Molecular Cancer Therapeutics

1α,25-Dihydroxyvitamin D3 Inhibits Esophageal Squamous Cell Carcinoma Progression by Reducing IL6 Signaling

Ping-Tsung Chen[1,2], Ching-Chuan Hsieh[1,3], Chun-Te Wu[4,5], Tzu-Chen Yen[6,7], Paul-Yang Lin[8], Wen-Cheng Chen[4,9], and Miao-Fen Chen[4,9]

Abstract

The aim of this study was to highlight the role of 1α,25-dihydroxyvitamin D3 (calcitriol) in esophageal squamous cell carcinoma (SCC). The human esophageal SCC cell lines CE81T and TE2 were selected for cellular and animal experiments to investigate the changes in tumor behavior after calcitriol supplementation and the underlying mechanisms. Moreover, we evaluated the relationship between calcitriol supplementation, myeloid-derived suppressor cell (MDSC) recruitment, IL6 levels, and tumor progression by a 4-nitroquinoline 1-oxide (4-NQO)–induced esophageal tumor animal model. In this study, we demonstrated that calcitriol supplementation inhibited aggressive tumor behavior both *in vitro* and *in vivo*. The underlying changes included increased cell death, a lower degree of epithelial–mesenchymal transition, and inhibited IL6 signaling. In the 4-NQO–induced esophageal tumor animal model, increased IL6 and MDSC recruitment were linked with invasive esophageal tumors. Supplementation with calcitriol attenuated the level of IL6, the induction of MDSCs, and the incidence of 4-NQO–induced invasive tumors. Moreover, the IL6-induced changes in C57 mice, including augmented MDSC recruitment, increased levels of ROS and p-Stat3 in MDSCs, and higher suppressive function of MDSCs in T-cell proliferation, which were abrogated by calcitriol supplementation. On the basis of our results, we concluded that calcitriol abrogated the IL6-induced aggressive tumor behavior and MDSC recruitment to inhibit esophageal tumor promotion. Therefore, we suggest that supplementation with vitamin D_3 may be a promising strategy for the prevention and treatment of esophageal SCC. *Mol Cancer Ther; 14(6); 1365–75. ©2015 AACR.*

Introduction

Esophageal cancer is an aggressive upper gastrointestinal malignancy that generally presents as a locally advanced tumor that requires multimodal treatment (1). The predominant histologic types of esophageal cancer are adenocarcinoma and squamous cell carcinoma (SCC; ref. 2). Although the prognosis for patients with either type of esophageal cancer is poor, some studies indicate that the outlook is worse for esophageal SCC patients than those with adenocarcinoma (3, 4). The identification of molecular mechanisms that can pinpoint biologically aggressive tumors will be important for the effective management of esophageal SCC.

Evidence shows that vitamin D deficiency is associated with increased incidences of prostate cancer, head and neck squamous cell carcinoma (HNSCC), and esophageal cancer (5, 6). Moreover, studies using model systems of lung, SCC, and breast cancers have shown that the administration of vitamin D3 analogs has significant anticancer effects (7–9). The most biologically active form of vitamin D, namely $1\alpha,25(OH)_2D_3$ (calcitriol), exerts potent anticancer and anti-inflammation effects in many cells in a cell- and tissue-specific manner (10–12). Although vitamin D3 is reported to exhibit antitumor abilities such as antiproliferative and prodifferentiating actions on various malignant cells and retards tumor growth in animal models of cancer, the role of vitamin D3 in esophageal SCC and its molecular mechanism need further investigation. To assess this issue, we evaluated the effects of calcitriol on esophageal SCC *in vitro* and *in vivo*.

Calcitriol is also reported to exhibit anti-inflammatory and immune-regulating effects, including suppression of prostaglandin (PG) action and the production of proinflammatory cytokines such as IL6 (13, 14). Accumulating evidence has indicated that inflammatory responses are important for cancer development (15, 16). An increase in inflammatory mediators, such as COX-2 and proinflammatory cytokines has been shown to lead to tumor promotion, invasion, angiogenesis, and inflammation-associated intestinal tumorigenesis (17–19). In our previous study, IL6 has been demonstrated to link with aggressive tumor behavior and poor prognosis of esophageal SCC (20). Moreover, IL6-induced myeloid-derived suppressor cells (MDSC) recruitment provides a

[1]Graduate Institute of Clinical Medical Sciences, College of Medicine, Chang Gung University, Taoyuan, Taiwan. [2]Department of Hematology and Oncology, Chang Gung Memorial Hospital, Chiayi, Taiwan. [3]General Surgery, Chang Gung Memorial Hospital, Chiayi, Taiwan. [4]College of Medicine, Chang Gung University, Taoyuan, Taiwan. [5]Department of Urology, Chang Gung Memorial Hospital, Keelung, Taiwan. [6]Nuclear Medicine Department, Chang Gung Memorial Hospital at Linkou, Linkou, Taiwan. [7]Center for Advanced Molecular Imaging and Translation, Chang Gung Memorial Hospital at Linkou, Linkou, Taiwan. [8]Department of Pathology, Chang Gung Memorial Hospital, Chiayi, Taiwan. [9]Department of Radiation Oncology, Chang Gung Memorial Hospital, Chiayi, Taiwan.

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

P.-T. Chen and C.-C. Hsieh contributed equally to this article.

Corresponding Author: Miao-Fen Chen, Chang-Gung Memorial Hospital, Chiayi, #6, Chia-Pu Rd., Putz City, Chia-Yi Hsien, Taiwan. Phone: 886-5362-1000, ext. 2011; E-mail: miaofen@adm.cgmh.org.tw

doi: 10.1158/1535-7163.MCT-14-0952

microenvironment conducive to tumor growth and the development of treatment resistance in esophageal SCC (21). Therefore, we also examine the role of calcitriol in cancer prevention and its linkage with IL6 in an animal esophageal tumor model to provide new insights into the development of immune-based therapies.

Materials and Methods

Cell culture, cell growth, and reagents

The human cancer cell lines CE81T, which is derived from a well-differentiated esophageal SCC and obtained from Bioresource Collection and Research Center (22), and TE2, which is derived from a poorly differentiated esophageal SCC and provided by Dr. S.-H. Li (23), were used in this study. No further authentication was conducted for the two cancer cell lines. The cytokine– IL6, IL6-neutralizing antibody, and calcitriol were purchased from R&D Systems and Sigma, respectively.

