Inhibitory effects of $1,25(OH)_2D_3$ on the proliferation of hepatocellular carcinoma cells through the downregulation of HDAC2

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Abstract. The inhibitory effects of $1,25(OH)_2D_3$ on the proliferation of a variety of cancer cell lines have been extensively reported. However, the underlying mechanisms remain largely unknown. In the present study, the effects of $1,25(OH)_2D_3$ on the *in vitro* proliferation of human hepatocellular carcinoma HepG2 cells and the mechanism involved were investigated. Flow cytometry and MTT assay revealed that $1,25(OH)_2D_3$ inhibited cell proliferation *in vitro*. Western blotting and real-time PCR indicated that $1,25(OH)_2D_3$ upregulated the expression of phosphatase and tensin homologue deleted on chromosome 10 (PTEN) and attenuated that of histone deacetylase 2 (HDAC2). Knockdown of HDAC2 completely mimicked the effects of $1,25(OH)_2D_3$ on PTEN gene expression. The influence of $1,25(OH)_2D_3$ on PTEN expression was reversed in the cells treated with a recombinant pEGFP-LV2-HDAC2 plasmid. Akt phosphorylation, which was downregulated by $1,25(OH)_2D_3$ treatment, was promoted by HDAC2 overexpression. These findings revealed that $1,25(OH)_2D_3$ inhibited cell growth possibly by HDAC2-mediated PTEN upregulation, Akt deactivation, and inhibition of the PI3K/Akt signaling pathway.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common human malignant tumors, with a high degree of malignancy and rapid progression, posing a serious threat to human health (1). The onset and progression of HCC are complex processes involving multiple factors, levels and genes. Uncontrollable cell proliferation caused by disorders of cell cycle regulation is one of the important mechanisms responsible for the occurrence of various tumors including HCC (2). Human phosphatase and tension homolog deleted on chromosome 10 (PTEN) is an anti-oncogene that regulates the cell cycle mainly by inhibiting progression from the G_1 to the S phase, thus suppressing cell proliferation. PTEN further regulates the changes of tumor cell proliferation and the cell cycle by negatively regulating the PI3K/Akt signaling pathway (3,4). Acetylation is another mechanism that regulates the activity of PTEN, and inhibiting the expressions of histone deacetylases (HDACs) upregulates that of PTEN (5). Pan *et al* found that trichostatin A inhibited the expression of HDAC while upregulating that of PTEN, indicating that histone acetylation is a crucial mechanism in the regulation of the activity of PTEN (6).

Histone acetylation is mainly regulated by histone acetylases (HATs) and HDACs simultaneously. The balance between HATs and HDACs stabilizes chromatin structures and gene expression, which, when broken, may lead to chromatin structural changes and transcriptional imbalance of genes related to cell proliferation, the cell cycle and apoptosis. This is a key molecular mechanism for tumor onset and progression (7). To date, HDACs have been found to be aberrantly expressed in various malignancies such as HCC, gastric, pancreatic and bladder cancer (8-10). HDAC2, as a member of the HDAC family, can widely regulate gene transcription and silencing. Noh *et al* reported that the HDAC2 gene was highly expressed in human HCC tissues, with its level increasing upon aggravation (11). Zhang *et al* found that after targeted downregulation of HDAC2, the expression level of PTEN was significantly upregulated, thereby inhibiting tumor cell proliferation (12).

As a lipid-soluble vitamin closely associated with human health, vitamin D functions physiologically through its *in vivo* metabolite $1,25(OH)_2D_3$. However, by regulating *in vivo* calcium and phosphorus metabolisms, $1,25(OH)_2D_3$ and its analogues can also inhibit tumor cell proliferation, promote differentiation, induce apoptosis and suppress tumor invasion and metastasis (13,14). Toropainen *et al* reported that $1,25(OH)_2D_3$ downregulated MYC gene expression, which was significantly decreased after interference with HDAC2 (15). We previously found that $1,25(OH)_2D_3$ upregulated the expression of PTEN and inhibited the proliferation of HCC cells (16). Therefore, we postulated that $1,25(OH)_2D_3$ inhibited the proliferation of HCC cells and arrested the cell cycle in the G_0/G_1 phase by downregulating HDAC2 and regulating the PTEN/PI3K/Akt

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signaling pathway. Thereby motivated, we evaluated the effects of aberrant HDAC2 expression on $1,25(OH)_2D_3$-inhibited HepG2 cell proliferation, and explored the possible mechanism.

