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Noncalcemic Vitamin D Hydroxyderivatives Inhibit Human Oral Squamous Cell Carcinoma and Down-regulate Hedgehog and WNT/β-Catenin Pathways

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Abstract. *Background/Aim: The hormonally-active form of vitamin D, 1,25(OH)$_2$D$_3$, demonstrated activity against oral squamous cell carcinoma (OSCC). Cytochrome P450scc (CYP11A1)-derived vitamin D hydroxyderivatives, such as 20(OH)D$_3$ and 1,20(OH)$_2$D$_3$, have overlapping beneficial effects with 1,25(OH)$_2$D$_3$ without causing hypercalcemia. This study sought to determine (i) whether 20(OH)D$_3$ and 1,20(OH)$_2$D$_3$ exhibit antitumor effects against OSCC comparable to those of 1,25(OH)$_2$D$_3$ and (ii) whether these effects may stem from down-regulation of sonic hedgehog (SHH) or WNT/β-catenin signaling pathways. Materials and Methods: Effects on CAL-27 cells were assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt and spheroid assays. Signaling pathways were assessed by immunofluorescence and western blotting. Results: 20(OH)D$_3$ and 1,20(OH)$_2$D$_3$ inhibited the growth of CAL-27 and demonstrated inhibition of WNT/β-catenin and the SHH signaling as evidenced by down-regulation of nuclear translocation of glioma-associated oncogene 1(GLI1) and β-catenin. Conclusion: Noncalcemic vitamin D hydroxyderivatives demonstrated antitumor activities against OSCC comparable to those of 1,25(OH)$_2$D$_3$. Their activities against SHH and the WNT/β-catenin pathways provide insight for a possible target for OSCC treatment.*

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Oral and pharyngeal cancer combined represent the sixth most common cancer globally. The estimated global incidence of oral cancer alone is 275,000, and about two-thirds of these occur in developing nations (1). The estimated incidence of oral cavity and oropharyngeal cancer in the USA was 53,000 in 2019 (2), and more than 90% of these were squamous cell carcinomas (OSCCs) (3). Greater than 50% of OSCCs arise from the tongue and the floor of the mouth but they can arise from anywhere within the mouth (4). CAL-27 is an oral adenosquamous cell carcinoma line derived from the tongue of a 56-year-old man with poorly differentiated disease (5, 6), and it is frequently used for *in vitro* studies of OSCC.

Systemic treatment options for OSCCs include docetaxel, cisplatin and fluorouracil, as well as cetuximab, an epidermal growth factor receptor (EGFR) inhibitor (7). Loss of cyclin-dependent kinase inhibitor 2A (*CDKN2A*), tumor protein p53 (*TP53*), transforming growth factor beta receptor 2 (*TGFBR2*)/SMAD (fusion of *Caenorhabditis elegans Sma* and *Drosophila* Mothers against decapentaplegic) family member 4 (*SMAD4*) have been implicated in the molecular pathogenesis of OSCC. Abnormal expression of genes associated with tumor survival, such as *EGFR*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) and caspase 8 (*CASP8*), have also been implicated (8).

Recently, members of the hedgehog signaling pathway have been identified as being up-regulated in OSCC. Sonic hedgehog (SHH), a ligand that binds to patched, was found

