

# 1 $\alpha$ ,25-Dihydroxyvitamin D3 Modulates CYP2R1 Gene Expression in Human Oral Squamous Cell Carcinoma Tumor Cells

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**Abstract** Oral squamous cell carcinomas (OSCC) are the most common malignant neoplasms associated with mucosal surfaces of the oral cavity and oropharynx. 1 $\alpha$ ,25-Dihydroxyvitamin D3 (1,25(OH)<sub>2</sub>D3) is implicated as an anticancer agent. Cytochrome P450 2R1 (CYP2R1) is a microsomal vitamin D 25-hydroxylase which plays an important role in converting dietary vitamin D to active metabolite, 25-(OH)<sub>2</sub>D3. We identified high levels of CYP2R1 expression using tissue microarray of human OSCC tumor specimens compared to normal adjacent tissue. Therefore, we hypothesize that 1,25(OH)<sub>2</sub>D3 regulates CYP2R1 gene expression in OSCC tumor cells. Interestingly, real-time RT-PCR analysis of total RNA isolated from OSCC cells (SCC1, SCC11B, and SCC14a) treated with 1,25(OH)<sub>2</sub>D3 showed a significant increase in CYP2R1 and vitamin D receptor (VDR) mRNA expression. Also, Western blot analysis demonstrated that 1,25(OH)<sub>2</sub>D3 treatment time-dependently increased CYP2R1 expression in these cells. 1,25(OH)<sub>2</sub>D3 stimulation of OSCC cells transiently transfected with the hCYP2R1 promoter (−2 kb)-luciferase reporter plasmid demonstrated a 4.3-fold increase in promoter activity. In addition, 1,25(OH)<sub>2</sub>D3 significantly increased c-Fos, p-c-Jun expression, and c-Jun N-terminal kinase (JNK) activity in these cells. The JNK inhibitor suppresses 1,25(OH)<sub>2</sub>D3, inducing CYP2R1 mRNA expression and gene promoter activity in OSCC cells. Furthermore, JNK inhibitor significantly decreased 1,25(OH)<sub>2</sub>D3 inhibition of OSCC tumor cell proliferation. Taken together, our results suggest that AP-1 is a downstream effector of 1,25(OH)<sub>2</sub>D3 signaling to modulate CYP2R1 gene expression in OSCC tumor cells, and vitamin

D analogs could be potential therapeutic agents to control OSCC tumor progression.

## Introduction

Head and neck squamous cell carcinoma (HNSCC) contributes to approximately 3 % of all malignancies in the USA [25]. More than 90 % of oral cancers are squamous cell carcinomas (OSCC) which contributes >40 % of HNSCC and are associated with mucosal surfaces of the oral cavity and oropharynx [18]. The etiology of OSCC is strongly associated with certain environmental, lifestyle risk factors including tobacco, alcohol consumption, chronic inflammation, and viral infections. Genetic alteration and molecular events such as cytogenic abnormalities, inactivation of tumor suppressor genes, and changes in intracellular signaling pathways are involved in OSCC tumor progression [39]. OSCC show a potent activity of local bone invasion, which dramatically impacts the patients' recovery and quality of life [32]. OSCC tumor cells have been shown to invade maxillary and mandibular bone in a murine model [28]. It has been reported that TGF- $\beta$  signaling in the tumor–bone microenvironment facilitates cancer cell invasion of bone [15]. Nuclear factor kappaB (NF- $\kappa$ B) expression is upregulated in OSCC gradually from premalignant lesions to invasive cancer [26]. It has been shown that MMP-1 and MMP-9 are highly expressed in BHY cells, derived from an SCC which had deeply invaded into the mandible [10]. Some of the chemoattractants present in the bone matrix play a pivotal role in bone invasion. Recently, we demonstrated that CXCL13 plays an important role in OSCC invasion of bone/osteolysis in athymic mice [29].

1 $\alpha$ ,25-Dihydroxyvitamin D3 (1,25(OH)<sub>2</sub>D3; calcitriol) is the most biologically active form of vitamin D metabolite with

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high affinity to the vitamin D receptor (VDR) and has been implicated as an anticancer agent [23]. Previously, it has been reported that vitamin D3 and 13-*cis* retinoic acid have equipotent antiproliferative effects on tongue squamous cell carcinoma (SCC-25) cells [9]. Also, 1,25(OH)<sub>2</sub>D3 has been shown to inhibit the growth of HNSCC cells through upregulation of cell cycle inhibitor p18 expression [14]. Single nucleotide polymorphisms associated with VDR have been shown to increase the risk of OSCC [24]. Vitamin D 25-hydroxylase (CYP2R1) is a member of the cytochrome P450 superfamily of monooxygenases, which are involved in drug metabolism and synthesis of cholesterol, steroids, and lipids. CYP2R1 is a microsomal enzyme that converts vitamin D into 25-(OH)D3 [4]. The physiologic significance of CYP2R1 was established by the finding in two Nigerian brothers that a homologous inactivating L99P mutation of the *CYP2R1* gene was associated with rickets caused by isolated 25(OH)D deficiency [4]. In this study, we demonstrated CYP2R1 expression and 1,25(OH)<sub>2</sub>D3 transcriptional regulatory mechanism in oral squamous cell carcinoma tumor cells.