Materials and methods

Cell line and main reagents. Human HCC HepG2 cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). PCR primers were designed and synthesized by Invitrogen (Shanghai, China). Lentiviruses for HDAC2 interference and overexpression were packaged by Shanghai GeneChem Co., Ltd. (Shanghai, China). $1,25(OH)_2D_3$ was purchased from Sigma-Aldrich (St. Louis, MO, USA). Thiazolyl blue (MTT) was purchased from Amresco LLC (Solon, OH, USA). The cell cycle detection kit was obtained from BD Biosciences (San Jose, CA, USA). Rabbit anti-human PTEN, PI3K and p-PI3K monoclonal antibodies were purchased from Abcam (Cambridge, MA, USA). Rabbit anti-human Akt and p-Akt monoclonal antibodies were purchased from Cell Signaling Technology Inc. (CST; Danvers, MA, USA). Rabbit anti-human β-actin polyclonal antibody was obtained from Bio-World (Dublin, OH, USA). Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody was purchased from Beijing Bioss Antibodies Co., Ltd. (Beijing, China). BCA protein quantification kit was purchased from Shanghai Generay Biotech Co., Ltd. (Shanghai, China). Pre-stained protein Rainbow marker was obtained from Beijing Solarbio Life Sciences Co., Ltd. (Beijing, China). Total RNA extraction, SYBR® Premix Ex Taq™ and PrimeScript® RT reagent kits were obtained by Takara (Shiga, Japan).

Cell culture. Human HCC HepG2 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum and 100 U/ml penicillin-streptomycin, and incubated in an incubator at 37˚C with 5% CO_2. $1,25(OH)_2D_3$ was dissolved in 100% ethanol and stored at -80˚C.

Cell proliferation assay. HepG2 cells in the logarithmic growth phase were collected and inoculated into 96-well plates at the density of $5x10^4$ cells/ml. After 24 h of adherent growth, the cells were starved in serum-free DMEM for 24 h and $1,25(OH)_2D_3$ was added at final concentrations of 10, 100 and 1,000 nM. Meanwhile, the control and zero wells were set, and five replicate wells were set up for each group. Then, they were cultured in the incubator at 37˚C with 5% CO_2 for 24, 48 and 72 h. After 10 μl of MTT solution was added into each well, they were further incubated for 4 h. Finally, the culture medium was carefully pipetted, and 50 μl of dimethyl sulfoxide (DMSO) was added into each well. The plates were then shaken on a shaking table at low speed for 10 min to completely dissolve the formed crystals. The optical density (OD) of each well was measured at 490 nm by a microplate reader. The cell proliferation rate (%) was calculated as follows: $= (OD_{experimental} - OD_{blank})/(OD_{control} - OD_{blank})$.

Cell cycle assay. HepG2 cells were seed into 6-well plates at the density of $2x10^5$ cells/ml. After 72 h of exposure at 100 nM of $1,25(OH)_2D_3$, the cells were collected by centrifugation after trypsin digestion, washed twice with phosphate-buffered saline (PBS), fixed in 4˚C 70% ethanol for 30 min, and centrifuged to discard the supernatant. The cells were then washed twice with PBS, gently mixed with 150 μl of RNase and propidium iodide (PI) in the dark, and then left still at room temperature for 30 min and subjected to cell cycle detection using a flow cytometer.