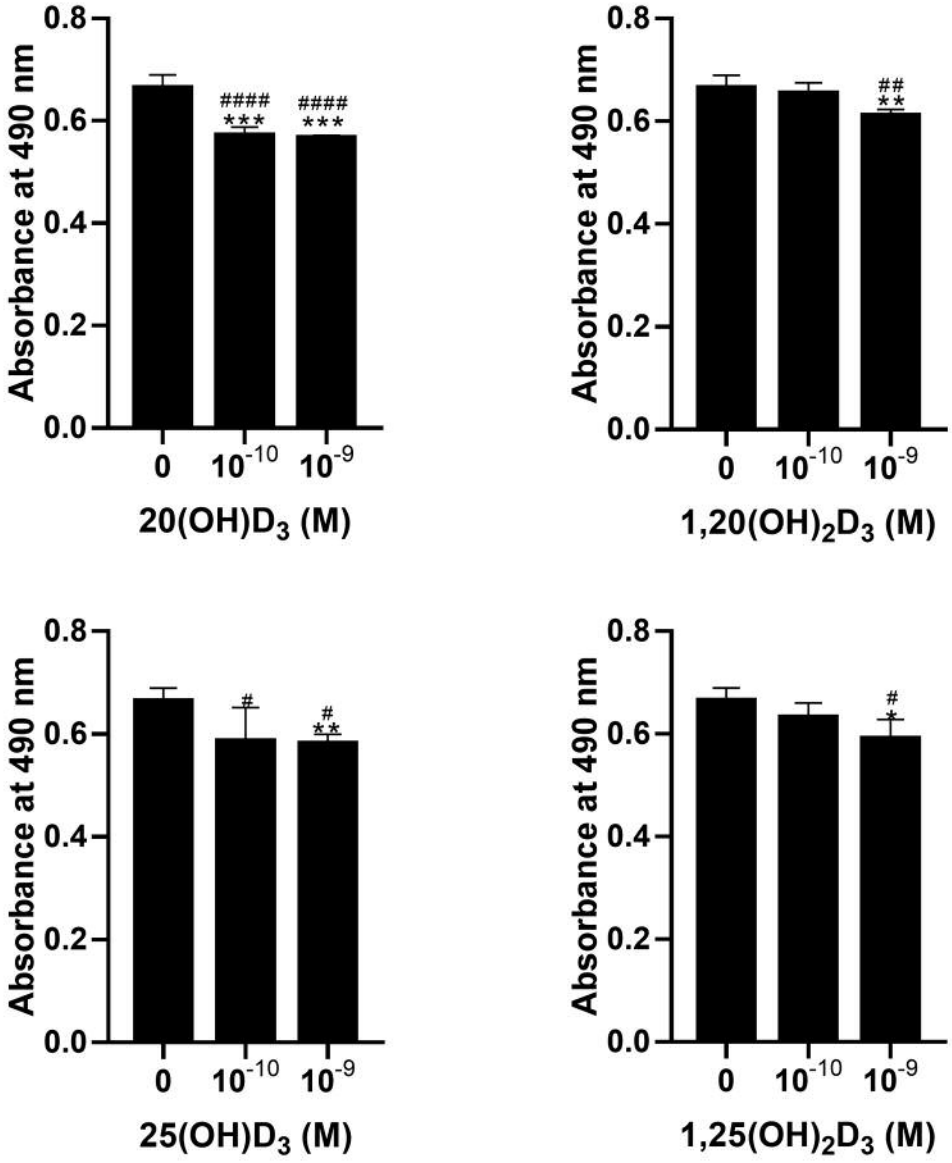

Figure 1. *Hydroxyderivatives of vitamin D, 20(OH)D_3 and 1,20(OH)$_2D_3$, exhibit antiproliferative effects against human oral squamous cell carcinoma cells at levels comparable to those of 25(OH)D_3 and 1,25(OH)$_2D_3$. CAL-27 cells were treated with vitamin D hydroxyderivatives in 10% charcoal-treated fetal bovine serum for 48 h. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt/phenazine methosulfate solution was added 3 h prior to absorbance measurement. Data are presented as the mean±SD (n≥3). Significantly different at: *p≤0.05, **p≤0.01, ***p≤0.001 by Student's t-test; #p≤0.05, ##p≤0.01, and ####p≤0.0001 by analysis of variance.*

to be highly up-regulated in human tongue OSCC, and other members of the hedgehog signaling pathway, such as patched, glioma-associated oncogene 1 (GLI1) and GLI2, were found to be up-regulated in microvascular cells in the tumor invasive front (9). A significantly higher expression of SHH by immunohistochemistry was identified from patient biopsy samples of OSCC, and the pattern of expression highlighted increased staining in the invasive tumor front of well-differentiated OSCCs (10).

The WNT/β-catenin pathway, a regulator of key molecular functions such as proliferation, differentiation and stem cell renewal, had also been implicated in migration and invasion of OSCC. OSCC cells with mutated β-catenin exhibited increased invasion and migration in transwell chamber assay, and increased expression of matrix metalloproteinase-7 identified by reverse transcriptase-polymerase chain reaction (11).