## Materials and Methods

**Reagents and Antibodies** Cell culture and DNA transfection reagents were purchased from Invitrogen (Carlsbad, CA). 1,25(OH)<sub>2</sub>D3 was purchased from Enzo Life Sciences (Farmingdale, NY). Anti-CYP2R1, anti-c-Fos, anti-p-c-Jun, and peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Supersignal enhanced chemiluminescence (ECL) reagent was obtained from Amersham Bioscience (Piscataway, NJ), and nitrocellulose membranes were purchased from Millipore (Bedford, MA). A luciferase reporter assay system was obtained from Promega (Madison, WI). Protease inhibitor cocktail was purchased from Sigma Chemical Co. (St. Louis, MO), and c-Jun N-Terminal Kinase (JNK) inhibitor (SP600125) was purchased from Calbiochem (San Diego, CA).

**Cell Lines and Cell Cultures** Human OSCC-derived cell lines SCC1, SCC11B, and SCC14a were generously provided by Dr. Thomas E. Carey (University of Michigan, Ann Arbor, MI) and were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10 % fetal bovine serum (FBS) and supplemented with L-glutamine, penicillin, and streptomycin. All cells were incubated at 37 °C in 5 % CO<sub>2</sub>.

**Quantitative Real-Time Reverse Transcription PCR** CYP2R1 mRNA expressions in OSCC cells were measured by real-time reverse transcription (RT)-PCR as described previously [34]. Briefly, total RNA was isolated from human OSCC cells stimulated with and without 1,25(OH)<sub>2</sub>D3 (10<sup>-8</sup> M) for 0–48 h, using

RNAzol reagent (Biotecx Labs, Houston, TX). A reverse transcription reaction was performed using poly-dT primer and reverse transcriptase in a 25-μl reaction volume containing total RNA (2 μg), 1× PCR buffer and 2 mM MgCl<sub>2</sub>, at 42 °C for 15 min and followed by 95 °C for 5 min. The quantitative real-time PCR was performed using IQ™ SYBR Green Supermix in an iCycler (iCycler iQ Single-Color Real-Time PCR Detection System; Bio-Rad, Hercules, CA). The primer sequences used to amplify the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were 5' CCTACCCCAATGTATCCGTTG TG-3' (sense) and 5'-GGAGGAATGGGAGTTGCTGTTGAA-3' (antisense); human CYP2R1 mRNA were 5' GAAAGCAG AGCCAGGTGTACG 3' (sense) and 5' TCATGAATAAAGGA AGGCATGG 3' (antisense); and human VDR mRNA were 5' CCAACTCCAGACACTCC 3' (sense) and 5' AGATTG GAGAAGCTGGACGA 3' (antisense). Thermal cycling parameters were 94 °C for 3 min, followed by 40 cycles of amplifications at 94 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min, and 72 °C for 5 min as the final elongation step. Relative levels of CYP2R1 mRNA expression were normalized in all the samples analyzed with respect to GAPDH amplification.

**Cloning and Characterization of hCYP2R1 Gene Promoter** The CYP2R1 gene promoter region (−1 to −2 kb relative to the transcription start site) was PCR-amplified using the template human genomic DNA and CYP2R1 gene-specific primers, 5'-GAGCTCGAGTTATTGATTAA TAAGAATTTT-3' (sense) and 5'-GAGCTCGAGCGGCC CGAGCTGGAGCTGGAGGTGCGAAC-3' (antisense) (GenBank™ accession no. AY800276.1). Sequences underlined are the XhoI restriction enzyme site added for subcloning purposes. The PCR-amplified CYP2R1 promoter fragment was subcloned into the pGL2 Basic vector, and the resulting plasmid termed pGL2 Basic-hCYP2R1-luciferase and the promoter sequence were analyzed using the web-based TF database search.

SCC14a cells were cultured in DMEM supplemented with 10 % FBS and 100 units/ml of penicillin/streptomycin. DNA transfections were performed using Lipofectamine-Plus transfection reagent (Invitrogen, Inc., San Diego, CA). Cells were transiently transfected with pGL2 Basic-hCYP2R1-luciferase plasmid and cultured in the presence or absence of 1,25(OH)<sub>2</sub>D3 (10<sup>-8</sup> M) for 48 h. A 20-μl aliquot of total cell lysates was mixed with 100 μl of the luciferase assay reagent. The light emission was measured for 10 s of integrated time using Sirius Luminometer following the manufacturer's instructions (Promega, Madison, WI). The transfection efficiency was normalized by co-transfection with pRSV β-gal plasmid and measuring the β-galactosidase activity in these cells.

