Antitumor effects and mechanisms of 1,25(OH)_2D3 in the Pfeiffer diffuse large B lymphoma cell line

JING HAN¹,², YONGHONG TANG², MEIZUO ZHONG² and WENLIN WU³

¹Department of Oncology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430030; ²Department of Oncology, Xiangya Hospital, Central South University, Changsha, Hunan 410011; ³Department of Pediatrics, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou, Guangdong 510000, P.R. China

Received October 8, 2018; Accepted August 6, 2019

DOi: 10.3892/mmr.2019.10756

Abstract. Diffuse large B cell lymphoma (DLBCL) represents the most common subtype of non-Hodgkin lymphoma in China. 1,25-Dihydroxyvitamin D₃ [1,25(OH)₂D₃] has been shown to possess significant antitumor potential and is degraded by 25-hydroxyvitamin D-24-hydroxylase (CYP24A1). In the present study, the role of CYP24A1 and autophagy, and their underlying mechanisms in the anticancer effects of 1,25(OH)₂D₃ in DLBCL cells, were investigated. It was found that the levels of CYP24A1 in DLBCL lymph node tissues were higher than in hyperplasia lymphadenitis tissue. Moreover, the expression of CYP24A1 was positively associated with the Ann Arbor stage and the International Prognostic Index in patients with DLBCL, and negatively associated with the clinical response to treatment. Patients >60 years of age were found to have a higher level of CYP24A1. 1,25(OH)₂D₃ inhibited the proliferation of the Pfeiffer DLBCL cell line and increased the G1 phase population of Pfeiffer cells. Rapamycin (RAPA) in combination with 1,25(OH)₂D₃ increased the G1 phase distribution of Pfeiffer cells. Furthermore, RAPA blocked the increase of CYP24A1 and vitamin D receptor (VDR) expression induced by 1,25(OH)₂D₃. 1,25(OH)₂D₃ induced the formation of autophagosomes, increased the expression of autophagy related protein light chain (LC)3II/LC3I and reduced the expression of the ubiquitin binding protein P62. In addition, 1,25(OH)₂D₃ decreased the phosphorylation of AKT and mammalian target of rapamycin (mTOR), and downstream targets eukaryotic translation initiation factor 4E-binding protein 1 and ribosomal protein S6 kinase β-1 in Pfeiffer cells. The results from the present study suggested that CYP24A1 may be a novel prognostic indicator for DLBCL. 1,25(OH)₂D₃ may be a novel prognostic indicator for DLBCL. 1,25(OH)₂D₃ inhibited proliferation and induced autophagy of Pfeiffer cells. In addition, 1,25(OH)₂D₃ increased the G1 phase population of Pfeiffer cells. These effects may be mediated by inhibition of the AKT/mTOR/Pi3K signaling pathway. RAPA increased the cell cycle arrest induced by 1,25(OH)₂D₃ by blocking the upregulated expression of CYP24A1 and VDR.

Introduction

Diffuse large B cell lymphoma (DLBCL) represents the most common subtype of non-Hodgkin lymphoma (1) and accounts for ~40% of newly diagnosed cases of non-Hodgkin lymphoma annually in China (2). DLBCL is an aggressive form of lymphoma, with a highly variable clinical manifestation, histology, outcome and prognosis (1). Despite the fact that patients with DLBCL who receive comprehensive treatment, including radiotherapy, chemotherapy and combined therapy with molecular targeted therapy, have a high chance of achieving partial or complete remission, some patients are resistant to first line therapy or relapse, leading to reduced survival rates, psychological and physical pain (1). Furthermore, the medical cost of DLBCL is high (1). Therefore, identifying new strategies for the treatment of DLBCL is needed.

B lymphocyte receptor (BCR) signaling is important for B cell lymphoma proliferation (3). BCR signaling can activate the PI3K signaling pathway, leading to the production of phosphatidylinositol 3,4,5-trisphosphate, which initiates a large number of signaling cascades, including those involving serine/threonine kinase AKT (4). This can lead to uncontrolled growth by inhibiting the function of the cell cycle inhibitors p21 and...
The concentration of 1,25(OH)2D3 available in tissues depends on the balance between its synthesis and degradation. Carried out by 25-hydroxvvitamin D-1 α-hydroxylase and 25-hydroxyvitamin D-24-hydroxylase (CYP24A1), respectively. A previous study found that the basal expression of CYP24A1 was higher in several tumor types, including colon, breast, lung, ovarian and cervical tumors (42). The high expression of CYP24A1 was found to be associated with the increased expression of replication licensing factors and tumor progression (43-47). However, little is known concerning the relationship between the clinical features of patients with DLBCL and the expression of CYP24A1, or how the antitumor effect of 1,25(OH)2D3 in DLBCL is influenced by the expression of CYP24A1.

The present study aimed to investigate the relationship between the clinical features of patients with DLBCL and the expression of CYP24A1. In addition, the roles of the PI3K/AKT/mTOR signaling pathway and autophagy in the antitumor effects of 1,25(OH)2D3 in DLBCL cells were investigated.

