1,25-Dihydroxyvitamin D_3 enhances the susceptibility of anaplastic thyroid cancer cells to adriamycin-induced apoptosis by increasing the generation of reactive oxygen species

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Abstract. Anaplastic thyroid cancer (ATC) is a very aggressive malignancy that is resistant to various types of chemotherapy in humans. Most patients with late-stage ATC cannot undergo surgery and receive chemotherapy drugs. The present study investigated the influence of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) pretreatment on adriamycin (ADM) chemotherapy efficacy in the 8305c and 8505c ATC cell lines. The apoptotic effects of ADM on ATC cells pretreated with $1,25(OH)_2D_3$ were evaluated. Cell viability was identified by using the Cell Counting Kit-8 assay. Apoptosis was assessed by flow cytometry and staining with Hoechst 33342. The expression of the apoptotic protein cleaved caspase-3 was tested with a colorimetric assay kit and by western blotting. Reactive oxygen species (ROS) generation was assessed with the antioxidant N-acetyl-L-cysteine (NAC) and the assay H_2-DCFDA. In addition, ROS production could be reversed by NAC treatment. The present study demonstrated that $1,25(OH)_2D_3$ enhanced ADM-induced apoptosis in 8305c and 8505c cell lines. Furthermore, $1,25(OH)_2D_3$ improved the ADM-induced ROS production and expression of cleaved caspase-3. NAC treatment inhibited the expression of cleaved caspase-3 in ATC cells, and reduced apoptosis in cells that were pretreated with $1,25(OH)_2D_3$ and ADM. These results demonstrated that $1,25(OH)_2D_3$ may enhance ADM-induced apoptosis by increasing ROS generation in ATC cells.

Introduction

Anaplastic thyroid cancer (ATC) accounts for 2% of all thyroid cancers in the worldwide (1). ATC is a very aggressive malignancy in humans; it is resistant to numerous chemotherapy treatments and is associated with a high prevalence of mortality (2). The prognosis of ATC is poor. The majority of patients succumb within 5 months following the diagnosis; <20% survive for >1 year (3). Most patients with ATC are diagnosed at an advanced stage and can develop distant metastasis as ATC progresses (4). There is a lack of standard therapy for ATC (5). A multimodal approach based on extensive resection followed by adjuvant chemotherapy and radiotherapy may improve the prognosis (6). However, numerous patients with late-stage ATC cannot undergo surgery and receive therefore chemotherapy drugs only (7,8).

Adriamycin (ADM) is the main chemotherapy drug used to treat ATC. The guidelines set by the American Thyroid Association and National Comprehensive Cancer Network state that ADM should be considered a first-line anticancer drug in ATC treatment (3,9). However, ADM causes several serious adverse drug reactions, including cardiotoxicity, myelosuppression and digestive tract injury, which depend on the dose (10). Increasing attention has therefore been applied to the development of novel synergistic formulations in order to increase the specific anti-tumor efficacy and to reduce ADM toxicity.

The role of Reactive oxygen species (ROS) in apoptosis induction has been described for a diverse collection of xenobiotics. ROS plays an important role in apoptosis induced by chemo drug, 1,25(OH)2D3 can modulate ROS production. ROS production is believed to be the major mechanism underlying its cytotoxicity to cancer cells in most major organs, including thyroid (11). Increased levels of ROS may activate the mitochondrial cascade of apoptosis. The role of oxidative stress is ambivalent in cancer. The proper amount of reactive oxygen species in normal cells can protect cells from damage. However, the excessive generation of ROS in the drug treatment may result in cell damage via apoptosis, necrosis and autophagy. It has been demonstrated that 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) increases the anticancer effects of numerous chemotherapy drugs in synergistic or additive manners, including ADM, cisplatin, paclitaxel and docetaxel, in various types of malignant somatic cell *in vitro* and *in vivo* (12,13). In addition, $1,25(OH)_2D_3$ can intensify the apoptotic effect of chemotherapy drugs, including ADM, the dose of which can be reduced when combined with $1,25(OH)_2D_3$ (14,15). However, whether $1,25(OH)_2D_3$ can

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promote the ADM-induced apoptosis of ATC cells remains known.

The present study aimed to investigate the apoptotic effects of $1,25(OH)_2D_3$ pretreatment upon ADM treatment in ATC cells *in vitro*. In addition, the molecular mechanism of action of this drug combination was examined as a novel therapeutic strategy.

Materials and methods

Cell lines and cultures. The ATC cell lines 8305c and 8505c were obtained from the Department of Endocrinology and Metabolism, Institute of Endocrinology, Liaoning Provincial Key Laboratory of Endocrine Diseases from the First Affiliated Hospital of China Medical University. Cells were cultured in minimum essential medium (MEM; HyClone; GE Healthcare Life Sciences) supplemented with 10% fetal bovine serum (PAN-Biotech GmbH) and placed at 37˚C in a humidified incubator containing 5% CO_2 (Thermo Fisher Scientific, Inc.).

