

## Original Article

# Effects of calcitriol on the growth of PANC-1 cell line with vitamin D receptor (VDR) gene silencing

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Received November 28, 2015; Accepted August 13, 2016; Epub October 15, 2016; Published October 30, 2016

**Abstract:** Treatment with calcitriol or its analogues may be a potential effective chemotherapy for pancreatic cancer. Vitamin D Receptor (VDR) plays an important role in antitumor. However, the precise function and mechanism of VDR in Pancreatic cancer remain undefined. To address this question, we investigated the effects of calcitriol on PANC-1 cell line with VDR gene silencing or not. We found evidence that calcitriol could induce growth inhibition, block the G1/S transition and result in apoptosis in PANC-1 cell line. But those effects were weakened in the VDR gene silencing group. Caspase-3, p21 and p27 levels were noticeably up-regulated after treatment with calcitriol, while Bax/Bcl-2 ratio changed not obviously. In VDR gene silencing groups, caspase-3 expression was equally up-regulated after treatment with calcitriol, but not obviously; Bax/Bcl-2 ratio declined after treatment with calcitriol; p21 and p27 levels were down-regulated. These results were further confirmed at the mRNA levels. Therefore, our results revealed that calcitriol exerted anticancer effects may be mainly via VDR pathway. The VDR gene expression may have a close relationship with the expression of cycle and apoptosis-related molecules, like caspase-3, p21 and p27. However, further precise antitumor mechanisms of calcitriol still need to be elucidated.

**Keywords:** Calcitriol, apoptosis, VDR, cell cycle, pancreatic cancer

## Introduction

Pancreatic carcinoma is the fourth leading cause of cancer death and one of the most deadly malignancies worldwide [1]. The high case-fatality rate of pancreatic cancer is due to the extremely poor prognosis, the absence of specific symptoms and the less effective response to conventional therapy. The 5-year relative survival rate of pancreatic cancer patients is only about 5%, and the median survival time after diagnosis is about 6 months [2, 3]. In the present, surgical resection of pancreatic cancer is still the most effective treatment, but only 15%-20% of the cases are resectable [4, 5]. Alternatively, radiotherapy and chemotherapy are utilized to treat pancreatic cancer in high proportion of patients. However, neither radiotherapy nor chemotherapy substantially improve 5-year survival rates [6-8]. Thus, novel effective therapeutic modalities are critical to treat those nonsurgical patients. There has been rising evidence showed that activated

vitamin D and its analogues are effective agents controlling cellular differentiation, proliferation and apoptosis in many different kinds of cancers [9-11].

Vitamin D<sub>3</sub> and its analogues coupled with their low toxicity, have been considered to be a therapeutic agent in the treatment of cancers, including pancreatic cancer [12]. The vitamin D receptor (VDR) mediating the biological activity has been identified in malignant tissue [13-15]. Furthermore, It also has been demonstrated that pancreatic cancer cells which express the VDR responded to the treatment with these agents as a general phenomenon [16, 17]. But high and systematically administered doses treatment with vitamin D<sub>3</sub> always leads to a risk of hypercalcemia which delay their clinical application [18]. Previous studies indicated that some new analogues are efficient in reducing pancreatic cancer cell growth without inducing hypercalcemia [19, 20]. However, to be used as therapy, the precise drug action mechanism

is still unclear. Previous studies show that Vitamin D<sub>3</sub> and its analogues controlling the cellular differentiation and proliferation of some pancreatic cancer cell lines is associated with G<sub>1</sub> phase cell cycle arrest [19]. Some reports also suggest that the differentiation-inducing activity of vitamin D has a close relationship with the signaling system of cell cycle-regulating agents, such as retinoblastoma protein and p21/27 protein [17, 21-23]. Accumulating evidence demonstrates that Vitamin D<sub>3</sub> and its analogues induced apoptosis might be mainly involved in the mitochondria-mediated pathway [11, 24, 25]. This pathway is triggered by Bak or Bax and inhibited by Bcl-2 and its family members. Calcitriol, an active form of vitamin D<sub>3</sub>, has been declared to induce differentiation and inhibit the proliferation of various types of cancer cells [26-29]. Rely on these premises, we supposed to determine the growth inhibitory effects of calcitriol on pancreatic cancer cells and to clarify the possible relationships between VDR gene expression. To clarify the precise effects, we studied PANC-1 cell line which is one type of pancreatic cancer cell lines.

The aim of the present study was to investigate the antitumor activity and mechanisms of calcitriol on PANC-1 cell line and to clarify their possible relationship between VDR gene expression.

## Materials and methods

### *Cell line and reagents*

Human pancreatic cancer cell line PANC-1 was purchased from Shanghai Institutes for Biological Sciences. PANC-1 was maintained in RPMI 1640 (Gibco) containing 10% FBS (Hyclone) and incubated at 37°C in 5% CO<sub>2</sub> humidified incubator. Revert Aid First Strand cDNA Synthesis Kit was obtained from Fermentas (Thermo Fisher Scientific Inc, Waltham, USA). Antibodies against Bcl-2 (sc492), Bax (sc6236) and caspase-3 (sc7148) were purchased from Santa Cruz (Santa Cruz, CA, USA). Antibodies against p21 (2947) and p27 (3688) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against VDR (ab8756) were purchased from Abcam (Cambridge, MA, USA). Calcitriol Injection was bought from Abbott Laboratories Limited (Canada). Lipofectamine 2000, and TRIzol were purchased from Invitrogen (Carlsbad, CA, USA).

