1,25(OH)\textsubscript{2}D\textsubscript{3} inhibits the progression of hepatocellular carcinoma via downregulating HDAC2 and upregulating P21(WAF1/CIP1)

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Abstract. Vitamin D, termed 1,25(OH)\textsubscript{2}D\textsubscript{3} in it's active form, activity is associated with a reduced risk of hepatocellular carcinoma (HCC) and is an important immune regulator. However, the detail molecular mechanisms underlying the effects of 1,25(OH)\textsubscript{2}D\textsubscript{3} on the progression of HCC are widely unknown. Histone deacetylase 2 (HDAC2) is usually expressed at high levels in tumors, and its downregulation leads to high expression levels of cell cycle components, including p21(WAF1/Cip1), a well-characterized modulator, which is critical in cell senescence and apoptosis. The present study investigated whether vitamin D inhibits HCC via the regulation of HDAC2 and p21(WAF1/Cip1). Firstly, the toxic concentrations of 1,25(OH)\textsubscript{2}D\textsubscript{3} were determined according to trypan blue and \textsuperscript{3}H\textsuperscript{2}thymidine incorporation assays. Secondly, HCC cells lines were treated with different concentrations of 1,25(OH)\textsubscript{2}D\textsubscript{3}. The expression of HDAC2 was either silenced via short hairpin (sh)RNA or induced by transfection of plasmids expressing the HDAC2 gene in certain HCC cells. Finally the mRNA and protein levels of HDAC2 and p21(WAF1/Cip1) were measured using reverse transcription-quantitative polymerase chain reaction and western blot analyses. The results revealed that 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment reduced the expression of HDAC2 and increased the expression of p21(WAF1/Cip1), in a dose-dependent manner, resulting in the reduction of HCC growth. Elevated levels of HDAC2 reduced the expression of p21(WAF1/Cip1), resulting in an increase in HCC growth. HDAC2 shRNA increased the expression of p21(WAF1/Cip1), resulting in reduction in HCC growth. Thus, 1,25(OH)\textsubscript{2}D\textsubscript{3} exerted antitumorigenic effects through decreasing the expression levels of HDAC2 and increasing the expression of p21(WAF1/Cip1), which inhibited the development of HCC and may indicate the possible underlying mechanism. These results suggest that vitamin D may be developed as a potential drug for effective therapy in the treatment of HCC.

Introduction

Hepatocellular carcinoma (HCC) accounts for 90% of all primary liver malignancies and is resistant to chemotherapy (1,2). The survival rates of patients with HCC is usually <1 year following diagnosis (3). Only 10-20% of patients with HCC are suitable for resective surgery, and the five-year relapse-free survival rate is no more than 20-30% (4). Developments in the techniques used to treat HCC have improved the survival rates of patients with HCC (5), however, this remains far from satisfactory. Therefore, novel effective therapeutic strategies are in high demand.

Epidemiological and clinical studies have shown that the downregulation of vitamin D is associated with an increased risk of various types of cancer; whereas a high level of 1,25(OH)\textsubscript{2}D\textsubscript{3} decreases the risk of cancer (6). Other investigations have also demonstrated that 1,25(OH)\textsubscript{2}D\textsubscript{3} can modulate calcium and skeletal homeostasis, and exert a marked effect on the growth and differentiation of various tissues (7). Further evidence indicates that 1,25(OH)\textsubscript{2}D\textsubscript{3} modulates the bioactivity of various immune cells (8). Of note, protein modification, and the acetylation of histones and non-histone proteins mediates gene expression levels and cell signaling. The removal of acetyl groups from acetylated histones by histone deacetylase 2 (HDAC2) reverses the bioactivity of histone acetyltransferases and returns histones to a basal state, which is followed by suppression of the activities of gene transcription. There is evidence indicating that HDAC2 controls the functions of key proteins associated with the cell cycle (9). Furthermore, the overexpression of HDAC2 is found in various types of cancer, resulting in the deregulation of uncontrolled proliferation (10). HDAC inhibitors have been reported to exert anticancer activities in multiple types of solid and hematologic malignancies (11,12). The expression levels of HDAC2 are enhanced between non-tumor tissues and histopathological grades of HCC (13). Thus, 1,25(OH)\textsubscript{2}D\textsubscript{3}
may exert antitumor activities in HCC via mediating the levels of HDAC2. Aberrant regulation of HDAC2 deletion leads to the upregulation of p21(WAF1/Cip1) (14), which is one of the well-characterized modulators critical in cell senescence and apoptosis (15). Therefore, the present study investigated the effects of 1,25(OH)2D3 on the expression levels of HDAC2 and p21(WAF1/Cip1). It is possible that 1,25(OH)2D3 exhibits antitumor properties against HCC and may be a potential therapeutic agent for the treatment of HCC.