Real-time PCR. Total RNA was extracted using a Total RNA extraction kit (Takara) according to the instructions. Then, the purity and concentration of RNA were detected by a UV spectrometer. According to the instructions of PrimeScript® RT reagent kit and SYBR® Premix Ex Taq™ kit, reverse transcription and target gene amplification were performed. The conditions for PCR amplificationwere : 95˚C for 30 sec, pre-denaturation for 30 sec, 1 cycle; 95˚C for 5 sec, 60˚C for 30 sec, 40 cycles. The relative mRNA expression level was expressed as $2^{-\Delta\Delta Ct}$. All experiments were performed in triplicate and repeated at least three times. The primer sequences for HDAC2 and p21 and PTEN and Akt were as follows: HDAC2 forward, 5'-ATAAAGCCACTGCCGAAGAA-3' and reverse, 5'-TCCTCCAGCCCAATTAACAG-3'; p21 forward, 5'-CATGGGTTCTGACGGACAT-3' and reverse, 5'-AGTCAGTTCCTTGTGGAGCC-3'; PTEN forward, 5'-GCTAGCCTCTGGATTTGACG-3', and reverse, 5'-ACCAGGACCAGAGGAAACCT-3'; Akt forward, 5'-TGA AGGTGCCATCATTCTTG-3' and reverse, 5'-ATGAGCGAC GTGGCTATTGT-3'.

Western blotting. Total protein concentration was detected using the BCA method. Protein (20 μg) was subjected to SDS-PAGE, and the gel was then transferred to a nitrocellulose membrane that was blocked in Tris-buffered saline and Tween-20 (TBST) containing 5% skimmed milk for 2 h. Then incubation with primary antibodies against HDAC2 (1:1,000) and β-actin (1:2,000) overnight at 4˚C followed and subsequently with secondary antibodies for 2 h. Finally the membranes were reacted with enhanced chemiluminescent (ECL) reagent in dark for 1-3 min, and then exposed by X-ray film, developed and scanned. The grey values of the target protein bands were analyzed by a UVP gel imaging system.

HDAC2 RNA interference (RNAi) and overexpression analysis. The targeted HDAC2 sequences were, 5'-GCTGGAGCT GTGAAGTTAAAC-3' (forward) and 5'-GTTTAACTTCACA GCTCCAGC-3' (reverse). HepG2 cells were transfected with packaged lentiviruses for interference and overexpression, and divided into an HDAC2 interference group, an HDAC2 overexpression group, a blank control and a negative control group. The cells were collected 48 h after transfection, and HDAC2 mRNA and protein expression were detected by real-time PCR and western blotting, respectively. HDAC2 RNAi or overexpression in combination with $1,25(OH)_2D_3$ treatment were used to determine cell proliferation, the cell cycle and related protein expression. The HDAC2 interference and HDAC2 overexpression groups were collected 48 h after transfection, inoculated into 96- or 6-well plates, and treated with 100 nM $1,25(OH)_2D_3$ for 72 h after adherent growth. Then, the cells were divided into a blank control, a negative control, an HDAC2 interference, an HDAC2 overexpression and a $1,25(OH)_2D_3$ group, and a $1,25(OH)_2D_3$ in combination with HDAC2 overexpression group. The proliferation activity of each group was detected using MTT assay, and the cell cycle was detected by flow cytometry. PTEN,

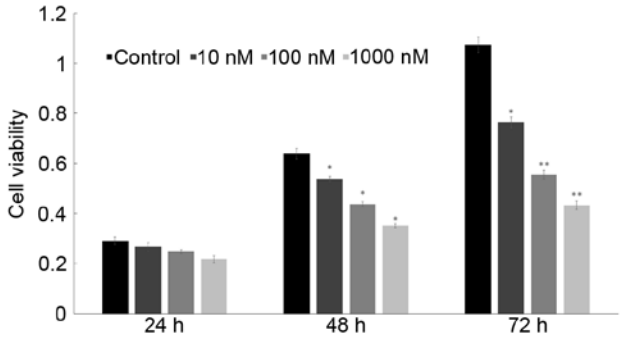

Figure 1. Effects of $1,25(OH)_2D_3$ on the viability of HepG2 cells are detected by MTT assay; $^{*}P<0.05$, $^{**}P<0.01$ vs. the control. Statistical analysis was performed using ANOVA.

PI3K and Akt mRNA and protein expression were detected by real-time PCR and western blotting, respectively.

Statistical analysis. All data were analyzed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA), and expressed as the mean ± standard deviation (mean ± SD). Inter-group mean comparisons were performed by one-way analysis of variance (ANOVA). P<0.05 was considered statistically significant.

