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Research paper

$1,25(OH)_2D_3$ induced apoptosis of human hepatocellular carcinoma cells *in vitro* and inhibited their growth in a nude mouse xenograft model by regulating histone deacetylase 2

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ABSTRACT

The aim of this study was to evaluate the effects of $1,25(OH)_2D_3$ on the apoptosis of human hepatocellular carcinoma (HCC) cells. The effects of $1,25(OH)_2D_3$ on the proliferation and apoptosis of human HCC cells by regulating histone deacetylase 2 (HDAC2) were assessed by MTT assay and flow cytometry. The effects of $1,25(OH)_2D_3$ on the expressions of related proteins in the apoptosis pathway by regulating HDAC2 as well as the mechanism were studied by Western blot and quantitative real-time PCR. A nude mouse model of HCC xenograft was established. A control group, a $1,25(OH)_2D_3$ treatment group, a $1,25(OH)_2D_3$ + HDAC2 overexpression group, an HDAC2 interference group and an HDAC2 overexpression group were set. The tumor volume was recorded, and histopathological changes were observed by HE staining. $1,25(OH)_2D_3$ inhibited the proliferation of HepG2 cells and induced their apoptosis. Overexpression of HDAC2 attenuated the inhibitory effects of $1,25(OH)_2D_3$ on the proliferation of HepG2 cells and its ability to induce apoptosis. In the $1,25(OH)_2D_3$ + HDAC2 overexpression group, the expressions of p53, Bax, DR5 and caspase 8 were significantly lower but the expression of Bcl-2 was significantly higher than those of the $1,25(OH)_2D_3$ treatment group ($P < 0.05$). Compared with the control group, $1,25(OH)_2D_3$ treatment and HDAC2 interference groups had significantly decreased tumor volumes and promoted apoptosis of HCC cells in tumor tissues. Overexpression of HDAC2 weakened the inhibitory effects of $1,25(OH)_2D_3$ on tumor volume and its ability to induce apoptosis in tissues. A large area of tumor cells underwent necrosis in $1,25(OH)_2D_3$ treatment and HDAC2 interference groups. In the $1,25(OH)_2D_3$ + HDAC2 overexpression group, both the area of necrosis and cell volume decreased. In conclusion, $1,25(OH)_2D_3$ inhibited the proliferation of HCC cells and induced their apoptosis by down-regulating the expression of HDAC2, up-regulating p53, and regulating its downstream mitochondria-mediated pathway and the exogenous DR-mediated pathway.

1. Introduction

Hepatocellular carcinoma (HCC) severely threatens human health worldwide. The mortality rate of HCC in China accounts for 50% of the global one. At present, surgery is still an effective strategy for increasing the survival rate of HCC, but low resection rate together with high recurrence and metastasis rates mainly affect the clinical treatment outcomes [1,2]. Therefore, it is urgent to find better prevention, diagnosis and treatment methods for HCC.

In recent years, epigenetic regulation abnormalities, which play an important role in the onset and progression of tumors, have gradually attracted attention. By regulating the level of histone acetylation, the transcription of related genes can be modulated to induce tumor cell apoptosis or differentiation, finally treating tumors [3–9]. Histone deacetylase 2 (HDAC2) is a member of the HDACs protein family, which is highly expressed in most malignancies. As a popular target for antitumor drug design, HDAC2 can regulate genes related to cell proliferation, cycle and apoptosis, as well as transcription of oncogenes and anti-oncogenes, affecting the onset and progression of tumors [10]. Also, HDAC2 can induce cell

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apoptosis through exogenous DR-mediated apoptosis and endogenous mitochondria-mediated apoptosis. Schüler et al. found that in pancreatic cancer cells with HDAC2 knockout, TRAIL receptor DR5 significantly increased, and Bcl-2 expression was up-regulated, inducing apoptosis. The release of cytochrome C to the cytoplasm induced by mitochondrial membrane permeation plays a key role in endogenous mitochondria-mediated apoptosis, which is mainly regulated by Bcl-2 family proteins. HDAC2 evidently affects this pathway by regulating the activity of p53 protein [3,11–15].

Over the past decades, vitamin D has been studied mainly regarding the regulatory effects on calcium and phosphorus metabolisms. In recent years, vitamin D has been proven to inhibit the proliferation of various types of cells, to induce apoptosis and differentiation, and to regulate functions of the immune system [16,17]. Although the antitumor effects of vitamin D on a variety of HCC cells have been well-documented [18,19], whether its deficiency is related to the onset and progression of HCC or the specific mechanism remains unclear. We have previously reported that $1,25(OH)_2D_3$ significantly inhibited the proliferation of human HCC HepG2 cells and down-regulated the expression of HDAC2 [20]. Thus, we postulated that $1,25(OH)_2D_3$ induced apoptosis by down-regulating HDAC2 and regulating the DR-mediated pathway and/or the mitochondria-mediated pathway. The aim of this study was to evaluate the effects of $1,25(OH)_2D_3$ on the apoptosis of human HepG2 cells and its antitumor activity against HepG2 cell xenograft in a nude mouse model, and to explore the mechanism by which cell apoptosis was induced.