Cell viability assessment. Cell viability was assessed using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.). ATC cells were transferred to 96-well plates ($2x10^3$ cells/well) and incubated with $1,25(OH)_2D_3$ (Selleck Chemicals; dissolved in DMSO) at final concentrations of 0, 1, 10 and 100 nM at 37˚C for 48 h.

Subsequently, the cells were washed with PBS for twice and incubated in fresh MEM containing 100 ng/ml ADM (Selleck Chemicals; dissolved in sterile distilled water) for 24 h. In order to assess cell viability,10 μl CCK-8 solution was added into the medium and the ATC cells were incubated for another 2.5 h at 37˚C before the absorbance at 450 nm was measured with a microplate reader.

Apoptosis assessment. Cells were seeded in 6-well plates ($1x10^5$ cells/well). Following overnight incubation, cells were treated with $1,25(OH)_2D_3$ at final concentrations of 0 or 10 nM for 48 h at 37˚C. Treatment was then removed and replaced by a solution of ADM (100 ng/ml) dissolved in MEM for 24 h at 37˚C. Subsequently, cells were suspended in PBS and stained by 5 μl Annexin V-FITC and 5 μl Propidium Iodide (PI; BD Biosciences) in the dark for 15 min at 25˚C. Positively stained cells were detected by flow cytometry (BD Biosciences) within 1 h. Compensation and data analysis were conducted using FlowJo 7.6.1 (FlowJo LLC).

Nuclear morphology was evaluated by staining the cells with Hoechst 33342 (Beyotime Institute of Biotechnology). Briefly, cells were seeded in 6-well plates ($1x10^5$ cells/well), treated with the appropriate drugs as aforementioned, were stained with Hoechst 33342 at room temperature for 5 min and immediately imaged with a fluorescence microscope (magnification, x1,000; Olympus Corporation). Cells with changes in nuclear morphology, including condensation and fragmentation of chromatin, were considered to be apoptotic. Data were presented as the percentage of apoptotic cells.

Caspase-3 activity assessment by ELISA. Caspase-3 activity was determined using a Caspase-3 ELISA kit (c1115; Beyotime Institute of Biotechnology) according to the manufacturer's instructions. To evaluate caspase-3 activity, cells were treated with the aforementioned drugs, and cell lysates were prepared and homogenized in 100 ml fluorescent substrate Ac-DEVD-pNA (Beyotime Institute of Biotechnology) containing 10 μl caspase-3 substrate (2 mM) and incubated at 37˚C overnight. Caspase-3 activity was detected with an enzyme-linked immunosorbent assay at 405 nm using a microplate reader. Caspase-3 activity was expressed as the percentage of enzyme activity compared with control.

Measurement of intracellular reactive oxygen species (ROS). ATC cells were seeded into 96-well flat-bottom plates, cultured with MEM containing 10% fetal bovine serum and $1,25(OH)_2D_3$ (10 nM) and placed for 48 h at 37˚C in a humidified incubator containing 5% CO_2. ADM (100 ng/ml) was added to the wells, and the ROS level in each well was measured by the cell-permeant probe H2-DCFDA (Beyotime Institute of Biotechnology) at 2 h after adding ADM. After incubation with 10 μM H2-DCFDA at 37˚C for 30 min, fluorescence intensity was measured using a fluorescence microplate reader (excitation wavelength, 488 nm; emission wavelength, 525 nm). Data were presented as the mean of three independent experiments.

Western blotting. ATC cells were treated with $1,25(OH)_2D_3$ (10 nM) for 48 h and with ADM (100 ng/ml) for 24 h as aforementioned. Cells were then washed twice in PBS. To determine protein expression by western blotting, cells were harvested and lysed in RIPA buffer (Beyotime Institute of Biotechnology) containing 1 mM phenylmethylsulfonyl fluoride (Nanjing KeyGen Biotech Co., Ltd.) on ice for 1 h. Cell lysate was mixed with 5X loading buffer (1:5; CW Biotech) and immediately boiled for 10 min. Protein concentrations were quantified by a BCA assay kit (Beyotime Institute of Biotechnology). Proteins (10 μg) were separated on 12% SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc.) and blocked in 5% skimmed milk for 2 h at room temperature. Membranes were incubated with antibodies against caspase-3 (1:1,000 dilution; 9962, Cell Signaling Technology, Inc.), cleaved caspase-3 (1:1,000 dilution; 9964, Cell Signaling Technology, Inc.) and β-actin (1:1,000; 4790, Cell Signaling Technologies, Inc.) at 4˚C overnight. Then, the membranes were incubated with horseradish peroxidase (HRP) labelled secondary antibody (1:10,000 dilution; ZB-2301, OriGene Technologies, Inc.) for another 45 min at 37˚C. Proteins were visualized using an enhanced chemiluminescence system (Thermo Fisher Scientific, Inc.). Bands intensity was normalized to expression of the internal control β-actin.