Results

Effects of $1,25(OH)_2D_3$ on HepG2 cell proliferation and the cell cycle. An MTT assay (Fig. 1) revealed that after treatment with 10, 100 and 1,000 nM of $1,25(OH)_2D_3$, the 24-h inhibition rates of HepG2 cells were 8.6, 14.8 and 25.1%, respectively, the 48-h ones were 15.9, 31.5 and 45.1%, respectively, and the 72-h ones were 28.8, 48.3 and 59.7%, respectively. Therefore, $1,25(OH)_2D_3$ markedly inhibited the proliferation activity of HepG2 cells in a dose-dependent manner. Since 72 h at 100 nM of $1,25(OH)_2D_3$ treatment significantly inhibited the proliferation, the dose and time were selected thereafter.

$1,25(OH)_2D_3$ downregulates HDAC2 expression in HepG2 cells. To investigate the inhibitory effect of $1,25(OH)_2D_3$ in HDAC-dependant mechanisms, HepG2 cells were treated with different concentrations of $1,25(OH)_2D_3$. The results revealed that HDAC2 mRNA and protein expression levels in the HepG2 cells were dose-dependently and significantly downregulated compared with those in the control group (P<0.05). Therefore, to further confirm the inhibition of HDAC by $1,25(OH)_2D_3$, the acetylation status of histone protein H3 was detected. The expression of the ac-H3 protein was significantly increased (P<0.05), however that of the total H3 protein remained unchanged (Fig. 2).

Expression of PTEN is regulated by HDAC2 in HepG2 cells. Real-time PCR and western blotting revealed that compared with the negative control group, PTEN gene expression in the HDAC2-interference group was significantly upregulated. While PTEN expression was obviously decreased in the HepG2 cells transfected with the pEGFP-LV2-HDAC2 plasmid in comparison to the low-level expression of endogenous PTEN in HepG2 cells. The expression of ac-H3 was substantially increased when HDAC2 was blocked (Fig. 3).

Effects of HDAC2 interference and overexpression in the HepG2 cell cycle. After transfection with interference and overexpression plasmids and 72 h of $1,25(OH)_2D_3$ (100 nM) treatment, significantly more HepG2 cells in the HDAC2 gene interference group were arrested in the G_0/G_1 phase, but fewer cells were in the S phase than those in the negative control