Materials and methods

Cell lines and cell culture. The HpG2 hepatocellular carcinoma cell line was obtained from China Center for Typical Culture Collection (Wuhan, China) and cultured in Dulbecco's modified Eagle's medium (DMEM; Beijing Solarbio, Beijing, China) supplemented with 10% heat-inactivated fetal bovine serum (GE Healthcare Life Sciences, Logan, UT, USA; cat. no. F4135), 100 U/ml penicillin (Macgene Co., Ltd., Beijing, China), and 100 U/ml streptomycin (Macgene Co., Ltd., Beijing, China). During the experiments, 3×104 cells were plated in culture flasks and grown in a humid atmosphere (37˚C; 5% CO2). The cells were cultured with different concentrations (0, 0.001, 0.01, 0.1, 1 and 10 nM) of 1,25(OH)2D3 (Biofriendship, Inc., Beijing, China) or vehicle (dimethyl-sulfoxide).

Cell viability. The viability of the HpG2 cells was measured using a trypan blue uptake assay (Beyotime Institute of Biotechnology, Beijing, China) and a [3H]thymidine incorporation assay (Atom HighTech, Co., Ltd., Beijing, China) following treatment of the cells with different concentrations of 1,25(OH)2D3 (0, 0.001, 0.01, 0.1, 1, 10, 100 and 1,000 nM) (Beyotime, Beijing, China) for 24 h. The numbers of trypan blue-positive cells were calculated among 400 cells in two separate microscopic fields using an optical microscope (CX41; Olympus Corporation, Tokyo, Japan). To avoid bias caused by the disappearance of nonviable cells, the total cell number was counted at different times, with the results presented as the percentage of viable cells, reflecting data collected from four fields.

Short hairpin (sh)RNA constructs for HDAC2 gene silencing. The pT7U6+1 plasmids were provided by Chongqing Medical University (Chongqing, China). According to the principles of shRNA design (16), and using the HDAC2 cDNA sequence, 19-21 nt DNA fragments were designed, which spanned the TTGG insertion sequence, with an identical sequence to that of the HDAC2 gene. The HDAC2 coding sequence and the reverse complementary sequence were designed as follows: siHDAC2, forward 5'-GACTTGCATGC GACCCCTCTTCTGACTGCCATGTGAGCTGCTACAGTCAAGGAGGT GAGTTTTTT-3' and reverse 5'-AGTCTC TAGACTAGAAAAACCTCTTCTGACTGTCCACAGCAATGGGCTG CCTACATGGCGTACAGTCAAGGAGGGG-3'. The Sa/I and XbaI (underlined; Takara Biotechnology Co., Ltd.) restriction sites were added to either end of the oligos for constructing the pT7U6+1-HDAC2 vectors.

shRNA expression levels of HDAC2 and HDAC2. The cells were transfected with 1 µg of the HDAC2 or HDAC2-shRNA expression vector. Following 48 h transfection, Geneticin G418 (Invitrogen; Thermo Fisher Scientific, Inc.) was added to the DMEM (500 µg/ml), and the G418-resistant colonies were selected. The medium was replaced every 2 days. After 3 weeks, the G418-resistant cells were selected and cultured separately.

