Abstract. 1,25-Dihydroxyvitamin D3 (1,25D3) is the active form of vitamin D with antineoplastic effects. The glutathione peroxidase-1 (GPX1) gene is associated with tumour progression. The present study aimed to explore the role of GPX1 in 1,25D3-mediated progression of salivary adenoid cystic carcinoma (SACC). Downregulating GPX1 expression inhibited SACC cell proliferation, chemoresistance, motility, and uPA secretion, but promoted apoptosis via the NF-κB pathway. Pre-processing 1,25D3 inhibited expression of NF-κB/GPX1/uPA, which subsequently suppressed cell motility and cisplatin-resistance in ACC-2 cells. In conclusion, 1,25D3 works as a modifier of NF-κB/GPX1/uPA expression, inhibiting cisplatin-resistance and cell invasive ability of SACC cells. The present study comprehensively elucidated the potential mechanism underlying the effects of vitamin D on chemoresistance and invasive potential in SACC.

Introduction

Epidemiological studies have indicated that salivary adenoid cystic carcinoma (SACC) is one of the most common types of salivary gland cancers in China, accounting for 11% of epithelial tumours and 27% of malignant tumours (1). Clinical data have shown that the low long-term survival rate of SACC is associated with perineural invasion, local recurrence and distant metastasis (2). Chemotherapy is a necessary adjuvant to surgery; however, ~30% of new cases and 70% of recurrent cases have platinum-based chemoresistance (3). Thus, there is a growing interest in determining the mechanistic basis for SACC chemoresistance and invasive ability to identify potential therapeutic targets.

GPX1 belongs to the glutathione peroxidase (GPxs) family, the members of which protect the cell membrane from oxidative DNA damage and maintain the body's balance of intracellular redox systems by removing excess reactive oxygen species (ROS) (4). GPX1 modulates many pathophysiologic processes, and overexpression of GPX1 can promote cell invasion, migration and cisplatin resistance in breast, lung, bladder and prostate cancers (5-7). Nuclear factor-kappa B (NF-κB) may regulate GPX1 transcription and expression by combining with the GPX1 promoter region (8-10). However, few studies have reported the tumour-promoting role of GPX1 in head and neck cancers.

1,25-Dihydroxyvitamin (1,25D3) is the active form of vitamin D, which acts as the steroid hormone calcitriol and carries out multiple cellular functions, and 1,25D3 regulates...
numerous cellular pathways related to cancer risk and prognosis (11). Clinical studies have suggested that vitamin D deficiency increases the risk of developing cancer and that abundant vitamin D can reduce cancer incidence and improve cancer prognosis and outcome (12,13). Furthermore, it was reported that 1,25D3 could inhibit NF-κB expression in B lymphocytes, oral squamous cell carcinoma, prostate cancer and melanoma (14-17).

In the present study, we first investigated the biological effects of GPX1 on SACC cell lines. Next, we detected whether the NF-κB pathway was involved in these effects. Finally, we explored whether 1,25D3 alleviates SACC progression by suppressing GPX1 expression through the NF-κB signalling pathway.

Materials and methods

The present research was conducted in accordance with the Declaration of Helsinki and the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United National Institutes of Health. It has also been approved by the authors' institutional review board.

Cell lines and cell cultures. SACC cell lines (ACC-M, SACC-83 and ACC-2) were purchased from the Cell Bank of the Experimental Animal Center (Sun Yat-Sen University, Guangzhou, China). The cells were cultured in RPMI-1640 medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) in a 37°C humidified incubator containing 5% CO2.

Reagents and chemicals. The vitamin D metabolite 1,25D3 (Sigma, St. Louis, MO, USA) was dissolved at a concentration of 400 µM in anhydrous alcohol (AA) for preservation. Immediately prior to use, the stock was diluted to a final concentration of 30 nM in culture medium. An NF-κB inhibitor (BAY 11-7082) was purchased from Beyotime Institute of Biotechnology (Shanghai, China).

Small interfering RNA (siRNA) and overexpression vector. The GPX1 overexpression vector, the GPX1 antisense, and the scrambled siRNA were designed and synthesized by Invitrogen (Carlsbad, CA, USA). The GPX1 siRNA sequences used are listed in Table I. Full-length GPX1 coding sequences were PCR-amplified and cloned into a pcDNA3.1 expression vector (Invitrogen) according to the manufacturer's guidelines. DNA sequencing was used to verify the constructs. siRNA transfection was performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Subsequent real-time polymerase chain reaction or western blot analysis was performed to verify changes in GPX1 expression. In the present study, the experimental group was transfected with the antisense GPX1 siRNA or GPX1 overexpression vector, whereas the control group was transfected with a corresponding scrambled sequence.