**Tissue Microarray** Tissue microarray (TMA) of 60 primary human OSCC tumor specimens were obtained from the Head and Neck Cancer Tissue Array Initiative at the NIDCR, NIH

[27], and 14 control adjacent normal tissues were obtained from the Hollings Cancer Center Tissue Biorepository, Medical University of South Carolina, in accordance with an Institutional Review Board (IRB)-approved protocol. Serial 5- $\mu\text{m}$  sections were cut on a modified Leica RM 2155 rotary microtome (Leica Microsystems, Richmond Hill, ON, Canada). TMA blocks were deparaffinated in xylene for 10 min and rehydrated by successive transfers in alcohol with decreasing concentration and finally in  $\text{H}_2\text{O}$ . Then, sections were washed for 5 min in 3 %  $\text{H}_2\text{O}_2$  to inhibit endogenous peroxidase. The slides were incubated with goat polyclonal antibody against CYP2R1 for 3 h at room temperature. Immunohistochemical (IHC) staining was performed with HRP-labeled secondary antibody and diaminobenzidine (Vector Laboratories, Burlingame, CA, USA). The slides were briefly counterstained with hematoxylin and dehydrated through graded alcohols to xylene and were cover slipped with a permanent mounting media. CYP2R1 IHC semiquantification was determined using the modified *H* score which consists of the sum of percent of tumor cells staining multiplied by an ordinal value corresponding to the staining intensity level (0=none, 1=weak, 2=moderate, and 3=strong). IHC scores were determined by taking the product of the estimated staining intensity and area of tissue (tumor or normal) stained ( $<1/3=1$ ;  $1/3-2/3=2$ ;  $>2/3=3$ ), giving a range of possible scores between 0 and 9. IHC scores were averaged to determine a composite score for each group as described [19].

**Cell Proliferation Assay** OSCC tumor cells were stimulated with  $1,25(\text{OH})_2\text{D}_3$  ( $10^{-8}$  M) alone and in the presence of a 2- $\mu\text{M}$  concentration of JNK inhibitor (SP600125) for 24 h, then incubated with alamarBlue reagent (Life technologies, Grand Island, NY) for 4 h at 37 °C. The fluorescence intensity was read at 560 nm of excitation and at 590 nm of emission, and alamarBlue reduction was calculated as per the manufacturer's protocol. Background absorbance was subtracted using a control media.

**Western Blot Analysis** OSCC cells were stimulated with  $1,25(\text{OH})_2\text{D}_3$  ( $10^{-8}$  M) for an indicated time point (0–72 h) and lysed in a buffer containing 20 mM Tris-HCl at pH 7.4, 1 % Triton X-100, 1 mM EDTA, 1.5 mM  $\text{MgCl}_2$  10 % glycerol, 150 mM NaCl, and 0.1 mM  $\text{Na}_3\text{VO}_4$ . The protein content of the samples was measured using the BCA protein assay reagent (Thermo Fisher Scientific Inc., Rockford, IL). Protein (20  $\mu\text{g}$ ) samples were then subjected to SDS-PAGE using 12 % Tris-HCl gels and blot transferred onto a nitrocellulose membrane and immunoblotted with anti-CYP2R1, anti-c-Fos, and anti-p-c-Jun antibodies. The bands were detected using the enhanced chemiluminescence detection system. The band intensity was quantified by densitometric analysis using the NIH ImageJ Program.

**JNK Activity Assay** SCC14a cells were stimulated with  $1,25(\text{OH})_2\text{D}_3$  ( $10^{-8}$  M) for 0–6 h. Cells were resuspended in cell lysis buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM  $\beta$ -glycerol phosphate, 1 mM sodium vanadate, 1  $\mu\text{g}/\text{ml}$  leupeptin, 1 mM phenylmethyl sulfonyl fluoride (PMSF), and 1 % Triton X-100), followed by a brief sonication. Cell lysates were cleared by centrifugation for 3 min at 1,200g, and the supernatants were assayed for N-terminal JNK activity using a solid-phase GST-c-Jun (1–89 amino acids) fusion protein. Briefly, JNK was co-precipitated with its substrate conjugated to glutathione-S-sepharose beads at 4 °C. After overnight incubation, the precipitates were washed twice with cell lysis buffer followed by a kinase buffer (25 mM Tris (pH 7.5), 5 mM  $\beta$ -glycerol phosphate, 1 mM sodium vanadate, 2 mM dithiothreitol (DTT), and 10 mM  $\text{MgCl}_2$ ). After the last wash, pellets were resuspended in 50  $\mu\text{l}$  of kinase buffer. The reaction was carried out at 30 °C for 30 min in the presence of 100  $\mu\text{M}$  of ATP and stopped by adding sample buffer. Proteins were separated by SDS-PAGE (15 %) and blot transferred onto a nitrocellulose membrane. p-c-Jun expression was detected with a specific anti-phospho-c-Jun antibody following the manufacturer's instructions (New England Biolabs, Beverly, MA, USA).

