

CYP24A1 depletion facilitates the antitumor effect of vitamin D3 on thyroid cancer cells

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Abstract. It has been demonstrated that 25-hydroxyvitamin-D3-24-hydroxylase (CYP24A1) is a key enzyme that neutralizes vitamin D activity, which may have an anti-tumor effect. Therefore, the aim of the current study was to explore the effect of the active metabolite of vitamin D, 1,25-dihydroxyvitamin D (1,25-D3) on thyroid cancer cells following the downregulation of CYP24A1. A Cell Counting Kit-8 assay identified that CYP24A1 knockdown enhanced the anti-proliferative effects of 1,25-D3 on thyroid cancer cells. Furthermore, the results of the scratch wound and Transwell assays indicated that CYP24A1 knockdown enhanced the inhibitory effect of 1,25-D3 on cell migration. The results from reverse transcription-quantitative polymerase chain reaction and western blot analysis indicated that treatment with 1,25-D3 and CYP24A1 knockdown synergistically enhanced the expression of the epithelial-related gene E-cadherin and decreased the expression of the mesenchymal-related genes N-cadherin and vimentin. Following CYP24A1 knockdown and treatment with 1,25-D3, the expression of matrix metalloproteinase 2 and metalloproteinase inhibitor 1 were significantly decreased and increased, respectively, compared with the group that underwent treatment with 25-D3 alone. Furthermore, protein kinase B (Akt) and β -catenin activity was significantly decreased by this synergistic effect compared with the group that underwent treatment with 1,25-D3 alone. The results of the current study suggest that CYP24A1 knockdown contributes to the anti-tumor effect of 1,25-D3 and that this effect may be due to deactivation of the Akt and β -catenin signaling pathways. Therefore, CYP24A1 knockdown and

1,25-D3 treatment may be used synergistically as a novel therapeutic strategy to treat patients with thyroid cancer.

Introduction

Thyroid cancer is one of the most common endocrine tumors and its incidence is increasing globally (1). The first symptom of thyroid cancer is usually the development of thyroid nodules. More than 90% of these nodules are benign; however, malignant thyroid nodules account for >90% of all endocrine malignancies (2). The aggressiveness of thyroid cancer increases with the reduction of cell adherence (3) Anaplastic thyroid cancer (ATC) is the most aggressive type of thyroid cancer and there are currently no effective methods of treating patients with ATC. However, understanding of the molecular pathogenesis of this cancer is increasing and a number of clinical trials have been performed to try and identify novel methods of treating this type of thyroid cancer (4).

Vitamin D is a fat-soluble prohormone that exerts important roles in calcium metabolism and homeostasis, and its active metabolite is 1,25-dihydroxyvitamin D (1,25-D3) (5). The primary effect of 1,25-D3 is to regulate bone and calcium homeostasis. However, this active form of vitamin D3 also exhibits non-classical effects, including regulation of the cell cycle and various anti-tumor effects, and is therefore attracting increased attention (6-8). It has been demonstrated that 1,25-D3 is implicated in various types of cancer (9-11), including thyroid cancer, where its concentration was reduced (12,13). Active vitamin D may serve as a ligand of the vitamin D receptor (VDR). Vitamin D signaling affects the expression of its target genes via the vitamin D response element (VDRE). A previous study has demonstrated the interaction of vitamin D signaling and nuclear receptor ligands in certain types of cancer (14). However, the results of clinical trials have indicated that the anti-tumor efficacy of 1,25-D3 is disappointing (15-17). Furthermore, the administration of large amounts of 1,25-D3 may induce hypercalcemia, which limits the scope of its use (18).

It remains controversial whether vitamin D exhibits an anti-cancer effect (19-22); therefore, it is important to investigate the factors that may affect the anti-cancer effect of vitamin D in cancer cells. The metabolism of vitamin D is highly complex and a series of enzymes are involved in

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its synthesis, activation and inactivation (23). The enzyme 25-hydroxyvitamin-D3-24-hydroxylase (CYP24A1), is responsible for neutralizing active 1,25-D3 and is an important component of the mitochondrial enzyme cytochrome P450 (24). This 1,25-D3-inactivating enzyme is critical in determining the antitumor activity of 1,25-D3. It has been demonstrated that high expression of CYP24A1 facilitates carcinogenesis in colorectal cancer (25). Thus, the aim of the current study was to identify whether CYP24A1 affects the anti-tumor effect of 1,25-D3 in thyroid cancer.

The protein kinase B (Akt) signaling pathway enhances cell survival and tumorigenesis (26-28). Abnormal activation of Akt may be associated with the progression of thyroid tumors (29,30). In addition, β -catenin, another important signaling molecule in cells, has attracted extensive attention due to its critical role in differentiation and regulation of patterning (31). The activity of β -catenin in tumors is enhanced by various mutations (32-34). Furthermore, the alteration of β -catenin activity has been identified in the majority of aggressive thyroid tumors (35). Based on these results, the current study sought to explore the effect of 1,25-D3 on the proliferation and metastasis of thyroid cancer cells following the inhibition of CYP24A1 and to determine its molecular mechanisms of action.

Materials and methods

Cell culture. The anaplastic thyroid cancer cell line KAT-18 was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were kept in RPMI 1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA) with 10% fetal bovine serum (FBS Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified incubator containing 5% CO₂.

Cell transfection. To knockdown CYP24A1 expression, cells were seeded in 24-well plates at a density of 2x10⁵/well. When cells reached 50-60% confluence, they were cultured in serum-free medium overnight. Cells were then transfected with 100 nM human CYP24A1siRNA (cat. no. sc-44652; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or 100 nM scramble control siRNA (cat. no. sc-37007; Santa Cruz Biotechnology, Inc.) using X-tremeGENE™ siRNA transfection reagent (Roche Diagnostics, Basel, Switzerland) following the manufacturer's protocol. Following 36 h incubation, cells were treated with or without 100 nM 1,25-D3 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 4 h; this concentration of 1,25-D3 was selected according to the results of previous studies (36,37). Cells in all relevant groups received the same concentration of 1,25-D3 (100 nM). All subsequent experiments were independently performed ≥ 3 times.