Up-regulation of vitamin D-binding protein was identified in OSCC (12), and a functional single nucleotide polymorphism

in the gene for vitamin D receptor (*VDR*) was associated with reduced overall survival in a study examining 110 patients with OSCC and 122 healthy controls (13). Furthermore, higher expression of VDR was seen in squamous intraepithelial neoplasia I-III, as well as in OSCC, compared to that of normal oral mucosa (14). It had also been reported that eldecalcitol, an analog of $1,25(OH)_2D_3$, inhibits oral squamous cell carcinoma *in vitro*, and causes downregulation of the nuclear factor of the κ-chain in B-cells (NF-κB) pathway (15). It was also demonstrated that calcitriol treatment was able to partially reverse cisplatin sensitivity in OSCCs (16).

Based on prior results reporting the activity of $1,25(OH)_2D_3$ activities against OSCC, it was hypothesized that novel noncalcemic hydroxyderivatives of vitamin D, $20(OH)D_3$ and $1,20(OH)_2D_3$, will have similar antitumor effects. These novel secosteroids inhibit proliferation of keratinocytes, stimulate their differentiation, and downregulate the NF-κB pathway (17-19). Both hydroxyderivatives are metabolites of cytochrome P450scc (CYP11A1), the first enzyme of the steroid biosynthetic pathway in mitochondria, which besides cholesterol can also hydroxylate vitamins D_3 and D_2, and their precursors 7-dehydrocholesterol and ergosterol (20-23), respectively. CYP11A1 and its products are endogenously present in human skin; they have been identified in cultured keratinocytes, dermal fibroblasts and the epidermis (20, 21, 24, 25). Many novel secosteroids derived from the CYP11A1-mediated pathway inhibit proliferation and induce differentiation with efficacies comparable to those of $1,25(OH)_2D_3$ and $25(OH)D_3$ (7). Two best characterized of these novel secosteroids, $20(OH)D_3$ and $20,23(OH)_2D_3$, are noncalcemic at pharmacological doses (26, 27). Administration of a high dose (30 μg/kg) of $20(OH)D_3$ for 14 days to C57BL/6 mice did not raise the serum calcium level or cause any toxicity detectable *via* histological analysis or serum chemistry (27). On the other hand, $1,25(OH)_2D_3$ induced renal and cardiac calcium deposits at 0.8 μg/kg (28).

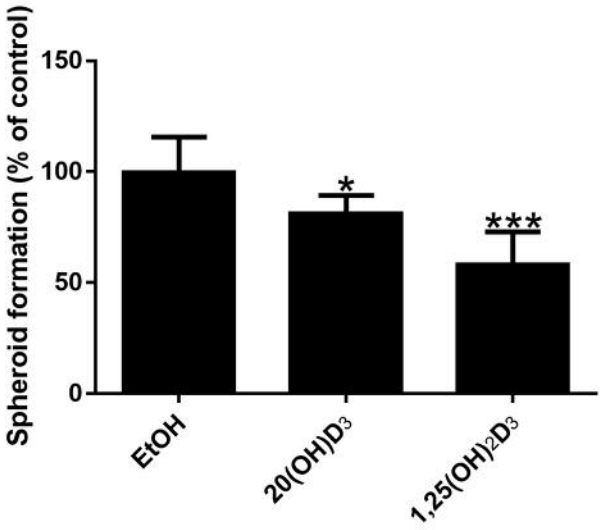

Figure 2. *Hydroxyderivative of vitamin D, $20(OH)D_3$, inhibits spheroid formation by CAL-27 cells. CAL-27 cells were seeded at 500 cells/well and treated with 10^{-7} M of $20(OH)D_3$, $1,25(OH)_2D_3$ or vehicle control (ethanol, EtOH), and incubated for 7 days at 37˚C. Spheroids that were greater than 50 μm were counted. Data are presented as mean±SD (n≥4). Significantly different at: *p≤0.05, and ***p≤0.001 by Student's t-test.*

Materials and Methods

Secosteroids. $20(OH)D_3$ and $1,20(OH)_2D_3$ were enzymatically synthesized with a reconstituted CYP11A1-mediated system using vitamin D_3 and 1α-hydroxyvitamin D_3 from Sigma-Aldrich (St. Louis, MO, USA) as substrates, respectively (29, 30). Both were purified by thin-layer chromatography, followed by reversed-phase high-performance liquid chromatography. Their molecular identities were confirmed by mass and UV spectra as described previously (31), and were stored until use at −80˚C. Commercially available $1,25(OH)_2D_3$ and $25(OH)D_3$, were purchased from Sigma-Aldrich.