**Statistical Analysis** Results are presented as mean $\pm$ SD for three independent experiments and were compared by Student's *t* test. Paired *t* test was used to compare the mean *H* score of CYP2R1 levels in OSCC and normal adjacent tissues. Values were considered significantly different for  $p<0.05$ .

## Results

### CYP2R1 Expression in Oral Squamous Cell Carcinoma

Cytochrome P450 (CYP) enzymes play an important role in the conversion of inactive vitamin D into active  $1,25(\text{OH})_2\text{D}_3$ . CYP2R1 catalyzes the initial step converting vitamin D into 25-hydroxyvitamin D3 (25-(OH)D3) in the liver. It has been shown that several cancer cell types are capable of producing  $1,25(\text{OH})_2\text{D}_3$  to regulate their growth [33]. Therefore, we examined the CYP2R1 abundance in OSCC tumor specimens. Interestingly, immunohistochemical staining using a TMA of 60 human OSCC tumor specimens identified that 56 of 60 (>90 %) stained strongly positive for CYP2R1 abundance with an average *H* score >7 as described in "Materials and Methods." In contrast, 14 normal adjacent tissues demonstrated very low levels of CYP2R1 expression with an average *H* score <2. A representative OSCC tumor specimen demonstrated an abundance of CYP2R1 compared

to the normal adjacent tissue. Immunostaining of OSCC tumor specimen with a nonspecific IgG served as negative control (Fig. 1).

We next examined CYP2R1 expression in OSCC-derived cell lines (SCC1, SCC11B, and SCC14a). Real-time RT-PCR analysis of total RNA isolated from these cells showed that 1,25(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-8</sup> M) significantly increased CYP2R1 mRNA expression in these cells. SCC14a cells showed a high level of CYP2R1 mRNA expression compared to other cell lines SCC1 and SCC11B (Fig. 2a). Western blot analysis of total cell lysates further confirmed that 1,25(OH)<sub>2</sub>D<sub>3</sub> stimulates CYP2R1 abundance (4.8-fold) in a time-dependent (0–72 h) manner in SCC14a cells (Fig. 2b). We next examined the vitamin D receptor (VDR) mRNA expression in OSCC cells. SCC14a cells were stimulated with 1,25(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-8</sup> M) for 0–48 h, and total RNA isolated was subjected to real-time RT-PCR analysis for VDR mRNA expression. As shown in Fig. 2c, 1,25(OH)<sub>2</sub>D<sub>3</sub> significantly increased (4.6-fold) VDR mRNA expression in a time-dependent manner. These results suggest that 1,25(OH)<sub>2</sub>D<sub>3</sub> modulates VDR and CYP2R1 expression in OSCC tumor cells. We then tested the effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on OSCC tumor cell growth. OSCC cells (SCC1, SCC11B, and SCC14a) were stimulated with 1,25(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-8</sup> M) for 24 h and assayed for proliferation. As shown in Fig. 2d, 1,25(OH)<sub>2</sub>D<sub>3</sub> significantly inhibit OSCC tumor cells proliferation.

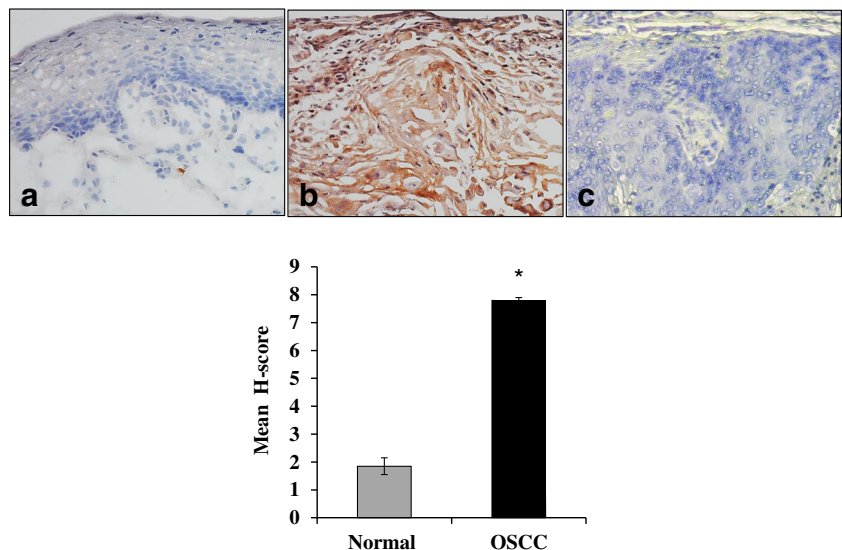
#### 1,25(OH)<sub>2</sub>D<sub>3</sub> Transcriptional Control of CYP2R1 Gene Expression