Cell growth

To measure cell growth, 1×10^4 cells per well were plated into 6-well dishes. At the indicated time points, the cells were trypsinized and collected, and the surviving cells were counted using Trypan blue exclusion.

Immunoblotting

To determine the *in vitro* effects of calcitriol, proteins were extracted from cells incubated in the presence of indicated dosage of calcitriol or ethanol only for 48 hours.

The cells were treated with lysis buffer (Calbiochem). Equal amounts of protein were loaded in a 4%–20% gradient SDS-PAGE gel, separated and transferred onto nitrocellulose filters. The blots were blocked with 2% BSA in TBST for 1 hour and incubated overnight (4°C) with antibodies against the target protein(s) and then with the respective HRP-conjugated secondary anti-goat, anti-mouse, or anti-rabbit antibodies (dilution 1:1,000–1:2,000) for 1 hour at room temperature. The proteins were visualized by ECL, exposed to an X-ray film, and developed with an X-ray processor. The membranes were reprobed with an antibody against r-tubulin or nuclear lamin to normalize protein loading.

Cell invasion and wound-healing assay

Capacities for cell invasion were determined by Cell Invasion Assay (Trevigen). After treatment with calcitriol or ethanol for 48 hours, cells were plated in the top chambers. The top chambers were precoated with basement membrane extract (derived from EHS tumor and provided in the kit). After incubation for 24 hours, the number of cells in the bottom chamber was determined by measuring the fluorescent anion calcein released from intracellular calcein acetoxymethylester. Wound-healing assays were also done, as described previously (20). A 2 mm wide scratch was drawn across each cell layer using a pipette tip. The plates were photographed at the times indicated.

Immunofluorescence staining

Cells demonstrating exponential growth were seeded onto cover slips for immunofluorescence staining after treatment. To determine the *in vitro* effects, expressions of target proteins were evaluated using cells incubated in the presence of indicated dosage of calcitriol or p38 inhibitor for 48 hours. At the indicated times after treatment, the cells were fixed, permeabilized with 2% paraformaldehyde for 5 minutes, and washed with PBST. The slides were incubated for 1 hour at room temperature with antibodies against E-cadherin, IL6, and p-STAT3 and for 1 hour with a FITC or Texas Red–conjugated secondary antibody. The slides were counterstained with DAPI to visualize the nuclei. After two washes with PBST, the specific target proteins were visualized using a fluorescence microscope.

Tumor xenografts

Eight-week-old male athymic nude mice were used and all of the animal experiments conformed to the protocols approved by the experimental animal committee of our hospital. In the ectopic tumor implantation model, CE81T and TE2 cancer cells (1×10^6 cells per implantation, 3 animals per treated group) were subcutaneously implanted into the dorsal gluteal region. In the orthotopic tumor implantation model, human cancer cells (1×10^6 cells per implantation, 3 animals per treated group) were intraoperatively injected into the wall of the esophagus. The extent of orthotopic tumor invasion was measured at the indicated time points. The effects of calcitriol on tumor growth and aggressiveness were also investigated *in vivo*. In the treated group, the intraperitoneal injection of calcitriol (0.5 μg/kg per mice, three times per week) was started 1 day before tumor implantation and continued until the indicated times. For animal experiments, calcitriol treatment did not induce significant increase in serum calcium levels evaluated by Calcium Assay Kit (Abnova). Triplicate experiments were performed for the tumor growth and invasion analyses.

Induction of esophageal tumors in mice

Six-week-old male C57BL/6J mice and IL6 knockout mice on a C57BL/6J background (B6.129S2-Il6tm1Kopf/J; IL6KO) were used for the 4-NQO–induced esophageal tumor model (21, 24). Briefly, the mice were allowed access to drinking water containing 100 μg/mL 4-NQO (tumor-induced group) or solvent only (control group) at all times during the treatment period. After 16 weeks of treatment, the mice were analyzed for lesions in the esophagus at different times for up to 14 weeks. The Experimental Animal Ethics Committee of our hospital approved this animal protocol. To analyze the effects of calcitriol on the esophageal tumor formation *in* 4-NQO–treated mice and its link with IL6 signaling, an intraperitoneal injection of calcitriol (0.5 μg /kg per mice, twice a week for 6 weeks) or IL6 (5 μg/kg per mice, thrice a week for 6 weeks) was started 12 weeks after the initiation of 4-NQO treatments. Furthermore, to evaluate whether calcitriol attenuated the IL6-induced MDSC recruitment, we examined the percentage of MDSCs in IL6-treated mice (intraperitoneal injections of 5 μg/kg IL6 per mice, thrice a week for 2 weeks) with or without calcitriol (0.5 μg/kg per mice, thrice a week for 2 weeks) compared with that in control mice. For the experiments of 4-NQO–induced esophageal tumor, 5 animals were used per group and duplicate experiments were performed.

Small-animal PET imaging of tumor

As described previously (21, 25), the 4-NQO–induced esophageal tumors were detected using PET. Briefly, the esophageal tumors were detected after the injection of 7.4–9.25 MBq of 2-deoxy-2-[F-18]fluoro-D-glucose via the tail vein 60 minutes before the PET scan, which was performed on an Inveon system (Siemens Medical Solutions Inc.) located in the Molecular Image Center of our hospital. All of the mice underwent micro-CT imaging for anatomic registration following

micro-PET imaging. The mean radioactivity concentration within the tumor or organ was obtained from the mean pixel values within the multiple regions of interest (ROI) volumes and was expressed as the standardized uptake value. The standard tracer uptake value ratio (SUVR, tumor-to-muscle) was calculated to represent the tumor lesion and for statistical comparisons. After PET examination, gross and tissue examinations were performed to evaluate the esophageal lesions in the 4-NQO–treated mice. The gross lesions were identified and photographed. A pathologist analyzed the esophageal lesions histologically. Examination of the tissue sections revealed pathologies, including hyperplasia (thickened epithelium with prominent surface keratinization), papilloma (noninvasive exophytic growth of neoplastic cells), and invasive carcinoma (lesion with invasion into the subepithelial tissues).