**Materials and methods**

*Reagents.* 1,25(OH)2D3 and RAPA were purchased from Sigma-Aldrich; Merck KGaA. 3-Methyladenine was purchased from Selleck Chemicals. The FITC Annexin V Apoptosis Detection kit, propidium iodide (PI) and ribonuclease A (RNase A) were purchased from BD Biosciences. The Cell Counting Kit-8 (CCK-8) was purchased from 7Sea Biotech. The ECL Western kit was purchased from Advansta, Inc. The following primary antibodies were purchased from Cell Signaling Technology, Inc.: mTOR (cat. no. 2972), phosphorylated (p)-mTOR (Ser2448; cat. no. 2971), AKT (cat. no. 4691), p-AKT (Ser473; cat. no. 4060), p-4E-BP1 (Thr37/46; cat. no. 2855), 4E-BP1 (cat. no. 9452), p-p70S6K (Thr389; cat. no. 9234), p70S6K (cat. no. 2708), ubiquitin binding protein P62 (P62; cat. no. 8025), LC3I/II (cat. no. 4108). The goat monoclonal antibody against CYP24A1 was purchased from Abcam (cat. no. ab189322). A rabbit monoclonal antibody against the vitamin D receptor (VDR) was purchased from Santa Cruz Biotechnology (cat. no. sc-13133), Inc. α-Tubulin was purchased from ProteinTech Group (cat. no. 11224-1-AP), Inc. Horseradish peroxidase (HRP)–conjugated goat-anti-mouse IgG (cat. no. A-11001) and HRP-conjugated goat-anti-rabbit IgG (cat. no. A-11034) were obtained from Thermo Fisher Scientific, Inc. 1,25(OH)2D3 was dissolved and stored in ethanol and RAPA was dissolved and stored in DMSO at -20°C. A biotinylated goat-anti-mouse antibody (cat. no. PV-9003) was purchased from Origene Technologies, Inc. A fluorescein-conjugated goat anti-rabbit secondary antibody (cat. no. Andy Fluor™ 594) was purchased from GeneCopoeia, Inc. Fetal calf serum was purchased from Biological Industries.

**Clinical specimens.** In total, 57 formalin-fixed (overnight at 4°C), paraffin-embedded (FFPE) DLBCL lymph node tissues from diagnostic biopsies (age, 31-70 years; male:female, 30:27) and 21 FFPE lymphonoditis lymph node tissues from diagnostic biopsies (age, 35-66 years; male:female, 12:9) were collected. All the samples were obtained between January 2010 to June 2013 from Xiangya Hospital. The Institutional Ethical Review Board of Xiangya Hospital approved the present study and written informed consent was obtained from all patients for the use of their biopsy samples. No patient had received any antitumor treatments before the biopsy sample was collected. The Ann Arbor staging system was used to assess the stage of the patient and the International Prognostic Index (IPI) was used to assess the risk of the patient (48). The serum lactate dehydrogenase (LDH) level was measured by clinical laboratory staff using the Hitachi 7170 biochemical analyzer (Hitachi, Ltd.).
Cell culture. The human Pfeiffer DLBCL cell line was purchased from the American Type Culture Collection (cat. no. ATCC® CRL-2632™) and cultured in RPMI-1640 (HyClone; GE Healthcare Life Sciences) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin and streptomycin (HyClone; GE Healthcare Life Sciences) at 37°C in a humidified 5% CO₂ incubator. Cells were passaged every 2-3 days to maintain a density between 1-2x10⁵ cells/ml.

Immunohistochemistry. Sections (thickness, 4 mm) obtained from the 57 FFPE DLBCL specimens were deparaffinized, rehydrated and the endogenous peroxidase activity was blocked using 3% H₂O₂ in methanol for 15 min at room temperature. The sections were microwaved for 4 min with trisodium citrate dihydrate solution (0.125%, pH 6.0) and then soaked with PBS three times for 5 min for citrate-mediated high-temperature antigen retrieval. Non-specific binding was blocked by pre-incubating with 5% BSA for 30 min at room temperature. Sections were incubated with anti-CYP24A1 (1:300) at 4°C overnight and then incubated with a biotinylated secondary antibody (1:5,000) for 30 min at room temperature. Sections were incubated with a streptavidin-HRP complex (Origene Technologies, Inc.) at room temperature for 5 min and 3,3'-diaminobenzidine (Origene Technologies, Inc.) at room temperature for 5 min, sections were then counterstained with hematoxylin at room temperature for 20. Positive cells were counted in 10 randomly selected fields with a x40 objective (Olympus CX23; Olympus Corporation). All sections were scored by two pathologists. The staining index was calculated as the product of staining intensity (Score: 0, no staining; 1, weak/light yellow; 2, moderate/yellow-brown; 3, strong/brown). The number of stained cells was scored as 0 (0-5%), 1 (6-25%), 2 (26-50%), 3 (51-75%) or 4 (76-100%). The sum of the intensity and number scores was used as the final staining score (0-7). Low expression was defined as a final staining score >3.

Transmission electron microscopy. To morphologically observe the induction of autophagy in 1,25(OH)₂D₃-treated Pfeiffer cells, ultrastructural analysis was performed. After treatment with 100 nM 1,25(OH)₂D₃ for 48 h, cells were washed twice with PBS and fixed with ice-cold glutaraldehyde (3% in 0.1 M cacodylate buffer, pH 7.4) for 30 min. Cells were post-fixed in 1% osmium tetroxide at room temperature for 2 h and embedded in Epon812 (SERVA Electrophoresis GmbH) before being cut (60 nm) and stained with 2% uranyl acetate/2.5% lead citrate and incubated for 15 min at room temperature. The formation of autophagosomes was assessed using the Tecnai electron microscope (FEI; Thermo Fisher Scientific, Inc.) at a magnification of x1,700 and x5,000.