To characterize the transcriptional control of human CYP2R1 gene expression in OSCC cells, we analyzed the CYP2R1 promoter region for potential transcription factor binding motifs by web-based TF search. We thus identified several AP-1 binding sites (-118, -245, and -1,318 bp) in the hCYP2R1

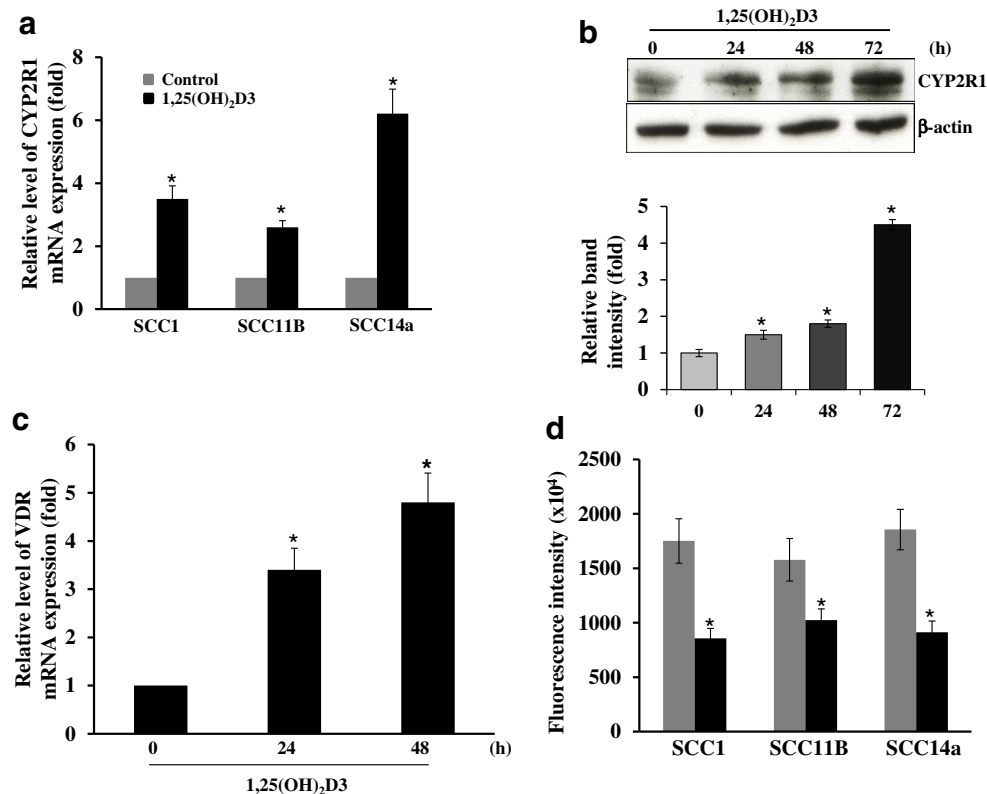
gene promoter (-2 kb relative to start codon) region. It has been shown that 1,25(OH)<sub>2</sub>D<sub>3</sub> upregulates c-Jun/c-Fos (AP-1) expression in human osteosarcoma [41]. Therefore, we examined the 1,25(OH)<sub>2</sub>D<sub>3</sub> modulation of c-Fos and c-Jun expression. SCC14a cells were stimulated with 1,25(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-8</sup> M) for 0–6 h. Total cell lysates were subjected to western blot analysis for c-Fos and p-c-Jun expression. As shown in Fig. 3a, 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment increased the levels of c-Fos (5-fold) and p-c-Jun (7-fold) protein in a time-dependent manner in OSCC cells. Also, 1,25(OH)<sub>2</sub>D<sub>3</sub> stimulation elevated (4.5-fold) JNK activity in these cells (Fig. 3b). Furthermore, 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment to OSCC cells transiently transfected with AP-1-luciferase reporter plasmid significantly increased (3.2-fold) AP-1 activity in these cells (Fig. 3c). We next examined the potential of AP-1 modulation of CYP2R1 gene expression in SCC14a cells. Cells were treated with JNK inhibitor SP 600125 (2 μM) for 30 min, followed by 1,25(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-8</sup> M) for 48 h. Total RNA isolated from these cells was subjected to real-time RT-PCR analysis for CYP2R1 mRNA expression. As shown in Fig. 4a, JNK inhibitor significantly decreased 1,25(OH)<sub>2</sub>D<sub>3</sub>, inducing CYP2R1 mRNA expression in these cells.