2. Materials and methods

2.1. Materials

$1,25(OH)_2D_3$ was purchased from Sigma (USA). Rabbit anti-human Bcl-2, rabbit anti-human Bax and mouse anti-human DR5 monoclonal antibodies were bought from Abcam (USA). Rabbit anti-human p53 and caspase 8 antibodies were obtained from Cell Signaling Technology (USA). Total RNA extraction kit, SYBR® Premix Ex TaqTM kit and PrimeScript® RT reagent kit were provided by Takara (Japan). Annexin V/PI apoptosis detection kit was purchased from BD (USA). RIPA effective protein lysis buffer was bought from Beijing Solarbio Science & Technology Co., Ltd. (China). Horseradish peroxidase-labeled goat anti-rabbit secondary antibody was obtained from Beijing Biosynthesis Biotechnology Co., Ltd. (China). BCA protein quantification kit was provided by Shanghai Generay Biotech Co., Ltd. (China).

2.2. Cell culture and viability assay

HepG2 cells were purchased from ATCC (USA). They were cultured in high-glucose DMEM containing 5% fetal bovine serum and penicillin/streptomycin (100 U/ml), and placed in a 37 °C incubator with 5% CO_2. Cell apoptosis was detected with an MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay kit, and the absorbance was measured by a microplate reader at 490 nm.

2.3. RNA interference and plasmid construction

siRNA oligonucleotide sequences were synthesized by Shanghai Genechem Co., Ltd. (China) as: FW-5′-GCTGGAGCTGTGAAGTTAAAC-3′, REV-5′-GTTTAACTTCACAGCTCCAGC-3’. They were then ligated with lentivirus GV248 to construct a lentivirus plasmid LV-HDAC2-RNAi. HDAC2 overexpression vector was constructed by Shanghai Genechem Co., Ltd. (China).

2.4. Detection of cell apoptosis by flow cytometry

HepG2 cells in the logarithmic growth phase were inoculated into 12-well plates at the density of 2×10^5/well and observed 24 h later. After the confluence reached over 80%, they were starved in serum-free DMEM for 24 h, added $1,25(OH)_2D_3$ at the final concentration of 100 nM, cultured for 72 h, digested by trypsin, collected and centrifuged at 1000 r/min for 5 min. The supernatant was discarded. Then the cells were washed twice to three time by PBS, resuspended by adding 400 μL of 1 × binding buffer, stained with 5 μL of annexin V-FITC in dark at 4 °C for 15 min, and finally stained with 10 μL of PI solution in dark at 4 °C for 5 min. Cell apoptosis was detected by a flow cytometer.

2.5. Quantitative real-time PCR

Total RNA was extracted from HepG2 cells according to the

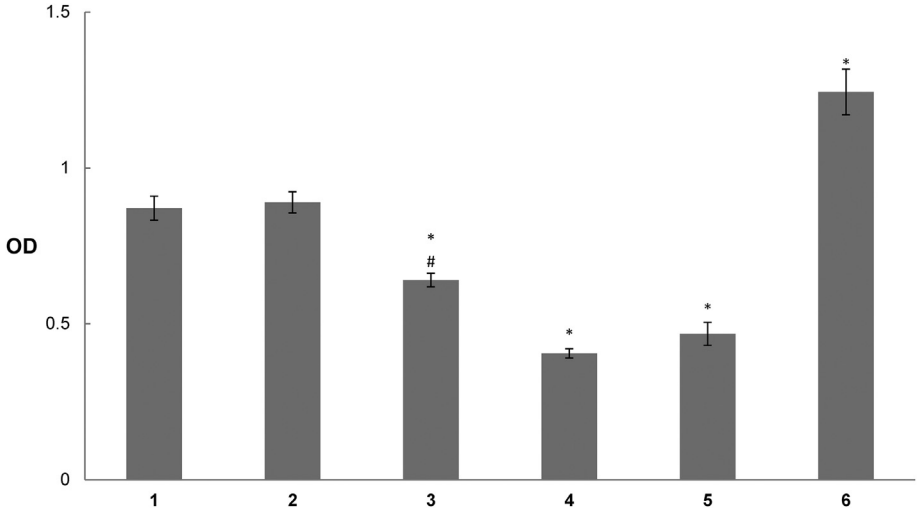

Fig. 1. Proliferation activities of different groups of HepG2 cells detected by MTT assay. *P < 0.05 versus control group, #P < 0.05 versus $1,25(OH)_2D_3$ treatment group. 1: Blank control group; 2: negative control group; 3: $1,25(OH)_2D_3$ + HDAC2 overexpression group; 4: $1,25(OH)_2D_3$ treatment group; 5: HDAC2 interference group; 6: HDAC2 overexpression group.