Western blot analysis. Proteins were extracted using a nuclear and cytoplasmic protein extraction kit (Beyotime Institute of Biotechnology). Protein concentrations were determined using the bicinchoninic acid method. Proteins (30 µg) were separated using 6% SDS-PAGE under reducing conditions and then transferred onto PVDF membranes. The membranes were blocked in 5 mg/ml BSA for 2 h at room temperature. The following primary antibodies were used: VDR (1:1,000), CYP24A1 (1:1,000), AKT (1:1,000), p-AKT (1:1,000), mTOR (1:1,000), p-mTOR (1:1,000), LC3II (1:1,000) and P62 (1:1,000), P70S6K (1:1,000), p-P70S6K (1:1,000), 4EBP1 (1:1,000), α-Tubulin (1:5,000) was used as a loading control. The primary antibodies were incubated with the membranes overnight at 4°C. HRP-conjugated secondary antibodies (1:5,000) were incubated with the membranes at room temperature for 1-2 h. Protein bands were visualized using ECL and the Chemi Doc™ MP Imaging System (Bio-Rad Laboratories, Inc.). Band densitometry was assessed using ImageM software 1.8.0 (National Institutes of Health).

Statistical analysis. All statistical analysis was performed using SPSS 22.0 software (IBM Corp.). Data are present as the mean ± SD. Data were analyzed using the χ² test, Student's t-test, or one- or two-way ANOVA and the Tukey's test when applicable. Data were representative of three independent
experiments. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of CYP24A1 is different in patients with DLBCL and lymphnoditis. To examine whether the expression of CYP24A1 protein in DLBCL and lymphnoditis tissues, immunohistochemistry was performed on the 57 paraffin-embedded DLBCL lymph node tissue sections and 21 lymphnoditis lymph node tissue sections to evaluate the expression of CYP24A1 (Fig. 1A-D). High levels of CYP24A1 expression were detected in 9.5% of patients with lymphnoditis (n=21), whereas high levels of CYP24A1 expression were detected in 35.1% of patients with DLBCL (n=57). Low CYP24A1 expression was detected in 90.5% of patients with lymphnoditis, while low CYP24A1 expression was detected in 74.9% of patients with DLBCL. The expression levels of CYP24A1 between patients with lymphnoditis and DLBCL was significantly different (P<0.001). The patients with DLBCL were stratified into different groups, using factors such as age and sex, to investigate associations with the expression of CYP24A1 (Table I). There was no significant different in the expression of CYP24A1 between the Ann Arbor stage I/II patients and the Ann Arbor stage III/IV patients (P=0.28), however, the expression of CYP24A1 was significantly higher in patients with a higher IPI (P<0.05). Serum LDH levels were also significantly associated with CYP24A1 expression. The patient whose clinical treatment response was stable or showed complete remission had a lower level of CYP24A1 (P<0.05). The expression of CYP24A1 was significantly higher in patients over the age of 60 (P<0.05).

1,25(OH)2D3 inhibits the proliferation of Pfeiffer cells. To investigate the growth inhibitory properties of 1,25(OH)2D3, CCK-8 assays were performed, which is based on the ability of viable cells to reduce CCK-8 to formazan crystals. As shown in Fig. 1E, Pfeiffer cell proliferation was significantly inhibited by 10 nM (P<0.01) and 100 nM 1,25(OH)2D3 (P<0.001). The rate of inhibition was calculated as 16.8±3.4% for cells treated with 10 nM 1,25(OH)2D3 and 28.2±2.9% for cells treated with 100 nM 1,25(OH)2D3 for 48 h.

1,25(OH)2D3 induces cell cycle arrest in Pfeiffer cells. To determine whether 1,25(OH)2D3 induces cell cycle arrest in Pfeiffer cells, the effect of 1,25(OH)2D3 on the cell cycle distribution was analyzed using PI staining. Pfeiffer cells were treated with different concentrations of 1,25(OH)2D3 for 48 h and then subjected to cell cycle analysis. As shown in Fig. 2A and B, 22.70±0.40% of cells in the control group were in the G1 phase, while the cells treated with 100 nM 1,25(OH)2D3 showed a significant increase in the proportion of cells in the G1 phase (26.11±0.64%; P<0.05). In addition, whether 1,25(OH)2D3 increased the G1 phase arrest induced by RAPA was investigated. It was found the addition of RAPA increased the number of cells in the G1 phase compared with 1,25(OH)2D3 treatment alone (Table II; Fig. 2A and B).

