1,25-Dihydroxyvitamin D$_3$ up-regulates expression of hsa-let-7a-2 through the interaction of VDR/VDRE in human lung cancer A549 cells

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A B S T R A C T

Aims: We aim to investigate the relationship between 1,25-(OH)$_2$VD$_3$ and hsa-let-7a in lung cancer A549 cells.

Methods: Real-time PCR and luciferase reporter assays were used to detect the influence of 1,25-(OH)$_2$VD$_3$ on the expression of hsa-let-7a-2 after A549 cells were treated with 1,25-(OH)$_2$VD$_3$ (10$^{-8}$ - 10$^{-6}$ mol/L). Analysis of the 5.0 Kb upstream sequence of the pre-let-7a-2 showed that one vitamin D response element (VDRE) is located in -2066/-2042 bp of pre-let-7a-2. Electrophoretic mobility shift assays (EMSA), chromatin immunoprecipitation (ChIP) and luciferase reporter assays were performed to determine whether 1,25-(OH)$_2$VD$_3$ activating vitamin D receptor (VDR) could bind to this VDRE to promote hsa-let-7a-2 expression.

Results: We found that 1,25-(OH)$_2$VD$_3$ could up-regulate the expression of hsa-let-7a-2 in a dose-dependent manner. The results of EMSA and ChIP demonstrated that 1,25-(OH)$_2$VD$_3$/VDR could interact with the VDRE in the upstream of pre-let-7a-2. Luciferase reporter assay showed that this VDRE is a functional cis-element mediating the up-regulation of hsa-let-7a-2 expression induced by 1,25-(OH)$_2$VD$_3$.

Conclusions: Our data indicated that 1,25-(OH)$_2$VD$_3$ could up-regulate the transcription of hsa-let-7a-2 in lung cancer cells, and the up-regulation of hsa-let-7a-2 expression induced by 1,25-(OH)$_2$VD$_3$ might mediate the anti-proliferation effects of 1,25-(OH)$_2$VD$_3$ in lung cancer cells.

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1. Introduction

1,25-Dihydroxyvitamin D$_3$ (1,25-(OH)$_2$VD$_3$), an active form of vitamin D, regulates cell differentiation and proliferation (Pendás-Franco et al., 2007; Samuel and Sitrin, 2008; Wang et al., 2010; Yang et al., 2010). Such a regulation is mediated by the nuclear vitamin D receptor (VDR), a ligand-dependent transcription factor (Ali and Vaidya, 2007). 1,25-(OH)$_2$VD$_3$ activates VDR to form a complex containing VDR, retinoic X receptor (RXR), and the accessory transcription factor. Then the complex binds to the vitamin D responsive element (VDRE) of target gene to affect the cell growth, differentiation and apoptosis (Goczek and Studzinski, 2009; Pan et al., 2010). It is reported that expression of VDR is decreased in lung cancers and the nuclear vitamin D receptor expression is associated with improved survival in non-small cell lung cancer (Srinivasan et al., 2011).

It is found that some miRNAs correlate with lung cancer closely (Eder and Scherr, 2005). hsa-let-7a is a lung cancer related tumor suppressor. Several cellular protein targets of hsa-let-7a have been identified, such as IRS1, IRS2, EGFR, RAF1, PAK1, BCL2 and RAS (Jiang et al., 2010; Kefas et al., 2008; Saydam et al., 2011; Xiong et al., 2011) and (Johnson et al., 2005). Many pieces of research evidence show that hsa-let-7 functions as a tumor suppressor in lung cancer (Johnson et al., 2007). Over-expression of let-7a was shown to be related to increases in overall survivals of NSCLC patients. hsa-let-7 is also a prognostic factor indicating the prevention of recurrence in surgically resected NSCLC patients (Yanaihara et al., 2006; Yu et al., 2008). It is reported that the expression levels of hsa-let-7 are frequently reduced in lung cancers (Takamizawa et al., 2004). Furthermore, lung cancer patients with reduced hsa-let-7a expression were found to have significantly worse prognosis (He et al., 2010; Ortholan et al., 2009; Raponi et al., 2009). The hsa-let-7a inhibits growth of lung cancer cells in vitro (He et al., 2009; Kumar et al., 2008).