N-acetyl-L-cysteine (NAC) treatment. The antioxidant NAC (Beyotime Institute of Biotechnology) was dissolved in PBS to produce a 1 mM stock solution. This solution was used to decrease ROS production. Cell viability, caspase 3 activity and expression, intracellular ROS level measurement and apoptosis were measured in cell cultures that were treated in the presence or absence of 1 mM NAC for 24 h.

Statistical analyses. Statistical analyses were conducted using SPSS v21.0 (IBM Corp.) and Prism v5.0 (GraphPad Software, Inc.). Data are the means ± standard error of the mean. Each experiment was repeated independently three

times. Differences between the groups were analyzed using the Student's t-test. The data from three or more groups were analyzed by one-way analysis of variance followed by Bonferroni's multiple comparison test. P<0.05 was considered to indicate a statistically significant difference.

Results

$1,25(OH)_2D_3$ enhances ADM-induced cell toxicity in ATC cell lines. $1,25(OH)_2D_3$ treatment alone did not reduce ATC cell viability compared with control; however, ADM significantly inhibited ATC cell viability (Fig. 1A and B). In addition, ATC cell viability was significantly reduced by various concentrations of $1,25(OH)_2D_3$ followed by ADM treatment (Fig. 1A and B) in a dose-dependent manner. Notably, pretreatment with 10 nM 1,25(OH)2D_3 followed by treatment with 100 ng/ml ADM significantly reduced ATC cell viability compared with cells treated with ADM alone (P<0.05). A greater effect was achieved with 100 ng/ml ADM combined with 100 nM $1,25(OH)_2D_3$ (P<0.05). In addition, 8505c was more sensitive than 8305c to the treatments. These results suggested that $1,25(OH)_2D_3$ increased the potent toxic effect of ADM on ATC cells.

$1,25(OH)_2D_3$ increases ADM-induced apoptosis. Apoptosis was evaluated to determine the mechanism underlying ATC cell viability inhibition. The results demonstrated that $1,25(OH)_2D_3$ did not induce ATC cell apoptosis (Fig. S1). However, ADM significantly increased ATC cell apoptosis (Fig. 2A). No increase in apoptosis was observed in cells incubated with 1 nM $1,25(OH)_2D_3$ followed by ADM compared with ADM alone (data not shown); however, apoptosis was significantly increased in cells pretreated with 10 nM $1,25(OH)_2D_3$ followed by ADM. Morphologic changes were detected by staining cells with Hoechst 33342. Cells incubated with ADM displayed apoptotic nuclei with DNA fragmentation, chromatin condensation and apoptotic body formation. Cells incubated with 1,25(OH)2D3 followed by ADM (P<0.05; Fig. 2B) presented a higher number of apoptotic nuclei.

$1,25(OH)_2D_3$ increases ADM-induced caspase-3 activation. $1,25(OH)_2D_3$ treatment did not increase caspase-3 activation in ATC cells; however, ADM significantly increased caspase-3 activation compared with the control. In addition, caspase-3 activity was significantly increased following $1,25(OH)_2D_3$ treatment combined with ADM (P<0.05; Fig. 3A). These results indicated that ADM induced ATC cell apoptosis through caspase-3 activation. In addition, $1,25(OH)_2D_3$ pretreatment enhanced ADM-induced caspase-3 activation.

$1,25(OH)_2D_3$ increases ADM-induced ROS production. To determine whether the apoptotic effect of $1,25(OH)_2D_3$ combined ADM was caused by an increase in ROS production, the impact of ADM alone or $1,25(OH)_2D_3$ plus ADM on intracellular ROS generation was measured. The results demonstrated that $1,25(OH)_2D_3$ did not increase intracellular ROS production in ATC cells. However, ADM significantly increased ROS generation compared with the control. In addition, $1,25(OH)_2D_3$ (10 nM) followed by ADM significantly increased intracellular ROS generation (P<0.05; Fig. 3B).