Cell grouping. Cells were divided into different groups as follows: A control group (untreated cells); cells treated with 100 nM 1,25-D3 for 4 h (VD3); cells transfected with scramble control siRNA and treated with 100 nM 1,25-D3 (siscr+VD3); cells transfected with CYP24A1 siRNA and treated with 100 nM 1,25-D3 (siCYP+VD3); cells transfected with scramble control siRNA (siscr) and cells transfected with CYP24A1siRNA (siCYP).

Cell proliferation assay. Cells were seeded at 1x10⁵ cell/well in 24-well plates. A Cell Counting Kit 8 (CCK-8) assay was used to measure the proliferation of cells in each group. The CCK-8 solution from the Cell Counting Kit (Beijing Solarbio Science & Technology, Co., Ltd., Beijing, China) was added to each well (10 μ l/well). Cells were then maintained at 37°C for 4 h. The absorbance of the reaction reagent was determined at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cell migration assay. Cells (2 ml) at a density of 1x10⁵/ml were placed on the upper cell culture chambers containing Transwell inserts (Corning Incorporated, Corning, NY, USA) and maintained in medium containing 0.2% FBS. The lower chamber was supplemented with the medium containing 15% FBS. The chambers were incubated for 24 h and then cells retained on the upper chamber were removed. Cells that migrated to the lower chamber were fixed with 4% paraformaldehyde at room temperature for 20 min. Cells were then stained with 0.1% crystal violet at room temperature for 20 min and observed using an optical microscope at a magnification of x200.

Scratch wound assay. Following transfection, cells were cultured in complete medium for a further 12 h. A sterile pipette tip was used to scratch the wells and cells were then incubated in serum-free solution following washing with PBS. The distance of the scratch was measured at 0 and 24 h following incubation using an inverted microscope at a magnification of x100.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The purification and concentration of RNA was measured at 260/280 nm using an ultra-micro spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). The integrity of RNA was detected using 1% agarose gel electrophoresis. A total of 1 μ g RNA was reverse transcribed using M-MLV reverse transcriptase (Promega Corporation, Madison, WI, USA), ribonuclease inhibitor and oligo(dT18) (Takara Bio, Inc., Otsu, Japan), 5X buffer (Takara Bio, Inc.) and dNTP (Takara Bio, Inc.) in 20 μ l volumes. The temperature protocol used for reverse transcription was: 25°C for 10 min; 42°C for 50 min; and 70°C for 15 min. A SYBR® Premix Taq™ II kit (Takara Bio, Inc.) was used to amplify cDNA on a Mx3000 platform (Agilent Technologies, Inc., Santa Clara, CA, USA) following the manufacturer's protocol. The thermocycling conditions were as follows: 95°C for 4 min; 35 cycles of 95°C for 30 sec; 60°C for 30 sec; and 72°C for 10 min. The relative expression level was calculated using the 2^{- $\Delta\Delta$ C_t} method (38). The primers used were as follows: E-cadherin, forward, 5'-TCA CATCCTACACTGCCAG-3' and reverse, 5'-AGTGTCCCT GTTCCAGTAGC-3'; N-cadherin, forward, 5'-ATATTTCCA TCCTGCGCGTG-3' and reverse, 5'-GTTTGGCCTGGC GTTCTTTA-3'; vimentin, forward, 5'-GAGAGGAAGCCG AAAACACC-3' and reverse, 5'-TTCTGAATCTGAGC CTGCA-3'; matrix metalloproteinase (MMP) 2, forward, 5'-ACCACAGCCAACTACGATGA-3' and reverse, 5'-GCT CCTGAATGCCCTTGATG-3'; MMP9, forward, 5'-GCG TCTCCCCTTCACTTTC-3' and reverse, 5'-ATAGGGTAC

ATGAGCGCCTC-3'; metalloproteinase inhibitor (TIMP) 1, forward, 5'-ACTACCTGCAGTTTTGTGGC-3' and reverse, 5'-CTGGAAGCCCTTTTCAGAGC-3'; CYP24A1 forward, 5'-CCGTAATCCCCAAGTGCAAC-3' and reverse, 5'-CCCAGAACTGTTGCCTTGTC-3'; VDR, forward, 5'-ACTCCA CCACCCAAAAGTCA-3' and reverse, 5'-GCATTCCCCAAA CTCAAGCA-3'; β -actin, forward, 5'-CTCCATCCTGGCCTC GCTGT-3' and reverse, 5'-GCTGTCACCTTACCGTTCC-3'.

Western blot analysis. Radioimmunoprecipitation assay lysis buffer containing protease inhibitors (Roche Diagnostics, Basel, Switzerland) was used to extract the total protein. Protein concentration was determined using Bio-Rad protein assay kits (Bio-rad Laboratories, Inc.). Proteins (20 μ g/lane) were resolved using 8-10% SDS-PAGE and transferred onto a PVDF membrane (EMD Millipore, Billerica, MA, USA). Subsequently, membranes were blocked using a blocking buffer (20 mM, Tris-base, 150 mM NaCl) containing 0.1% Tween-20 and non-fat milk for 2 h at room temperature. Following washing with PBS, the membrane was incubated with primary antibodies against CYP24A1 (cat. no. sc-365700; 1:700; Santa Cruz Biotechnology, Inc.), VDR (cat. no. sc-13133; 1:500; Santa Cruz Biotechnology, Inc.), Akt1/2 (cat. no. sc81434; 1:1,000; Santa Cruz Biotechnology, Inc.), phosphorylated (p)-Akt (cat. no. ab81283; 1:5,000; Abcam, Cambridge, UK), β -catenin (cat. no. ab32572; 1:5,000; Abcam), N-cadherin (cat. no. C2542; 1:100; Sigma-Aldrich; Merck KGaA), E-cadherin (cat. no. SAB4503751; 1:1,000; Sigma-Aldrich; Merck KGaA), vimentin (cat. no. V5255; 1:500; Sigma-Aldrich; Merck KGaA), MMP-2 (cat. no. 40994; 1:1,000; Cell signaling technology, Inc., Danvers, MA, USA), MMP-9 (cat. no. 13667; 1:1,000; Cell signaling technology, Inc.), TIMP1 (cat. no. 8946; 1:1,000; Cell signaling technology, Inc.) and β -actin (cat. no. 3700; 1:1,000; Cell signaling technology, Inc.) overnight at 4°C. Subsequently, the membrane was incubated with horseradish peroxidase conjugated secondary antibodies (cat. no. ab6728; 1:2,000; Abcam) for 1 h at room temperature. Immunoblots were visualized using enhanced chemiluminescence (Beyotime Institute of Biotechnology, Haimen, China). ImageJ software version 1.46 (National Institutes of Health, Bethesda, MD, USA) was used to quantify protein expression.