1,25(OH)2D3 induced the expression of CYP24A1 mediated by VDR, which is the receptor of 1,25(OH)2D3, and CYP24A1 causes the degradation of 1,25(OH)2D3. This is an important regulatory mechanism to control the level of 1,25(OH)2D3 (30,31). In order to investigate whether RAPA increases the cell cycle arrest induced by 1,25(OH)2D3,

| Parameter | CYP24A1 expression | P-value |
|-----------|--------------------|---------|
| Age, years | Low | High |
| <60       | 32 | 11 | 0.008a |
| ≥60       | 5  | 9  |       |
| Sex       | Low | High |
| Male      | 18 | 12 | 0.37   |
| Female    | 19 | 8  |       |
| Ann Arbor stage | Low | High |
| I/II      | 10 | 3  | 0.28   |
| III/IV    | 27 | 17 |       |
| LDH       | Low | High |
| Normal    | 32 | 11 | 0.015a |
| Elevated  | 5  | 9  |       |
| IPI       | Low | High |
| 0-2       | 18 | 8  | 0.008a |
| 3-5       | 19 | 20 |       |
| Clinical treatment response | Low | High |
| CR/PR     | 26 | 8  | 0.028a |
| SD/PD     | 11 | 12 |       |
by reducing the degradation of 1,25(OH)₂D₃, the levels of CYP24A1 and VDR were determined in Pfeiffer cells after exposure to either 1,25(OH)₂D₃ or RAPA using western blot analysis. It was found that RAPA suppressed the expression of CYP24A1 and VDR induced by 1,25(OH)₂D₃ (Fig. 2C and D). This suggested that RAPA increased the cell cycle arrest in Pfeiffer cells induced by 1,25(OH)₂D₃ by suppressing the expression of CYP24A1 and VDR.

1,25(OH)₂D₃ induces autophagy in Pfeiffer cells. In order to investigate whether 1,25(OH)₂D₃ regulates autophagy in Pfeiffer cells, the induction of autophagy was analyzed. The
induction of autophagy in Pfeiffer cells after treatment with or without 1,25(OH)2D3 (10 nM, 100 nM) for 48 h was analyzed. The extent of autophagosome formation is associated with the modification of LC3I to LC3II and the degradation of P62. A significant increase in LC3II/LC3I and a reduction in P62 was induced by 100 nM 1,25(OH)2D3, as assessed using western blot analysis (Fig. 3c and d). Furthermore, the aggregation and localization of LC3 plays an important role in the maturation and transport of the autophagosome; therefore, immunofluorescence experiments were performed to observe changes in LC3 aggregation. A notable increase in green LC3 puncta was observed in the 100 nM 1,25(OH)2D3 treatment group compared with the control group (Fig. 3B). In addition, it was found that 100 nM 1,25(OH)2D3 enhanced the formation of autophagosomes, as observed using transmission electron microscopy (Fig. 3A). These findings indicated that 1,25(OH)2D3 activates autophagy in Pfeiffer cells.

1,25(OH)2D3 inhibits the AKT/mTOR signaling pathway. Having established that 1,25(OH)2D3 inhibited proliferation and induced autophagy in Pfeiffer cells, the molecular mechanisms underlying these biological effects were investigated. Activation of the AKT/mTOR signaling pathway increases the proliferation of Pfeiffer cells and is one of the major targets in the process of autophagy (51). Western blot analysis showed that p-AKT and p-mTOR were downregulated after 1,25(OH)2D3 exposure. The activation of downstream targets of mTOR, including p70S6K and 4E-BP1, were also decreased following 1,25(OH)2D3 treatment (Fig. 3E and F).

Discussion

In the present study, it was found that the levels of CYP24A1 in DLBCL lymph node tissues were higher than in lymphnoditis tissues. There was no significant difference in the expression of CYP24A1 between Ann Arbor stage I/II patients and Ann Arbor stage III/IV patients (P=0.28), however, the expression of CYP24A1 was significantly higher in patients with a higher IPI. Moreover, CYP24A1 expression was negatively associated with clinical treatment response. Patients over the age of 60 had a higher level of CYP24A1 expression. Furthermore, it was found 1,25(OH)2D3 inhibited proliferation and increased the G1 phase population of Pfeiffer cells. RAPA led to a further increase in the G1 population and decreased the expression of CYP24A1 and VDR induced by 1,25(OH)2D3. 1,25(OH)2D3 induced the formation of autophagosomes and increased the levels of the autophagy related protein LC3II/LC3I and reduced the expression of P62. In addition, 1,25(OH)2D3 decreased the phosphorylation of AKT, mTOR, and its downstream targets 4E-BP and p70S6K in Pfeiffer cells.

To the best of our knowledge, the present study was the first to show higher levels of the vitamin D-degrading enzyme
Figure 3. Analysis of autophagy and the AKT/mTOR signaling pathway in Pfeiffer cells after treatment with 1,25(OH)2D3. (A) Representative transmission electron microscope images [magnification, x1,700 (left) and x5,000 (right)] of the ultrastructure of Pfeiffer cells after treatment with or without 100 nM 1,25(OH)2D3 for 48 h. (B) Changes in the level and localization of LC3 examined by confocal microscopy after treatment with or without 100 nM 1,25(OH)2D3 for 48 h. Scale bar =100 µm. (C) Western blot analysis of the expression of the autophagy related proteins LC3 and P62 after treatment with 100 nM 1,25-D3 for 48 h and (D) the quantification. (E) Pfeiffer cells were cultured without or with 1,25(OH)2D3 for 1 h. The phosphorylation levels of AKT/mTOR signaling proteins were evaluated using western blotting. A representative example of three independent experiments is shown. (F) Quantification of western blotting. *P<0.05 vs. control. LC3, protein light chain 3; 1,25(OH)2D3, 1,25-dihydroxyvitamin D3; mTOR, target of rapamycin; p-, phosphorylated; 4E-BP, eukaryotic translation initiation factor 4E-binding protein 1; p70S6K, ribosomal protein S6 kinase β-1; P62, ubiquitin binding protein P62.
CYP24A1 in DLBCL tissues compared with lymphnoditis tissues, which was used as a normal control for the lymphoma tissues. CYP24A1 degrades 1,25(OH)\(_2\)D and 25(OH)D, which is a substrate required for the synthesis of 1,25(OH)\(_2\)D. Therefore, CYP24A1 is an important factor in determining the local concentration of 1,25(OH)\(_2\)D and regulates the antiproliferative effect of 1,25(OH)\(_2\)D (52). The higher expression of CYP24A1 may be one explanation for the significantly lower serum concentration of 25(OH)D in patients with DLBCL (53).