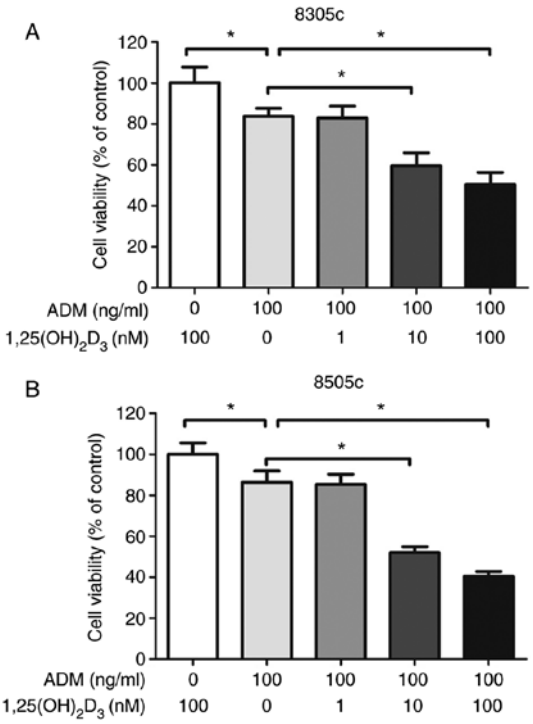

Figure 1. Effects of ADM of ATC cell viability are increased following $1,25(OH)_2D_3$ pretreatment. $1,25(OH)_2D_3$ enhanced ADM-induced viability inhibition of (A) 8305c and (B) 8505c ATC cell lines. Cells were incubated with ADM (100 ng/ml) for 24 h with or without various concentrations of $1,25(OH)_2D_3$ for 48 h prior to assessing cell viability using a Cell Counting Kit-8 assay. Results are the means ± standard error of the mean of three independent experiments. *P<0.05. $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; ADM, Adriamycin; ATC, anaplastic thyroid cancer. Cells were treated with 100 ng/ml ADM or 10 nM of $1,25(OH)_2D_3$ followed by ADM. (A) Flow cytometric analyses of cell apoptosis in anaplastic thyroid cancer cell lines. Annexin V-FITC/PI double-staining was used to label cells. (B) Morphologic assessment of apoptosis detected by Hoechst 33342 under fluorescence microscopy. Following staining, cells presenting nuclear fragmentation were counted as apoptotic cells (white arrow). Magnification, x1,000. Scale bar, 10 μm. Results are the means ± standard error of the mean of three independent experiments. *P<0.05. $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; ADM, adriamycin; PI, propidium iodide.

NAC attenuates the increased ROS generation and apoptosis induced by $1,25(OH)_2D_3$ plus ADM. To establish the association between the increased oxidative stress and the apoptosis induced by the combined drugs, the antioxidant NAC was used as an intracellular ROS scavenger. Cell viability was examined via a CCK-8 assay (Fig. 4A) and apoptosis was detected by flow cytometry and western blotting. The results demonstrated that NAC abolished the apoptotic effects induced by treatment with $1,25(OH)_2D_3$ followed by ADM (Fig. 4B). In addition, NAC attenuated the ROS production and upregulation of cleaved caspase-3 mediated by the combined drug treatment (Fig. 5A and B). These results indicated that $1,25(OH)_2D_3$ increased ADM-induced apoptosis in ATC cells, which may be mediated by an increase in intracellular ROS generation.

Discussion

ADM belongs to the anthracycline family of antitumor antibiotics (16). Most patients with late-stage ATC cannot undergo surgery and receive ADM treatment. However, patients treated with high doses of ADM usually suffer from serious