Statistical analysis. Data are presented as the mean \pm standard deviation. One-way analysis of variance followed by a Turkey's post-hoc test was performed to compare differences between groups. $P < 0.05$ was considered to indicate a statistically significant difference. GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla, CA, USA) was used to perform data analysis.

Results

The effect of CYP24A1 knockdown and 1,25-D3 treatment on cell proliferation. The association between enhanced CYP24A1 activity and tumorigenesis has been previously reported (8). Following transfection with CYP24A1 siRNA, CYP24A1 expression was significantly downregulated (Fig. 1A-C), meaning that the combined effect of CYP24A1 and 1,25-D3 on thyroid cancer cells could be explored

effectively. The results of the CCK-8 assay indicated that the proliferation of thyroid cancer cells was significantly inhibited in the VD3 group and the siCYP group, compared with the control (Fig. 1D). Furthermore, cell proliferation was significantly inhibited in the siCYP+VD3 group compared with the VD3 and siCYP groups (Fig. 1D). These results suggest that CYP24A1 knockdown and 1,25-D3 treatment synergistically suppress cell proliferation.

The effect of CYP24A1 knockdown and 1,25-D3 treatment on the expression of CYP24A1 and VDR. Impaired 1,25-D3-VDR signaling serves an important role in the progression of thyroid cancer (39). It has also been demonstrated that the expression of CYP24A1 is increased by 1,25-D3 (8,40). Thus, levels of CYP24A1 and VDR in the presence of 1,25-D3 were measured. The results revealed that 1,25-D3 treatment increased CYP24A1 mRNA (Fig. 2A) and protein (Fig. 2B and C) expression whereas CYP24A1 expression was significantly decreased following treatment with siCYP. By contrast, VDR expression did not change significantly following treatment with 1,25-D3 or transfection with siCYP (Fig. 2).

The effect of CYP24A1 knockdown and 1,25-D3 treatment on cell migration. The migration of thyroid cancer cells was measured using Transwell and scratch assays. The number of migrating cells significantly decreased following treatment with 1,25-D3 or transfection with CYP24A1 siRNA, compared with untreated cells (Fig. 3). Furthermore, the number of migrating cells was significantly lower in the group of cells that underwent transfection with CYP24A1 siRNA and treatment with 1,25-D3, compared with the group that underwent treatment with 1,25-D3 alone (Fig. 3B).

The results of the scratch wound assay demonstrated that the relative migration distance was 0.5 ± 0.06 mm in the control group, 0.78 ± 0.11 mm in the VD3 group, 0.74 ± 0.09 mm in the siCYP+VD3 group, 0.98 ± 0.13 mm in the siCYP+VD3 group, 0.47 ± 0.06 mm in the siCYP group and 0.57 ± 0.08 mm in the siCYP group (Fig. 4). Significant differences were observed in the relative wound width between the VD3, siCYP+VD3, siCYP+VD3 groups compared with the control group. Furthermore, the migration distance was significantly higher in the siCYP+VD3 compared with the VD3 group.

The effect of CYP24A1 knockdown and 1,25-D3 treatment on the expression of epithelial-mesenchymal transition (EMT)-associated genes. The EMT is considered to be responsible for tumor cell migration and invasion, and production of the extracellular cell matrix (ECM) during tumor progression (41,42). E-cadherin is highly expressed in epithelial cells whereas N-cadherin and vimentin are abundant in mesenchymal cells (43). The results of the western blotting revealed that the expression of E-cadherin was significantly increased in cells treated with 1,25-D3 or CYP24A1 siRNA (Fig. 5A and B). Furthermore, treatment with 1,25-D3 and CYP24A1 siRNA synergistically enhanced the mRNA expression of this epithelial related gene (Fig. 5C). By contrast, the expression of N-cadherin and Vimentin significantly decreased following treatment with 1,25-D3 or CYP24A1, with the greatest decrease in the group that underwent treatment with 1,25-D3 and CYP24A1.