FACS for MDSCs

Current data suggest that MDSCs are not a defined subset of cells but rather a group of phenotypically heterogenous myeloid cells that have common biologic activity. The coexpresssion of myeloid-cell lineage differentiation antigen Gr1 and CD11b characterize MDSCs in mice (26). Therefore, in the current study, MDSCs are defined as $CD11b^+Gr1^+$ in mice. FACS was carried out on single-cell suspensions prepared from murine spleen after digestion and immunostaining for CD11b and Gr1 with fluorescence-labeled monoclonal antibodies (BD PharMingen). The percentage of MDSC was measured by multicolor flow cytometry with the abovementioned monoclonal antibodies. Isotype-specific antibodies were used as negative controls in FACS. For MDSC isolation, the tissue specimen was cut into pieces and further digested in RPMI1640 medium containing 0.05 mg/mL liberase and 0.1 mg/mL DNAse in an incubator at 37°C for 40 minutes. The cells were then suspended in PBS and layered on 40% Percoll (Gibco BRL) solution. Myeloid cells were then isolated from the cocultures using anti-CD11b magnetic microbeads and LS column separation (Miltenyi Biotec) as per manufacturer's instructions.

T-cell suppression assay

The suppressive function of MDSCs was measured by their ability to inhibit the proliferation of autologous T cells in the following suppression assay. For $CD8^+$ T-cell isolation, the spleen tissue specimen was cut into pieces and further digested. T cells were then isolated from the cocultures using anti-CD8 magnetic microbeads and LS column separation (Miltenyi Biotec) as per manufacturer's instructions.

The isolated T cells were CFSE-labeled (3 μmol/L, Sigma) and seeded in 96-well plates (at 2×10^5 cells/well) with MDSC isolated previously with 2:1 ratio. T-cell proliferation was induced by anti-CD3/CD28 stimulation beads (Invitrogen). Suppression assay was analyzed by flow cytometry for T-cell proliferation after 3 days.

ELISA of IL6 levels

The levels of IL6 in the cell supernatants and murine serum samples were analyzed using an IL6 Quantikine ELISA Kit (R&D Systems). To measure the IL6 levels in cellular supernatants, the cells were cultured in 1 mL of serum-free medium for 24 hours in 6-well plates. The medium was collected and clarified by centrifugation at $3{,}000 \times g$. To measure the circulating IL6 levels *in vivo*, blood was removed from the heart and serum. The samples were stored frozen before being subjected to the IL6 assay.

Results

Effects of calcitriol on tumor growth

As determined through viable cell counting over a period of 6 days and observation of xenograft tumors, calcitriol significantly inhibited tumor growth *in vitro* (Fig. 1A) and *in vivo* (Fig. 1B). Calcitriol also decreased the proliferation index *in vivo* demonstrated by nuclear staining with Ki-67. The cell death rate increased from $5.6\% \pm 1.2\%$ to $15.2\% \pm 2.1\%$ in CE81T cells and from $6.6\% \pm 1.3\%$ to $16.5\% \pm 1.9\%$ in TE2 cells after treatment with calcitriol has measured by flow cytometry (Fig. 1C), and immunofluorescence with cleaved caspase-3 staining (Supplementary Fig. S1A). Furthermore, we found that treatment with calcitriol for 48 hours resulted in cell-cycle arrest (Supplementary Fig. S1B). As shown in Fig. 1D and E, calcitriol induced changes in apoptosis- and cell-cycle–related proteins, including Bax, Bcl-2, p21, and p27 *in vitro* and *in vivo*.

Effects of calcitriol on tumor invasion and underlying mechanisms

As demonstrated through the invasion assay and wound-healing assay, calcitriol attenuated the abilities of invasion and migration in esophageal cancer cells (Fig. 2A and Supplementary Fig. S2A). Moreover, an orthotopic tumor implantation technique was used to examine the effect of calcitriol on invasion ability *in vivo*. As demonstrated in Fig. 2B, the intraperitoneal injection of calcitriol decreased the invading rate of implanted tumor into the surrounding tissues and was associated with a smaller tumor size. The epithelial-to-mesenchymal transition (EMT) is a key event in invasiveness. At the molecular marker level, EMT is characterized by the loss of E-cadherin with increased invasion- and angiogenesis-related factors (27). As shown in Fig. 2C and Supplementary Fig. S2B, incubation with calcitriol for 48 hours increased E-cadherin expression for esophageal cancer cells. Furthermore, the decreased levels of VEGF and MMP-9 by calcitriol treatment were demonstrated *in vitro* (Fig. 2D) and xenograft tumors in mice (Fig. 2E and Supplementary Fig. S2C). Therefore, we suggested that reversing EMT changes may, at least in part, be responsible for the attenuated tumor invasiveness induced by calcitriol in esophageal cancer.

Effects of calcitriol on IL6 signaling

We previously reported that IL6 is significantly associated with aggressive tumor behavior, EMT, and poor prognosis in esophageal SCC (20). As demonstrated in Supplementary Fig. S3, the changes induced by blocking IL6 similar to that induced by calcitriol treatment, including the changes of tumor cells growth, cell death, and EMT-related proteins. Therefore, the correlation between the levels of IL6/phosphorylated STAT3 and calcitriol treatment were investigated. As determined through mRNA and protein analyses *in vitro*, calcitriol inhibited IL6 in association with an attenuation of STAT3 activation (Fig. 3A and B and Supplementary Fig. S4A). Moreover, immunohistochemistry and immunoblotting analysis from xenograft tumors (Fig. 3C and Supplementary Fig. S4B) confirmed the *in vitro* findings. The inhibition of p38 signaling is classically anti-inflammatory and has been hypothesized to contribute to some of the activities of vitamin D in cancer prevention (14). By protein analysis *in vitro* and *in vivo*,