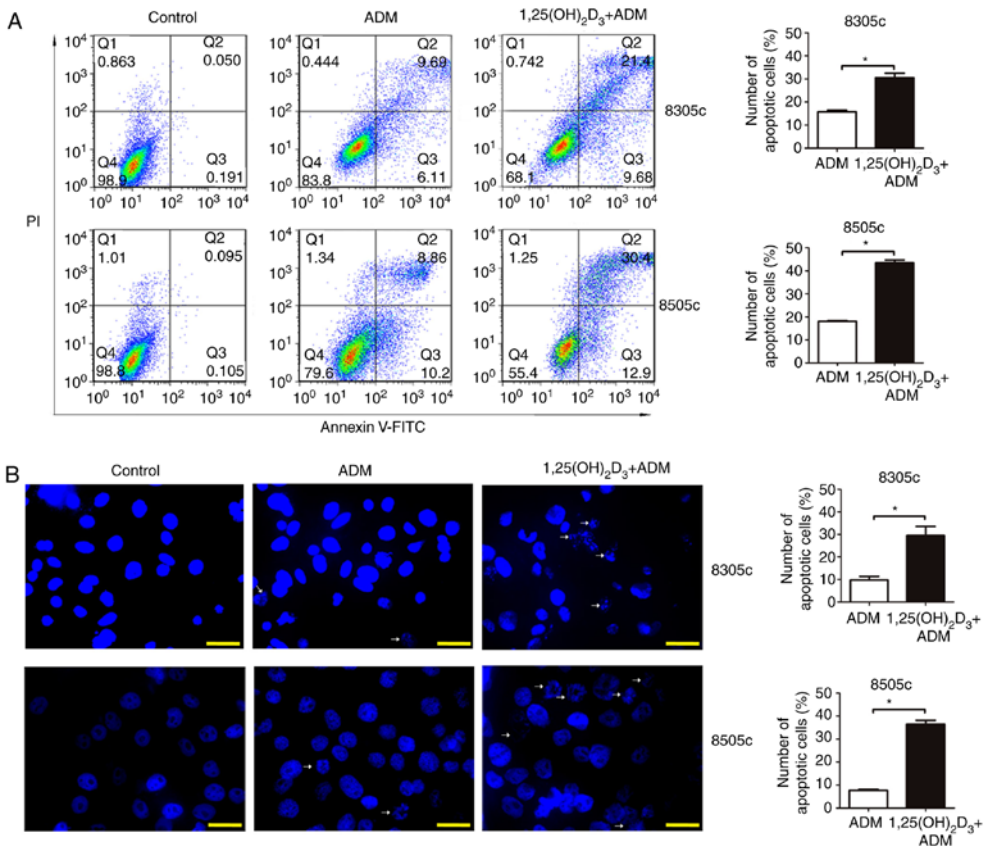

Figure 2. 1,25(OH)$_2$D$_3$ pretreatment promotes ADM-induced apoptosis. Cells were treated with 100 ng/ml ADM or 10 nM of 1,25(OH)2D3 followed by ADM. (A) Flow cytometric analyses of cell apoptosis in anaplastic thyroid cancer cell lines. Annexin V-FITC/PI double-staining was used to label cells. (B) Morphologic assessment of apoptosis detected by Hoechst 33342 under fluorescence microscopy. Following staining, cells presenting nuclear fragmentation were counted as apoptotic cells (white arrow). Magnification, x1,000. Scale bar, 10 μm. Results are the means ± standard error of the mean of three independent experiments. *P<0.05. 1,25(OH)2D3, 1,25-dihydroxyvitamin D3; ADM, adriamycin; PI, propidium iodide.

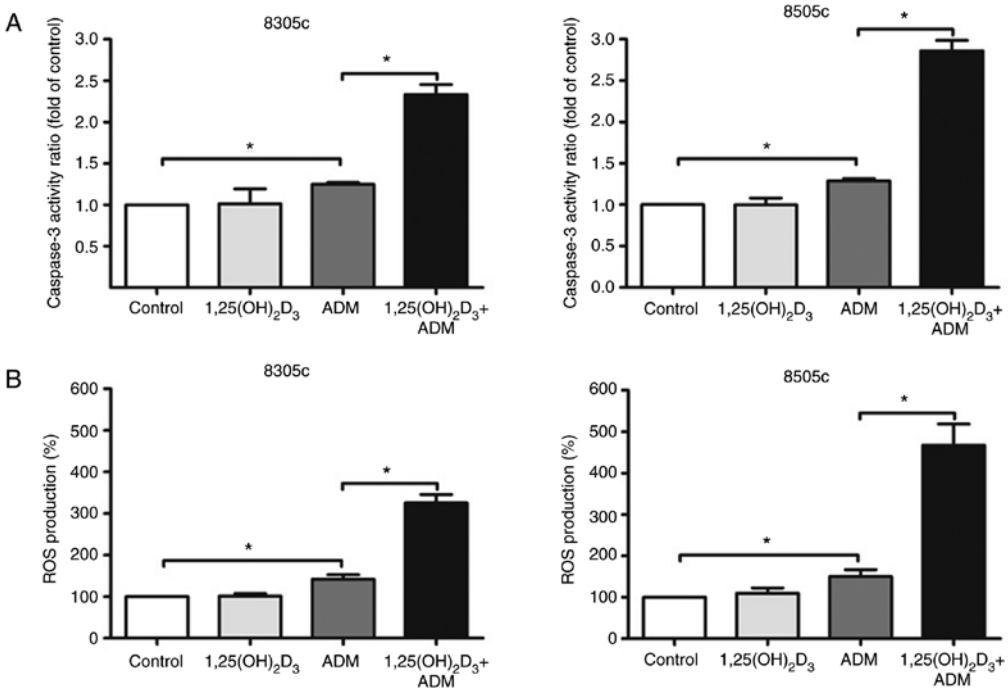

Figure 3. 1,25(OH)$_2$D$_3$ pretreatment promotes ADM-induced caspase-3 activation and ROS production. (A) Caspase-3 activity was determined using the fluorescent substrate Ac-DEVD-pNA. Quantification of caspase-3 activity in 8305c and 8505c ATC lines. Results are the means ± standard error of the mean of three independent experiments. *P<0.05. (B) Production of intracellular ROS in ATC cell lines was determined by H_2-DCFDA. Results are the means ± standard error of the mean of three independent experiments. *P<0.05. 1,25(OH)$_2$D$_3$, 1,25-dihydroxyvitamin D$_3$; ADM, adriamycin; ATC, anaplastic thyroid cancer; ROS, reactive oxygen species.