So far, little is known about the role and the regulatory mechanisms of hsa-let-7a-2 expression in lung cancers. In the present study, we found that 1,25-(OH)$_2$VD$_3$ up-regulates hsa-let-7a-2 gene expression.
in a transcriptional level in A549 cancer cells. Our work is focused on the regulatory mechanisms of hsa-let-7a-2 expression induced by 1,25-(OH)₂VD₃.

2. Materials and methods

2.1. Plasmid construction

According to the hsa-let-7a-2 target sequence in mirBase (http://www.mirbase.org/) Targets, the corresponding DNA sequence (5′-agctTGAAGTATAGTTGATAGTgacgt-3′) and the complementary sequence were synthesized. The double-stranded sequence was generated by annealing equal amounts of the sense and antisense sequences at 95 °C for 10 min, then cooling to room temperature. The double-stranded sequence with an overhanging Hind III site (agctt) and Sac I site (gagct) was inserted into the 3′ end of the luciferase gene in PMIR-Report™ Luciferase reporter vector (Ambion, USA) to generate the pMIR-Report-let7a2T plasmid. The construct was confirmed by DNA sequencing.

The upstream sequence of pre-let-7a-2 was analyzed by Maltinspector 2.2 (http://www.genomatix.de). One VDRE sequence in -2066/-2042 bp upstream of pre-let-7a-2 was found. The wildtype sequence (CACAGTGTCACGGAAGTGATGA) or mutant VDRE sequence (CACAGTGTCACGGAAGTGATGA) of the dual-tandem VDRE were synthesized in vitro and inserted into the upstream of the TATA box in pGL4-luc2 [minp] vector (Ambion, USA) to generate the VDRE/pGL4-luc2 [minp] vector. For competition reaction, unlabelled double-stranded VDRE sequence or mutant VDRE sequence (VDREM) in 250-fold excess were added to the binding reaction mixture and incubated. For super-shift assays, anti-human VDR (Santa Cruz) was used. Bound and free oligonucleotide probes were resolved by electrophoresis on an 8% non-denaturing polyacrylamide gel. Electroblotting and chemiluminescence detection were performed by the DIG gel shift kit (Roche, Penzberg, Germany) according to the manufacturer’s instructions.

2.2. Cell culture

Lung cancer A549 cells were cultured in F-12 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 100 μg/ml ampicillin and 100 μg/ml streptomycin at 37 °C with 5% CO₂. For induction, the cells were treated with 1,25-(OH)₂VD₃ (10⁻⁸ – 10⁻⁶ mol/L). Solvent vehicles (DMSO) were added to the control cells at a concentration equal to that for the treated cells.

2.3. Transient transfection

A549 cells were seeded in 24-well plates 1 day prior to transfection, and transfected with FuGENE HD (Roche, Germany) according to the manufacturer’s instruction. 0.5 μg of plasmid, 1 μl of FuGENE HD and 500 μl of F-12 medium without antibiotics were added to each well. After transfection, the cells were treated with 1,25-(OH)₂VD₃ (10⁻⁸ – 10⁻⁶ mol/L) or DMSO for 48 h.

2.4. Luciferase reporter gene assay

The luciferase reporter assay was performed 48 h after transfection. The cells were lysed by 1 × Reporter Lysis Buffer (RLB) and harvested by manually scraping. Luminescence was detected using Mithras LB 940 (Berthold Technologies). Firefly luciferase activity of pMIR-Report plasmid was measure 1 (M1), and the β-galactosidase activity (internal control) of pMIR-REPORT β-gal plasmid (Promega) or Renilla luciferase activity (internal control) of pRL-TK plasmid (Promega) was Measure 2 (M2). The relative luciferase activity was calculated as the ratio of M1/M2.

2.5. RNA extraction and real-time-PCR analysis

Total RNA was extracted from A549 cells using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. First strand cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Fermentase). The real-time-PCR primers for amplification of pri-let-7a-2 are pri7a2F (5′-CATGTTGACTGATCGTCC-3′) and pri7a2R (5′-TTGTTAGTCGAAGACCC-3′). Quantitative PCR was carried out using SYBR® Premix EX Taq (TaKaRa). Samples were analyzed in triplicates, and their relative expression levels were normalized to the expression of 5S rRNA.