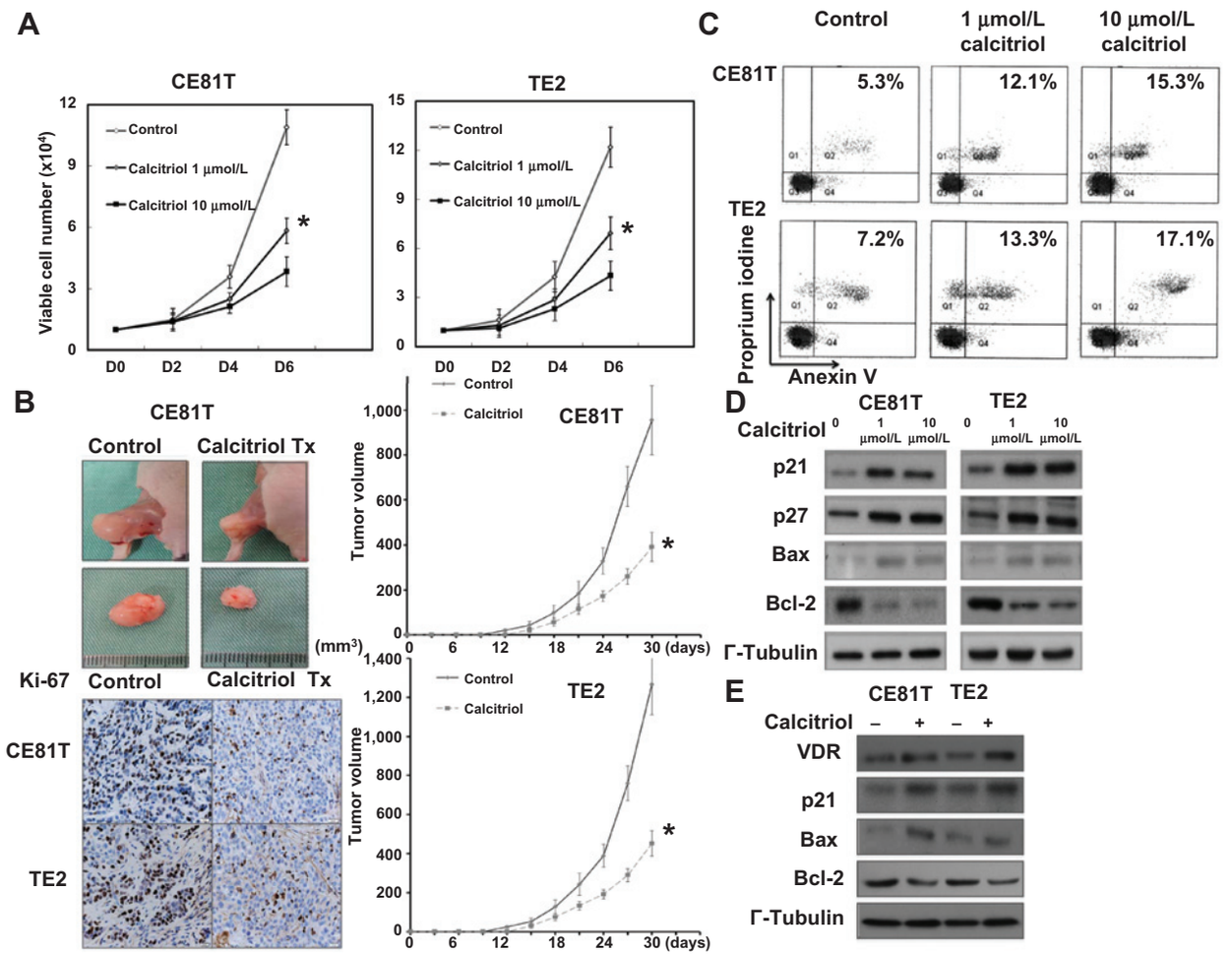

Figure 1.
Effects of calcitriol on tumor growth *in vitro* and *in vivo*. A, effect of calcitriol on the proliferation of CE81T and TE2 cancer cells *in vitro* as determined by viable cell counting. The *y*-axis represents the viable cell number. Data represent the means ± SEM. *, $P < 0.05$. B, effect of calcitriol treatment on ectopic tumor growth. Data represent the means ± SD of three independent experiments (9 animals per treated group in total). *, $P < 0.05$. Representative slides using Ki-67 nuclear staining in tumors 21 days after implantation was also shown with magnification ×400. C, effect of calcitriol treatment on cell death, as assessed by FACS with Annexin V–PI staining using cells under control condition or treated with calcitriol for 72 hours. The results of representative slides are shown. D, the changes of apoptosis and cell-cycle–associated proteins *in vitro* were evaluated by immunoblotting. E, immunoblotting analysis of VDR, p21, Bax, and Bcl-2 expressions using proteins extracted from tumor samples 21 days after implantation.

calcitriol treatment inhibits p38 phosphorylation in cells (Fig. 3D and Supplementary Fig. S4C). To test whether inhibition of p38 signaling is responsible for the inhibited IL6/p-stat3 induced by calcitriol, we evaluated the expressions of IL6 and p-stat3 in cells incubated with calcitriol or p38 inhibitor (SB203580; 10 μmol/L). The data revealed that the inhibition of IL6 signaling induced by calcitriol was similar to that led by the p38 MAPK inhibitor *in vitro* (Fig. 3E and F and Supplementary Fig. S4D). Therefore, we suggested that the inhibition of IL6 signaling induced by calcitriol might be medicated by attenuated p38 signaling, at least in part.

Inflammation linked with esophageal tumor formation in immunocompetent mouse model

The potential to inhibit inflammation is considered an important mechanism responsible for the antitumor activity of vitamin D3 (14). Therefore, we examined the relationship between calcitriol, inflammation, and tumor progression using a 4-NQO–induced esophageal tumor mouse model. Twelve to 14 weeks after the 16-week 4-NQO treatment, the mice were subjected to micro-PET and analyzed and the esophagus was removed for further evaluation. As shown in Fig. 4A, tumor lesions on the esophagus were noted by gross examination and pathologic analysis of tissue sections. Pathologic evidence of esophageal lesions, including hyperplasia, papilloma, and invasive carcinoma, was found in the 4-NQO–treated mice. The validity of micro-PET for detecting esophageal tumors in mice and the SUVR calculated as a representation of the tumor lesion are shown in Fig. 4B. The data revealed that a significantly higher value of SUVR was noted in esophageal carcinoma than those detected in esophageal lesion with hyperplasia or papilloma. It has been reported that the IL6-induced MDSC recruitment provides a microenvironment conducive to tumor growth. We therefore assessed the link between tumor progression, the circulating IL6 levels, and MDSC recruitment in 4-NQO–treated mice. Figure 4C