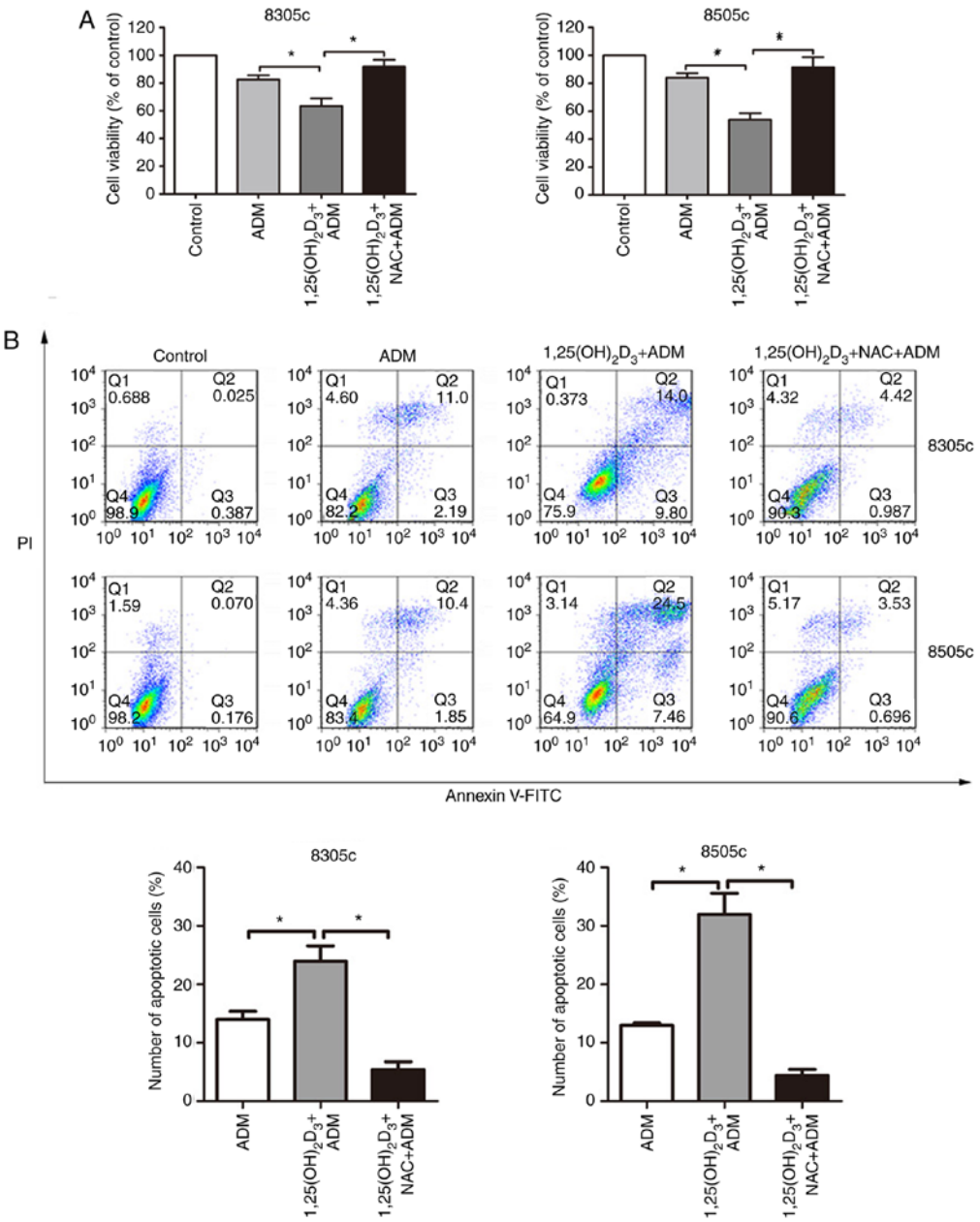

Figure 4. NAC attenuates $1,25(OH)_2D_3$ pretreatment-induced enhancement of ADM cytotoxicity. Cells were treated with 100 ng/ml ADM, 10 nM $1,25(OH)_2D_3$ followed by ADM, or $1,25(OH)_2D_3$ plus ADM and 1 mM NAC. (A) Cell viability was measured using Cell Counting Kit-8 assay. (B) Apoptosis was detected by flow cytometry. Results are the means ± standard error of the mean of three independent experiments. *P<0.05. $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; ADM, adriamycin; NAC, N-acetyl-L-cysteine.