### *Cell proliferation and apoptosis analysis*

The cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT; Cell Proliferation Kit I) assay according to the manufacturer's protocol (Roche, Mannheim, Germany). Briefly, 5×10<sup>3</sup> cells were plated in 96-well plates in growth media for 24 h before the serum starvation procedure. After starving with the serum-free medium (SFM) for 24 h, cells were treated with different concentrations of calcitriol for 24 h, 48 h, and 72 h as indicated. Then, cells were co-incubated with MTT reagent (5 mg/ml) for approx. 4 h. The reaction was stopped by the addition of acidified sodium dodecyl sulfate (10% SDS) buffer and the plates were incubated overnight in the humidified atmosphere (37°C, 5% CO<sub>2</sub>). Cell viability was determined on a plate reader (BioTek Instruments, USA) by measuring the absorbance (test wavelength 595 nm, reference wavelength 650 nm). The percentages of cell viability compared with control group cells were considered as cell growth proliferation rates.

### *VDR gene silencing*

All the siRNA molecules were chemically synthesized and purchased from Shanghai GenePharma (Shanghai, China). Three siRNA targeting to VDR was designed according to the characterization of siRNA by Shanghai GenePharma. siRNA VDR-homo-1103 (sense: 5'-CCU GCU CAG AUC ACU GUA UTT-3' and antisense: 5'-AUA CAG UGA UCU GAG CAG GTT-3'), siRNA VDR-homo-1346 (sense: 5'-GUG CCA UUG AGG UCA UCA UTT-3' and antisense: 5'-AUG AUG ACC UCA AUG GCA CTT-3'), and siRNA VDR-homo-643 (sense: 5'-CCA CUG GCU UUC ACU UCA ATT-3' and antisense: 5'-UUG AAG UGA AAG CCA GUG GTT-3') targeted to human VDR mRNA sequences, respectively. Negative control duplexes of siRNA (sense: 5'-UUC UCC GAA CGU GUC ACG UTT-3' and antisense: 5'-ACG UGA CAC GUU CGG AGA ATT-3') did not target any known mammalian genes. Transfection was performed with Lipofectamine 2000 according to the manufacturer procedures.

### *Morphological studies of apoptosis*

Following transfection for 24 h, PANC-1 cells in logarithmic growth were seeded in 24-well plates by density of 5×10<sup>4</sup>/ml. Calcitriol treatment group (50 nmol/L) and control group were

**Table 1.** Prime pairs used in Real-time-PCR

Genes	Primers (5'-3')	Product Size (BP)
VDR	Forward	ATCTGTGGGGTGTGTGGAGAC
	Reverse	GAATAGTGCCTTCCGCTTCAT
Bcl-2	Forward	ATGTGTGTGGAGAGCGTCAAC
	Reverse	AGACAGCCAGGAGAAATCAAAC
Bax	Forward	GGTTGTGCGCCCTTTTCTACTTT
	Reverse	GTGAGGAGGCTTGAGGAGTCT
Caspase-3	Forward	TCCTTTTCCTTTGACGCTACTT
	Reverse	CCACCAACCAACCATTTCTTTA
P21	Forward	GGAAGGGACACACAAGAAGAAG
	Reverse	AACACAGAGATAACCCCACTCAA
P27	Forward	CTGCTGTGCCTCCAAATACA
	Reverse	TTCTCAGGGGCTTCTCTTAGTG
GAPDH	Forward	TGACTTCAACAGCGACACCCA
	Reverse	CACCCTGTTGCTGTAGCCAAA

as the percentage of cells in G1, S, and G2.

#### Real-time PCR

Total RNA was extracted from cultured cells with TRIzol Reagent following the manufacturer's protocol. The RNA quality was verified using spectrophotometric and agarose gel electrophoresis. cDNA was synthesized with the Revert Aid First Strand cDNA Synthesis Kit using 1 µg RNA. Quantitative analyses of VDR, Bax, Bcl-2, caspase-3, p21 and p27 mRNA expression were performed using SYBR Green

Real-time-PCR Master Mix (Toyobo) on 7500 Real-time-PCR System (Applied Biosystems, Carlsbad, California, USA) with an initial denaturation step at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 5 s and 60°C for 30 s. At the end of the amplification, a melting curve was executed to ensure that only a single specific product was amplified. The primers were designed and synthesized by Shanghai Sangon Biotech (Shanghai, China) and were listed in **Table 1**.

#### Western blotting

Total cellular protein was extracted from cells using RIPA buffer containing protease and phosphatase inhibitor (Beyotime, China) according to the manufacturer's instructions. Proteins were quantified by the BCA Protein Assay Kit (Beyotime, China). Then proteins were separated by SDS gel electrophoresis and transferred to the PVDF membrane (Bio-Rad). After blocking the membranes with 5% nonfat dry milk for about 2 h, proteins were incubated with relevant antibodies overnight at 4°C and then incubated with a secondary antibody conjugated with horseradish peroxidase. Target band signals were detected using ECL (Advansta, Menlo Park, California, USA) and exposed on films. The density of protein bands was measured by Quantity One.

#### Statistical analysis

The obtained data were presented as means ± SD from all the experiments and statistically evaluated by one-way ANOVA among groups of

cultured for 24, 48 h. Cells were fixed and stained with 2.5 mg/ml Hoechst 33258. Variations in cell morphology were observed by fluorescence microscopy (Olympus, Tokyo, Japan).