In addition, previous studies have described the moderate impact of 1,25(OH)\(_2\)D on the proliferation of certain DLBCL cell lines, including DOHH2 and K442 (50,49). The present study found higher levels of CYP24A1 expression in DLBCL lymph node tissues, suggesting that the actual concentration of vitamin D available and the concentration of 1,25(OH)\(_2\)D in these tissues may be low, and that this may attenuate the antitumor effects of 1,25(OH)\(_2\)D in vivo. The increased expression of CYP24A1 was also found in some solid tumors, including colon, lung, prostate, thyroid, breast and ovarian cancer cells (43-47). However, little is known regarding the mechanism underlying the increased expression of CYP24A1 in these cancer cells. Understanding the mechanism that leads to the upregulation of CYP24A1 in tumors may provide strategies for targeting CYP24A1 in the future.

A combination of CYP24A1 inhibitors, such as liarozole, genistein (a soy isoflavone that directly inhibits CYP24A1 activity and increases the sensitivity of cells to 1,25(OH)\(_2\)D) or ritonavir (which inhibits the expression of CYP24A1), may increase the level and calcemic activity of 1,25(OH)\(_2\)D, increasing the risk of hypercalcemic side effects (54,55). Structural non-calcemic action analogs of calcitriol that resist CYP24A1 may be more biologically active and more useful in cancer therapy and for the treatment of DLBCL, which expresses a high level of CYP24A1.

In the present study, it was found that 1,25(OH)\(_2\)D\(_3\) inhibits the proliferation of DLBCL Pfeiffer cell line. In a previous study into the antiproliferative effect of 1,25(OH)\(_2\)D\(_3\) in other DLBCL cell lines, 1,25(OH)\(_2\)D\(_3\) was found to have an antiproliferative effect on the Pfeiffer cell line (50).

CYP24A1, which degrades 1,25(OH)\(_2\)D\(_3\) and 25(OH)D, is important for lowering the levels of 1,25(OH)\(_2\)D\(_3\) and 25(OH)D. Vitamin D deficiency is a risk factor for autoimmune diseases, such as rheumatoid arthritis and system lupus erythematosus (SLE) (56,57). A recent study reported that vitamin D deficiency and the upregulation of CYP24A1 have a combined role in the transition to SLE (58). Patients with autoimmune diseases, in particular those mediated by B lymphocytes, have a higher risk of developing DLBCL, with a more aggressive nature, possibly due to the misregulated production of several cytokines, including interleukin (IL)-6, IL-10 and tumor necrosis factor-\(\alpha\), that contribute to the pathogenesis of DLBCL (59-62). 1,25(OH)\(_2\)D also plays an important role in the regulation of immune function by inhibiting the production of cytokines (63,64). In future research, whether the increased production of cytokines contribute to the pathogenesis of DLBCL, and whether this is associated with the upregulation of CYP24A1, should be investigated. Furthermore, whether inhibiting the production of cytokines using 1,25(OH)\(_2\)D\(_3\) is beneficial for the treatment of some cases of DLBCL should be investigated in in vivo studies.

Autophagic cell death is a survival mechanism to deal with metabolic stress and is a caspase-independent mechanism of cell death (65). Nevertheless, previous studies have indicated that autophagy and apoptosis may be interconnected process (66,67). The present study found that 1,25(OH)\(_2\)D\(_3\) induced autophagy in Pfeiffer cells. These results suggested that 1,25(OH)\(_2\)D\(_3\) induced cell killing in a caspase-independent autophagy-mediated manner in Pfeiffer cells.

Activation of the PI3K/AKT/mTOR signaling pathway suppresses autophagy (68,69). The data from the present study showed that 1,25(OH)\(_2\)D\(_3\) decreased the phosphorylation levels of AKT and mTOR in Pfeiffer cells, consistent with a previous study that found that 1,25(OH)\(_2\)D\(_3\) is involved in mTOR signaling (70). These results suggested that 1,25(OH)\(_2\)D\(_3\) induces autophagy in Pfeiffer cells by inhibiting the PI3K/AKT/mTOR signaling pathway. In addition, the PI3K/AKT/mTOR signaling pathway also increases mRNA translation, protein synthesis and cellular proliferation (9,10).

The aberrant activation of mTOR is frequently associated with poorer prognosis and has been well described in non-Hodgkin lymphoma (4,71-73). Suppressing mTOR in Pfeiffer cells may extend the therapeutic applications of 1,25(OH)\(_2\)D to the treatment of DLBCL. A previous study reported that 1,25(OH)\(_2\)D\(_3\) inhibits the mTOR signaling pathway by stimulating the expression of DDIT4, a potent suppressor of mTOR activity (39). The present study showed that 1,25(OH)\(_2\)D\(_3\) decreased the phosphorylation of downstream factors in the PI3K/AKT/mTOR signaling pathway in Pfeiffer cells, including 4EBP and p70S6K. p70S6K is an important regulator of protein synthesis; blocking the activation of p70S6K, for example using Saquinavir-NO, interrupts protein synthesis, leading to decreased cancer cell proliferation (74-77).