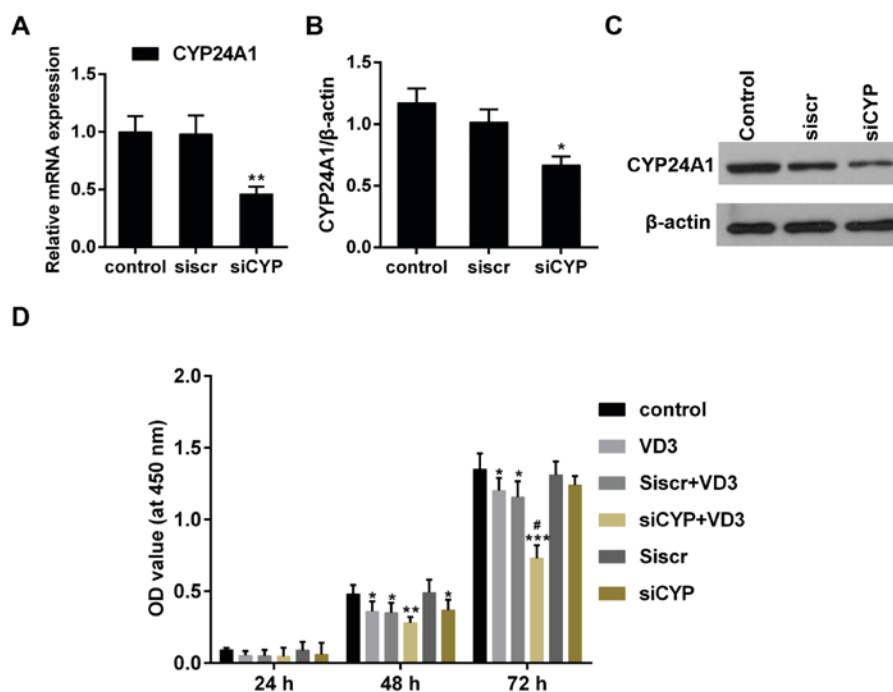


Figure 1. (A) Quantitative analysis of CYP24A1 mRNA expression following transfection with siRNA. ** $P < 0.01$ vs. control. (B) Determination of CYP24A1 protein expression following transfection with siRNA. * $P < 0.05$ vs. control. (C) Western blot analysis of CYP24A1 expression following transfection with siRNA. β -actin was used as a sample loading control. (D) Determination of cell proliferation using a Cell Counting Kit-8. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. control; # $P < 0.05$ vs. VD3 group. Control, untreated cells; VD3, cells treated with 1,25-D3 for 4 h; siscr+VD3, cells transfected with scramble control siRNA and treated with 1,25-D3; siCYP+VD3, cells transfected with CYP24A1 siRNA and treated with 1,25-D3; siscr, cells transfected with scramble control siRNA; siCYP, cells transfected with CYP24A1 siRNA; siRNA, small interfering RNA; CYP24A1, 25-hydroxyvitamin-D3-24-hydroxylase; 1,25-D3, 1,25-dihydroxyvitamin D.

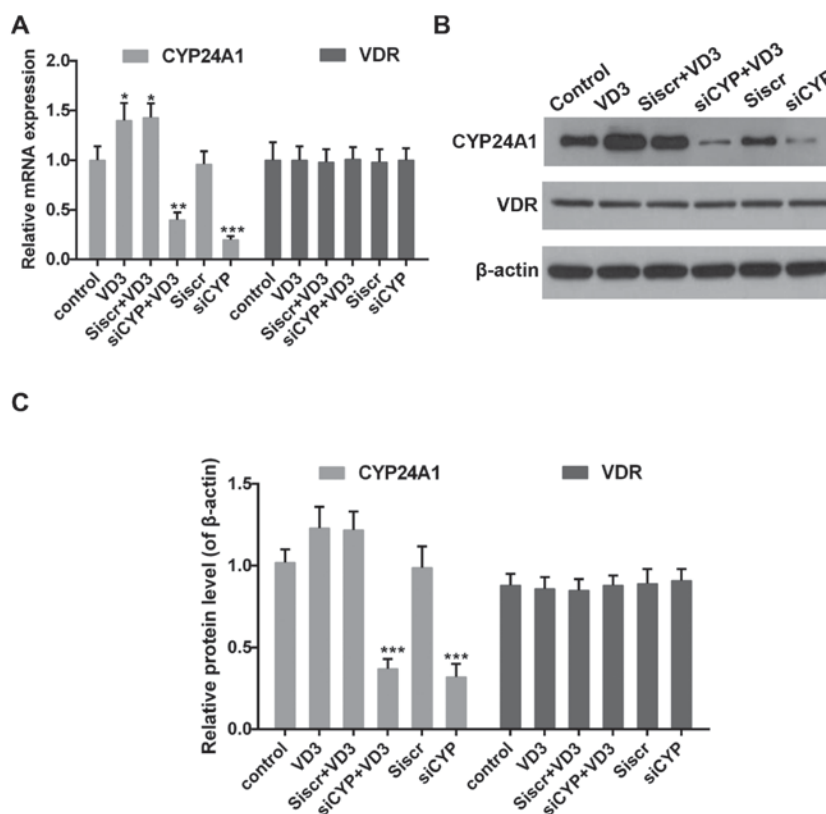


Figure 2. (A) Quantitative analysis of CYP24A1 and VDR mRNA expression. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. control. (B) Western blotting and (C) quantification of CYP24A1 and VDR protein expression. β -actin was used as the sample loading control. *** $P < 0.001$ vs. control. Control, untreated cells; VD3, cells treated with 1,25-D3 for 4 h; siscr+VD3, cells transfected with scramble control siRNA and treated with 1,25-D3; siCYP+VD3, cells transfected with CYP24A1 siRNA and treated with 1,25-D3; siscr, cells transfected with scramble control siRNA; siCYP, cells transfected with CYP24A1 siRNA; siRNA, small interfering RNA; CYP24A1, 25-hydroxyvitamin-D3-24-hydroxylase; VDR, vitamin D receptor; 1,25-D3, 1,25-dihydroxyvitamin D.