Reverse transcription-quantitative (RT-qPCR). Total RNA was isolated from the cells using a Takara MiníBEST Universal RNA Extraction kit (Takara Biotechnology Co., Ltd.), according to the manufacturer's protocol. cDNA was amplified using a Transcriptor First Strand cDNA Synthesis kit (Takara Biotechnology Co., Ltd.). RT-qPCR was performed on a qTOWER2 (Analytik Jena AG, Jena, Germany), according to the manufacturer's protocol. The following PCR primers were used: HDAC2 (NCBI Reference Sequence: NM_001527.3), forward 5'-GGT CAGAGGAGGT GAGTTTTTT-3' and reverse 5'-GCACTA GGTTGATACATCTC-3'. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (NCBI Reference Sequence: TGACCTCCTCTTG-3') was used as an internal control.
NM_001289746.1), forward 5'-GATCCCCTCCAAAATCAAGTG-3' and reverse 5'-ATGATCTTGAGCTGT TGTC-3') was used as a loading control for normalizing data.

Two master mixes (HDAC2 and GAPDH) were used for qPCR analysis. Each mix contained 10 µl SYBR Green (Bioline Ltd., London, UK), 0.5 µl of each of primer (100 pm), and 8 µl Aqua Dest distilled water/per well of a 96-well plate (Frame Star Fast Plate 96; 4titude, Surrey, UK). A total of 19 µl master mix and 1 µl cDNA were used, and two replicates for the expression levels of HDAC2 and GAPDH were used for each individual. Consequently, 21 animals were measured per plate, together with 12 non-template controls. These controls contained RNAse-free water instead of cDNA. The plates were sealed using q-stick adhesive for qPCR (4titude) and were centrifuged for a few seconds to ensure all liquid was inside the well (MPS 1000; Labnet, Edison, NJ, USA). Subsequently, the plates were incubated at 95°C for 2 min and run on an Applied Biosystems 7500 Real-Time PCR system (Invitrogen; Thermo Fisher Scientific, Inc.) in 45 cycles of 5 sec at 95°C, followed by 30 sec at 60°C and 30 sec at 72°C. The mRNA expression levels were normalized against the levels of GAPDH.

Western blot analysis. The cells were lysed using radioimmunoprecipitation assay buffer (Shanghai Sigma High-Tech Co., Ltd., Shanghai, China), containing 20 mM TrisHCl (pH 8.0), 100 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin and 0.5% sodium orthovanadate. Polyacrylamide gels (10%) for SDS-PAGE (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were used for the separation of protein samples. Following SDS-PAGE, the proteins were transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Following the blotting, the membranes were blocked with 5% skim milk in Tris-buffered saline with Tween 20 (TBS-T) for 1 h at room temperature. The membranes were incubated with rabbit anti-human anti-HDAC2 antibody (1:1,000; cat. no. SC-7872; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4°C. Following three washes with TBS-T, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (1:10,000; cat. no. G21234; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. Signals were measured using chemiluminescent HRP substrate (EMD Millipore). The relative expression of HDAC2 was measured using Image J software (Windows version 1.49; National Institutes of Health, Maryland, MD, USA).

Cell growth assay. The HpG2 cells (1x10⁴) were cultured in 24-well plates with DMEM, with or without 1,25-(OH)₂D₃. The number of viable cells were examined by adding 2 mg/ml 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (Sigma-Aldrich, St. Louis, MO, USA) in each well for 3 days consecutively. The medium was discarded after 2 h, and the formazan crystals were dissolved in 100 µl dimethyl-sulfoxide/per well. The absorbance values were read at 590 nm using a microplate reader (WD-2102A; Beijing Liuyi Instrument Factory, Beijing, China).

Statistical analysis. All experiments were performed three times and all samples were measured in triplicate. The results are presented as the mean ± standard deviation. The statistical difference between groups was examined using Student's t-test. Statistical comparisons were made using a two-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference. All data were analyzed using the SPSS 20.0 software package (IBM SPSS, Armonk, NY, USA).