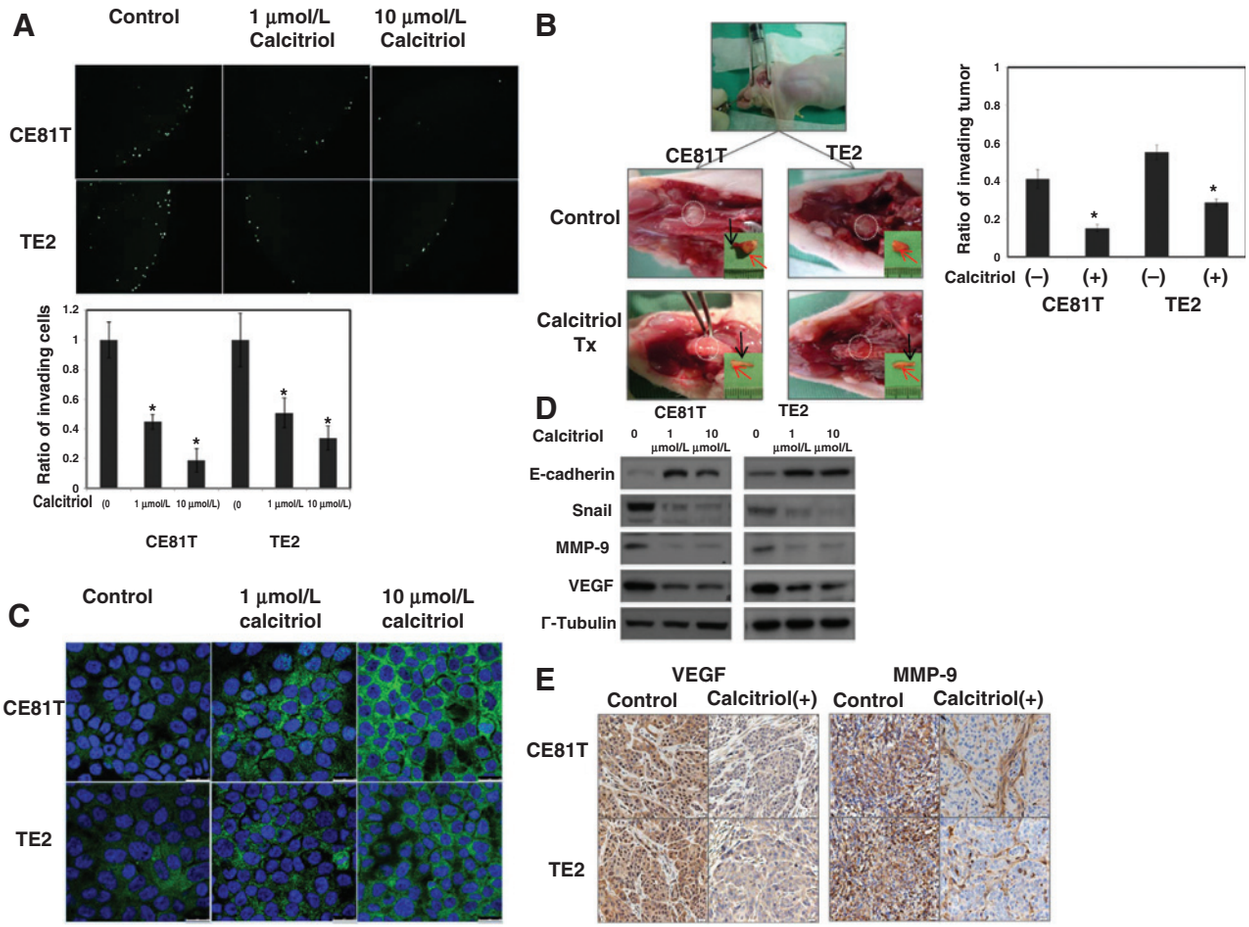

Figure 2.
Effects of calcitriol on aggressive tumor behavior and EMT changes. A, effect of calcitriol on the invasiveness of esophageal cancer cells *in vitro*. Representative slides and quantitative data (*y*-axis shows the relative ratio, normalized to the number of invading cells under control conditions) are shown. *, $P < 0.05$. B, effect of calcitriol treatment on the invasive capacities was evaluated by murine orthotopic tumor implantation. The results are shown by representative slides and quantitative data. The *y*-axis represents the relative ratio of esophageal tumor invading into adjacent structure 2 weeks after implantation. *, $P < 0.05$ (white circle, excision location; black arrow, esophagus; red arrow, tumor). C, the changes of E-cadherin expression were evaluated by immunofluorescence *in vitro*, and the results of representative slides are shown. Scale bars, 25 μm. D, effect of calcitriol treatment on EMT-associated proteins. The changes in the levels of EMT-associated proteins were evaluated by immunoblotting *in vitro*. E, levels of VEGF and MMP-9 expression evaluated using immunohistochemical analysis in tumors 21 days after implantation. The results of representative slides are shown with magnification ×400.

and D revealed that the level of IL6 and the percentage of MDSCs were correlated significantly with invasive carcinoma formation in the treated mice.

Effect of calcitriol on tumor progression *in vivo* and its linkage to IL6

As shown in Fig. 5A–C, calcitriol treatment decreased the SUVR in PET associated with a lower incidence of esophageal invasive carcinoma formation, and suppressed MDSC recruitment compared with that found in the 4-NQO–treated mice. Moreover, as determined by real-time RT-PCR, immunofluorescence, and ELISA analysis (Fig. 5D and Supplementary Fig. S5), calcitriol significantly attenuated the increased IL6 noted in 4-NQO–treated mice, including serum and esophageal lesions. We previously reported that IL6 is associated with MDSC accumulation and invasive tumor progression *in vivo* (21). Moreover, our data suggested that the inhibition of IL6 was linked to the antitumor effect induced by calcitriol. Accordingly, to provide direct evidence to demonstrate the decreased IL6 underlined the inhibition of esophageal tumor formation in 4-NQO–treated mice induced by calcitriol; we examined the changes including MDSC recruitment and the incidence of esophageal carcinoma in 4-NQO–treated mice with direct regulation of IL6. As shown, IL6 stimulation augmented the increases in the SUVR of esophageal lesion, the incidence of esophageal invasive carcinoma and MDSC recruitment noted in 4-NQO–treated mice. Furthermore, we used IL6 KO mice to abrogate microenvironment-produced IL6. Figure 5A–C showed that the absence of IL6 decreased the SUVR in PET images of the esophageal lesions, the recruitment of MDSCs, and the formation of esophageal invasive carcinoma, similar to that induced by calcitriol in the 4-NQO–induced esophageal tumor model using C57 mice. Therefore, we suggest that decreased IL6 is responsible for the inhibition of esophageal tumor formation induced by calcitriol.