2.6. Electrophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared from A549 cells using a nuclear extraction kit (Active Motif, Carlsbad, CA, USA) following the manufacturer’s instructions. Oligonucleotides corresponding to the VDRE sequence in the upstream of hsa-let-7a-2 were synthesized as probes, and labeled with digoxigenin (DIG) (Roche). Binding reaction of nuclear extract with VDRE probes was performed for 20 min at room temperature. For competition reaction, unlabelled double-stranded VDRE sequence or mutant VDRE sequence (VDREM) in 250-fold excess were added to the binding reaction mixture and incubated. For super-shift assays, anti-human VDR (Santa Cruz) was used. Bound and free oligonucleotide probes were resolved by electrophoresis on an 8% non-denaturing polyacrylamide gel. Electroblotting and chemiluminescence detection were performed by the DIG gel shift kit (Roche, Penzberg, Germany) according to the manufacturer’s instructions.

2.7. Chromatin immunoprecipitation (ChIP)

In vivo binding of VDR to the VDRE in the upstream of the hsa-let-7a-2 gene was detected using the ChIP assay kit (Upstate Biotechnology, Inc., Lake Placid, NY, USA). In general, confluent A549 cells (1 × 10⁷) were treated with 1,25-(OH)₂VD₃ for 48 h and ChIP analysis was performed as described previously (Liu et al., 2008) using anti-human VDR (Sigma, USA) or rabbit IgG (Sigma, USA). Immunoprecipitated DNA fragments were analyzed by PCR using a pair of primers (F: CCTGCTTTGTGATCCATATAAG and R: GTCCTTGCTACTAGTGTC ACT) that span the VDRE sequence in the upstream of the hsa-let-7a-2 gene. PCR products were analyzed in 1% agarose gel and sequenced.

2.8. Statistical analysis

The data represents the mean ± standard deviation (SD). The data analysis was performed using the SPSS version 11.0. Statistical significance was considered if P < 0.05.

3. Results

3.1. Expression of hsa-let-7a-2 in A549 cells was up-regulated by 1,25-(OH)₂VD₃

To determine whether the expression of has-let-7a-2 was regulated by 1,25-(OH)₂VD₃, A549 cells were treated with different concentration of 1,25-(OH)₂VD₃ for 48 h, and the expression of pri-let-7a-2 was detected. The result of real-time PCR showed that the expression of pri-let-7a-2 was up-regulated by 1,25-(OH)₂VD₃ in a dose-dependent manner (Fig. 1a). Moreover, expression of mature let-7a-2 was detected by dual-luciferase reporter gene assay. A549 cells were transfected with pMIR-reporter-vector or pMIR-reporter-7a2T with pMIR-REPORT β-gal plasmid as internal control, and treated with 1,25-(OH)₂VD₃. As shown in Fig. 1b, 1,25-(OH)₂VD₃ treatment decreased the relative luciferase activity of pMIR-reporter-7a2T through increasing mature let-7a-2 expression and the interaction of let-7a-2 with has-let-7a target sequence in pMIR-reporter-7a2T, while 1,25-(OH)₂VD₃ treatment didn’t influence the luciferase activity of pMIR-reporter-vector.
3.2. VD3/VDR interacts with the VDRE from the upstream of pre-let-7a-2 in vitro

To identify the mechanism that 1,25-(OH)₂VD₃ up-regulates the expression of hsa-let-7a-2, MaltInspection 2.2 was used to find the potential VDR binding sites in the upstream sequences of pre-let-7a-2, and one VDRE locates in −2066/−2042 bp of pre-let-7a-2 was detected. Next this VDRE sequence was synthesized and used as probe in EMSA to investigate the interaction of VD3/VDR with the VDRE upstream of pre-let-7a-2. Nuclear extracts from A549 cells treated with 1,25-(OH)₂VD₃ (10⁻⁸ – 10⁻⁶ mol/L) were incubated with labeled VDRE. Result of EMSA indicated that the VDRE could bind with VDR in vitro. Furthermore, the binding was proven to be specific, which was partially blocked by a 250-fold excess of unlabeled VDRE and by anti-VDR antibody, but not by unlabeled mutant VDRE (Fig. 2).