Results

Effects of 1,25-(OH)₂D₃ toxicity on HpG2 cells. To avoid the presence of cytotoxic effects of 1,25-(OH)₂D₃ on the HCC cells, the concentration of 1,25-(OH)₂D₃ was investigated prior to performing experiments to assess its effects. Exposure of the HpG2 cells to 1,25-(OH)₂D₃, in the presence of >1,000 nM, resulted in loss of cell viability, as observed by the uptake of trypan blue (P<0.05). At concentrations <100 nM, the cell viability was >90% (Fig. 1). These effects of 1,25-(OH)₂D₃ were not noted immediately following addition. The percentage viabilities of the cells in the 1,25-(OH)₂D₃ groups were 100, 98 and 96%, compared with the controls, after 6, 12 and 24 h culture, respectively (Fig. 1). Toxicity was also evaluated by examining the ability of cells to incorporate [³H]thymidine into DNA following exposure to different concentrations of 1,25-(OH)₂D₃. As shown in Fig. 1B, this method was a more sensitive index of cell injury. A significant reduction in [³H]thymidine uptake due to a significant increase in cell death was observed in the presence of 1,25-(OH)₂D₃. These results indicated that 1,25-(OH)₂D₃ was toxic to HpG2 cells.
was measured at concentrations >100 nM (P<0.05). Based on these results, the lower concentrations (0.001, 0.01, 0.1, 1 and 10 nM) of 1,25(OH)$_2$D$_3$ were used for the subsequent culture of HpG2 cells.

**Treatment with 1,25(OH)$_2$D$_3$ reduces the mRNA levels of HDAC2 and increase the levels of p21(WAF1/Cip1).** To understand the mechanism underlying the antitumor activity of 1,25(OH)$_2$D$_3$, **in vitro**, the mRNA levels of HDAC2 and p21(WAF1/Cip1) were investigated following treatment with 1,25(OH)$_2$D$_3$. The results showed that 1,25(OH)$_2$D$_3$ treatment reduced the mRNA levels of HDAC2 and increased the levels of p21(WAF1/Cip1) (Fig. 2). The mRNA level of HDAC2 was significantly enhanced when the HpG2 cells were transfected...
with pCDNA3.1-HDAC2. In addition, the mRNA level of p21(WAF1/Cip1) decreased significantly, compared with the cells without HDAC2 transfection. The changes in the levels of HDAC2 and p21(WAF1/Cip1) were also marginally affected by 1,25(OH)₂D₃ in a dose-dependent manner, according to the logistic regression (Fig. 2). By contrast, the mRNA expression...
levels of HDAC2 were almost completely absent when the HpG2 cells were transfected with HDAC2 shRNA, whereas the mRNA expression levels of p21(WAF1/Cip1) were significantly increased, compared with the cells without HDAC2 shRNA transfection. In addition, the change in the expression levels of HDAC2 and p21(WAF1/Cip1) were affected by 1,25(OH)$_2$D$_3$ in a dose-dependent manner, according to the logistic regression (Fig. 2). These results suggested that 1,25(OH)$_2$D$_3$ affected the development of HCC, predominantly by regulating the mRNA levels of HDAC2, which mediated the mRNA level of p21(WAF1/Cip1).

Treatment with 1,25(OH)$_2$D$_3$ reduces the protein levels of HDAC2 and increases the protein levels of p21(WAF1/Cip1). To further investigate the mechanism underlying the antitumor activity of 1,25(OH)$_2$D$_3$, the protein expression levels of HDAC2 and p21(WAF1/Cip1) were investigated following treatment with 1,25(OH)$_2$D$_3$. The results showed that 1,25(OH)$_2$D$_3$ treatment reduced the protein levels of HDAC2 and increased the protein levels of p21(WAF1/Cip1; Fig. 3). The protein expression of HDAC2 was significantly enhanced when the HpG2 cells were transfected with pCDNA3.1-HDAC2, whereas the protein expression of p21(WAF1/Cip1) was decreased significantly, compared with that in the cells without HDAC2 transfection. In addition, the changes in the levels of HDAC2 and p21(WAF1/Cip1) were also marginally affected by 1,25(OH)$_2$D$_3$ in a dose-dependent manner, according to the logistic regression (Fig. 3). By contrast, the protein expression of HDAC2 was almost completely eliminated when the HpG2 cells were transfected with HDAC2 shRNA. The protein levels of p21(WAF1/Cip1) were also significantly increased, compared with those in the cells without HDAC2 shRNA transfection, and the changes in the levels of HDAC2 and p21(WAF1/Cip1) were affected by 1,25(OH)$_2$D$_3$ in a dose-dependent manner, according to the logistic regression (Fig. 3). These results suggested that 1,25(OH)$_2$D$_3$ affected the development of HCC, predominantly by regulating the protein levels of HDAC2, which mediated the expression of p21(WAF1/Cip1).