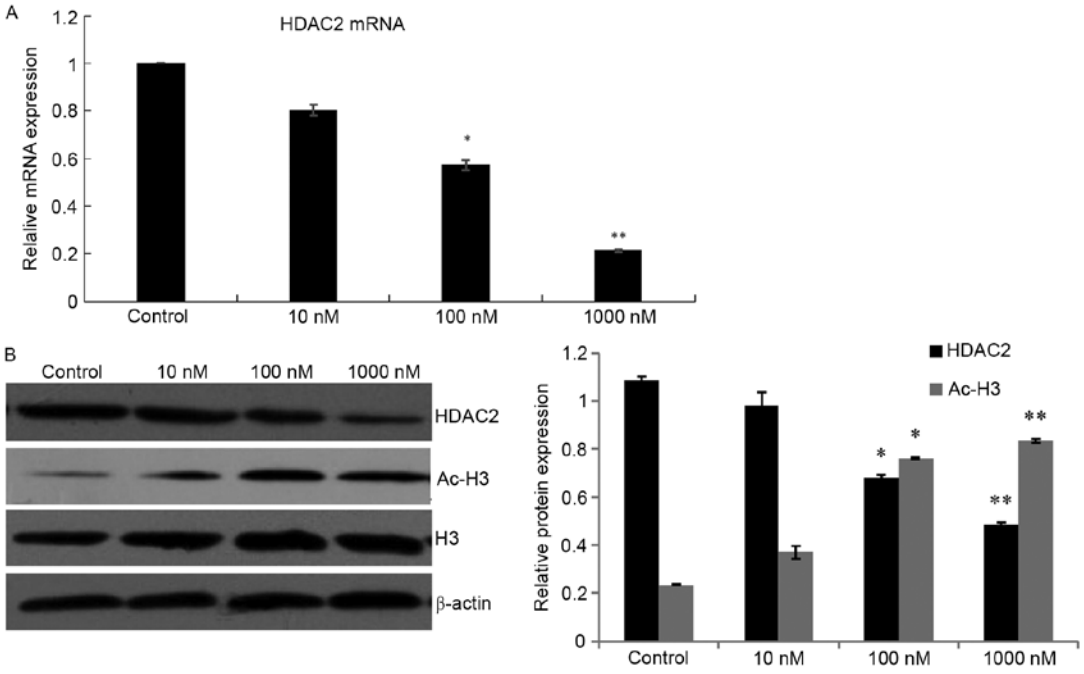

Figure 2. Inhibitory effects of $1,25(OH)_2D_3$ on the expression of H3 and HDAC2 proteins. HepG2 cells were treated with 10, 100 and 1,000 nM $1,25(OH)_2D_3$ for 72 h. (A) HDAC2 mRNA expression was analyzed using real-time PCR. (B) The protein expression levels of HDAC2 and ac-H3 were analyzed using western blotting. Representative images of at least three independent experiments are shown; $^{*}P<0.05$, $^{**}P<0.01$ vs. the control.

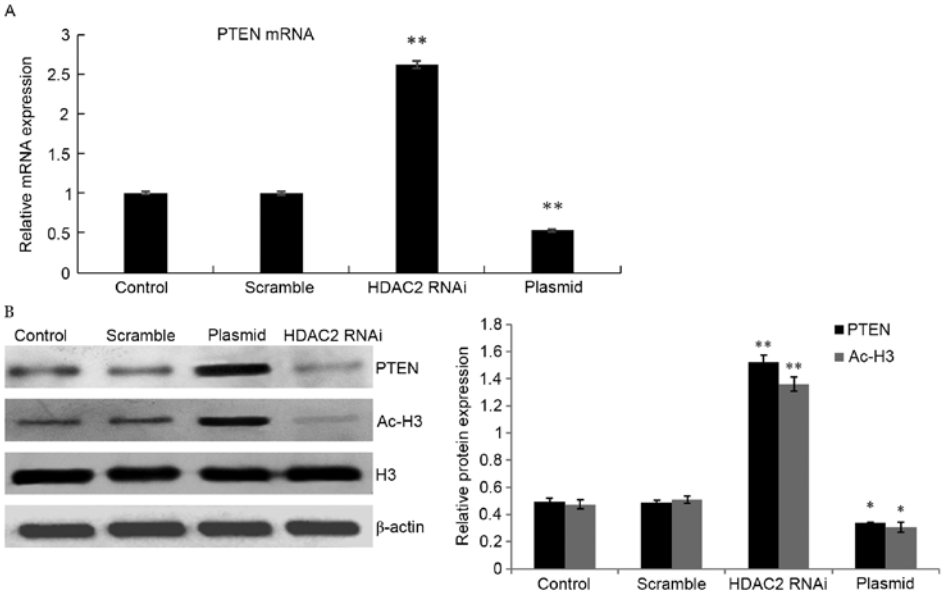

Figure 3. Effects of pEGFP-LV2-HDAC2 plasmid and mimicked HDAC2 knockdown on PTEN and histone H3 expression in HepG2 cells. HepG2 cells were transfected with HDAC2-shRNA and pEGFP-LV2-HDAC2 plasmid for 72 h. (A) PTEN mRNA expression was analyzed by RT-PCR. (B) The protein expression levels of ac-H3 and PTEN were analyzed using western blotting. *P<0.05, **P<0.01 vs. the control and scramble shRNA.