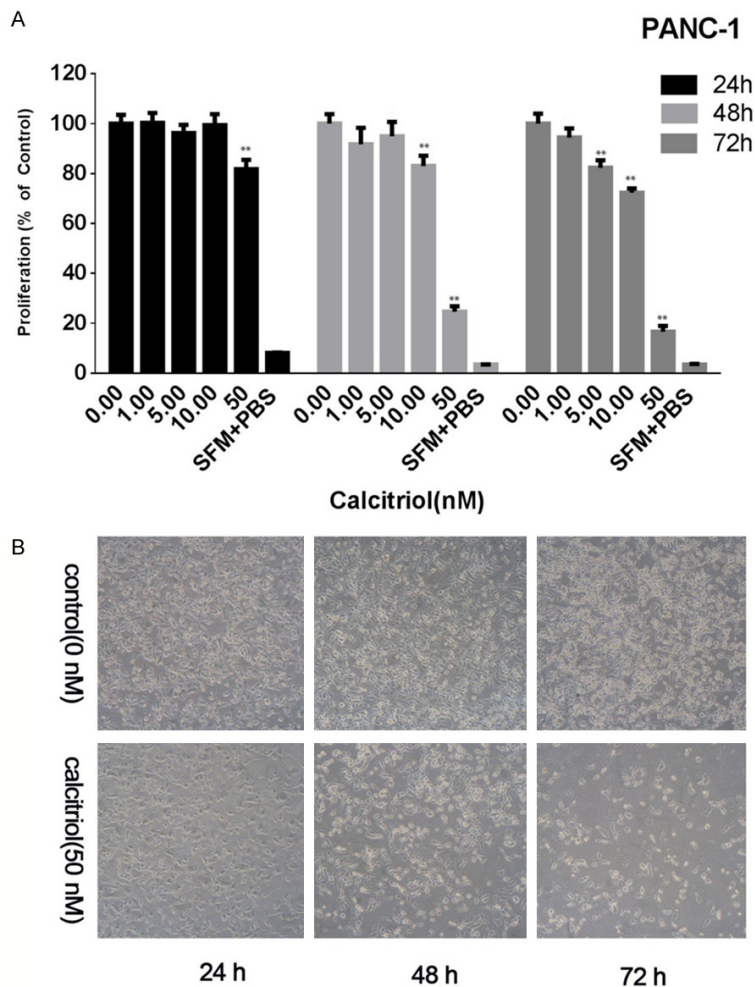
#### Flow cytometric analysis of apoptosis

The PANC-1 cells were seeded at  $1 \times 10^6$  in per 3.5 cm cell culture dishes and transfected as described before. 24 hours later, cells were treated with calcitriol (50 nM) for the indicated periods of time. After the treatment, the cells were harvested, stained with Annexin V-FITC/PI (1 µg/ml) (Sigma) to analyze apoptosis on the FACS Cali-bur flow cytometer (BD, CA, USA) using Cell Quest software. Cells with FITC (-) and PI (-) were deemed viable cells. Cells with FITC (+) and PI (-) were deemed early apoptosis. Cells with both FITC (+) and PI (+) were deemed late apoptosis. All of the samples were carried out in triplicate. The cell apoptosis rate was calculated following the formula:  $(N_{\text{apoptosis cells}}/N_{\text{total cells}}) \times 100\%$ .

#### Flow cytometric analysis of the cell cycle

To analyze cell-cycle distribution, cells were treated with 50 nM calcitriol for 24 h and 48 h. After trypsinization, cells were washed with PBS and fixed in 75% ethanol at 4°C for 2 hours. After fixation, the cells were washed with PBS and incubated with propidium iodide solution containing 10 µg/ml of DNase-free RNase A for 15 min. A total of  $1 \times 10^6$  cells were then subjected to cell-cycle analysis by flow cytometry according to the manufacturer's instructions. The cell-cycle distribution was illustrated

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**Figure 1.** Calcitriol affect cell viability and morphology. Calcitriol time and dose dependently reduce proliferation (A). Data are expressed as the mean of six replicate wells from one experiment repeated at least three times. Each bar represents the means  $\pm$  SD (n = 6). Photos of cell morphology changes of PANC-1 (100 $\times$ ) (B). (\*\*P<0.001).

cells. All analyses were conducted using SPSS 16.0 statistical software (IBM). The level of statistical significance was set at P<0.05.

### Results

#### Calcitriol affect cell viability and morphology

In MTT based cell proliferation experiment, calcitriol had a significant inhibitory effect on the growth of PANC-1 cell line in a time- and dose-dependent manner. The results revealed that the treatment of calcitriol (1, 5, 10 nM) for 24 h and 48 h did not change the viability of PANC-1 significantly. Cell viability was declined significantly after treatment of calcitriol at 50 nM (\*\*P<0.001), the proliferation rate of PANC-1 cells decreased to 80% at 24 h, decreased to

30% at 48 h, and dropped to 20% or less at 72 h (**Figure 1A**). Calcitriol also changed cell morphology. Cells without calcitriol had smooth cell membrane and rapidly increasing number, while cells co-incubated with calcitriol exhibited a large number of cellular debris and shrinking morphology in a time-dependent manner (**Figure 1B**).

#### VDR gene silencing

Inhibition of VDR expression was achieved by Lipofectamine 2000 transfers of VDR siRNA. Successfully transfected Cells shown green fluorescence which indicated high transfection efficiency (**Figure 2C**). In order to select one most effective siRNA from 3 candidates, VDR mRNA expression was tested by Real-time-PCR. In our experiment, VDR expression was reduced by approximately 30%, 88% and 91% in siRNA VDR-1346, siRNA VDR-643 and siRNA VDR-1103 transfected groups compared with the negative Control group (\*\*P<0.001) (**Figure 2A**). The results of our analysis were also confirmed by Western blotting. VDR protein expression significantly decreased in siRNA VDR-1103 group (\*\*P<0.001) (**Figure 2B**). Additionally, the morphological appearances of cells were still normal (**Figure 2D**).