3.3. VD3/VDR interacts with the VDRE located in the upstream of pre-let-7a-2 in vivo

To determine whether the binding of VDR to the VDRE sequence in the upstream of pre-let-7a-2 was true in vivo, we performed ChIP assays in A549 cells treated with 1,25-(OH)₂VD₃ or DMSO. After cross-linking with formaldehyde, cell lysates were prepared by sonication and immunoprecipitated with anti-VDR antibody or rabbit IgG (as a negative control). DNA purified from the coprecipitation was analyzed by PCR with primers spanning the VDRE sequence. The input DNA containing whole genomic DNA fragments was used as a positive control. As shown in Fig. 3, a stronger band about 200 bp in length was observed from the PCR product amplified with the DNA purified from anti-VDR precipitation complexes with 1,25-(OH)₂VD₃ treatment than that with DMSO treatment. No band was detected from that amplified with rabbit IgG precipitation complexes treated with 1,25-(OH)₂VD₃ or DMSO. The sequence of PCR product was confirmed to be VDRE sequence in the upstream of pre-let-7a-2 by DNA sequencing, indicating that VDR activated by 1,25-(OH)₂VD₃ could bind to the VDRE in the upstream of pre-let-7a-2 in live cells.

3.4. 1,25-(OH)₂VD₃ up-regulates hsa-let-7a-2 expression through the interaction between VDR with VDRE in the upstream of pre-let-7a-2

To further investigate whether the interaction between VDR and VDRE in vivo is functional for the regulation of let-7a2 expression, pGL4-luc2[minp] plasmid was modified to insert the VDRE consensus sequence (VDRE-pGL4-luc2[minp]) (as a positive control), the VDRE sequence in the upstream of pre-let-7a-2 (VDRE/let-7a-2-pGL4-luc2[minp]) or the mutant VDRE sequence in the upstream of pre-let-7a-2 (VDREM/let-7a-2-pGL4-luc2[minp]) into the upstream of TATA box. A549 cells was transfected with these pGL4-luc2[minp] constructs and pRL-TK plasmid as internal control, and treated with 1,25-(OH)₂VD₃ for 48 h. The interaction of 1,25-(OH)₂VD₃ activating VDR with VDRE in the upstream of pre-let-7a-2 should be analyzed by luciferase reporter attempts. As shown in Fig. 4, 1,25-(OH)₂VD₃ treatment could up-regulate luciferase activity of VDRE/let-7a-2-pGL4-luc2[minp] in a dose-dependent manner, which is similar to that of VDRE/let-7a-2-pGL4-luc2[minp]. There are no significant changes in LUC expression of VDREM/let-7a-2-pGL4-luc2[minp] under 1,25-(OH)₂VD₃ treatment. The results suggested that the VDRE in the upstream of pre-let-7a-2 is a functional cis-element that mediates VD3/VDR regulating hsa-let-7a-2 expression.

4. Discussion

1,25-(OH)₂VD₃ modulates gene expression through binding to VDR. 1,25-(OH)₂VD₃-bound VDR heterodimerizes with retinoid X
receptors (RXRs) and binds to specific DNA sequences in the promoter of target genes known as VDRE (Haussler et al., 1998; Mangelsdorf et al., 1995). Besides its well characterized role in calcium homeostasis (Jones et al., 1998), 1,25-(OH)2VD3 also inhibits growth and stimulates differentiation of cancer cells derived from a variety of tissues (Campbell and Koeffler, 1997; Hershberger et al., 1999; Zhuang and Bernstein, 1998).

Considerable studies have shown the anti-proliferative effects of 1, 25-(OH)2VD3 in lung cancer. It is reported that 1,25-(OH)2VD3 inhibits the growth of human lung cancer cell lines (Higashimoto et al., 1996) and represses lung tumor growth and lung cell metastases in some kind of cancers, including lung cancer (Kilkkinen et al., 2008; Rammath et al., 2011). It has also been reported that improved survival in the early stage of lung cancer is associated with higher circulating levels of 1,25-(OH)2VD3 (Zhou et al., 2005, 2007). Therefore, 1,25-(OH)2VD3 is a preventive factor in the metastasis of lung cancer (Nakagawa et al., 2005), and metastatic growth of lung cancer cells is extremely reduced in vitamin D receptor knockout mice (Nakagawa et al., 2004). The association between VDR polymorphisms and poor survival rate among lung cancer is also reported (Dogan et al., 2009; Zhou et al., 2006). Nuclear VDR expression is associated with improved survival rate in non-small cell lung cancer (Srinivasan et al., 2011). In summary, 1,25-(OH)2VD3 and VDR are important modulators of cellular proliferation and differentiation in a number of normal and malignant cells, and they can up- or down-regulate the expression of genes involved in cell proliferation, differentiation (Flores and Burnstein, 2010; Wang et al., 2008, 2011), and mineral homeostasis (Kanli and Savli, 2007). In this study, we showed that 1,25-(OH)2VD3 could up-regulate the transcription of hasa-let-7a-2 gene in lung cancer A549 cell line.