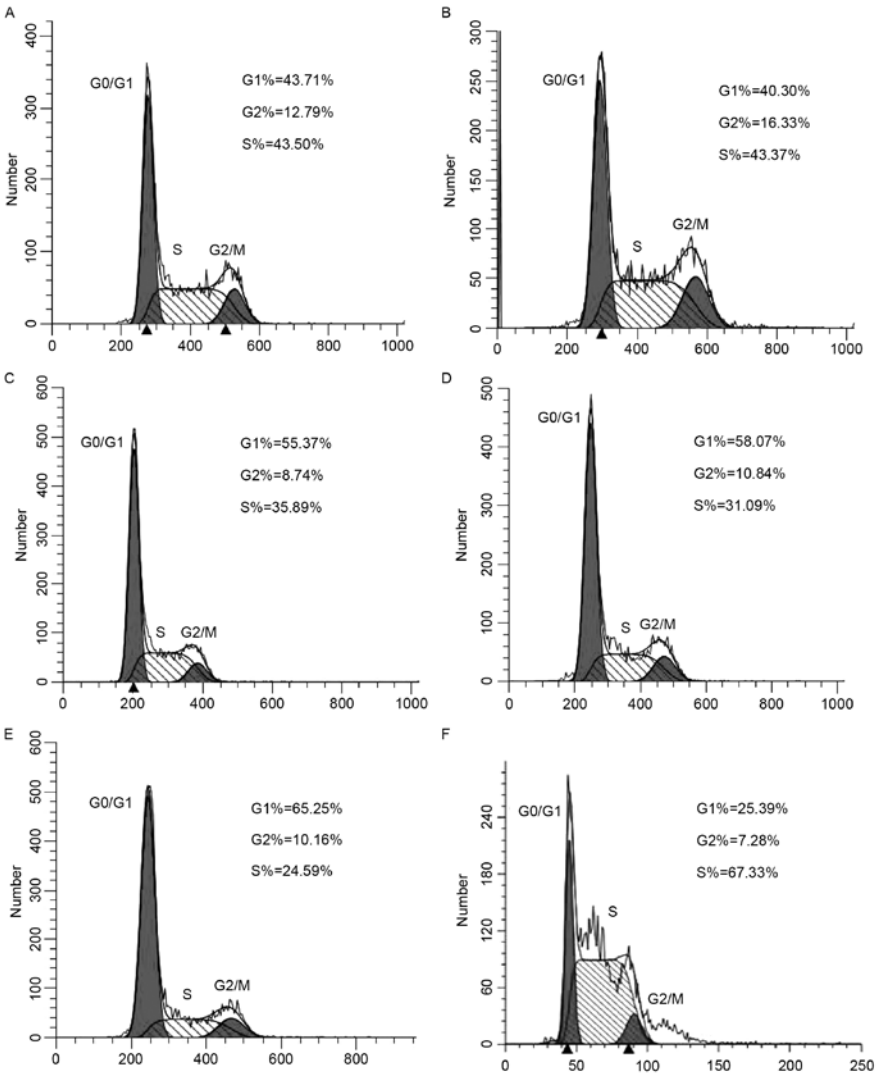

Figure 4. Effects of aberrant expression of HDAC2 on the cell cycle distribution of HepG2 cells. *P<0.05, **P<0.01 vs. the control and scramble shRNA. The groups were (A) control, (B) scramble shRNA, (C) 100 nM of 1,25(OH)$_2$D$_3$ and (D) plasmid, (E) 100 nM of 1,25(OH)$_2$D$_3$ with HDAC2 RNAi and (F) plasmid, respectively.