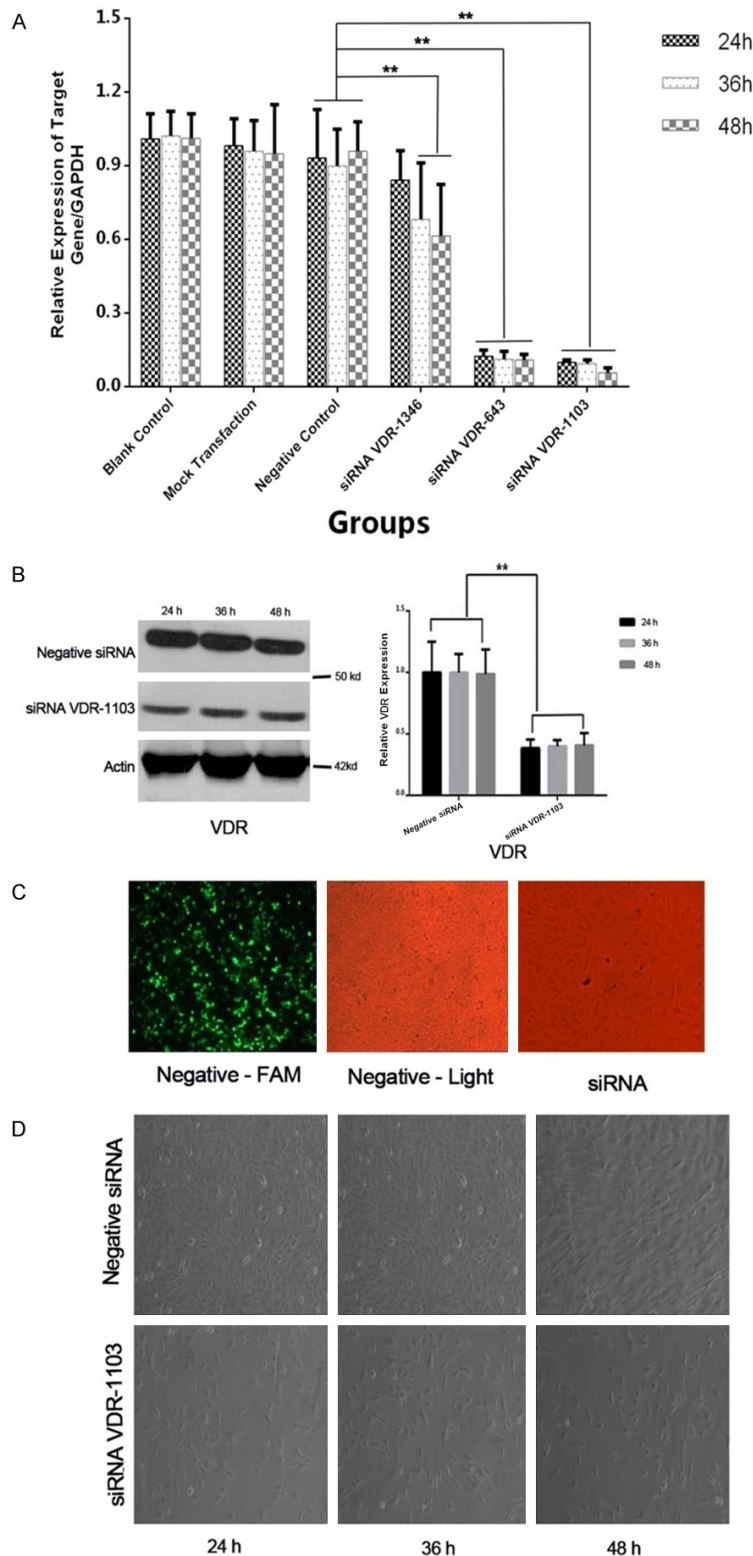
decreased in siRNA VDR-1103 group (\*\*P<0.001) (**Figure 2B**). Additionally, the morphological appearances of cells were still normal (**Figure 2D**).

#### Hoechst 33258 and Annexin V/PI double staining test cell apoptosis

To demonstrate the role of calcitriol in inducing cell apoptosis, we examined the morphologic changes by Hoechst 33258 staining (**Figure 3A**). When negative control group cells were cultured with 50 nM calcitriol, apoptotic morphologic changes were observed with a time-dependent manner compared with the medium control group. In medium control group, cells were round and homogeneously stained, while they were cultured with calcitriol for 24 h and