RNA extraction and PCR. Total RNA was extracted using TRIzol™ reagent (Sigma-Aldrich, Arklow, Ireland) according to the manufacturer's instructions and was then reverse-transcribed into cDNA using a PrimeScript RT reagent kit (Takara Bio, Inc., Shiga, Japan). The newly synthesized cDNA was then used as a template for detection. Quantitative PCR was carried out using the SYBR-Green method (Takara Bio) in a CFX96 Real-Time PCR Detection system (Bio-Rad Laboratories, Hercules, CA, USA). The primers (Table II) were purchased from Takara Bio, and GAPDH was used as an endogenous control. Amplification was performed according to the manufacturer’s protocol (Takara Bio).

Protein extraction and western blot analysis. For protein extraction, cells were washed twice with cold phosphate-buffered saline (PBS), harvested by scraping and lysed in lysis buffer (Beyotime Institute of Biotechnology). Following centrifugation, the supernatant was collected, and protein concentration was determined using a BCA protein assay kit (Pierce™, no. 23227).

For western blotting, 20 µl of protein was loaded and separated using 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Beyotime Institute of Biotechnology); the protein bands were then transferred to Immobilon-P transfer membranes (PVDF) (Beyotime Institute of Biotechnology). The membranes were blocked with 5%...
non-fat milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 for 1 h at room temperature. The blots were probed using antibodies against GPX1 (1:2,000; Abcam), NF-κB P65 (P65, 1:1,000; Cell Signaling Technology, Danvers, MA, USA), phospho-NF-κB P65 (P-p65, 1:1,000; Cell Signaling Technology), urokinase (uPA, 1:1,000; GeneTex, Inc., Irvine, CA, USA) and MMP-2 (1:1,000; GeneTex). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:2000; Cell Signaling Technology) was used as a loading control. After incubation with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G secondary antibody, an enhanced chemiluminescence detection method (Pierce ECL western blotting Substrate; Thermal Form & Function, Beverly, MA, USA) was used to visualize the proteins on the blots.

**Cell proliferation assay.** Cells were plated at a density of 5x10^3 cells/well in 96-well plates, incubated overnight and counted using Cell Counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). The medium in each well was removed, and a mixture of 10 µl CCK-8 and 90 µl RPMI-1640 medium was added. The plates were incubated for an additional 2.5 h, and absorbance was measured at 450 nm using a microplate spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA, USA).

For cisplatin sensitivity testing, cells (7.5x10^3) were seeded into 96-well plates. After overnight incubation, the cells were treated with various concentrations of cisplatin (0, 2.5, 5, 10, 20 and 40 µmol/l), and a CCK-8 assay was performed to examine the cytotoxicity of cisplatin after 48 h of treatment.

**Flow cytometry apoptosis assay.** After 24 h of transfection, cells were plated in 6-well plates and incubated with 7.5 µmol/l cisplatin for 48 h at 37°C. The cells were collected and washed with PBS, and cell apoptosis was analysed by Annexin V/fluorescein isothiocyanate and propidium iodide staining (Nanjing Keygen Biotech., Co., Ltd., Nanjing, China) using a BD FACSCalibur flow cytometer.

**In vitro migration and invasion assays.** To assay invasion, 3x10^3 transfected cells were seeded into the upper chamber of a polycarbonate Transwell plate (8-mm pore size; Corning Incorporated, Corning, NY, USA) that was pre-coated with Matrigel (Becton-Dickinson, Bedford, MA, USA). RPMI-1640 containing 20% FBS was used as a chemoattractant and was added to the lower chamber. After a 24-h incubation at 37°C, the cells on the upper surface of the filter were removed. Cells that had traversed the membrane were fixed in methanol at 4°C for 20 min and then stained with 0.1% crystal violet for 20 min. To microscopically quantify cell migration, the cells on the upper surface of the filter were removed. Cells were counted in 3 random fields (magnification, x200). To assay migration, a method similar to that used in the invasion assay was employed, with the following modifications: cells were seeded at 1.0x10^3/chamber into plates with no Matrigel coating and incubated in 600 µl RPMI-1640 medium with 10% FBS in the lower chamber for 18 h.