So far, little is known about the role and the regulatory mechanisms of hasa-let-7a-2 expression in lung cancer. In the present study, we report that 1,25-(OH)2VD3 could up-regulate hasa-let-7a-2 expression in the transcriptional level in A549 cancer cells. Real-time PCR analysis and luciferase reporter assays showed that 1,25-(OH)2VD3 up-regulates the expression level of hasa-let-7a-2. Analysis of the sequence in the upstream of pre-let-7a-2 showed that one VDRE sequence locates in -2066/-2042 bp of pre-let-7a-2. To determine whether 1,25-(OH)2VD3 activated VDR can bind to this VDRE sequence to promote hasa-let-7a-2 gene expression, EMSA and ChIP experiments were performed. The results of EMSA and ChIP demonstrated that VD3/VDR could interact with VDRE located in the upstream of pre-let-7a-2 both in vivo and in vitro. To further confirm the interaction between VDR and VDRE is required in vivo for the upregulation of hasa-let-7a-2 expression, pGL4-Luc2[minp] constructs in which the VDRE sequence was inserted into the upstream of TATA box were transfected into A549 cells and the cells were treated with 1,25-(OH)2VD3. The result showed that the VDRE in the upstream of pre-let-7a-2 is a functional cis-element mediating the up-regulation of hasa-let-7a-2 expression induced by 1,25-(OH)2VD3 and VD3/VDR.

Epidemiological studies indicate that low vitamin D levels may play a role in the carcinogenesis and progression of various human cancers, including lung cancer. Vitamin D has strong antiproliferative effects in prostate cancers, breast cancers, colorectal cancers, head/neck cancers, and lung cancers, as well as lymphoma and leukemia. Preclinical research indicates that 1,25-(OH)2D3 or vitamin D analogs might have potential as anticancer agents because their administration has antiproliferative effects (Cheung et al., 2012; Deeb et al., 2007). Increasing epidemiological and experimental evidence supports the development of 1,25-(OH)2D3 and vitamin D analogs as preventative and therapeutic anticancer agents. In conclusion, we reported that 1,25-(OH)2VD3 activating VDR interacts with the VDRE in the upstream of hasa-let-7a-2 gene to promote the hasa-let-7a-2 expression in lung cancer cells. Both 1,25-(OH)2VD3/VDR and hasa-let-7a are supposed to have antitumor effects. Some let-7a target genes such as E2F, EGR, IGF1R, USP, RAS and MAP4K are involved in cell proliferation. Our data presented here suggested that the up-regulation of hasa-let-7a-2 expression induced by 1,25-(OH)2VD3 might be involved in the anti-proliferation effects of 1,25-(OH)2VD3 in lung cancer cells.

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![Fig. 3. CHIP analysis of the interaction between VD3/VDR and the VDRE in the upstream of pre-let-7a2. A549 cells treated with 1,25-(OH)2VD3 (10−8 mol/L) or DMSO for 48 h were cross-linked by formaldehyde treatment and lysed. Cell lysates were subjected to immunoprecipitation with either anti-human VDR or rabbit IgG. Recovered DNA from the immunoprecipitation was amplified by a pair of primers spanning the VDRE in the upstream of pre-let-7a2. Input DNA was loaded as positive control.](image1)

![Fig. 4. Luciferase reporter assay showing that 1,25-(OH)2VD3 up-regulates let-7a-2 expression through the interaction between VDR with VDRE in the upstream of pre-let-7a-2. The wildtype or mutant sequences of dual-tandem VDRE from the up- stream of pre-let-7a-2 were synthesized in vitro and inserted into the upstream of the TATA box in pGL4-luc2[minp] vector to generate the VDRE/let-7a2-pGL4-luc2[minp] or VDREm/let-7a2-pGL4-luc2[minp] plasmids. pGL4-luc2[minp] and VDRE-pGL4-luc2[minp] containing a VDRE consensus sequence was used as the negative and positive control, respectively. The four construct was co-transfected with PRL-TK plasmid respectively into A549 cells, and the cells were treated with 1,25-(OH)2VD3 (10−8, 10−9 mol/L) or DMSO for 48 h. The ratio of pGL4-luc2 luciferase activity (M1) and the PRL-TK luciferase activity (M2) was expressed as relative luciferase activity. The data is presented as the means of six individual values ± SD. P < 0.05, * vs #.](image2)
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