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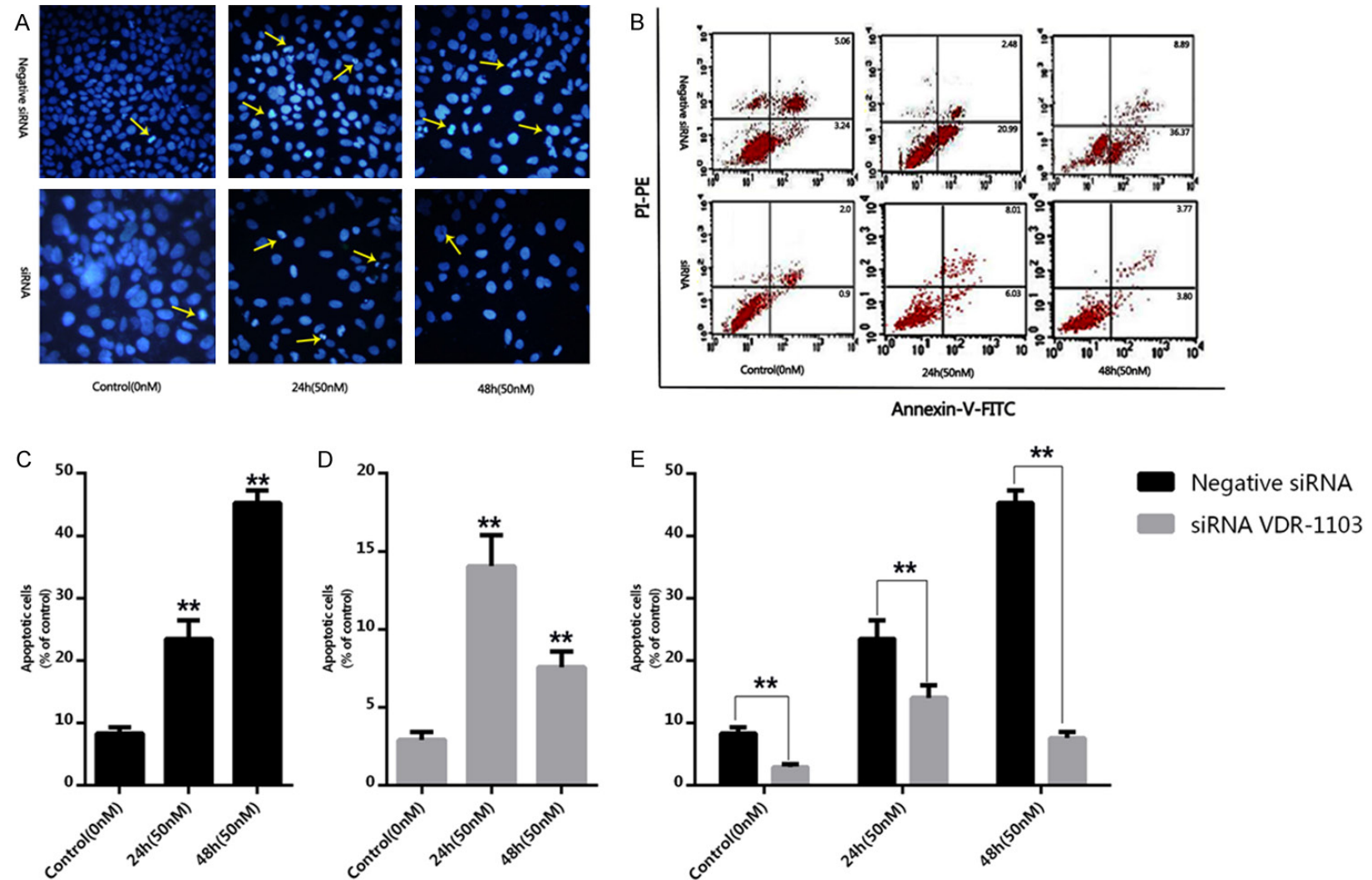
**Figure 2.** VDR Gene Silencing in PANC-1 cells. Effects of different siRNA transfection on expression of VDR mRNA (A). VDR protein expression levels in siRNA VDR-1103 transfected cells (B). Successfully transfected cells show green fluorescence (C). Photos of cell morphology changes of transfected PANC-1 cells (100×) (D). (\*\*P<0.001).

48 h cells showed marked granular apoptotic bodies (indicated by yellow arrows in **Figure 3A**). However, In VDR gene silencing groups (siRNA VDR-1103); no obvious apoptosis were seen no matter treated with or without calcitriol. We also used Annexin V/PI based flow cytometry to test the apoptosis in PANC-1 cells (**Figure 3B**). Treatment with 50 nM calcitriol for 24 hours the percentage of apoptosis obviously increased (23.47%), especially for 48 hours (45.26%) in negative siRNA group (**Figure 3C**). However, the percentage of apoptotic cells in VDR gene silencing group (siRNA VDR-1103) which treated with calcitriol were also significantly increased compared with the control group (**Figure 3D**), but the percentage of apoptotic cells were significantly decreased compared with the negative siRNA groups (**Figure 3E**).

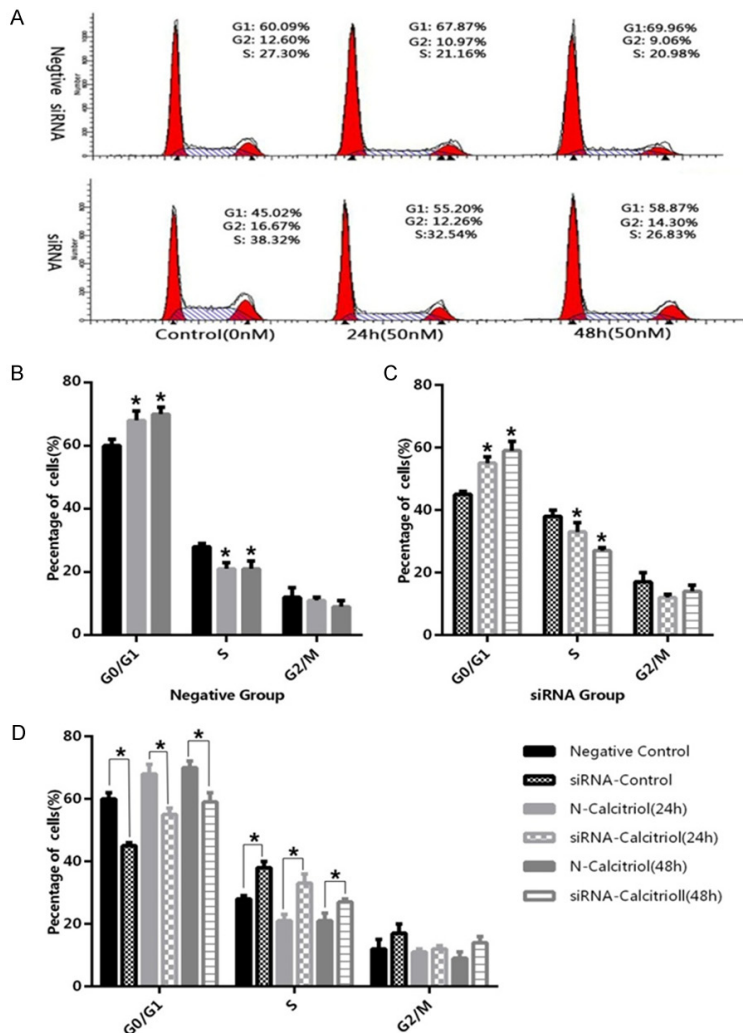
### VDR gene silencing affected the cell cycle arrest