**ELISA assay for uPA and MMP-2.** The supernatant of the culture medium of the ACC-2 cells was collected and centrifuged at 1,000 x g for 20 min at room temperature. The concentrations of uPA and MMP-2 in the supernatant were quantified using an uPA ELISA kit (Cloud-Clone Corp., Houston, TX, USA) and an MMP-2 ELISA kit (Shanghai ExCell, Biology, Inc., Shanghai, China) according to the recommended protocols. The detection limits of the assays were 15.6 pg/ml (uPA) and 0.6 ng/ml (MMP-2).

**In vivo tumorigenicity assay.** To explore the effects of 1,25D3 on tumour growth in vivo, ACC-2 cells were treated for 3 days with 30 nM 1,25D3, AA and culture medium in the experimental group, control group and blank group, respectively. Next, 2.0x10^6 ACC-2 cells were subcutaneously implanted into the right upper back of 5 BALB/c nude mice, and the mice were reared for 24 days. The tumours were measured every 3 to 4 days for tumour volume (mm^3), which equaled length x width^2 x 0.5. All mice were subsequently sacrificed, and pieces of tumour tissues were used to establish orthotopic implant models. When tumour volume reached ~50 mm^3, 30 nM 1,25D3, AA and PBS were mixed with 5 µM cisplatin and then intraperitoneally injected into the three groups. Tumour formation was observed and tumour growth curves were constructed. The mice were sacrificed 24 days after the injection of the ACC-2 cells, and the tumours were harvested, weighed and frozen or paraffin-embedded. Finally, tissue protein was extracted for analysis.

**Immunohistochemistry.** Immunohistochemistry was performed on paraffin-embedded tissue sections from tumorigenicity assays. The indicated antibodies (GPX1, 1:50; P65, 1:100; uPA, 1:50; MMP-2, 1:50; and Ki-67, 1:100; GeneTex) were used according to the EnVision HRP detection system (Dako, Carpinteria, CA, USA). After deparaffinization, antigen retrieval was conducted using 10 mM sodium citrate buffer (pH 8.0) in a pressure cooker at full power for 5 min. Tissue sections were then treated with 3% hydrogen peroxide for 10 min. The primary antibodies were diluted with a background-reducing diluent (Dako) according to the manufacturer's specifications and were incubated at 4°C overnight. Next, slides were incubated with the EnVision reagent for 30 min at 37°C. The slides were then developed with 3,3'-diaminobenzidine for 3 min, counterstained with Meyer's haematoxylin and mounted. The samples were rinsed with phosphate-buffered saline (PBS) between each step.

**Statistical analyses.** Statistical analyses were performed with SPSS 22.0 software (IBM Inc., Armonk, NY, USA). P-values <0.05 were considered to indicate a statistically significant result. Images were created with the Adobe Photoshop CS5 and the GraphPad Prism 5.

**Results.**

Overexpression of GPX1 promotes cell proliferation, invasion, migration and cisplatin resistance and increases apoptosis in SACC cells. We transfected siGPX1 into 3 SACC cell lines; ACC-2 cells showed relatively effective silence GPX1, with an 80% GPX1 reduction compared with other cells (Fig. 1A and G). Therefore, ACC-2 cells were selected for further experimentation. When we further transfected cells with GPX1 vector and negative control (NC) sequences, GPX1 increased by 40%. Twenty-four hours after transfection, we performed
CCK-8 assays to detect the effect of GPX1 on ACC-2 cell proliferation at three time-points (18, 36 and 54 h). Cell proliferative capacity was reduced in the siGPX1 group (Fig. 1C). As for cisplatin resistance, as the expression of GPX1 decreased, the cells displayed the same trend, particularly at concentrations of 5 and 10 µM (Fig. 1E). Next, we selected a 5 µM concentration of cisplatin for use in a flow cytometry apoptosis assay and found that the apoptosis rate in the siGPX1 cells (19.50%) was higher than that in the control (9.74%) (Fig. 1B). A Transwell assay was performed; as shown in Fig. 1D, siGPX1 cells displayed weak invasive and migratory abilities. In contrast, upregulation of GPX1 promoted proliferation (Fig. 2A), cisplatin resistance (Fig. 2C), invasion and migration (Fig. 2D) but decreased apoptosis (Fig. 2B) in ACC-2 cells. Taken together, these results indicated that GPX1 overexpression in ACC-2 cells could be responsible for enhanced cell proliferation, cisplatin resistance, invasion and migration.

