migration in the DDP + VitD + pc-SIRT1 group were markedly higher than those in the DDP + VitD group ($p < 0.01$). Moreover, T24/DDP and UMUC3R cells show obvious resistance to DDP, so the apoptosis rate of T24/DDP and UMUC3R cells treated with DDP was maintained at a low level. VitD treatment significantly induced T24/DDP and UMUC3R cell apoptosis, and DDP + VitD treatment significantly increased the cell apoptotic rate relative to that of the DDP alone group ($p < 0.01$). In contrast, treatment with DDP + VitD + pc-SIRT1 significantly decreased the cell apoptotic rate, which was significantly different from that of the DDP + VitD group ($p < 0.01$, Figure 4c). Additionally, the apoptotic morphology was viewed using electron microscopy. In the DDP + VitD+ pc-SIRT1 group and DDP alone group, T24/DDP and UMUC3R cells maintained the normal cell morphology; however, in the DDP + VitD group and DDP + VitD+ pc-NC group, cells showed the typical apoptotic morphology, including chromatin pycnosis, cytoplasm condensation and vacuoles, and appearance of apoptotic bodies (Figure 4d).

Discussion

ROS are powerful signalling molecules involved in cancer occurrence, progression and survival phenotypes by regulating certain redox-sensitive transcription factors, such as p53, HIF and Nrf-2[16,17]. Cancer cells demonstrate significantly enhanced ROS levels when compared with their normal counterparts, which is due to heightened metabolic activity and mitochondrial dysfunction, and escalated oxidative damage may contribute to genetic instability and malignant transformations, as well as tumour resistance to chemotherapy[18,19]. Others have reported beneficial anticancer effects of antioxidants; that is, prolonging gefitinib treatments is associated with cell cycle defects, mitochondrial dysfunction, enhanced ROS generation and epithelial-mesenchymal transition (EMT)[20]. Moreover, gefitinib combined with mTempo (a mitochondria-specific ROS scavenger) was sufficient to abolish EMT and restore mitochondrial functions[21]. Consistent with these results, Huang et al[22] showed a promoting effect of increased mitochondrial fission on ROS production in hepatocellular carcinoma cells. In this study, we found that the DDP-resistant cell Lines T24/DDP and UMUC3R increased cellular ROS levels, which may be closely related to the regulation of bladder cancer cell proliferation, survival, and drug resistance.

Autophagy can prevent the transformation of normal cells into cancerous cells but can also trigger drug resistance to chemotherapy; this opposite effect seems to be directly linked to autophagy levels and different intracellular or extracellular stressors, such as ROS generation[23-25]. Previous studies have shown that PARPi-induced autophagy is a potentially important source of adaptive drug resistance in ovarian cancer[26]. In addition, Ganguli et al[27] found that inhibition of autophagy by chloroquine enhanced the anticancer properties of artemisinin by promoting ROS-dependent apoptosis. Therefore, reprogramming cancer cells to produce cytotoxic autophagy, which in turn induces apoptosis of tumour cells, is an applicable strategy for tumour treatment. A previous study reported that the *SIRT1* gene regulates mitochondrial biogenesis and participates in the pathogenesis of tumours. SIRT1