Proliferation assay. CAL-27 cells, purchased from the American Type Culture Collection (Manassas, VA, USA), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.5% penicillin/streptomycin and 10% fetal bovine serum (FBS) in a humidified incubator with 5% CO_2 at 37˚C. CAL-27 cells were plated on 96-well plates with 10% charcoal-treated FBS (Atlanta Biologicals, Inc., Flowery Branch, GA, USA). After an overnight incubation, the medium was switched to serum-free medium to synchronize the cells for the subsequent 24 h. Cells were then treated with secosteroids in 10% charcoal-treated FBS and 0.5% penicillin/streptomycin for 48 h. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS)/phenazine methosulfate (PMS) solution (Promega, Madison, WI, USA) was added per the manufacturer's instructions. After incubation at 37˚C for 3 h, the absorbance at 490 nm was recorded using a Cytation 5 Cell Imaging Multi-Mode Reader (Winooski, VT, USA).

Immunofluorescence microscopy. CAL-27 cells (40,000 cells in 200 μl) were placed on autoclaved glass coverslips on a 6-well plate. After 4 h in 37˚C, the slides were incubated overnight at 37˚C with 2 ml of DMEM containing 0.5% penicillin/streptomycin. They were then treated with $20(OH)D_3$, $1,20(OH)_2D_3$, $25(OH)D_3$, $1,25(OH)_2D_3$ or vehicle (ethanol) at 10^{-7} M in DMEM with 0.5% bovine serum albumin (bovine serum albumin; Sigma-Aldrich), for 24 h at 37˚C. The cells were fixed with 2 ml of 4% paraformaldehyde for 15 min, then in 0.5% Triton X-100 in phosphate-buffered saline (PBS) for 5 min. They were then washed three times in PBS, followed by blocking for 1 h at 37˚C. Subsequently, the cells were incubated with mouse antibodies against β-catenin (BD Transduction Laboratories, Franklin Lakes, NJ, USA) or rabbit antibodies against GLI1 (Novus, St. Louis, MO, USA), at a dilution of 1:200 in 2% BSA in PBS, at 4˚C overnight. Stained cells were washed in PBS 3 times, then respectively treated with secondary anti-mouse antibodies and anti-rabbit antibodies at 1:200 (Santa Cruz, Dallas, TX, USA). These cells were mounted using propidium iodide (Vector Laboratories, Burlingame, CA,

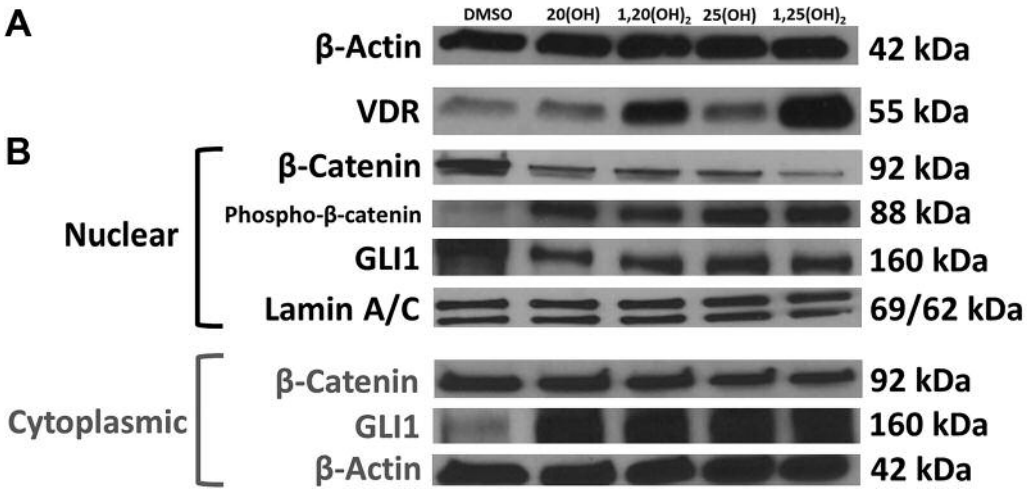

Figure 3. *Western blot analysis of the effects of secosteroids on the levels of vitamin D receptor (VDR) and nuclear and cytoplasmic forms of glioma-associated oncogene 1 (GLI1) and β-catenin. A: CAL-27 cells were incubated with hydroxyvitamin D derivatives (10^{-7} M for 24 h) and the level of VDR was measured in total cell extracts. Lamin A/C and β-actin served as loading controls. B: Cells were incubated for 24 h with 10^{-7} M of $20(OH)D_3$, $1,20(OH)_2D_3$, $25(OH)D_3$ or $1,25(OH)_2D_3$ and nuclear and cytoplasmic levels of GLI1 and β-catenin were determined. DMSO: Dimethyl sulfoxide.*