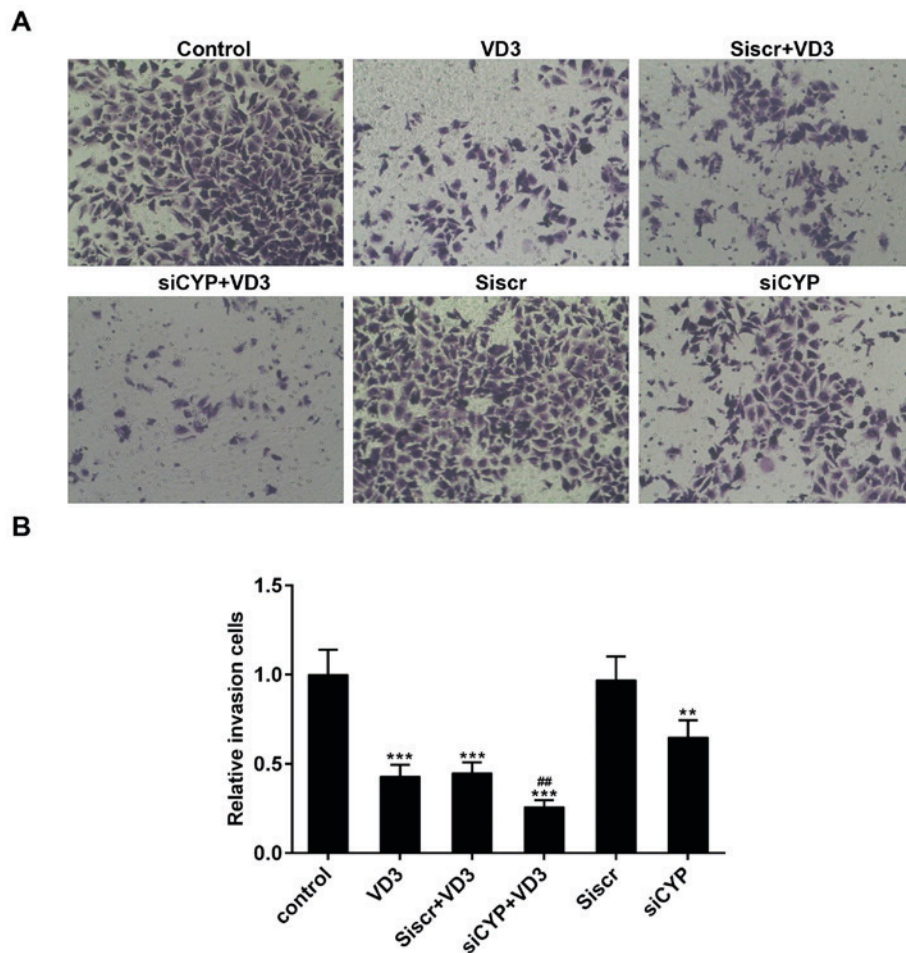


Figure 3. (A) Measurement of cell migration using a Transwell assay. Magnification, $\times 200$. (B) Relative numbers of migrating cells. ** $P < 0.01$ and *** $P < 0.001$ vs. control; ## $P < 0.01$ vs. VD3 group. Control, untreated cells; VD3, cells treated with 1,25-D3 for 4 h; siscr+VD3, cells transfected with scramble control siRNA and treated with 1,25-D3; siCYP+VD3, cells transfected with CYP24A1 siRNA and treated with 1,25-D3; siscr, cells transfected with scramble control siRNA; siCYP, cells transfected with CYP24A1 siRNA; siRNA, small interfering RNA; CYP24D1, 25-hydroxyvitamin-D3-24-hydroxylase; 1,25-D3, 1,25-dihydroxyvitamin D.

The effect of CYP24A1 knockdown and 1,25-D3 treatment on the expression of MMPs. MMPs serve a critical role in the migration and metastasis of tumor cells, which is essential for inducing degradation of the ECM (44). The expression of MMP-2 mRNA and protein was significantly decreased in the siCYP+VD3 group compared with VD3 and control groups (Fig. 6). By contrast, the expression of MMP-9 mRNA and protein was unaffected in all treatment groups; there was a slight decrease in MMP-9 expression in the siCYP+VD3 group, although this was not significant. The mRNA and protein expression of TIMP1, which inhibits the expression of MMPs (45), was increased following treatment with 1,25-D3 or CYP24A1 siRNA. This increase was significantly greater in the siCYP+VD3 group compared with the VD3 group.

The effect of CYP24A1 knockdown and 1,25-D3 treatment on the activity of AKT and β -catenin. AKT activation and β -catenin inhibition are correlated with the pathogenesis of thyroid cancer (31,46). As presented in Fig. 7A and B, the phosphorylation of AKT was significantly decreased in cells following treatment with 1,25-D3 or CYP24A1 siRNA. Furthermore, the expression of p-AKT was significantly decreased in the siCYP+VD3 group compared with the VD3

group. β -catenin activity was also significantly decreased in the siCYP+VD3 groups compared with the control and VD3 groups.

Discussion

To determine the influence of the enzyme CYP24A1 on the anti-tumor effect of vitamin D3, its expression was decreased using specific siRNA. The proliferation of thyroid cancer cells was assessed using a CCK-8 assay and it was demonstrated that thyroid cell proliferation was significantly inhibited following treatment with 1,25-D3 or CYP24A1 knockdown. Furthermore, CYP24A1 knockdown significantly enhanced the anti-proliferative effects of 1,25-D3 72 h after treatment, compared with cells that were treated with 1,25-D3 alone. It has been reported that the susceptibility of cancer cells to vitamin D3 gradually decreases during tumor progression (15); nevertheless, its mechanisms of action remain unclear. In the present study, the effect of 1,25-D3 on the expression of CYP24A1 and VDR, which are important factors that affect the anti-tumor effect of vitamin D3, was assessed. It was demonstrated that 1,25-D3 treatment increased CYP24A1 expression but had no influence on VDR expression. The elevated expression of