The inhibition of Pfeiffer cell proliferation by 1,25(OH)\(_2\)D\(_3\) may be due to the decreased phosphorylation of p70S6K. In future studies, the anticancer effects of other p70S6K inhibitors should be investigated in DLBCL, and their potential synergism with 1,25(OH)\(_2\)D\(_3\) should be tested.

A previous study reported that the mTOR signaling inhibitor RAPA and its analogs increase the antitumor effects of 1,25(OH)\(_2\)D in breast cancer cells and acute myelogenous leukemia (78,79). This effect of RAPA and its analogs may be due to the increased expression, or nuclear translocation, of VDR (79). Consistent with these previous studies, the data from the present study showed that 1,25(OH)\(_2\)D\(_3\) increased the G1 phase population of Pfeiffer cells and that this was potentiated by RAPA. However, it was found that RAPA blocked the increase in VDR and CYP24A1 expression induced by 1,25(OH)\(_2\)D\(_3\), 1,25(OH)\(_2\)D\(_3\) induced the expression of CYP24A1, mediated by VDR, which is the receptor of 1,25(OH)2D3, and CYP24A1 causes the degradation of 1,25(OH)\(_2\)D\(_3\). This is an important autoregulatory mechanism of 1,25(OH)\(_2\)D\(_3\). RAPA may potentiate the effect of 1,25(OH)\(_2\)D\(_3\) by reducing its degradation.

In conclusion, the results of the present study suggested that the expression of CYP24A1 may be a novel prognostic indicator for DLBCL. 1,25(OH)\(_2\)D\(_3\) inhibited the proliferation, and induced the autophagy, of Pfeiffer cells. In addition, 1,25(OH)\(_2\)D\(_3\) increased the G1 phase population of Pfeiffer cells. These effects may be mediated by inhibiting the PI3K/AKT/mTOR signaling pathway. RAPA may potentiate
the cell cycle arrest caused by 1,25(OH)2D3 by inhibiting the expression of CYP24A1 and VDR.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Natural Science Foundation of China (grant no. 81570200) and was partially supported by the fund from Guangzhou Institute of Pediatrics/Guangzhou Women and Children's Medical Center (grant no. YIP-2018-005). The funders had no role in the study concept, study design, data analysis, interpretation or reporting of the results. The authors had full control of the data and information submitted for publication.

Availability of data and materials

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

Authors' contributions

WW and JH contributed to the design of the study and were involved in performing the experiments, data analysis and the preparation of the manuscript. YT contributed to the data analysis. MZ contributed to data analysis and manuscript preparation. All authors contributed to the data interpretation and approved the final version of the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