1,25(OH)$_2$D$_3$ treatment significantly reduces the growth rate of HCC. To examine the antitumor effects of 1,25(OH)$_2$D$_3$ in HCC, the present study examined its antiproliferative effects in the human HpG2 HCC line. The cells were incubated with different concentrations of 1,25(OH)$_2$D$_3$ for 3 days and
the status of cell growth was measured. Consistent with previous evidence (17), cell growth in the 1,25(OH)\textsubscript{2}D\textsubscript{3} group was decreased in a dose-dependent manner, compared with the control group. The growth curve line (Fig. 4A) showed a significant proliferation inhibitory effect of 1,25(OH)\textsubscript{2}D\textsubscript{3}, which occurred in a dose-dependent manner.

The average growth rate of the pCDNA3.1-HDAC2-transfected HpG2 cells was increased, compared with the untransfected cells (Fig. 4). Additionally, expression of HDAC2 in the HpG2 cells reversed the inhibitory effect of 1,25(OH)\textsubscript{2}D\textsubscript{3} (Fig. 4). The average growth rate was also enhanced in the cells treated with a high concentration of 1,25(OH)\textsubscript{2}D\textsubscript{3} (10 nM/day). Of note, there was difference in growth rate between the cells treated with 1,25(OH)\textsubscript{2}D\textsubscript{3} and those without treatment when the expression of HDAC2 was induced by transfection (Fig. 4), although the inhibitory effects of HDAC2 remained, according to the logistic regression. Following shRNA transfection, the average growth rate of the HpG2 cells was reduced. Additionally, HDAC2 shRNA transfection of the HpG2 cells had a similar function as the inhibitory effect of 1,25(OH)\textsubscript{2}D\textsubscript{3} (Fig. 4). The average growth rate was also decreased, although the cells was not treated with high concentration of 1,25(OH)\textsubscript{2}D\textsubscript{3}. Of note, there was a difference in growth rate between the cells treated with 1,25(OH)\textsubscript{2}D\textsubscript{3} and the untreated cells when HDAC2 was silenced (Fig. 4), although the inhibitory effects of HDAC2 remained, according to the logistic regression.

Discussion

At present, epidemiological evidence indicates that decreased concentrations of vitamin D are associated with an enhanced risk of various types of cancer (18). Although previous studies have indicated that 1,25(OH)\textsubscript{2}D\textsubscript{3} inhibits the proliferation of HCC in a dose dependent manner (19), the detailed molecular mechanism underlying the function of 1,25(OH)\textsubscript{2}D\textsubscript{3} in preventing the progression of HCC remains to be fully elucidated.

Prior to commencement of the present study, the potential toxicity of high concentrations of 1,25(OH)\textsubscript{2}D\textsubscript{3} on HCC cells were examined. As observed inhibitory effects may be caused by toxicity, rather than by other molecular mechanisms, the toxicity of 1,25(OH)\textsubscript{2}D\textsubscript{3} was first measured using trypan blue and \([^3]H\)thymidine incorporation assays. As expected, the viability of the HCC cells was significantly affected when the concentration of 1,25(OH)\textsubscript{2}D\textsubscript{3} was >100 nM (Fig. 1). Therefore, the concentration used in the subsequent experiments was ≤10 nM. This ensured that any inhibitory effects were not caused by toxicity, but by other molecular mechanisms.