We also investigated the effects of calcitriol on the cell-cycle phase distributions by flow cytometric (**Figure 4A**). We discovered that calcitriol could delay cells in the G0/G1 phase; from entering S phase in negative siRNA groups (**Figure 4B**). As shown, the percentage of G0/G1 phase cells treated with 50 nM calcitriol for 24 h and 48 h increased to 67.87% and 69.96% respectively relative to 60.09% of the medium control group. Also, a marked increase of G0/G1 phase cells was observed in the VDR gene silencing group (siRNA VDR-1103) compared with the medium control group (**Figure 4C**). The increased percentage of G0/G1 indicates that siRNA group cells were also

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**Figure 3.** Hoechst 33258 staining of PANC-1 cells (A). Apoptotic cells were marked by yellow arrows. Hoechst 33258 stain.  $\times 200$ . Annexin V/PI based flow cytometry to test the apoptosis in cells (B). The percentage of apoptosis obviously increased (23.47%), especially for 48 hours (45.26%) in negative siRNA group (C). The percentage of apoptotic cells also significantly increased in the VDR gene silencing groups (D). The percentage of apoptotic cells significantly decreased compared with the negative siRNA groups (E). (\*\* $P < 0.001$ ).



**Figure 4.** Cell cycle arrest and inhibition in PANC-1 cells treated with 50 nM calcitriol. The percent cells in each phase are plotted and the histograms showing the percentage of different groups cells distribution in the G0/G1, S, and G2/M phases. (\* $P < 0.05$ ).

susceptible arrested (\* $P < 0.05$ ). However, the percentage of G0/G1 phase cells in siRNA groups was significantly decreased compared with the negative siRNA groups (Figure 4D). All increased proportion of cells in the G0/G1 phase of the cell-cycle together with a decrease in S phase was statistically significant (\* $P < 0.05$ ).

#### VDR gene silencing effected the Bax/Bcl-2 ratio and the expressions of caspase-3

In negative groups, we found the Bax/Bcl-2 ratio changed not obvious which treated with calcitriol compared with the medium control group (Figure 5B-D). In VDR gene silencing groups, the amounts of Bax mRNA expression were slightly decreased, but not significantly; the expression levels of Bcl-2 mRNA were

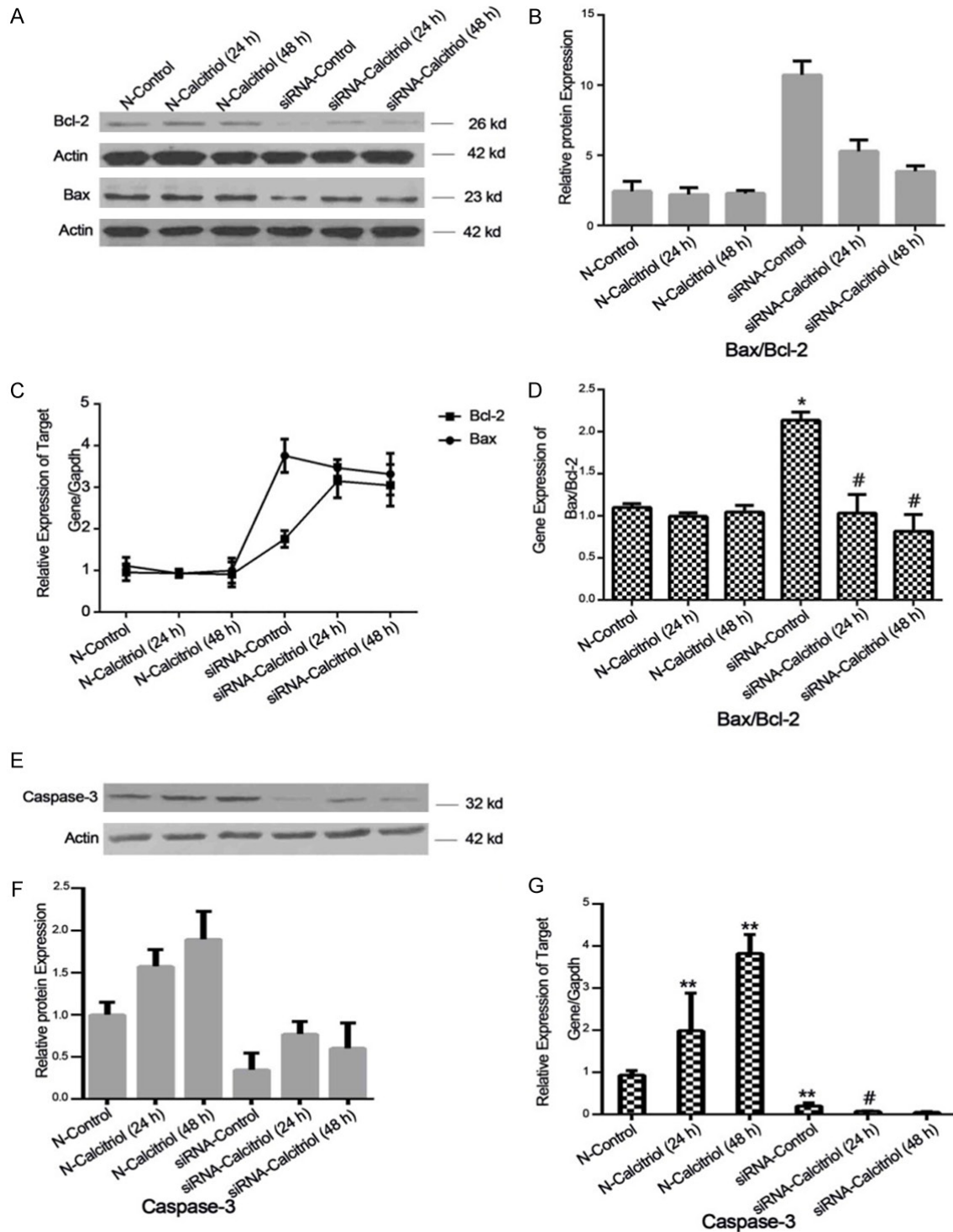
sharply increased (Figure 5C). So, the Bax/Bcl-2 ratio diminished after treatment with calcitriol (Figure 5D). These results were likewise confirmed at the protein levels (Figure 5B). In negative groups, we found the cells treated with calcitriol expressed higher levels caspase-3 compared with the medium control group (Figure 5E). As shown, the caspase-3 mRNA expression was up-regulated 2.0 times within 24 h and 3.8 times within 48 h (Figure 5G). In siRNA groups, caspase-3 expression was dramatically suppressed (Figure 5F). The mRNA expression levels of caspase-3 were also suppressed remarkably by calcitriol (Figure 5G). (Compared with the siRNA control group # $P < 0.05$ , compared with the negative control group \*\* $P < 0.001$ ).