The Institutional Ethical Review Board of the Xiangya Hospital approved the present study, and written informed consent was obtained from all patients for the use of their biopsy samples.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Li S, Young KH and Medeiros LJ: Diffuse large B-cell lymphoma. Pathology 50: 74-87, 2018.
2. Li JM, Wang L, Shen Y, Xia ZG, Chen Y, Chen QS, Chen Y, Zeng XY, You JH, Qian Y and Shen XJ: Rituximab in combination with CHOP chemotherapy for the treatment of diffuse large B-cell lymphoma in Chinese patients. Ann Hematol 86: 639-645, 2007.
3. He F, Wang L, Hu XB, Yin DD, Zhang P, Li GH, Wang YC, Huang SY, Liang YM and Han H: Notch and BCR signaling synergistically promote the proliferation of Raji B-lymphoma cells. Leuk Res 33: 798-802, 2009.
4. Uddin S, Hussain AR, Siraj AK, Manogaran PS, Al-Jomah NA, Muorji A, Alizadeh V, Al-Dayer F, Belgaumi A, El-Solh H, et al: Role of phosphatidylinositol 3-kinase/AKT pathway in diffuse large B-cell lymphoma survival. Blood 108: 4178-4186, 2006.
5. Liang J, Zavobit J, Petrocelli T, Kotchetkov R, Connor MK, Han K, Lee JH, Ciarallo S, Catavolos C, Beniston R, et al: PKB/Akt phosphorylates p27, impairs nuclear import of p27 and causes p27-mediated G1 arrest. Nat Med 11: 1153-1160, 2005.
6. Héron-Milhavet L, Franchkhauser C, Rana V, Bertheken C, Fisher D, Hemmings BA, Fernandez A and Lamb NJ: Only Akt1 is required for proliferation, while Akt2 promotes cell cycle exit through p21 binding. Mol Cell Biol 26: 8267-8280, 2006.
7. Brazil DP, Yang ZZ and Hemmings BA: Advances in protein kinase B signalling: AKTion on multiple fronts. Trends Biochem Sci 29: 233-242, 2004.
8. Cai SL, Tee AR, Short JD, Bergeron JM, Kim J, Shen J, Guo R, Johnson CL, Kiguchi K and Walker CL: Activity of TSC2 is inhibited by AKT-mediated phosphorylation and membrane positioning. J Cell Biol 173: 279-289, 2006.
9. Cantley LC: The phosphoinositide 3-kinase pathway. Science 296: 1655-1657, 2002.
10. Chang F, Lee JT, Novolanic PM, Steelman LS, Shelton JD, Blalock WL, Franklin RA and McCubrey JA: Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: A target for cancer chemotherapy. Leukemia 17: 590-603, 2003.
11. Nicoletti F, Fagone P, Meroni P, McCubrey JA and Bendtzen K: mTOR as a multifunctional therapeutic target in HIV infection. Drug Discov Today 16: 715-721, 2011.
12. Xu ZZ, Xia ZG, Wang AH, Wang WF, Liu ZY, Chen LY and Li JM: Activation of the PI3K/AKT/mTOR pathway in diffuse large B cell lymphoma: Clinical significance and inhibitory effect of rituximab. Ann Hematol 92: 1351-1358, 2013.
13. Hans CP, Weisenburger DD, Greiner TC, Gascoyne RD, Delabie J, Ott G, Muller-Hermelink HK, Campo E, Brazil P, et al: Classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood 103: 275-282, 2004.
14. Ott G, Ziepert M, Klocker W, Horn H, Szczepanowski M, Bernd HW, Thorns C, Feist AC, Lenze D, Hummel M, Jaffe ES, et al: Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood 103: 275-282, 2004.
15. Hans CP, Weisenburger DD, Greiner TC, Gascoyne RD, Delabie J, Ott G, Muller-Hermelink HK, Campo E, Brazil P, et al: Phase I pharmacokinetic and pharmacodynamic study of the pan-PI3K/mTORC vascular targeted pro-drug SF1126 in patients with advanced solid tumours and B-cell malignancies. Eur J Cancer 48: 3319-3327, 2012.
16. Bendel JC, Rodon J, Barrios HA, de Jonge M, Verweij J, Birle D, Demanse D, De Buck SS, Ril RU, Peters M, et al: Phase I, dose-escalation study of BKM120, an oral pan-Class I PI3K inhibitor, in patients with advanced solid tumors. J Clin Oncol 30: 282-290, 2012.
17. Cheng Y, Zhang Y, Zhang L, Ren X, Huber-Keener KJ, Liu X, Zhou L, Liao J, Keihack H, Yan L, et al: MK-2206, a novel allosteric inhibitor of Akt, synergizes with gefitinib against glioma via modulating both autophagy and apoptosis. Mol Cancer Ther 11: 154-164, 2012.
18. Donia M, Mangano K, Amoroso A, Mazzei MC, Imbesi R, Castrogiovanni P, Coco M, Meroni P and Nicoletti F: Treatment with rapamycin ameliorates clinical and histological signs of protracted relapsing experimental allergic encephalomyelitis in Dark Agouti rats and induces expansion of peripheral CD4+CD25+Foxp3+ regulatory T cells. J Autoimmun 33: 135-140, 2009.
19. Bagherpour B, Salehi M, Jafari R, Bagheri A, Kiani-Esfahani A, Esladati M, Kardi MT and Shaygannejad V: Promising effect of sirolimus study assessing double-masked uveitis treatment with rapamycin ameliorates clinical and histological signs of protracted relapsing experimental allergic encephalomyelitis in Dark Agouti rats and induces expansion of peripheral CD4+CD25+Foxp3+ regulatory T cells. Autoimmun 33: 135-140, 2009.
23. Donia M, McCreary JA, Bendtzen K and Nicoletti F: Potential use of rapamycin in HIV infection. Br J Clin Pharmacol 70: 784-793, 2010.

24. Nicoletti F, Caprini E, Donia M, Mccubrey JA, Bendtzen K and Nicoletti F: Potential of dabrafenib on the invasive behavior of melanoma cells with increased expression of cYP24a1 correlates with advanced stages of prostate cancer and can cause resistance to vitamin D receptor in patients with breast cancer. Radiat Oncol 140: 453-462, 2013.

25. Dona M, Mccubrey JA, Bendtzen K and Nicoletti F: Potential of dabrafenib on the invasive behavior of melanoma cells with increased expression of cYP24a1 correlates with advanced stages of prostate cancer and can cause resistance to vitamin D receptor in patients with breast cancer. Radiat Oncol 140: 453-462, 2013.

26. Yu Y, Yu X, Ma J, Tong Y and Yao J: Effects of NVP-BEZ235 on the proliferation, migration, apoptosis and autophagy in HT-29 human colorectal adenocarcinoma cells. Int J Oncol 49: 285-293, 2016.

27. Chang Z, Shi G, Jin J, Guo H, Guo X, Luo F, Song Y and Jia X: Dual PI3K/mTOR inhibitor NVP-BEZ235-induced apoptosis of hepatocellular carcinoma cell lines is enhanced by inhibitors of autophagy. Int J Mol Med 31: 1449-1456, 2013.

28. Papamolos E, Zelnak A and O'Regan R: Everolimus: Side effect profile and management of toxicities in breast cancer. Breast Cancer Res Treat 140: 453-462, 2013.

29. Lew S and Chamberlain SR: Risk of metabolic complications by rapamycin. Clin Exp Immunol 155: 1164-1174, 2014.

30. Marcinkowska E, Graffon G and Brown GR: The use of leu S and chamberlain SR: Risk of metabolic complications by rapamycin. Clin Exp Immunol 155: 1164-1174, 2014.