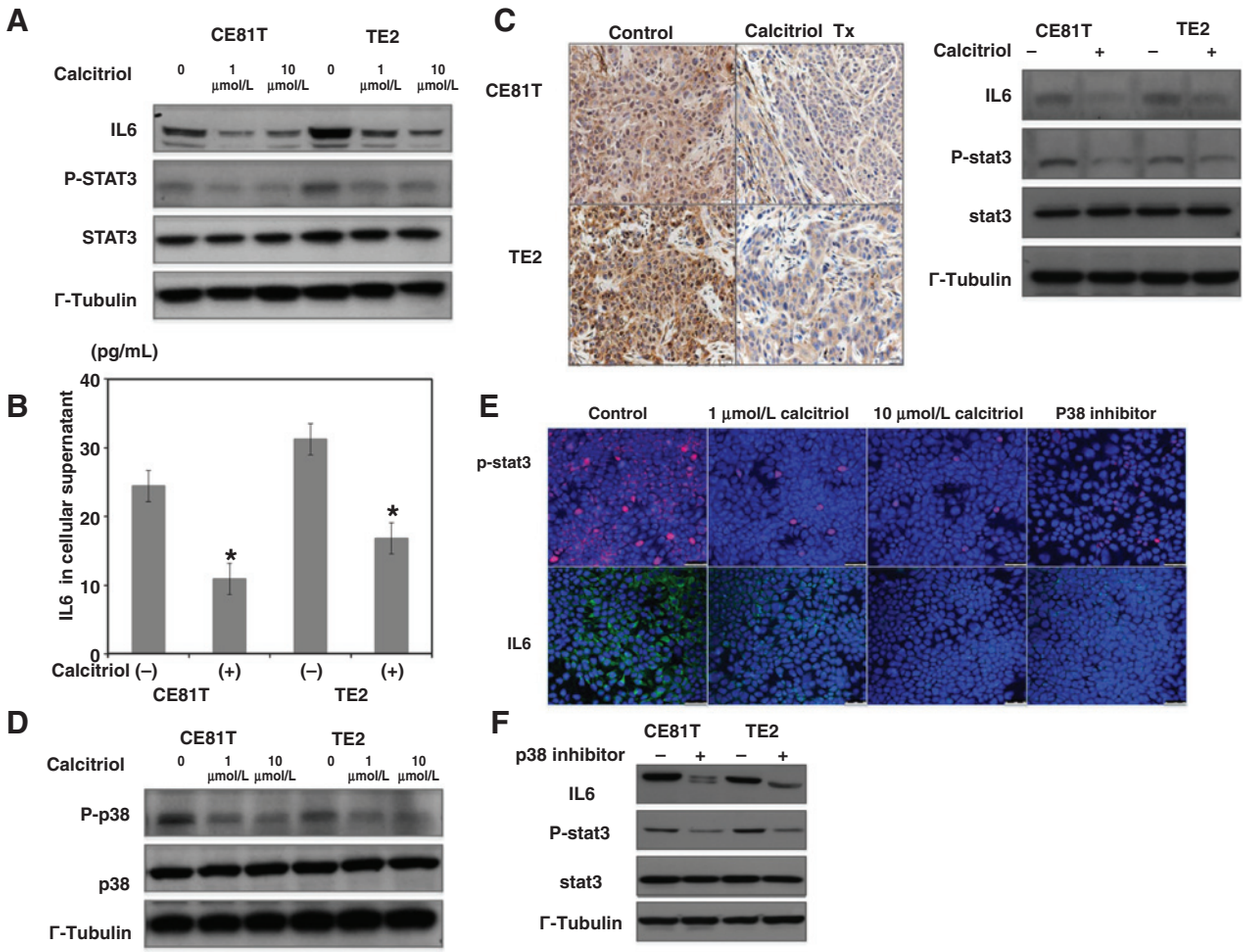

Figure 3.
Effects of calcitriol on IL6/STAT3 signaling in esophageal SCC. A, effect of calcitriol treatment on the expression of IL6 and activated STAT3 were evaluated by immunoblotting *in vitro*. B, the levels of IL6 in cellular supernatants were examined by ELISA *in vitro*. Columns represent the means ± SEM. *, $P < 0.05$. C, levels of IL6 and p-STAT3 evaluated using immunohistochemistry and immunoblotting analysis in ectopic tumors 21 days after implantation. The results of representative slides are shown with magnification ×400. Calcitriol treatment significantly decreased the expression of IL6. D, effect of calcitriol treatment on the expression of phosphorylated p38 was evaluated by immunoblotting. E, the expression levels of IL6 and p-STAT3 were evaluated by immunofluorescence using CE81T cells incubated in the presence of indicated dosage of calcitriol or p38 inhibitor for 48 hours and the results of representative slides are shown. Scale bars, 50 μm. F, effect of p38 MAPK inhibitor on the expression of IL6 and p-STAT3 was evaluated by immunoblotting.

Effects of calcitriol on the recruitment and function of MDSCs related to IL6 in immunocompetent mice

It has been reported that the recruitment and function of MDSCs play a role in tumor progression and IL6, and IL6 is an important regulator. Therefore, the effect of calcitriol on the recruitment and function of MDSCs and its relationship with IL6 were examined in IL6-stimulated or 4-NQO–treated mice. As shown in Fig. 6A, calcitriol abrogated the recruitment of MDSCs induced by IL6 stimulation in C57 mice. MDSCs are a heterogeneous cell population characterized by the ability to suppress T-cell functions including proliferation via a range of mechanisms, including reactive oxygen species (ROS) production and increases in the activated STAT3 levels (28–31). Therefore, to further assess whether calcitriol treatment impaired the function of the recruited MDSCs, we examined the levels of p-STAT3 and ROS and the suppressive ability of T-cell proliferation in MDSCs from IL6-stimulated or 4-NQO–treated mice with or without calcitriol treatment. As shown in Fig. 6B and C and Supplementary Fig. S6A and B, similar to that noted in 4-NQO–treated mice, the IL6-induced changes in C57 mice, including augmented MDSC recruitment, increased levels of ROS, and p-Stat3 in MDSCs, and higher suppressive function of MDSCs in T-cell proliferation, which could be abrogated by calcitriol supplementation.

Discussion

Some observational, preclinical, and clinical studies strongly suggest a beneficial role for vitamin D, but other studies do not support this hypothesis (32). Although the studies are inconsistent, accumulating evidence strongly suggest that vitamin D exhibit multiple anticancer actions and provide justification for the study of vitamin D_3 in cancer prevention and treatment (33). Esophageal SCC is a devastating disease with poor prognosis.