We further examined the participation of JNK in 1,25(OH)<sub>2</sub>D<sub>3</sub> transcriptional regulation of CYP2R1 gene promoter activity in SCC14a cells. PGL2 Basic-hCYP2R1 promoter-luciferase reporter plasmid was transiently transfected into SCC14a cells using the lipofectamine reagent. Cells were cultured with 1,25(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-8</sup> M) in the presence or absence of JNK inhibitor for 48 h. Total cell lysates obtained from these cells were analyzed for luciferase activity as described. As shown in Fig. 4b, JNK inhibitor suppressed 1,25(OH)<sub>2</sub>D<sub>3</sub> stimulation of hCYP2R1 gene promoter activity compared to unstimulated cells. Thus, our results suggest that AP-1 is a downstream regulator of vitamin D signaling to modulate CYP2R1 expression in OSCC tumor cells. We next,

**Fig. 1** CYP2R1 abundance in human OSCC tumor specimens. Immunohistochemical staining of CYP2R1 expression in a representative **a** normal adjacent tissue, **b** OSCC tumor specimen, and **c** control IgG as analyzed by tissue microarray. The mean *H* score of OSCC and adjacent normal tissue from 60 to 14 specimens respectively is illustrated as a graph. The data are shown as mean±SD (\**p*<0.05)







**Fig. 2** 1,25(OH)<sub>2</sub>D<sub>3</sub> enhances CYP2R1 mRNA expression in OSCC cell lines. **a** OSCC tumor cell lines SCC1, SCC11B, and SCC14a cells were stimulated with 1,25(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-8</sup> M) for 48 h. Total RNA isolated was subjected to real-time RT-PCR analysis for CYP2R1 mRNA expression. The relative levels of CYP2R1 mRNA expression were normalized with respect to the levels of GAPDH amplification. **b** SCC14a cells were stimulated with 1,25(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-8</sup> M) for 0–72 h. Total cell lysates obtained were subjected to western blot analysis for CYP2R1 levels. β-actin levels served as loading control. The band intensity was quantified

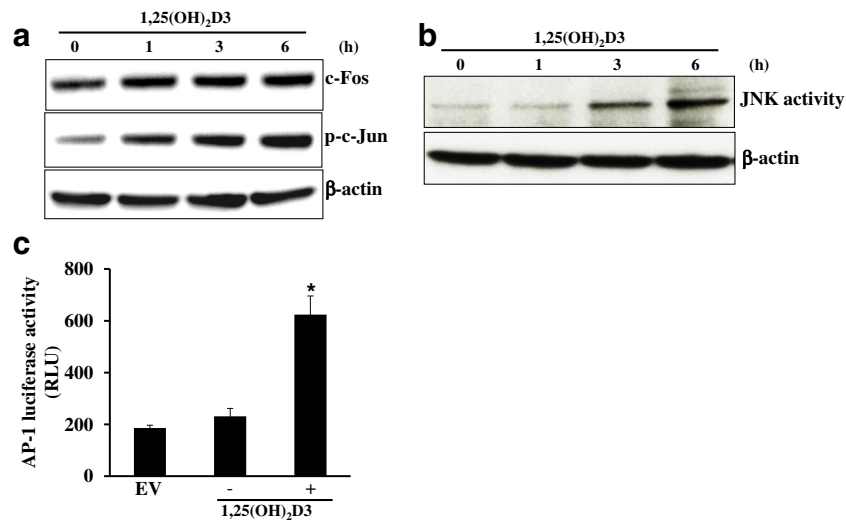
by densitometric analysis using the NIH ImageJ Program. **c** SCC14a cells were stimulated with 1,25(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-8</sup> M) for 0–48 h. Total RNA isolated was subjected to real-time RT-PCR analysis for VDR mRNA expression. The relative levels of VDR mRNA expression were normalized with respect to the levels of GAPDH amplification. **d** OSCC tumor cells were seeded (1 × 10<sup>4</sup> cells/well) in a 96-well plate. Cells were stimulated with 1,25(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-8</sup> M) for 24 h and incubated with alamarBlue reagent for 4 h at 37 °C and assayed for cell proliferation. Data represent triplicate studies and are shown as mean ± SD (\**p* < 0.05)

delineate the link between JNK activation and vitamin D inhibition of OSCC tumor cell proliferation. SCC14a cells were treated with JNK inhibitor (2 μM) for 30 min, followed by 1,25(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-8</sup> M) for 24 h. As shown in the Fig. 4c, JNK inhibitor significantly decreased vitamin D inhibition of OSCC cell proliferation. These data suggest the participation of JNK in anticancer activity of vitamin D.