GPX1-enhanced invasion and migration is associated with uPA. We speculated that the contribution of GPX1 to SACC motility might involve the downstream factors MMP-2 or uPA (18,19). At 24 h after transfection, the expression and secretion of uPA and MMP-2 were tested (Figs. 1F and G, and 2E and F). The results showed that uPA secretion was dramatically reduced, but MMP-2 remained stable when GPX1 was reduced. Meanwhile, uPA secretion increased when cells were...
transfected with the GPX1 overexpression vector. Based on these results, we considered that GPX1-enhanced invasion and metastasis in ACC-2 cells is associated with uPA.

The NF-κB pathway is related to GPX1-mediated SACC biological effects. To clarify the mechanism by which GPX1 exerts its effects, we investigated the role of the NF-κB pathway (20). Twenty-four hours after treatment with BAY 11-7082, western blotting showed that NF-κB (P65) expression was significantly downregulated (Fig. 3E). Cell proliferation (Fig. 3A), cisplatin resistance (Fig. 3B), and invasive and migratory ability (Fig. 3D) were all reduced; correspondingly, apoptosis increased when the concentration of cisplatin reached 5 µM (Fig. 3C). GPX1 expression and uPA expression were downregulated, but MMP-2 expression was sustained (Fig. 3E). Next, we performed ELISA to detect uPA and MMP-2 secretion. The results showed that uPA secretion was reduced, but MMP-2 showed no change (Fig. 3F). These findings suggested that the NF-κB pathway was related to proliferation, cisplatin resistance, invasion, migration and apoptosis in SACC via positively regulating GPX1 expression and uPA activation.

1,25D3 regulates SACC cell biological effects through the NF-κB pathway. We further investigated the biological role of 1,25D3 in SACC. After preprocessing with 1,25D3 for 3 days, the proliferative capacity (Fig. 4A) and cisplatin resistance (Fig. 4C) of ACC-2 cells were reduced. As for their invasive and migratory capacities, cells treated with 1,25D3 displayed weaker motility compared to controls (Fig. 4D). Cell apoptosis assays showed no significant difference (Fig. 4B) after 1,25D3 treatment. These results illustrated that 1,25D3 was able to reduce cell proliferation, cisplatin resistance and motility in ACC-2 cells.

We next examined if the reduced biological function of 1,25D3-treated ACC-2s was related to activation of NF-κB signalling and subsequent downregulation of GPX1 expression. To test this hypothesis, we examined NF-κB and GPX1 expression in ACC-2 cells after 1,25D3 treatment. As anticipated, NF-κB, GPX1 and uPA expression was inhibited...
Moreover, ELISA results showed that uPA secretion was reduced in 1,25D3-treated ACC-2 cells, whereas MMP-2 showed no change (Fig. 4F). Taken together, these results indicate that 1,25D3 may inhibit GPX1 expression to regulate biological functions in SACC cells through the NF-κB pathway.

In vivo, 1,25D3 alleviates SACC progression by inhibiting GPX1 expression through the NF-κB pathway. To further explore the potential function and mechanism of 1,25D3 in vivo, we performed tumorigenicity assays. On day 24, the mean tumour weight of the experimental group was lower than those of the two control groups (Fig. 5A). According to the tumour growth curves (Fig. 5B), obvious tumour nodules formed in the control and blank groups on day 4 after injection with ACC-2 cells, whereas in the experimental group this occurred on day 5. The surfaces of the tumours in the experimental group began to visibly fester on day 18 after injection with ACC-2 cells, and the tumours stopped growing. In contrast, tumours began to visibly fester on day 20 in the control groups, and the tumours continued to grow until day 24. H&E staining of the tumours revealed that the experimental group had more significant necrotic areas than the control groups (Fig. 5D). Furthermore, the expression of GPX1, P65, P-P65 and uPA in the experimental group was lower than that in the control and blank groups (Fig. 5C). Additionally, immunohistochemical analysis of the inoculated tumours revealed that the tumours of the experimental group expressed significantly lower levels of Ki-67, uPA and P65, with no change in MMP-2 expression and increased GPX1 expression (exclusive cytoplasmic expression; no nuclear expression) (Fig. 5E).