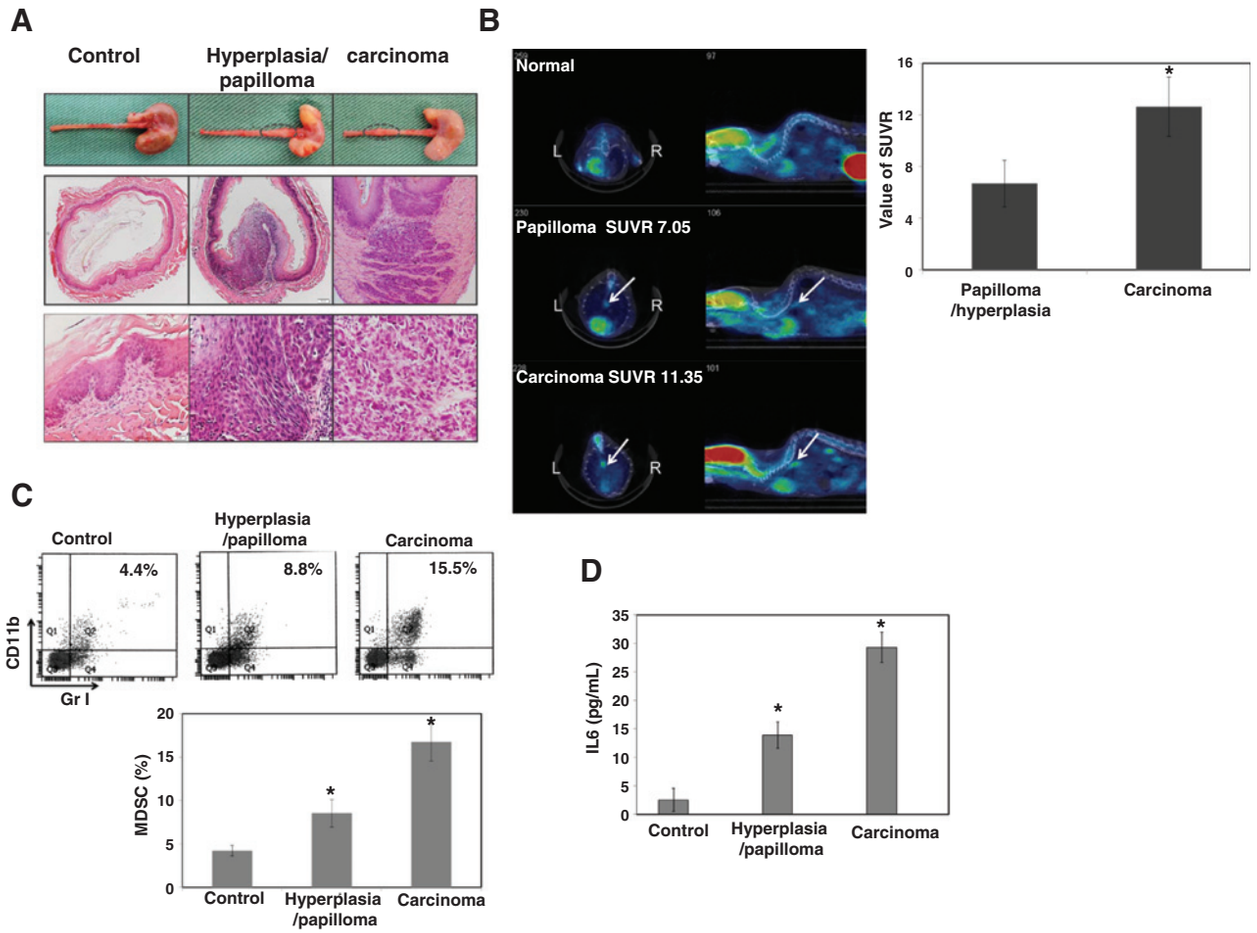

Figure 4.
Evaluation of esophageal tumor formation using histologic examination and animal micro-PET imaging and its relationship with IL6 levels and MDSC recruitment. A, images of the gross lesions and pathologic findings of tissue sections from animals treated with 4-NQO or vehicle for 16 weeks and then followed for 12 to 14 weeks. The representative slides are shown with magnification ×100 and ×400. B, representative PET images from mice treated with 4-NQO and diagnosed with invasive carcinoma, compared with benign lesions (hyperplasia/papilloma). The tumor-to-muscle SUVR was calculated from the micro-PET scans. Data points represent the means ± SEMs. C, flow cytometric analysis of $CD11b^{+}Gr1^{+}$ cells from mice exhibiting hyperplasia/papilloma or invasive carcinoma after histologic analysis. Representative images and quantitation are shown. Columns represent the means ± SEM. *, $P < 0.05$. D, IL6 levels in mice were measured using ELISA and quantitative data are shown. Columns represent the means ± SEM. *, $P < 0.05$.

Accordingly, we investigated whether vitamin D supplementation benefits the inhibition of esophageal SCC initiation and progression.

To demonstrate the effect of calcitriol in human esophageal SCC, we examined the changes in tumor cell growth *in vitro* and *in vivo*. Data obtained from cell counting and subcutaneous tumor observations revealed that calcitriol treatment resulted in decreased tumor growth, and the increased cell death and cell-cycle arrest were responsible for the slower growth rate. Moreover, the invasive ability was also significantly attenuated in the calcitriol-treated group, as determined by cellular invasion assays and an orthotopic tumor model. An orthotopic tumor model has been reported to increase the invasive and metastatic potential demonstrated by tumor cell lines following injection into their tissue of origin compared with subcutaneous tumor, which is an unrealistic growth location (34, 35). The transformation of an epithelial cell into a mesenchymal cell has been reported to be relevant to the invasive characteristics of epithelial tumors, including esophageal cancer (20, 36). Our data demonstrated that the calcitriol treatment attenuated EMT changes for esophageal cancer, which presented with increased E-cadherin levels and decreased MMP-9 and VEGF levels compared with the control group both *in vitro* and *in vivo*.