#### VDR gene silencing effected the expressions of p21 and p27

We also demonstrated that the expression levels of p21 and p27 were up-regulated in a time-dependent manner of negative group cells treated with 50 nM calcitriol (Figure 6B, 6C). Those results were equally confirmed at mRNA levels. In negative group, the expression levels of p21 mRNA were up-regulated 1.2 times in 24 h and 2.1 times in 48 h; the expression levels of p27 mRNA were up-regulated 4.2 times and 6.7 times (Figure 6D, 6E). VDR gene silencing resulted in significantly decreased levels of p21 and p27 compared to the control group (Figure 6B, 6C). The p21 and p27 mRNA expression levels also were suppressed significantly in siRNA group (Figure 6D, 6E). Our results showed that the amounts of p21 and p27 mRNA were reduced to 26% and 12% respectively compared to the negative control group (\*\* $P < 0.001$ ).

#### Discussion

Pancreatic cancer is the fourth leading cause of cancer death and it is one of the most deadly



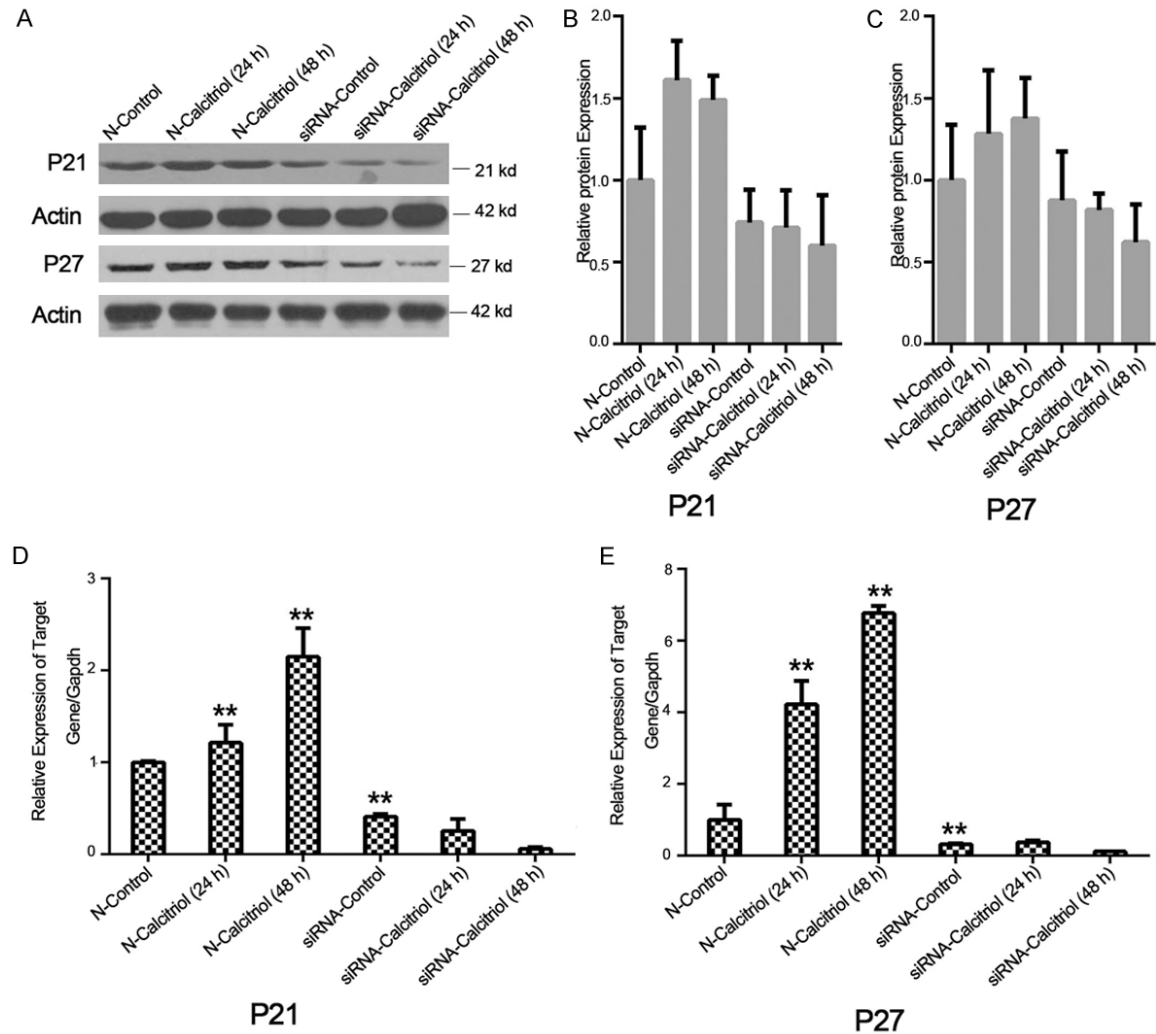
**Figure 5.** The Bax/Bcl-2 ratio changed not obvious in the negative groups and decreased after treatment with calcitriol in the siRNA groups (A-D). The caspase-3 expression was up-regulated markedly in negative groups and suppressed significantly in siRNA groups (E-G). (Compared with the siRNA control group # $P < 0.05$ , compared with the negative control group \* $P < 0.05$  compared with the negative control group \*\* $P < 0.001$ ).