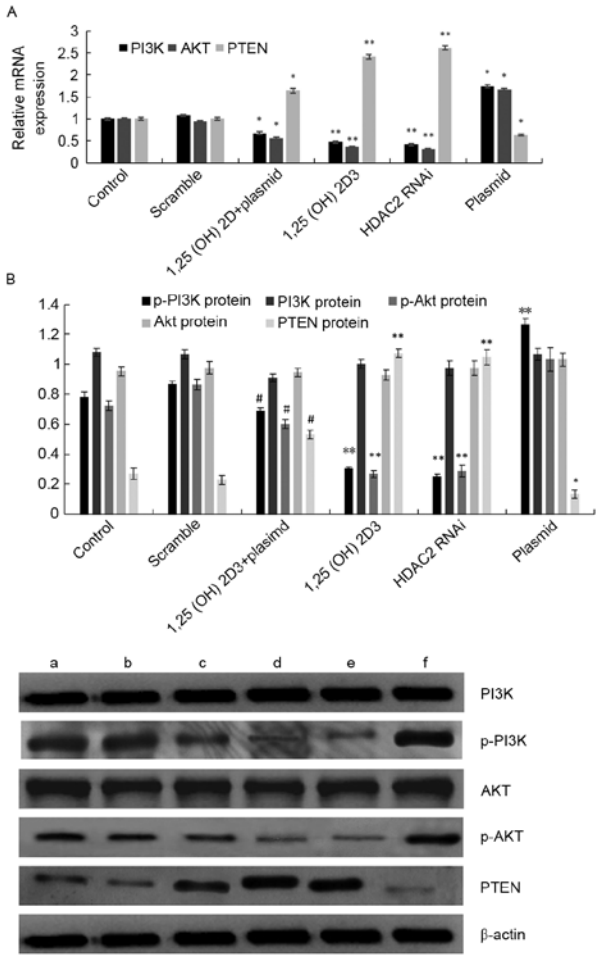

Figure 5. Effects of downregulation of the HDAC2 gene by $1,25(OH)_2D_3$ on PTEN, PI3K and Akt expression in HepG2 cells. HepG2 cells were treated with pEGFP-LV2-HDAC2 plasmid, pEGFP-LV2-HDAC2 plasmid and $1,25(OH)_2D_3$, 100 nM $1,25(OH)_2D_3$, HDAC2 RNAi and PEGFP-LV2-HDAC2 plasmid for 48 h. (A) PI3K, Akt and PTEN mRNA expression levels were analyzed by real-time PCR. (B) Phosphorylation of Akt and PI3k were detected by western blotting. a, Control; b, scramble; c, $1,25(OH)_2D_3$ + plasmid; d, 100 nM $1,25(OH)_2D_3$; e, HDAC2 RNAi; f, plasmid; **P<0.01 vs. the control and vector groups; $^{\#}$P<0.05 vs. pEGFP-LV2-HDAC2 plasmid group.

group (P<0.05) (Fig. 4). The HDAC2 gene overexpression group had exactly the opposite results (P<0.05). Compared with the $1,25(OH)_2D_3$ group, the $1,25(OH)_2D_3$-treated HDAC2 gene overexpression group had significantly fewer cells in the G_0/G_1 phase, but significantly more cells in the S phase (P<0.05). The blank control and negative control groups had similar results (P>0.05).

$1,25(OH)_2D_3$ promotes HDAC-mediated PTEN activation through downregulation of the Akt signaling pathway. To determine whether $1,25(OH)_2D_3$-inhibited cell proliferation is closely related to an Akt signal, we examined the mRNA and protein levels of Akt in cells following $1,25(OH)_2D_3$ treatment for 72 h. Real-time PCR (Fig. 5A) revealed that compared with the negative control group, the $1,25(OH)_2D_3$ and HDAC2-interference groups had significantly higher PTEN mRNA expression levels but significantly lower PI3K and Akt mRNA expression levels (P<0.05). However, compared with the $1,25(OH)_2D_3$ group, the PTEN mRNA expression of the $1,25(OH)_2D_3$-treated HDAC2 gene overexpression group was significantly decreased, whereas the PI3K and Akt mRNA expression levels were significantly increased (P<0.05). Western blotting (Fig. 5B) revealed that the $1,25(OH)_2D_3$ group did not affect the total Akt protein levels. However, Akt phosphorylation decreased in comparison to the control after treatment with $1,25(OH)_2D_3$ for 72 h. The PTEN expression in the HDAC2-knockdown group was upregulated more significantly than that in the scrambled-shRNA group. The expression of phosphorylated Akt and PI3K was markedly decreased in the HDAC2-knockdown group compared with the cells transfected with scrambled shRNA. To further analyze the interactions between $1,25(OH)_2D_3$ and HDAC2 in regulating the activation of the PI3K/Akt signaling pathway, we overexpressed HDAC2 in HepG2 cells and treated them $1,25(OH)_2D_3$. The phosphorylation levels of PI3K and Akt were significantly increased, but that of PTEN was markedly decreased, with unchanged total PI3K and Akt protein expression. Nevertheless, the phosphorylation levels of PI3K and Akt, which decreased after $1,25(OH)_2D_3$ treatment compared with the cells treated with the pEGFP-LV2-HDAC2 plasmid, still exceeded those of control and vector groups (Fig. 5B). Therefore, the PTEN gene may undergo deacetylation which enhances PI3K/Akt activation. Meanwhile, $1,25(OH)_2D_3$ inhibited the activation of Akt and downregulated the expression of phosphorylated Akt.