USA) as counterstain, and examined using a KEYENCE America BZ-X710 Fluorescence Microscope. The images were subsequently analyzed to calculate the corrected total cell fluorescence [CTCF=integrated density – (area of selected cell × mean of background fluorescence)] using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Spheroid formation. The spheroid formation ability of cells was assessed according to the protocol of Johnson and colleagues (32). CAL-27 cells were seeded at 1,000 cells/well on an ultra-low attachment 96-well plate, then treated with 10^{-7} M of $20(OH)D_3$, $1,25(OH)_2D_3$ or vehicle (ethanol), and incubated in medium enriched with B27 Supplement (Thermo Fisher Scientific, Waltham, MA, USA), bFGF, EGF, insulin and BSA (Sigma-Aldrich) for 7 days at 37˚C. Spheroids that were greater than 50 μm were counted using Cytation 5 Cell Imaging Multi-Mode Reader and statistically analyzed.

Western blot analysis. For analysis of β-catenin, phosphorylated β-catenin (Invitrogen, Carlsbad, CA, USA) and GLI1 expression, CAL-27 cells were incubated with 10^{-7} M of $20(OH)D_3$, $1,20(OH)_2D_3$, $25(OH)D_3$, $1,25(OH)_2D_3$ or vehicle (dimethyl sulfoxide) for 24 h at 37˚C prior to extraction of nuclear proteins. Protein extraction was carried out with Nuclear Extract kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's protocol. For analysis of VDR expression, extraction was carried out with RIPA buffer and proteins were further incubated for 3 h with anti-VDR (D-6; Santa Cruz Biotechnology). Primary antibody concentrations used for detection of β-catenin (E-5; Santa Cruz Biotechnology), phosphorylated β-catenin (Invitrogen), GLI1 and VDR were 1:1,000, 1:1,000, 1:1,000 and 1:200, respectively. Antibody to lamin A/C (N-18; Santa Cruz Biotechnology) was used at 1:200 as a control for nuclear proteins and antibody to β-actin at 1:20,000 (BD Transduction Laboratories, Franklin Lakes, NJ, USA) for cytoplasmic proteins and the non-fractionated mix for the VDR western blot.

Statistical analysis. Data analysis was performed using Graphpad Prism 7 (GraphPad Software, San Diego, CA, USA). Data are presented as means±SD. They were analyzed with unpaired two-tailed student's *t*-test or with one-way ANOVA using Dunnett's Multiple Comparison *post hoc* test (for more than two groups). Statistically significant differences were considered when $p \leq 0.05$, and were derived from Student's *t*-test and ANOVA.

Results

Noncalcemic vitamin D hydroxyderivatives exhibit antitumor effects. The MTS assay, which assesses metabolic activity in a 2D environment as an index of proliferation, and the spheroid assay, which assesses cell viability in a 3D environment, were used to investigate the effects of the vitamin D hydroxyderivatives on CAL-27 cells. Spheroid assays help mimic solid tumor characteristics, such as anchorage-independent growth, that are seen *in vivo* and are needed for tumor progression (33). A dose-dependent reduction in CAL-27 proliferation was seen with both $20(OH)D_3$ and $1,20(OH)_2D_3$ at levels similar to those of $25(OH)D_3$ and $1,25(OH)_2D_3$ (Figure 1). The incubation of cells with $20(OH)D_3$ also significantly reduced spheroid formation but the effect was slightly lower than that of $1,25(OH)_2D_3$ (Figure 2).