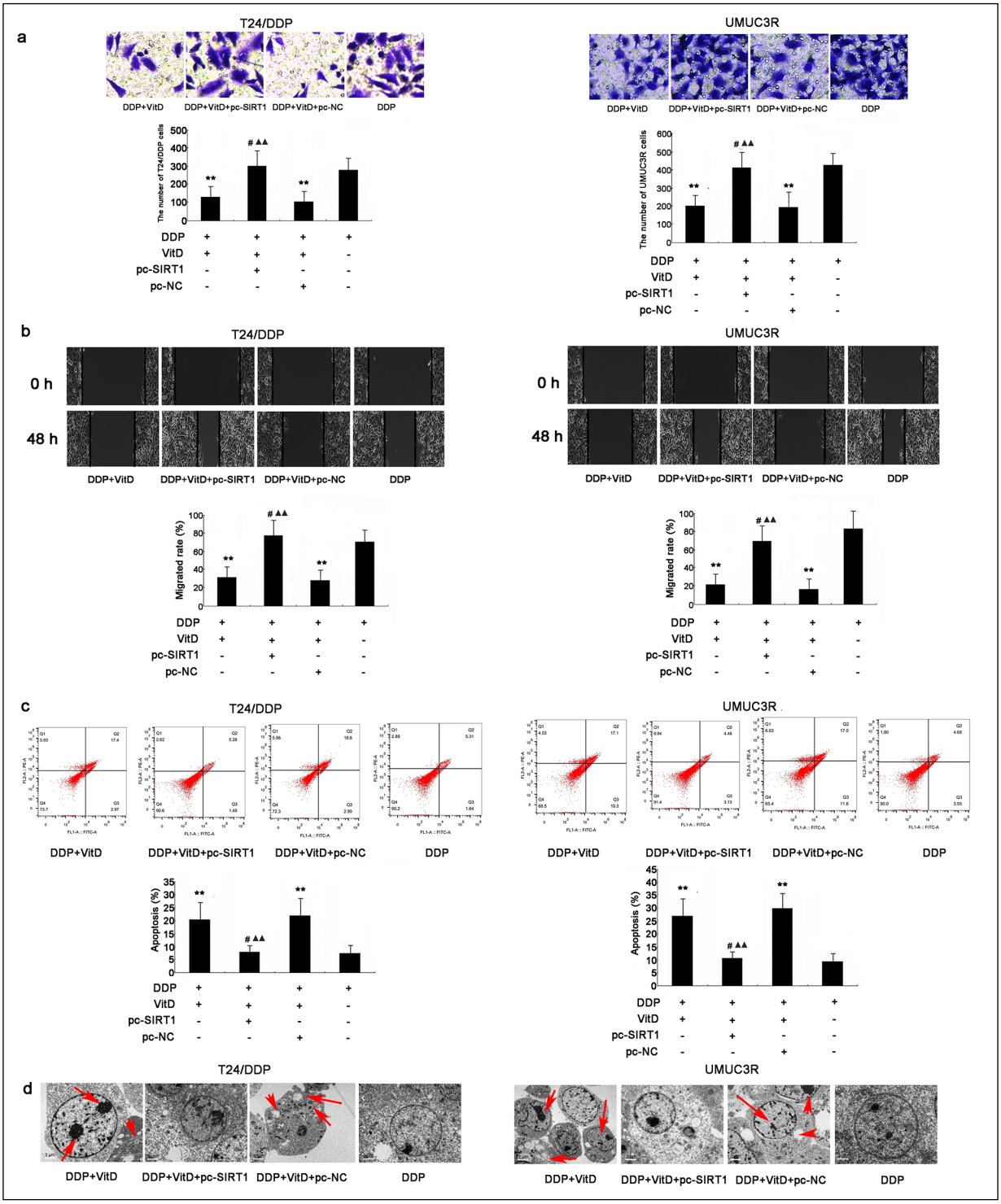

Figure 4. Effect of *SIRT1* gene on migration and apoptosis in Cisplatin (DDP) combined with VitD-treated T24/DDP and UMUC3R cells. T24/DDP and UMUC3R cells were separately exposed to 20 μM DDP in the absence or presence of VitD (100 nmol/L) for 24 h, and then transfected with pc-SIRT1 (2.5 μg/ml) or pc-NC (2.5 μg/ml) for another 24 h. **(a)** The invasion of T24/DDP and UMUC3R cells were measured using Transwell assay; **(b)** The migration of T24/DDP and UMUC3R was assessed using scratch healing analysis (200-fold magnification). **(c)** The apoptosis of T24/DDP and UMUC3R cells was detected by flow cytometric analysis. **(d)** The ultrastructural changes of T24/DDP and UMUC3R cells were observed by electron microscope (2500-fold magnification). The red arrow indicated that nuclear fragmentation and chromatin agglutination. Scale bar = 2 μm. $n = 3$, $^{**}p < 0.01$, $^{\#}p > 0.05$ *vs.* the DDP group; $^{\blacktriangle\blacktriangle}p < 0.01$ *vs.* the DDP + VitD group.

induces autophagy in gastric cancer through the PI3K/Akt/mTOR signalling pathways, which are associated with drug resistance[28]. We consistently found that treating T24 and UMUC3 bladder cancer cells overexpressing the *SIRT1* gene induced mitochondrial ROS accumulation, promoted the initiation of autophagosome formation and enhanced autophagic flux, suggesting that the *SIRT1* gene is involved in the occurrence and development of bladder cancer, which requires further investigation.