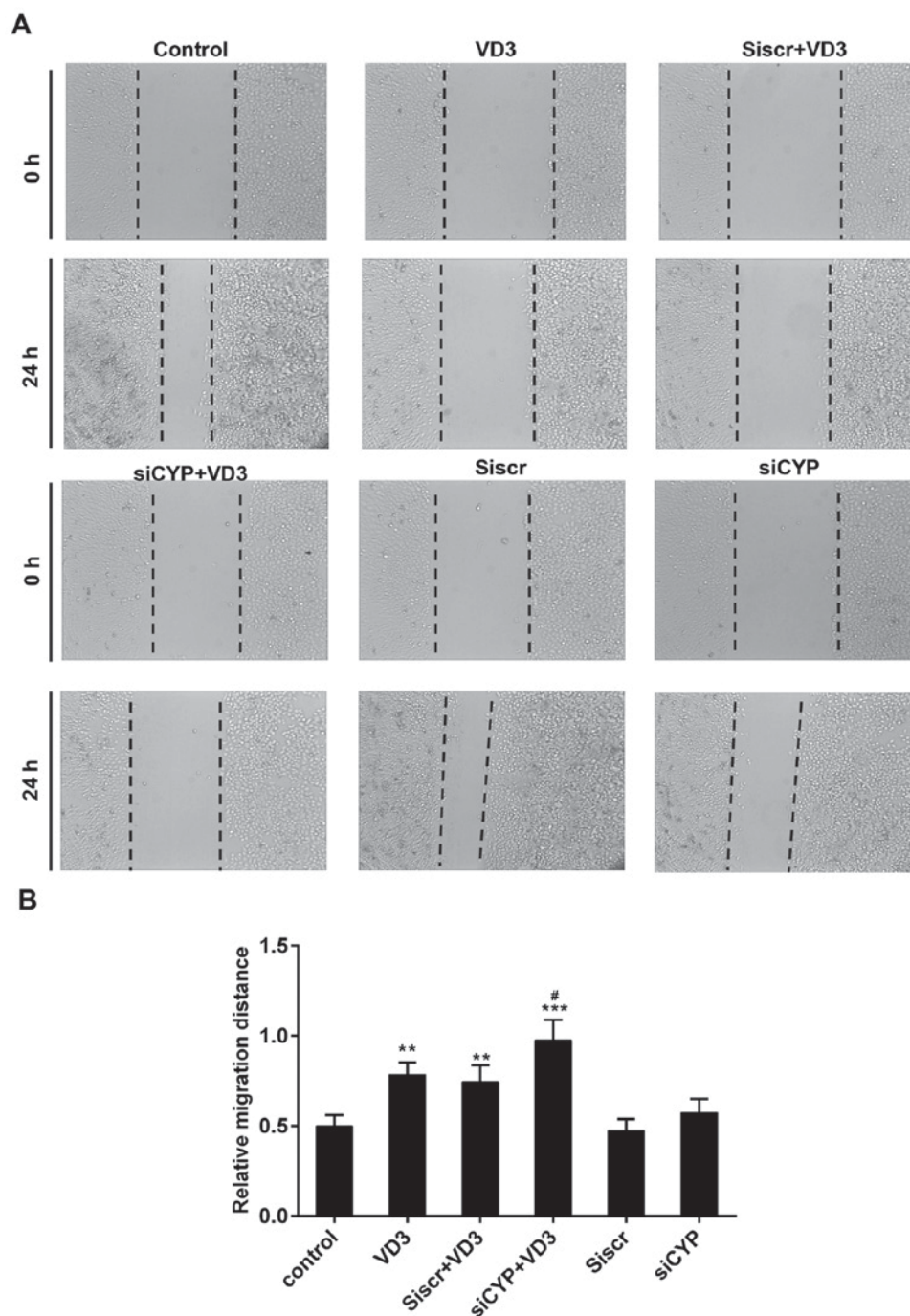


Figure 4. (A) Assessment of tumor cell migration using a scratch assay. Magnification, x100. (B) Relative wound width of tumor cells in each group. ** $P < 0.01$ and *** $P < 0.001$ vs. control; # $P < 0.05$ vs. VD3 group. Control, untreated cells; VD3, cells treated with 1,25-D3 for 4 h; siscr+VD3, cells transfected with scramble control siRNA and treated with 1,25-D3; siCYP+VD3, cells transfected with CYP24A1 siRNA and treated with 1,25-D3; siscr, cells transfected with scramble control siRNA; siCYP, cells transfected with CYP24A1 siRNA; siRNA, small interfering RNA; CYP24D1, 25-hydroxyvitamin-D3-24-hydroxylase; 1,25-D3, 1,25-dihydroxyvitamin D.

CYP24A1 following 1,25-D3 treatment was in line with a previous study (47). This may be a self-defense mechanism of cancer cells and may partly explain why the anti-tumor effect of 1,25-D3 is weakened following treatment.

Tumor metastasis is usually accompanied by tumor cell invasion and migration to other tissues, and the initiation of the EMT, which may lead to the loss of the epithelial phenotype and gain of fibroblast-like mesenchymal morphology (48). The results of a Transwell assay assessing cell migration indicated that 1,25-D3 treatment and CYP24A1 knockdown significantly

inhibited the migration of thyroid cancer cells. Cell migration was significantly decreased in the group that underwent CYP24A1 knockdown and treatment with 1,25-D3 compared with the groups that underwent 1,25-D3 treatment alone.

In addition, the results of a scratch wound assay indicated that the migratory ability of thyroid cancer cells was decreased following treatment with 1,25-D3, as well as following CYP24A1 knockdown. Likewise, CYP24A1 knockdown enhanced the inhibitory effect of 1,25-D3 on cell migration. The decrease in the expression of the epithelial-related