$20(OH)D_3$ and $1,20(OH)_2D_3$ up-regulate VDR expression. VDR expression was up-regulated after incubation with secosteroids for 3 h (Figure 3A). 1α-Hydroxylation potentiated

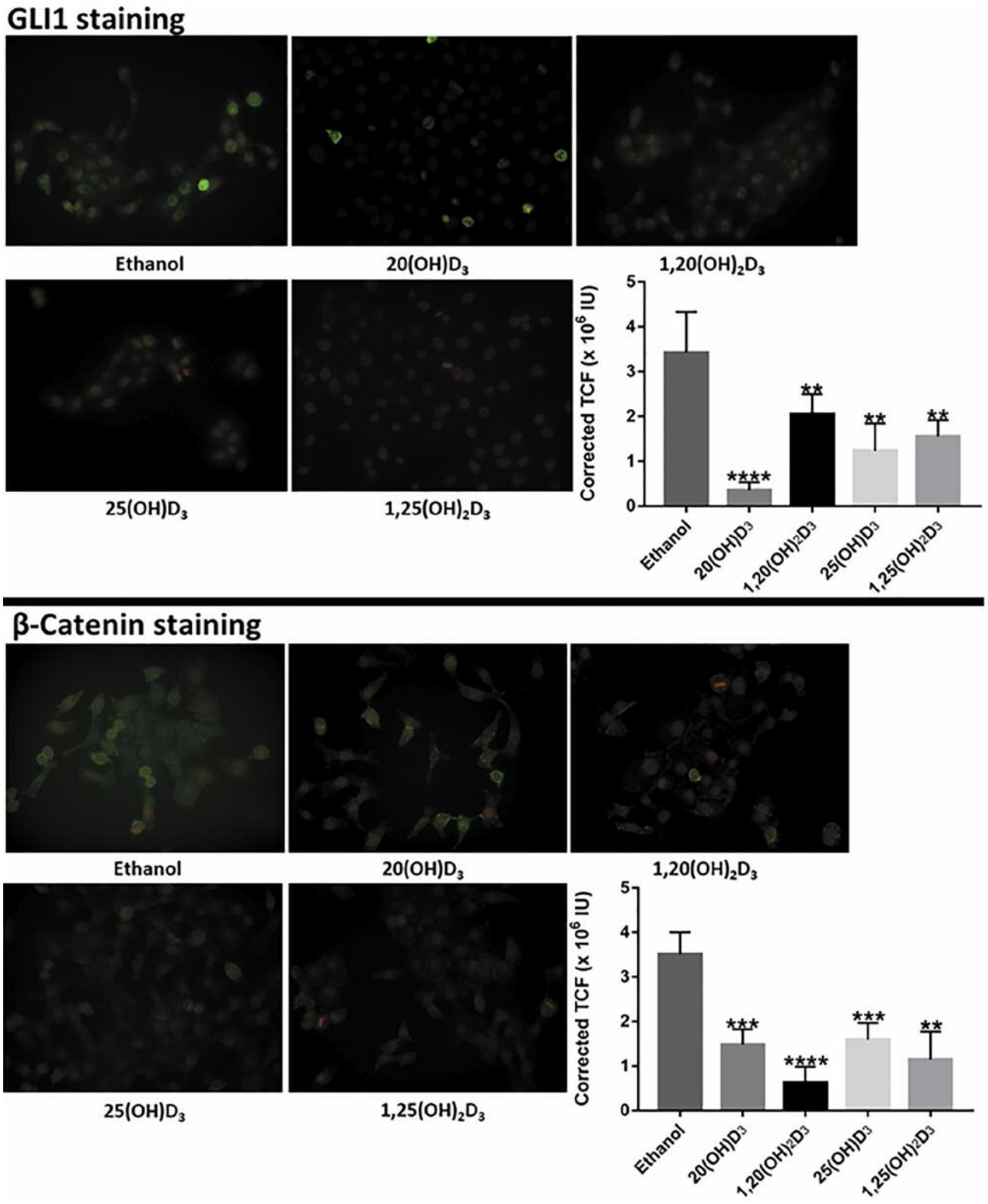

Figure 4. *Immunofluorescent staining (green) of β-catenin and glioma-associated oncogene 1 (GLI1) in CAL-27 cells treated with hydroxyvitamin D_3 derivatives (10^{-7} M for 24 hours) with propidium iodide (red) as counterstain. For both GLI1 and β-catenin, the bottom right panels shows the quantitation of florescence staining for treatment with the individual secosteroids shown in the other panels. TCF: Total cell fluorescence. Significantly different at: **p≤0.01, ***p≤0.001, and ****p≤0.0001 by Student's t-test.*

this effect since both $1,20(OH)_2D_3$ and $1,25(OH)_2D_3$ cause markedly higher levels of VDR to be expressed when compared to their respective precursors, $20(OH)D_3$ and $25(OH)D_3$.