malignancies worldwide [1]. Vitamin D<sub>3</sub> and its analogues coupled with their low toxicity, have

been considered to be a therapeutic agent in the treatment of cancers, including pancreatic



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**Figure 6.** VDR gene silencing effected the expressions of p21 and p27. The expression levels of p21 and p27 were up-regulated in a time-dependent manner of negative group cells and suppressed significantly in siRNA group (\*\* $P < 0.001$ ).

cancer [9, 10, 24, 25]. Calcitriol, an active form of vitamin D<sub>3</sub>, besides its role in the maintenance of calcium homeostasis and bone mineralization [26], has been declared to induce differentiation and inhibit the proliferation of various types of cancer cells [24, 29]. Preclinical studies also have shown that calcitriol has synergistical effect when used in combination with doxorubicin, cisplatin or gemcitabine [24]. Growth inhibition, cell apoptosis and cycle arrest were also observed of PANC-1 cell line treated with calcitriol in our experiments. In order to gain a better understanding of the mechanisms, we set out to silence the VDR gene expression and evaluate its influence on calcitriol antitumor function. In our experiment, the VDR gene expression was knocked down

specifically and effectively by siRNA VDR-1103.

The results of our study suggested that calcitriol induced the apoptosis of PANC-1 cells mainly through VDR expression. In VDR gene silencing PANC-1 cells, calcitriol induced only a modest decrease in the number of viable cells (7.57~14.04% reductions), substantially lower than that observed in negative group cells (23.47~45.26% reductions) as assessed by Annexin V/PI staining. Those results were compatible with the morphologic changes observed by Hoechst 33258 staining. In our experiment, we found the Bax/Bcl-2 ratio changed not obvious in negative groups. In VDR gene silencing groups, the Bax/Bcl-2 ratio was likewise de-

creased after treatment with calcitriol. As far as we know, the mitochondria pathway is inhibited by Bcl-2 or its other family members and activated by Bak or Bax [30, 31]. The cells survive or not after receiving apoptosis signals mainly depends on the ratio of Bax/Bcl-2 ratio [32]. Those results support that calcitriol inducing the apoptosis of PANC-1 cells may not be mainly through the mitochondria pathway in PANC-1 cell line. In our experiment, we also found calcitriol could induce higher expression levels of caspase-3 in negative groups. However, in si-RNA groups, caspase-3 expression was dramatically suppressed. These data suggest that the expression of the VDR gene, in majority part, renders cancer cells susceptible to calcitriol. This is in accordance with a previously published study on BxPC-3, AsPC-1, Hs700T, and Hs766T cells [19, 33-36]. However, the accurately mechanism of the regulation of caspase-3 is presently unknown. These results indicated that calcitriol induced cells apoptosis and inhibited proliferation mainly involved in the VDR-mediated pathway. Accordingly, other factors besides VDR states may also participate in the modulation of cell apoptosis induced by calcitriol.

VDR expression is under a close relationship with the signaling system of cell cycle-regulating agents, such as p21 and p27 proteins. In our study, the percentage of G0/G1 phase cells in siRNA groups was significantly decreased compared with the negative groups. Those results demonstrated that the growth inhibitory effects of calcitriol were mainly linked to VDR level and the induction of p21 and p27. Both p21 and p27 are members of cyclin-dependent kinase2 (cdk2) which involved in G1 cell cycle arrest [22, 37-40]. In our experiment showed marked increases in p21 and p27 content after treatment with calcitriol in Negative groups. These results are also consistent with the previously reported growth inhibitory effect of calcitriol on pancreatic cancer cells, characterized by the up-regulation of p21 and p27 in the BxPC-3, Hs700T and Hs766T cell lines [22, 41]. Furthermore, the increased percentage of G0/G1 phase cells in the VDR gene silencing groups suggested that calcitriol arrest the cell-cycle of PANC-1 cells may have any further possible mechanisms.

In conclusion, calcitriol exerts a conspicuous antitumor effect on PANC-1 cells, which mainly

mediated via the expression of the VDR gene and a series of genes which involved in cell apoptosis and cell cycle. The expression levels of the VDR gene and the antineoplastic effects of calcitriol, suggested that calcitriol induce apoptosis and inhibit cell proliferation is mainly via VDR pathway, but may not be only via this way. Other possible mechanisms including the calcitriol antitumor effects as possible mediators of these effects still were not elucidated. So, more antitumor mechanisms of calcitriol need to be elucidated further and should be confirmed in vivo studies.

## Acknowledgements

This study was financed by Wenzhou Municipal Sci-Tech Bureau. Funding for this work was supported by Wenzhou Municipal Sci-Tech Bureau. Project (No. Y20130097).

## Disclosure of conflict of interest

None.

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