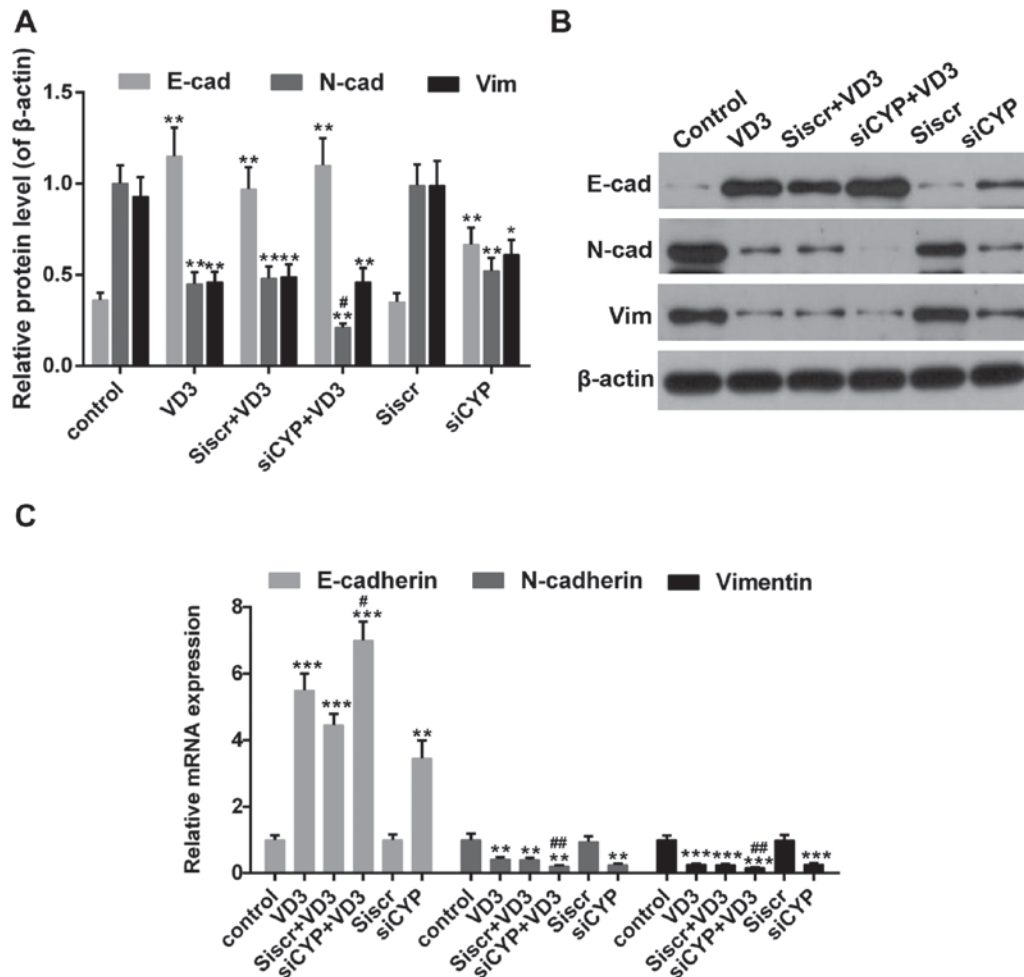


Figure 5. (A and B) Western blot analysis measuring E-cadherin, N-cadherin and vimentin expression. β -actin was used as sample loading control. (C) The expression of E-cadherin, N-cadherin and vimentin mRNA. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. control; # $P < 0.05$ and ## $P < 0.01$ vs. VD3 group. Control, untreated cells; VD3, cells treated with 1,25-D3 for 4 h; siscr+VD3, cells transfected with scramble control siRNA and treated with 1,25-D3; siCYP+VD3, cells transfected with CYP24A1 siRNA and treated with 1,25-D3; siscr, cells transfected with scramble control siRNA; siCYP, cells transfected with CYP24A1 siRNA; siRNA, small interfering RNA; CYP24D1, 25-hydroxyvitamin-D3-24-hydroxylase; 1,25-D3, 1,25-dihydroxyvitamin D.

protein E-cadherin and the increase in the expression of the mesenchymal-related proteins N-cadherin and vimentin were reversed by 1,25-D3 treatment and also by CYP24A1 knockdown. This effect was more pronounced in the group that underwent 1,25-D3 treatment and CYP24A1 knockdown compared with the group that underwent 1,25-D3 treatment alone, suggesting that a decrease in CYP24A1 expression inhibits the EMT.

MMPs are key enzymes that affect degradation of the ECM, an important step in the progression of the EMT (49). The results demonstrated that the expression of MMP-2 expression decreased and TIMP1 expression increased in the three treatment groups (VD3, siCYP and siCYP+VD3); MMP-9 expression was similar among all groups. Taken together, these results suggest that CYP24A1 knockdown facilitates the anti-tumor effect of 1,25-D3 by inhibiting the migration of thyroid cancer cells and the EMT.

A deep understanding of the underlying mechanisms is required to facilitate the development of precise targets during the intervention and treatment of thyroid cancer. Previous studies have identified the role of Akt and β -catenin in multiple biological events (31,46,50). In the present study,

it was noted that the treatment with 1,25-D3 suppressed Akt and β -catenin activity and that this effect was most prominent when 1,25-D3 treatment was initiated following CYP24A1 knockdown. These results are in line with those of a previous study, which also claimed that the deactivation of β -catenin could induce the expression of E-cadherin (51). However, in the current study, the association between β -catenin and EMT markers remained obscure; further studies are required to validate this. Furthermore, it has been reported that activation of the phosphoinositide 3-kinase/Akt pathway can phosphorylate β -catenin at a specific site, leading to its accumulation and stabilization in the nucleus (52). However, it remains unclear whether Akt affects the activity of β -catenin. It has been reported that β -catenin may be at the convergence of multiple signaling pathways in thyroid cancer (31). Therefore, a signaling pathway that was not assessed in the current study may participate in the regulation of thyroid cancer. It is important to undertake in-depth studies to determine the definite mechanisms of action of 1,25-D3 and CYP24A1. If the synergetic effect of 1,25-D3 and CYP24A1 is confirmed *in vivo*, it would be a big step forward in developing a novel therapeutic strategy to treat thyroid cancer.