Novel secosteroids down-regulate nuclear translocation of GLI1 and β-catenin. GLI1, a nuclear factor involved in the hedgehog signaling pathway, was down-regulated after incubation with $20(OH)D_3$ or with $1,20(OH)_2D_3$ for 24 h (Figure 4). Nuclear accumulation of GLI1 is required for activation of its downstream target genes. Western blot analysis revealed a decrease in GLI1 level in the nucleus and a corresponding increase in GLI1 level in the cytoplasm after treatment with vitamin D hydroxyderivatives (Figure 3B). The effect of $20(OH)D_3$ on GLI1 was greater than the effect of $1,25(OH)_2D_3$ based on a comparison of levels by immunofluorescence (Figure 4). Similarly, β-catenin is translocated from the cytoplasm to the nucleus, where it serves to activate the transcription of WNT/β-catenin target genes. Lower levels of β-catenin were observed by immunofluorescence (Figure 4) after treatment with the novel secosteroids for 24 h and a lower level was also observed in the nuclear fraction by western blot analysis (Figure 3A). Furthermore, increased levels of phospho-β-catenin were observed in the nuclear fraction after treatment with the secosteroids (Figure 3B). Phosphorylation of β-catenin triggers ubiquitination by E3 ubiquitin ligases and this process marks β-catenin for proteasome-mediated degradation (34).

Discussion

Nonclassical noncalcemic vitamin D hydroxyderivatives, $20(OH)D_3$ and $1,20(OH)_2D_3$, demonstrated dose-dependent antitumor activity against human OSCC cells at levels comparable to those of the classical vitamin D hydroxyderivatives, $25(OH)D_3$ and $1,25(OH)_2D_3$. $20(OH)D_3$ and $1,20(OH)_2D_3$ also up-regulated VDR expression, with the presence of the1α-hydroxy group potentiating this effect. Previously, it was shown that $20(OH)D_3$ and $1,20(OH)_2D_3$ have similar antiproliferative activity against human SKMel-188 melanoma cells (35). However, $1,20(OH)_2D_3$ had significantly more activity against normal melanocytes and it inhibited dendrite formation (36), whereas $20(OH)D_3$ did not. While 1α-hydroxylation may potentiate the biological effect of $20(OH)D_3$, it also increases its calcemic effect (26). This is consistent with the observation by Holick and colleagues that 1α-hydroxylation appears necessary for the physiological role of $1,25(OH)_2D_3$ in calcium regulation (37).

This study demonstrates that noncalcemic vitamin D hydroxyderivatives inhibit the sonic hedgehog signaling pathway, as well as the WNT/β-catenin signaling pathway. This is evident from the lower levels of their downstream transcription factors, GLI1 and β-catenin, after incubation with $20(OH)D_3$ or $1,20(OH)_2D_3$ (Figure 4). Furthermore, the nuclear translocation of GLI1 and β-catenin was also down-regulated (Figure 3). Schneider and colleagues reported in their study of adult tongue epithelium that SHH represses β-catenin transcriptional activity, and posited that smoothened (SMO) may inhibit nuclear β-catenin activity (38). Other studies have confirmed crosstalk between these two pathways and found that common modulators, such as Kirsten rat sarcoma viral oncogene homolog, phosphatase and tensin homolog, and p53, were involved in their regulation (39).

The inhibition of the growth of human OSCC cells by noncalcemic vitamin D analogs is in alignment with prior results documenting the activity of $1,25(OH)_2D_3$ against oral squamous cell carcinoma. Noncalcemic CYP11A1-derived vitamin D hydroxyderivatives, $20(OH)D_3$ and $1,20(OH)_2D_3$, seem to have antitumor activities against OSCC comparable to those of $25(OH)D_3$ and $1,25(OH)_2D_3$. Furthermore, their activities against the hedgehog signaling pathway and the WNT/β-catenin signaling pathway provide insight for a possible drug target for treatment of OSCC.

Conflicts of Interest

The Authors have no conflicts of interest to declare.

Authors' Contributions

A.T.S. conceived the study, designed the experiments and analyzed the data with input from M.A. A.S.W.O., T.K., A.A.B. and G.B. performed the experiments and analyzed the data. Z.J. provided technical guidance and analyzed the data. R.C.T. synthesized the novel noncalcemic hydroxyderivatives. A.S.W.O. wrote the article under A.T.S.'s supervision with input from all other Authors.

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