Discussion

$1,25(OH)_2D_3$ inhibits the proliferation of many types of cells, induces differentiation, promotes apoptosis and regulates various tumor and immune cells (17). In the present study, $1,25(OH)_2D_3$ effectively inhibited the proliferation activity of HCC cells in dose- and time-dependent manners, i.e. the inhibitory effects became more apparent with increasing drug concentration and treatment time. Possibly, $1,25(OH)_2D_3$ activated signal transduction molecules such as protein kinase C, mitogen-activated protein kinase, phospholipase A, protein kinase A and PI3K in VDR-independent manners. As a result, intracellular Ca^{2+} was rapidly changed, and proteins such as Bcl-2 and c-jun were activated or deactivated, ultimately affecting cell proliferation, differentiation and apoptosis (18,19).

As one of the important physiological functions of cells, proliferation, which is regulated by the cell cycle, proceeds by division. There are two key stages in the cell cycle: G_1 to S and G_2 to M. Regulating the two stages is thus of great significance to the in-depth understanding of cell development and growth as well as the control of tumor growth (20). In the present study, the effects of $1,25(OH)_2D_3$ on the cell cycle of HepG2 cells were evaluated by flow cytometry. After being treated with $1,25(OH)_2D_3$, the cells were arrested in the G_0/G_1 phase, accompanied by fewer cells in the S phase. Thus, $1,25(OH)_2D_3$ affected the cell cycle progression of HCC cells, which may partly contribute to the resistance to proliferation.

HDAC2 is a member of the HDACs protein family. Highly expressed in most malignant tumors, it can influence the onset and progression of tumors by regulating genes related to proliferation, cell cycle and apoptosis as well as transcription of oncogenes and anti-oncogenes, as is therefore a popular target for anticancer drug design (21). In the present study, the effects of the HDAC2 gene interference and overexpression on cell proliferation were

assessed by MTT assay. Compared with the control group, the proliferation ability of HepG2 cells was significantly decreased after HDAC2 gene interference. After overexpression of HDAC2 gene, the proliferation of HepG2 cells was significantly enhanced, being consistent with the results of Lee *et al* (22). Thus, HDAC2 played a vital role in regulating the proliferation of HCC cells. In addition, $1,25(OH)_2D_3$ herein downregulated the expression of HDAC2, whereas it enhanced the acetylation level of histone H3, thus we postulated that $1,25(OH)_2D_3$ inhibited the proliferation of HepG2 cells and induced their apoptosis possibly by downregulating HDAC2 gene expression. To confirm this hypothesis, HepG2 cells overexpressing the HDAC2 gene were treated with $1,25(OH)_2D_3$, and the resulting proliferation was detected by MTT assay. Compared with the $1,25(OH)_2D_3$-treated normal HepG2 cells, HDAC2 overexpression significantly weakened the inhibitory effects of $1,25(OH)_2D_3$.

PTEN, is one of the crucial antitumor genes in the post-p53 era. Upregulating PTEN can block the cell cycle and induce apoptosis. Furthermore, PTEN can also inhibit cell proliferation and induce apoptosis by negatively regulating the cell growth signaling pathway PI3K/Akt (4). Acetylation is another mechanism involved in the regulation of PTEN activity, and inhibiting the expression of HDACs can upregulate that of PTEN (23). In the present study, after targeted interference of the HDAC2 gene, the expression levels of both PTEN mRNA and protein were significantly upregulated while those of p-PI3K and p-Akt were downregulated, accompanied by a significantly increased acetylation level of histone H3. Hence, downregulating HDAC2 suppressed the proliferation of HCC cells by effectively inhibiting HDACs, boosting histone acetylation, upregulating the expression of PTEN and inhibiting activation of the downstream Akt signaling pathway, as reported by Zhang *et al* (12). In the present study, $1,25(OH)_2D_3$ increased the PTEN level via the PI3K/Akt signaling pathway, probably being linked to the downregulation of HDAC2. As suggest by our findings, the expression of HDAC2 was negatively correlated with that of PTEN.

In conclusion, we have demonstrated that $1,25(OH)_2D_3$ may have inhibitory effects in HepG2 cell cycle progression by HDAC2-mediated PTEN upregulation and inhibition of the PI3K/Akt signaling pathways. The present study may provide an attractive therapeutic modality for liver cancer.

Acknowledgements

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