## Discussion

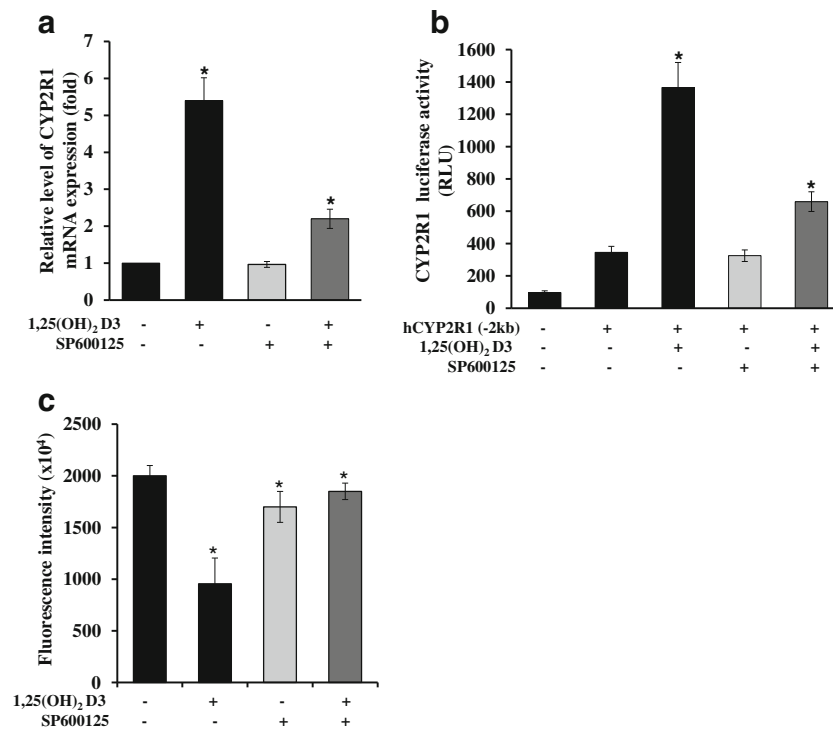
CYP2R1 is a microsomal vitamin D 25-hydroxylase. Although at least six CYP enzymes have been reported with 25-hydroxylase activity, CYP2R1 is the predominant type expressed at high levels in prostate and ovarian cancer cells [3, 8]. In this study, we demonstrated that human OSCC tumor cells express CYP2R1 at high levels and that physiologic concentrations of 1,25(OH)<sub>2</sub>D<sub>3</sub> modulate CYP2R1 levels in these cells. OSCC have been shown to produce elevated levels

of several cytokines/growth factors such as PTH, TNF-α, IL-1, and TGF-β [31]. Therefore, it is possible that besides 1,25(OH)<sub>2</sub>D<sub>3</sub>, tumor-derived cytokines may regulate CYP2R1 levels in OSCC cells. Recent evidence indicates that dietary vitamin D and 1,25(OH)<sub>2</sub>D<sub>3</sub> show equivalent anticancer activity in mouse xenograft models of breast and prostate cancer. 1,25(OH)<sub>2</sub>D<sub>3</sub> deficiency increases the cancer risk and mortality [35]. Environmental factors such as sunlight exposure which promotes 1,25(OH)<sub>2</sub>D<sub>3</sub> synthesis in skin lowers cancer risk [13, 37]. Furthermore, adequate levels of serum vitamin D are important to decrease the risk of prostate cancer [11]. Our findings that 1,25(OH)<sub>2</sub>D<sub>3</sub> modulates CYP2R1 abundance in OSCC tumor cells suggest that vitamin D3 deficiency enhances the risk of oral cancer. Although an inverse association between dietary components such as folate and oral cancer prognosis is noted [30], the contribution of vitamin D metabolism in OSCC is yet to be established. Vitamin D has been implicated in cellular proliferation, differentiation, and apoptosis of normal and cancer cells [7, 12]. In addition, 1,25



**Fig. 3**  $1,25(\text{OH})_2\text{D}_3$  enhances c-Fos and p-c-Jun levels in SCC14a cells. **a** Cells were stimulated with  $1,25(\text{OH})_2\text{D}_3$  (10<sup>-8</sup> M) for indicated time point (0–6 h). Total cell lysates were subjected to western blot analysis for c-Fos and p-c-Jun levels. **b** SCC14a cells were stimulated with  $1,25(\text{OH})_2\text{D}_3$  (10<sup>-8</sup> M) for 0–6 h. Total cell lysates were assayed for JNK activity using a solid-phase GST-c-Jun (1–89 amino acids) fusion protein as described in “Materials and Methods.” **c** SCC14a cells were

transfected with empty vector (EV) and AP-1-luciferase reporter plasmids and stimulated with  $1,25(\text{OH})_2\text{D}_3$  (10<sup>-8</sup> M) for 48 h. Total cell lysates prepared were assayed for luciferase activity. The transfection efficiency was normalized by  $\beta$ -galactosidase activity co-expressed in these cells. Values are expressed as mean  $\pm$  SD for three independent experiments (\**p* < 0.05)