**Discussion**

SACC is a malignant tumour arising from secretory epithelial cells in the salivary glands of the head and neck. The 5-year
Disease-free survival rate is ≤90%; however, the survival rate is reduced to 40% after 15 years (2). SACC has a poor prognosis, primarily owing to its insidious invasion into adjacent tissues and haematogenous spread to distant organs (lungs, bone and liver) (21-23). Therefore, it is necessary to identify and understand the mechanism behind SACC chemoresistance and metastasis to improve treatment strategies for SACC patients.

Recent studies have found that upregulation of GPx expression leads cancer cells to develop drug resistance by reducing ROS produced by platinum-based chemotherapy drugs, which promotes tumourigenesis (24,25). In the present study, we found that GPX1 acts as a key factor in regulating SACC progression, as GPX1 suppression reduced ACC-2 cell proliferation, cisplatin resistance and motility while increasing cell apoptosis. Upregulation of GPX1 shows the corresponding opposite effect. It has been reported that the generation of MMP-2 and uPA promotes tumour invasion and metastasis in squamous cell carcinoma of the head and neck (18,19). Following GPX1 downregulation, we detected a reduction of uPA expression and secretion in ACC-2 cells, whereas MMP-2 remained stable. In contrast, uPA secretion and activation increased when GPX1 was overexpressed.

In malignant tumours, NF-κB expression can regulate downstream protein expression, thereby regulating tumour chemoresistance and invasion (8,26-28). In the present study inhibiting the NF-κB pathway reduced ACC-2 cell growth, cisplatin resistance, and motility, while promoting apoptosis. To further analyse the role of NF-κB-regulated factors, the expression of GPX1 was decreased, and uPA secretion and expression showed the same trend. Thus, we established a correlation between NF-κB and GPX1 in ACC-2 cells and

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**Figure 4.** 1,25VD3 regulates ACC progression by suppressing GPX1 expression through the NF-κB pathway in vitro. After treatment with 1,25VD3, (A) ACC-2 cell proliferative capacity and (C) cisplatin resistance were reduced in comparison with the control groups, whereas no significant difference in cell apoptosis (B). 1,25VD3 reduced cell invasion and migration of ACC-2 cells (D, magnification, x200), nor the expressions of GPX1 and NF-κB (P65) (E). ELISA also showed that 1,25VD3 reduces the secretion and activation of uPA (F). *P<0.05 compared with the ACC-2 anhydrous alcohol (AA) group.
confirmed that GPX1-induced uPA secretion is mediated by an NF-κB-dependent pathway, which modulates SACC chemoresistance and invasion.

Vitamin D has widespread actions throughout the human body, and supplementation may be a strategy for preventing cancer incidence and/or tumour progression (29,30). Mechanistically it has been suggested that vitamin D regulates a wide range of factors, including interleukin-8, NF-κB, HBp17 and miR98, which subsequently inhibit tumour development (12,14-17,31,32). As expected, we found that the active metabolite of vitamin D, 1,25D3, inhibited ACC-2 cell proliferation, cisplatin resistance, invasion and migration and alleviated SACC progression in vivo. We analysed the mechanism by which 1,25D3 reduced the extent of SACC malignancy. After treatment with 1,25D3, NF-κB and GPX1 activation were responsively decreased in ACC-2 cells, and the secretion and expression of uPA were also reduced. Tumorigenicity assays confirmed that pre-applied 1,25D3 could inhibit the progres-
sion of SACC tumours; subsequent immunohistochemical examination also detected signal alterations among NF-κB, GPX1 and uPA. Our combined in vitro and in vivo experiments revealed that GPX1 is a target of 1,25D3, which alleviates SACC progression by suppressing GPX1 expression through the NF-κB pathway. This provides a theoretical basis for vitamin D supplementation in cancer management. Avoiding vitamin D deficiency and adding supplements may be an economical and effective way to reduce cancer incidence and improve cancer prognosis.

In conclusion, in the present study, we demonstrated that downregulation of GPX1 can suppress SACC cell proliferation, cisplatin-resistance, migration, and invasion and promote apoptosis through the NF-κB pathway and uPA activation. 1,25D3 achieved its antineoplastic function via the above-mentioned regulators. Collectively, establishing 1,25D3 as a modifier of NF-κB/GPX1/uPA expression provides a novel therapeutic strategy for the treatment of SACC.

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