adverse drug reactions, including cardiotoxicity, inhibition of bone-marrow function, nausea, vomiting, alopecia and increased transaminase levels (17). As these side effects are partly dose-dependent, a reduction in the ADM dose without reducing its therapeutic effect would therefore be of high clinical value. Therapeutic results could be improved if cancer cells were killed by ADM combined with a 'chemotherapy sensitizer' (18).

Previous studies reported that vitamin D receptors are highly expressed in ATC cell lines, which suggests that $1,25(OH)_2D_3$ could have a role in the treatment of malignant tumors (19-21). $1,25(OH)_2D_3$ could therefore be a chemotherapy sensitizer candidate for ADM and exert anticancer effects in the clinical treatment of ATC. The present study combined $1,25(OH)_2D_3$ with ADM to develop a therapeutic strategy that could allow ADM dose reduction, toxicity alleviation and potential inhibition of ATC cell proliferation. The results demonstrated that $1,25(OH)_2D_3$ amplified the apoptotic effect of ADM and reduced ATC cell viability in a synergistic manner.

The mechanisms underlying ADM-induced cytotoxicity are multiple such as cardiac injury and myelosuppression. However, ROS generation has been of high interest for a long time. Previous studies reported that ADM can stimulate intracellular ROS production and apoptosis in a dose-dependent manner (22,23). ROS are oxygen-containing chemically reactive species (24). The role of oxidative stress is ambivalent in cancer. A moderate increase in ROS production can promote