Epidemiological and experimental data show that low sunlight exposure and hypovitaminosis D are closely associated with the occurrence and progression of many cancers, such as lung, colon, breast, gastric and other cancers[29-32]. The reversal effect of VitD was also observed in everolimus-resistant hepatocellular carcinoma cells, and VitD directly regulated everolimus resistance by restoring the mesenchymal-like phenotype to the epithelial-like phenotype[33]. Similarly, the combined supplementation of VitD and epigallocatechin gallate (EGCG) seems to be an optimal approach for the treatment of myomas and correlated symptoms, showing synergistic effectiveness as a promising and novel treatment for myomas[34]. In the present study, we found that VitD was significantly downregulated in DDP-resistant bladder cancer patients. Furthermore, we verified that the synergistic effect of VitD and DDP had significant antitumor activity against T24/DDP and UMUC3R cells. Combined treatment with VitD and DDP inhibited mitochondrial ROS production and autophagy formation by regulating *SIRT1* gene expression.

Treatment with VitD reverts tamoxifen resistance by the Wnt/β-catenin signalling pathway in breast cancer cells, which is related to the upregulation of vitamin D receptor (VDR) protein expression[35]. The active form of VitD exerts its activity by binding to the intracellular VDR, and complex binding and interactions with target cell nuclei (at VDR elements) mediate transcriptional activation and repression of target genes[36]. Increasing scientific evidence supports the link between *VDR* gene polymorphisms and various types of cancer risk. The relevance of more *VDR* gene polymorphisms (including *FokI*, *BsmI*, *ApaI*, *TaqI* and *Cdx2*) for individual malignancies has been investigated, and *VDR* gene polymorphisms are considered a key factor for ethnic heterogeneity[37]. In the present experiment, the VDR polymorphism profile (TaqI/rs731236, BsmI/rs1544410, ApaI/rs7975232, FokI/rs2228570) was determined by using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method[38]. We found a significant difference in the allele distribution of the VDR polymorphism FokI/rs2228570 between human bladder cancer cell line (T24 and UMUC3) and DDP-resistant bladder cancer cell lines (T24/DDP and UMUC3R) (**Supplementary Figure 1**). Our findings suggested that the VDR polymorphism FokI/rs2228570 is associated with drug resistance. Further functional studies should be performed to elucidate the significance of the VDR gene polymorphism combined with VitD status assessment in the management of bladder cancer and the potential therapeutic implications.

Conclusions

Our data indicated that VitD treatment markedly repressed SIRT1 expression in T24 and UMUC3 bladder cancer cells and that overexpression of the *SIRT1* gene led to mitochondrial ROS generation, promoted the initiation of autophagosome formation and enhanced autophagic flux. DDP treatment in the presence of VitD inhibited cell invasion and migration and induced apoptosis and enhancing the *SIRT1* gene abolished the effect of VitD by regulating mitochondrial ROS accumulation and autophagosome formation. These data support a mechanism wherein the *SIRT1* gene plays a crucial role in VitD-mediated reversal of DDP resistance and may provide useful preventive and therapeutic applications in the future.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Ethics Approval

This study was performed in line with the principles of the Declaration of Helsinki. The research protocol was approved by the Medical Ethics Committee of Hongqi Hospital Affiliated to Mudanjiang Medical University (Data: 2017. 10. 06/No. 2017HQYY003-033).

Informed consent

Informed consent was obtained from all participants included in the study.

Availability of Data and Material

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Authors' Contribution

The conception and design of the study were performed by JS. The first draft of the manuscript was written by YCD. Material preparation, data collection and analysis were performed by ZHZ and YPS. All authors read and approved the final manuscript.

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