To investigate the molecular mechanism underlying the inhibitory effect of 1,25(OH)\textsubscript{2}D\textsubscript{3} in HCC, the present study transfected HCC cells with HDAC2. The resulting high expression levels of HDAC2 led to a significant reduction in the inhibitory effects of 1,25(OH)\textsubscript{2}D\textsubscript{3} on the HCC cells (Fig. 4). In addition, the expression of p21(WAF1/Cip1) was reduced. By contrast, when the HCC cells were transfected with HDAC2 shRNA, leading to silencing of the HDAC2 gene, similar inhibitory effects on HCC cell growth were observed in the cells, which were not treated with 1,25(OH)\textsubscript{2}D\textsubscript{3}.

Furthermore, exogenous 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment decreased the expression levels of HDAC2 and enhanced the expression levels of p21(WAF1/Cip1) in a dose-dependent manner. Therefore, 1,25(OH)\textsubscript{2}D\textsubscript{3} inhibited the development of HCC via the downregulation of HDAC2, which mediated the levels of p21(WAF1/Cip1).

There is increasing evidence reinforcing the hypothesis that excessively and chronically expressed HDAC2 contributes to the development of HCC (13,14). HDAC2 is a major deacetylase, and its expression is inversely associated with changes in the acetylation state of histone H4K5. RNA interference-mediated gene silencing of HDAC2 leads to hyperacetylation of histone H4 and a developmental delay, although the levels of HDAC3 remain high (20,21). Increased expression levels of p21(WAF1/Cip1) may contribute to the observed developmental delay. HDAC2 regulates a number of biological processes in cells. Although HDAC2 inhibits the growth of the liver in aged mice, the levels of HDAC2 are also increased in HCC (10). Increased levels of HDAC2 lead to its interaction with p21(WAF1/Cip1) via the Sp1 binding sites of the p21(WAF1/Cip1) gene promoter, and inhibition of acetylated histone H3 on these sites (22). Histone H3 is a conserved protein in the nucleus, which can be readily modified post-translationally, and the state of acetylation shows clinical diagnostic significance in HepG2 cells. The levels of acetylated peptides are associated with malignant transformation in the liver (23). The results of the present study indicated that 1,25(OH)\textsubscript{2}D\textsubscript{3} inhibited HDAC2, and its downregulation was involved in the upregulation of p21(WAF1/Cip1), which resulted in the inhibition of HCC proliferation.

Although 1,25(OH)\textsubscript{2}D\textsubscript{3} has potent inhibitory effects in several liver diseases (24,25), the molecular mechanism underlying the inhibitory effect of 1,25(OH)\textsubscript{2}D\textsubscript{3} in HCC development remains to be fully elucidated. The present study demonstrated the novel finding that downregulating the expression of HDAC2 resulted in an increase in the expression of p21(WAF1/Cip1), which eventually inhibited HCC development. HDAC2 ablation suppressed HCC development and enhanced the expression of p21(WAF1/Cip1), which was similar to the inhibitory effects of 1,25(OH)\textsubscript{2}D\textsubscript{3} on HCC (Figs. 2-4). In addition, elevated levels of HDAC2 promoted the development of HCC and decreased the activity of p21(WAF1/Cip1), which reversed the inhibitory effects of 1,25(OH)\textsubscript{2}D\textsubscript{3} on the development of HCC, even when the cells were treated with a high dose of 1,25(OH)\textsubscript{2}D\textsubscript{3}. Treatment with 1,25(OH)\textsubscript{2}D\textsubscript{3} led to the downregulation in the expression of HDAC2, which mediated the level of p21(WAF1/Cip1). Further investigations of the effects of 1,25(OH)\textsubscript{2}D\textsubscript{3} on hepatocarcinogenesis may provide further insights into the detailed molecular mechanism underlying vitamin D in antitumor activity. The results of the present study support the potential development of 1,25(OH)\textsubscript{2}D\textsubscript{3} as an effective drug for the treatment of HCC.

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Aspirin minimized delayed and prolonged histone hyperacetylation with hepatic arterial remodeling. Revisiting liver disease.

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