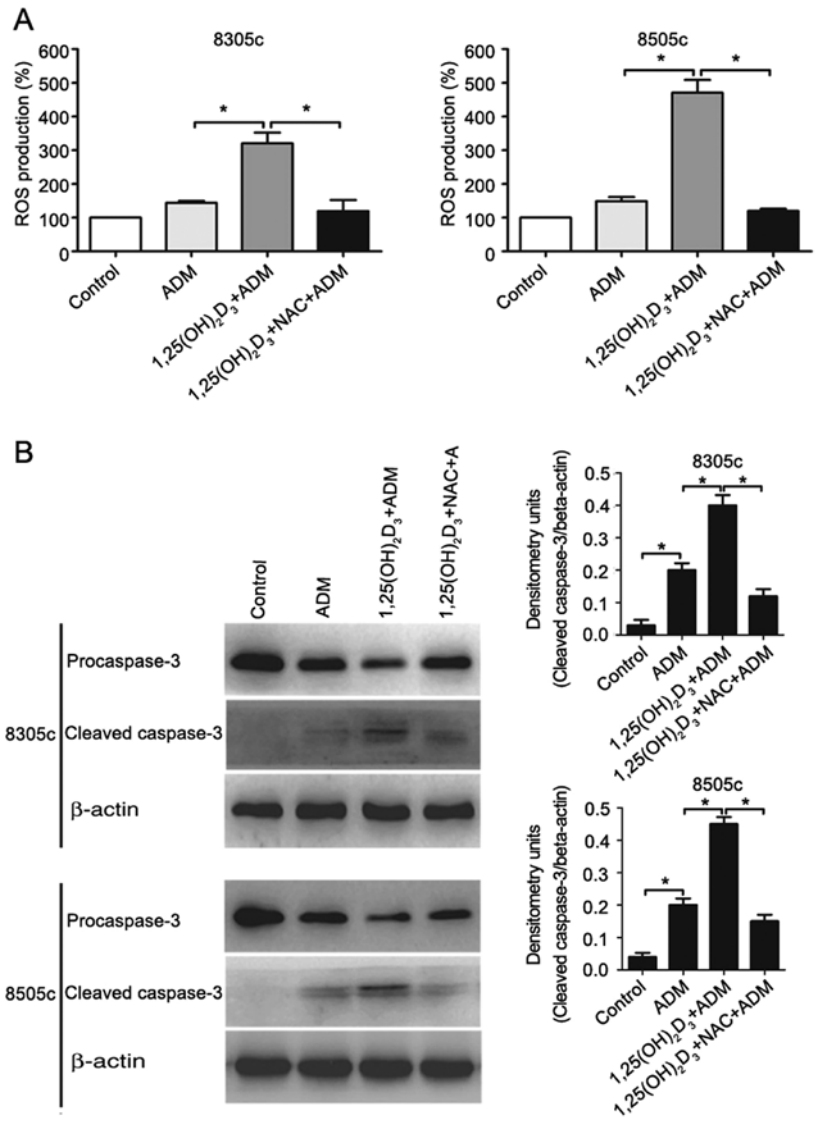

Figure 5. NAC attenuates 1,25(OH)$_2$D$_3$ pretreatment-induced enhancement of ADM-induced ROS production and cleaved caspase-3. Cells were treated with 100 ng/ml ADM, 10 nM 1,25(OH)$_2$D$_3$ followed by ADM, or 1,25(OH)$_2$D$_3$ plus ADM and 1 mM NAC. (A) Intracellular ROS production in anaplastic thyroid cancer cell lines was determined by H_2-DCFDA. (B) Expression of cleaved caspase-3 was measured using western blotting and β-actin protein was used for normalization. Results are the means ± standard error of the mean of three independent experiments. *P<0.05. 1,25(OH)$_2$D$_3$, 1,25-dihydroxyvitamin D$_3$; ADM, adriamycin; NAC, N-acetyl-L-cysteine; ROS, reactive oxygen species.

cell proliferation and differentiation (25). However, an acute and excessive increase in ROS production can induce cell apoptosis by activating DNA damage response and the p53 pathway (26). The role of ROS in cancer is not completely understood; however, certain studies have suggested that ROS serve vital roles in the proliferation, differentiation and apoptosis of cancer cells through the regulation of protein activity (27,28).

To the best of our knowledge, the present study reported for the first time that 1,25(OH)$_2$D$_3$ enhanced ADM susceptibility in ATC cells. Subsequently, whether the drugs worked in synergy was investigated. The results demonstrated that ATC cells pretreatment with 1,25(OH)$_2$D$_3$ significantly enhanced ADM-induced cytotoxicity in a dose-dependent manner. Furthermore, by using Annexin V-FITC/PI double-staining and Hoechst 33342, this study demonstrated that 1,25(OH)$_2$D$_3$ enhanced ADM-induced apoptosis. In addition, ADM induced ATC cell apoptosis, potentially by generating increased levels of ROS. The results demonstrated that 10 nM 1,25(OH)$_2$D$_3$ did not increase ROS production compared with control; however, when combined with ADM, ROS generation was increased in ATC cells. This finding has been previously demonstrated in breast cancer cell lines (29). In addition, the apoptotic cell rate in ATC cells was reduced following NAC addition in the two-drug combination group. Furthermore, cleaved caspase-3 expression was significantly reduced following NAC addition in the two-drug combination group. Taken together, these results suggested that ROS may induce ATC cell apoptosis through cleaved caspase-3 activation. In addition, the enhancement of ADM-induced apoptosis by 1,25(OH)$_2$D$_3$ in ATC cells may be closely associated with ROS. It has been demonstrated that ROS is implicated in the crosstalk between 1,25(OH)$_2$D$_3$ and ADM (30). The specific mechanism underlying ADM-induced ROS generation by 1,25(OH)$_2$D$_3$ remains unknown; however, it has been hypothesized that 1,25(OH)$_2$D$_3$ could inhibit superoxide dismutase expression and therefore

increase ROS generation (31). The excessive generation of ROS in the drug treatment can also be involved in promoting the activation of multiple signaling mechanisms. ROS generation precedes apoptosis and is required for the progression to apoptosis (32). ROS could subsequently affect the function of apoptosis-associated proteins, including proteins from the caspase family (33,34).

The present study demonstrated that combination of ADM chemotherapy drug with 1,25(OH)2D3 increased ADM-induced apoptosis. Pretreatment with $1,25(OH)_2D_3$ followed by ADM may achieve the same therapeutic effect against ATC and reduce the ADM-associated adverse reactions. These data suggested that $1,25(OH)_2D_3$ may be considered as a sensitizer for ADM chemotherapy and allow a reduction in ADM dose, which may be a novel therapeutic strategy for ATC. Whether the combination of $1,25(OH)_2D_3$ and ADM in the same cell culture, or $1,25(OH)_2D_3$ pretreatment followed by ADM administration can affect ATC cells and induce autophagy will be further investigated, alongside with the downstream molecular mechanisms involved.

In conclusion, ADM had high antineoplastic activity and induced apoptosis through activation of cleaved caspase-3 in ATC cells and increased ROS generation. The present study illustrated that $1,25(OH)_2D_3$ increased ADM-induced apoptosis in ATC cells, potentially by increasing ROS production.

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Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HZ conceived the study and edited the final manuscript. TZ contributed significantly to the study design and preparation of the manuscript. LH and WS conducted data collection and literature research. YQ and PZ analyzed data. All authors approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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