31. Holick MF: Vitamin D and bone health. J Nutr 126 (Suppl 4): 1711-1716, 2006.

32. Abu El Maaty MA and Wöhrle J: The two-faced cytokine il-6 in autoimmunity. Clin Rev Allergy Immunol 8: 207-210, 2015.

33. Barreto SG and Neale RE: Vitamin D and pancreatic cancer. Cancer Lett 368: 1-6, 2015.

34. Castronovo C, Castronovo V, Nikkels A and Peulen O: Vitamin D receptor binding protein reveals a role for vitamin D in osteoblast mTOR signaling. FASEB J 25: 937-947, 2011.

35. Fujiwara T, Yasuda K, Kitajima S, Yamamoto K and Chen TC: Cytochrome P450 12-beta-hydroxysteroid dehydrogenase type 2 (CYP12A1) is a potential biomarker for the progression and prognosis of human colorectal cancer. Hum Pathol 50: 101-108, 2016.

36. Sakai T, Yasuda K, Kittaka A, Yamamoto K and Chen TC: CYP24A1 as a potential target for cancer therapy. Anticancer Agents Med Chem 14: 97-108, 2014.
65. Lockshin RA and Zakeri Z: Caspase-independent cell deaths. Curr Opin Cell Biol 14: 727-733, 2002.

66. Matsui Y, Takagi H, Qu X, Abdellatif M, Sakoda H, Asano T, Levine B and Sadoshima J: Distinct roles of autophagy in the heart during ischemia and reperfusion: Roles of AMP-activated protein kinase and Beclin 1 in mediating autophagy. Circ Res 100: 914-922, 2007.

67. Takagi H, Matsui Y, Hirotni S, Sakoda H, Asano T and Sadoshima J: AMPK mediates autophagy during myocardial ischemia in vivo. Autophagy 3: 405-407, 2007.

68. Maiuri MC, Tasdemir E, Criollo A, Morselli E, Vicencio JM, Carnuccio R and Kroemer G: Control of autophagy by oncogenes and tumor suppressor genes. Cell Death Differ 16: 87-93, 2009.

69. Wullischler S, Loewith R and Hall MN: TOR signaling in growth and metabolism. Cell 124: 471-484, 2006.

70. Lisse TS and Hewison M: Vitamin D: A new player in the world of mTOR signaling. Cell Cycle 10: 1888-1889, 2011.

71. Peponi E, Drakos E, Reyes G, Leventaki V, Rassidakis GZ and Medeiros LJ: Activation of mammalian target of rapamycin signaling promotes cell cycle progression and protects cells from apoptosis in mantle cell lymphoma. Am J Pathol 169: 2171-2180, 2006.

72. Rudelius M, Pittaluga S, Nishizuka S, Pham TH, Fend F, Jaffe ES, Quintanilla-Martinez L and Raffeld M: Constitutive activation of Akt contributes to the pathogenesis and survival of mantle cell lymphoma. Blood 108: 1668-1676, 2006.

73. Hasselblom S, Hansson U, Olsson M, Torén L, Bergström A, Nilsson-Ehle H and Andersson PO: High immunohistochemical expression of p-AKT predicts inferior survival in patients with diffuse large B-cell lymphoma treated with immunotherapy. Br J Haematol 149: 560-568, 2010.

74. Maksimovic-Ivanic D, Fagone P, McCubrey J, Bendtzen K, Mijatovic S and Nicoletti F: HIV- protease inhibitors for the treatment of cancer: Repositioning HIV protease inhibitors while developing more potent NO-hybridized derivatives? Int J Cancer 140: 1713-1726, 2017.

75. Rothweiler F, Michaelis M, Brauer P, Otte J, Weber K, Fehse B, Doerr HW, Wiese M, Kreuter J, Al-Abed Y, et al: Anticancer effects of the nitric oxide-modified saquinavir derivative saquinavir-NO against multidrug-resistant cancer cells. Neoplasia 12: 1023-1030, 2010.

76. Maksimovic-Ivanic D, Mojic M, Bulatovic M, Radojkovic M, Kuzmanovic M, Ristic S, Stosis-Grujicic S, Miljkovic D, Cavalli E, Libra M, et al: The NO-modified HIV protease inhibitor as a valuable drug for hematological malignancies: Role of p70S6K. Leuk Res 39: 1088-1095, 2015.

77. Pearce LR, Alton GR, Richter DT, Kath JC, Lingardo L, Chapman J, Hwang C and Alessi DR: Characterization of PF-4708671, a novel and highly specific inhibitor of p70 ribosomal S6 kinase (S6K1). Biochem J 431: 245-255, 2010.

78. Guo LS, Li HX, Li CY, Zhang SY, Chen J, Wang QL, Gao JM, Liang JQ, Gao MT and Wu YJ: Synergistic antitumor activity of vitamin D3 combined with metformin in human breast carcinoma MDA-MB-231 cells involves m-TOR related signaling pathways. Pharmazie 70: 117-122, 2015.

79. Yang J, Ikezoe T, Nishioka C, Ni L, Koeffler HP and Yokoyama A: Inhibition of mTORC1 by RAD001 (everolimus) potentiates the effects of 1,25-dihydroxyvitamin D(3) to induce growth arrest and differentiation of AML cells in vitro and in vivo. Exp Hematol 38: 666-676, 2010.