**Fig. 4** Inhibition of JNK suppresses  $1,25(\text{OH})_2\text{D}_3$ -stimulated CYP2R1 expression in SCC14a cells. **a** Cells were treated with a 2- $\mu$ M concentration of JNK inhibitor (SP600125) for 30 min, followed by stimulation with  $1,25(\text{OH})_2\text{D}_3$  (10<sup>-8</sup> M) for 48 h. Total RNA isolated was subjected to real-time RT-PCR analysis for CYP2R1 mRNA expression. The relative levels of CYP2R1 mRNA expression were normalized with respect to the levels of GAPDH amplification. **b** Cells were transfected with empty vector (EV) and pGL2 Basic-hCYP2R1 promoter-luciferase reporter plasmid and stimulated with  $1,25(\text{OH})_2\text{D}_3$  (10<sup>-8</sup> M) for 48 h in

the presence and absence of JNK inhibitor. Total cell lysates were assayed for luciferase activity. The transfection efficiency was normalized by  $\beta$ -galactosidase activity co-expressed in these cells. **c** JNK inhibitor suppresses vitamin D inhibition of OSCC cell proliferation. SCC14a cells were stimulated with  $1,25(\text{OH})_2\text{D}_3$  (10<sup>-8</sup> M) for 24 h in the presence and absence of JNK inhibitor (2  $\mu$ M) and incubated with alamarBlue reagent for 4 h at 37 °C and assayed for cell proliferation. Data represent triplicate studies and are shown as mean  $\pm$  SD (\**p* < 0.05)

(OH)<sub>2</sub>D<sub>3</sub> inhibits hepatocellular carcinoma through reducing inflammatory cytokines [16]. Recently, vitamin D status, intake, and metabolism are implicated in oral cancer risk [45]. Vitamin D activation and VDR binding has been shown to modulate TGF-β which exerts antiproliferative activity of cancer cells [21]. 1,25(OH)<sub>2</sub>D<sub>3</sub> showed antiproliferative effect on thyroid cancer cell lines that demonstrated high levels of CYP2R1 expression [1]. CYP2R1 has been shown to be predominantly expressed in prostate cancer cells, suggesting that vitamin D metabolites play an important role in regulating prostate cancer growth [3]. Conversely, renal clear cell cancer cells showed suppression of VDR and CYP2R1 expression during malignant transformation [2]. Recently, 1,25(OH)<sub>2</sub>D<sub>3</sub> has been shown to modulate several cytokine levels in plasma from patients with HNSCC [38]. Therefore, it is possible that 1,25(OH)<sub>2</sub>D<sub>3</sub> regulation of cytokine/growth factor concentration in OSCC cells may affect the CYP2R1 gene expression in tumor cells.

Previously, a potential role for c-Jun and c-Fos protein levels in malignant transformation of oral mucosa has been reported [36]. Also, OSCC have been shown to constitutively express low levels of JNK activity [42]. NF-κB has been shown to be constitutively activated in squamous cell carcinoma [22]. It has been reported that antiproliferative actions of 1,25(OH)<sub>2</sub>D<sub>3</sub> involve VDR-mediated activation of MAPK signaling molecules and AP-1/p21/waf1 upregulation in human osteosarcoma [41]. Evidence also indicates that patients with oral and pharyngeal cancers harbor polymorphisms and mutations in VDR [17]. Gene polymorphisms associated with VDR and other cytochrome P450 family members CYP27B1 and CYP24A1 may affect susceptibility to OSCC [43]. Although CYP2R1 gene promoter contains a putative vitamin D response element (−1,969 to −1,953 bp relative to start codon), our findings using JNK inhibitor confer the specificity for AP-1 transcription factor to enhance CYP2R1 abundance in OSCC cells. Previously, it has been reported that vitamin D<sub>3</sub> and 13-*cis* retinoic acid have equipotent antiproliferative effects on tongue squamous cell carcinoma (SCC-25) cells [9]. Also, 1,25(OH)<sub>2</sub>D<sub>3</sub> has been shown to inhibit the growth of HNSCC cells through upregulation of cell cycle inhibitor p18 expression [14]. Consistently, we demonstrated that 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits OSCC tumor cell proliferation. Furthermore, our results that JNK inhibitor blocks vitamin D inhibition of OSCC tumor cell proliferation suggest that c-Jun activation plays an important role in anticancer activity. Therefore, the vitamin D analogs may have an adjunctive role to play in the management of certain cancers [20, 40]. It has been shown that 5-FU and 13-*cis* retinoic acid combination potently inhibits OSCCs than 5-FU and vitamin D alone [6]. Recently, MART-10, a vitamin D analog has been shown to inhibit HNSCC cells growth [5]. Vitamin D effects may be cell line-specific, and lung adenocarcinoma cancer cells with distinct EGFR mutations have been shown to be more

responsive to vitamin D [44]. However, in this study, we demonstrated that vitamin D inhibits the growth of different OSCC tumor-derived cell lines. Thus, our results suggest that AP-1 is a downstream effector of 1,25(OH)<sub>2</sub>D<sub>3</sub> signaling to modulate CYP2R1 expression and inhibits OSCC tumor cell proliferation. Therefore, the vitamin D analogs could be potential therapeutic agents to control OSCC tumor progression.

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**Conflict of Interests** The authors declare no conflict of interest.

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