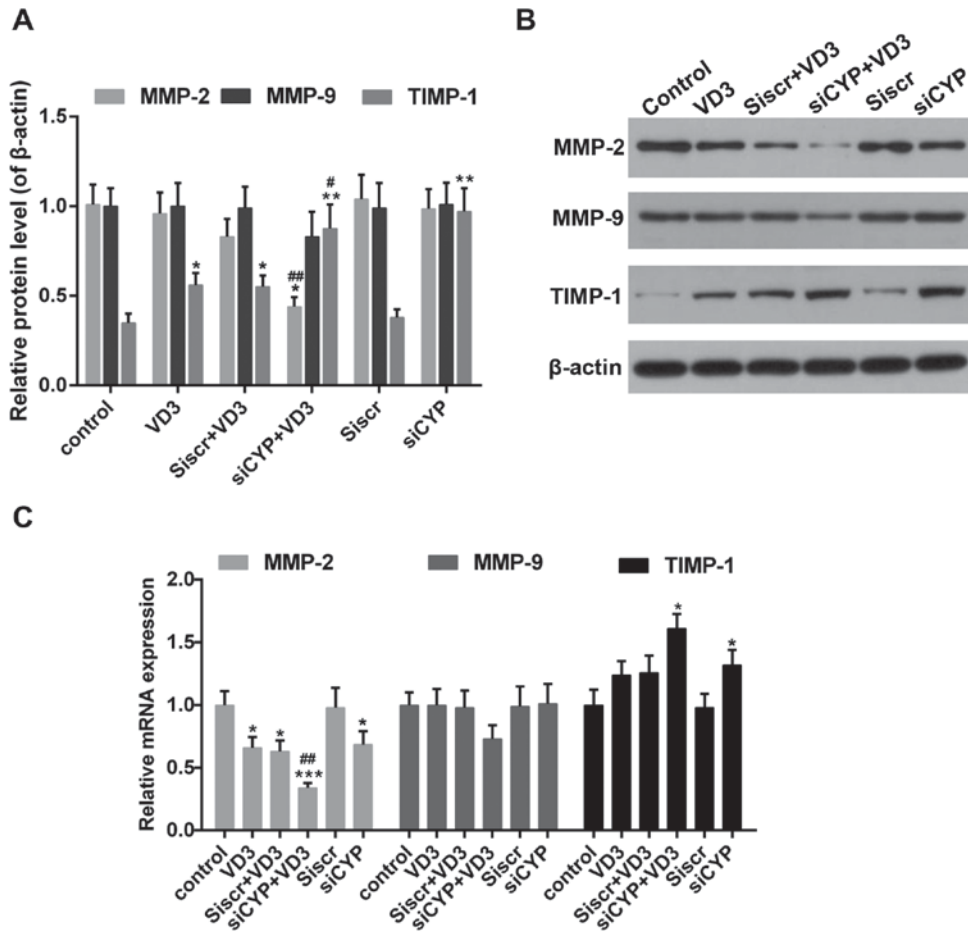


Figure 6. (A and B) Western blot analysis measuring MMP-2, MMP-9 and TIMP1 expression. β -actin was used as sample loading control. (C) The expression of MMP-2, MMP-9 and TIMP1 mRNA. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. control; # $P < 0.05$ and ## $P < 0.01$ vs. VD3 group. Control, untreated cells; VD3, cells treated with 10 nM 1,25-D3 for 4 h; siscr+VD3, cells transfected with scramble control siRNA and treated with 1,25-D3; siCYP+VD3, cells transfected with CYP24A1 siRNA and treated with 1,25-D3; siscr, cells transfected with scramble control siRNA; siCYP, cells transfected with CYP24A1 siRNA; siRNA, small interfering RNA; CYP24D1, 25-hydroxyvitamin-D3-24-hydroxylase; 1,25-D3, 1,25-dihydroxyvitamin D; MMP, matrix metalloproteinase; TIMP, metalloproteinase inhibitor.

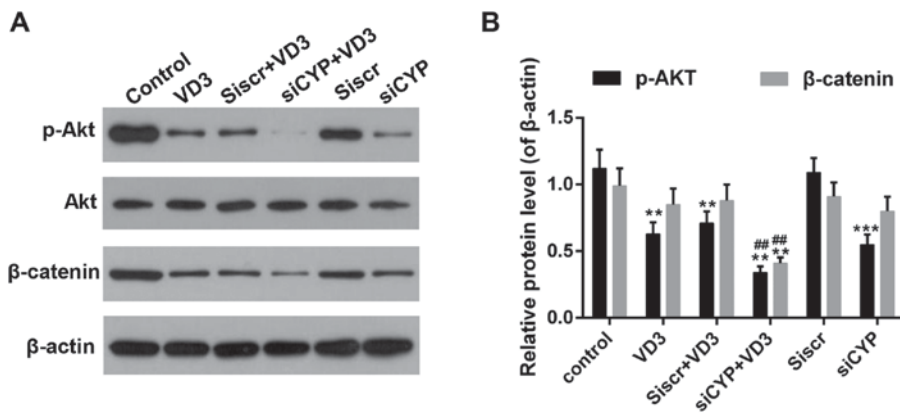


Figure 7. (A and B) Western blot analysis of p-Akt and β -catenin expression. β -actin was used as sample loading control. ** $P < 0.01$ and *** $P < 0.001$ vs. control; ## $P < 0.01$ vs. VD3 group. Control, untreated cells; VD3, cells treated with 10 nM 1,25-D3 for 4 h; siscr+VD3, cells transfected with scramble control siRNA and treated with 1,25-D3; siCYP+VD3, cells transfected with CYP24A1 siRNA and treated with 1,25-D3; siscr, cells transfected with scramble control siRNA; siCYP, cells transfected with CYP24A1 siRNA; siRNA, small interfering RNA; CYP24D1, 25-hydroxyvitamin-D3-24-hydroxylase; 1,25-D3, 1,25-dihydroxyvitamin D; p-phosphorylated; Akt, protein kinase B.

In conclusion, the results of the present study demonstrated that the downregulation of CYP24A1 facilitated the anti-tumor effect of vitamin D3. This anti-tumor effect primarily

occurred via the suppression of tumor cell proliferation and migration. Furthermore, the expression of the EMT-related genes E-cadherin, N-cadherin and Vimentin, and MMP-2 and

TIMP1 were regulated following treatment with 1,25-D3 and CYRP24A1 knockdown. This effect may be induced via the deactivation of Akt and β -catenin.

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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

NH performed the experiments and data analysis and contributed significantly to the preparation of the manuscript. HZ contributed to the experimental design.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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