Recent research studies indicate that vitamin D_3 has anti-inflammatory actions that likely contribute to its beneficial effects in multiple cancers (14, 33). Inflammatory mediators, such as COX-2 and cytokines, enhance tumorigenesis through the activation of multiple signaling pathways in tumor tissue (17, 37). IL6 is a major proinflammatory cytokine that participates in inflammation-associated carcinogenesis (38, 39) and has been reported to be positively linked with angiogenesis, EMT, and poor prognosis in esophageal cancer (20). By cellular experiments, we found inhibition of IL6 decreased tumor growth and attenuated EMT similar to that induced by calcitriol. Accordingly, we further investigated the effect of calcitriol on IL6 signaling in esophageal cancer. As shown by the data obtained from the mRNA and

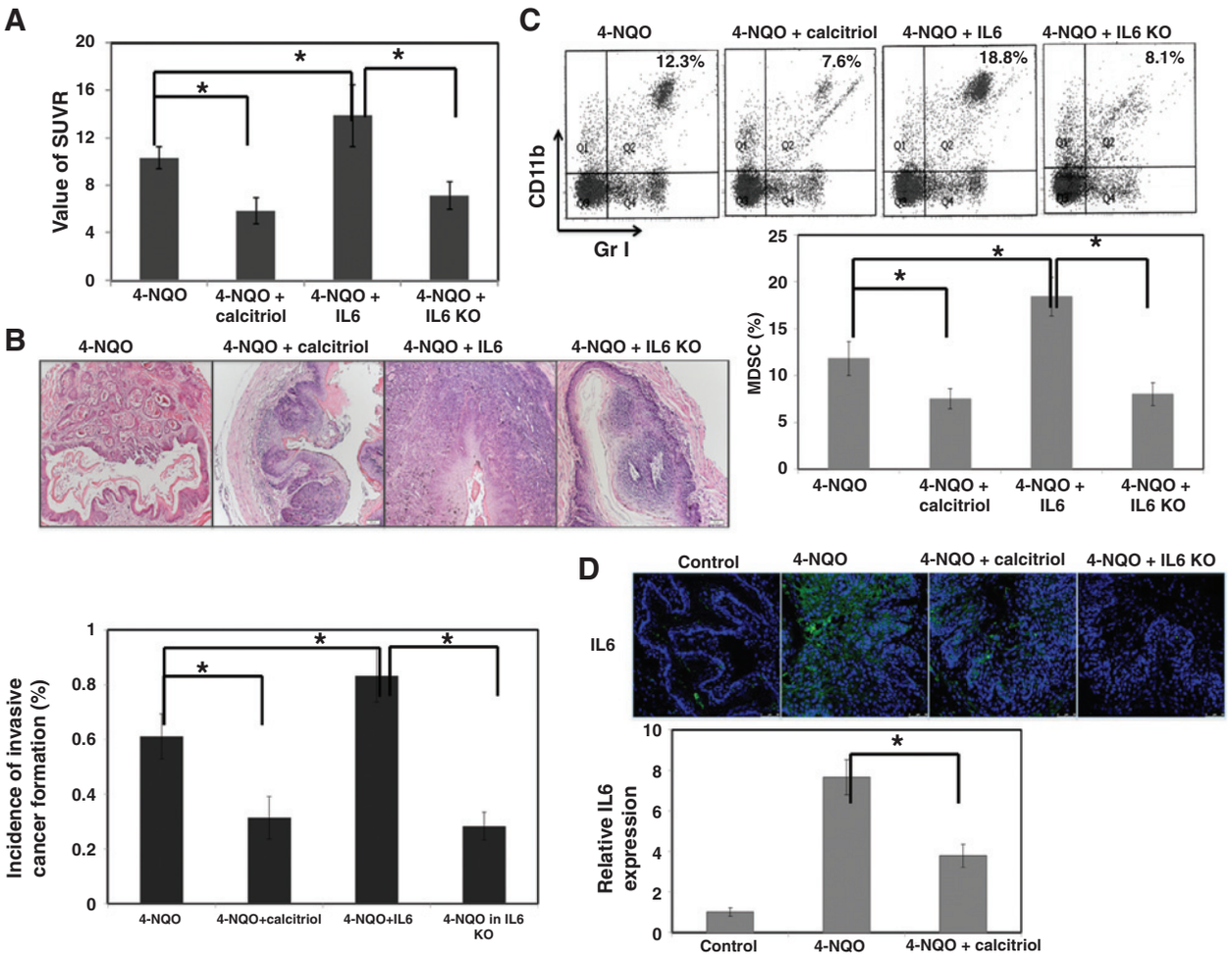

Figure 5.
Effect of calcitriol on tumor progression related to IL6 signaling *in vivo*. A, quantitative data of SUVR of esophageal lesion in 4-NQO–treated mice with or without calcitriol treatment or regulation of IL6. The tumor-to-muscle SUVR was calculated from the micro-PET scans. Columns represent the means ± SEM. B, representative images of gross lesions and the pathologic findings on sectioned tissue samples from 4-NQO mice with or without calcitriol or regulation of IL6. Quantitative data assessing the incidence of invasive esophageal tumors are shown. Columns represent the means ± SEM. *, $P < 0.05$. C, flow cytometric analysis of $CD11b^{+}Gr1^{+}$ cells from 4-NQO–treated mice with or without calcitriol or regulation of IL6. Representative images and quantitative data are shown. Columns represent the means ± SEM. D, effect of calcitriol treatment on the expression of IL6 in esophageal specimen from mice (control, C57 mice with vehicle only; 4NQO, 4-NQO–treated mice; 4-NQO + calcitriol, 4-NQO–treated mice with calcitriol injection; 4-NQO + IL6 KO, 4-NQO–treated IL6 KO mice) were evaluated by immunofluorescence and real-time RT-PCR. *, $P < 0.05$.

protein analyses, calcitriol suppressed IL6 expression and attenuated the activation of Stat3. Vitamin D_3 exhibits several anti-inflammatory effects, including inhibition of p38 stress kinase signaling and the subsequent production of proinflammatory cytokines (14). P38, a member of the MAPK family, has been reported to play a key role in the malignant transformation and is required for the invasive capacity of human hepatocellular carcinoma and HNSCC cell lines (40, 41). The ability of calcitriol to reduce the production of proinflammatory cytokines, such as IL6, by inhibiting p38 signaling has been demonstrated in cancer cells (42). As shown through protein analysis *in vitro* and *in vivo*, calcitriol treatment significantly decreased the expression of p-p38 associated with attenuated IL6 signaling. Moreover, inhibition of IL6 signaling led by calcitriol was similar to that seen by the p38 MAPK inhibitor. Therefore, we suggested that the inhibition of p38 phosphorylation may be the underlying mechanism responsible for the suppressed IL6 signaling induced by calcitriol in esophageal cancer, at least in part.

Novel immune-based therapies for the treatment of cancer are currently under development and emerging as a promising treatment approach for many solid tumors (19, 28). Many of these approaches are likely to be most effective in immunocompetent tumor-bearing individuals who are minimally immunosuppressed. IL6 has been reported linked with the recruitment of MDSCs and tumor progression in esophageal SCC (20, 21). MDSCs constitute an immature population of myeloid cells thought to be an important subset of cells that contribute to an immunosuppressive tumor microenvironment (43, 44) and are significantly increased in number in cancer patients. Therefore, to further investigate the link between

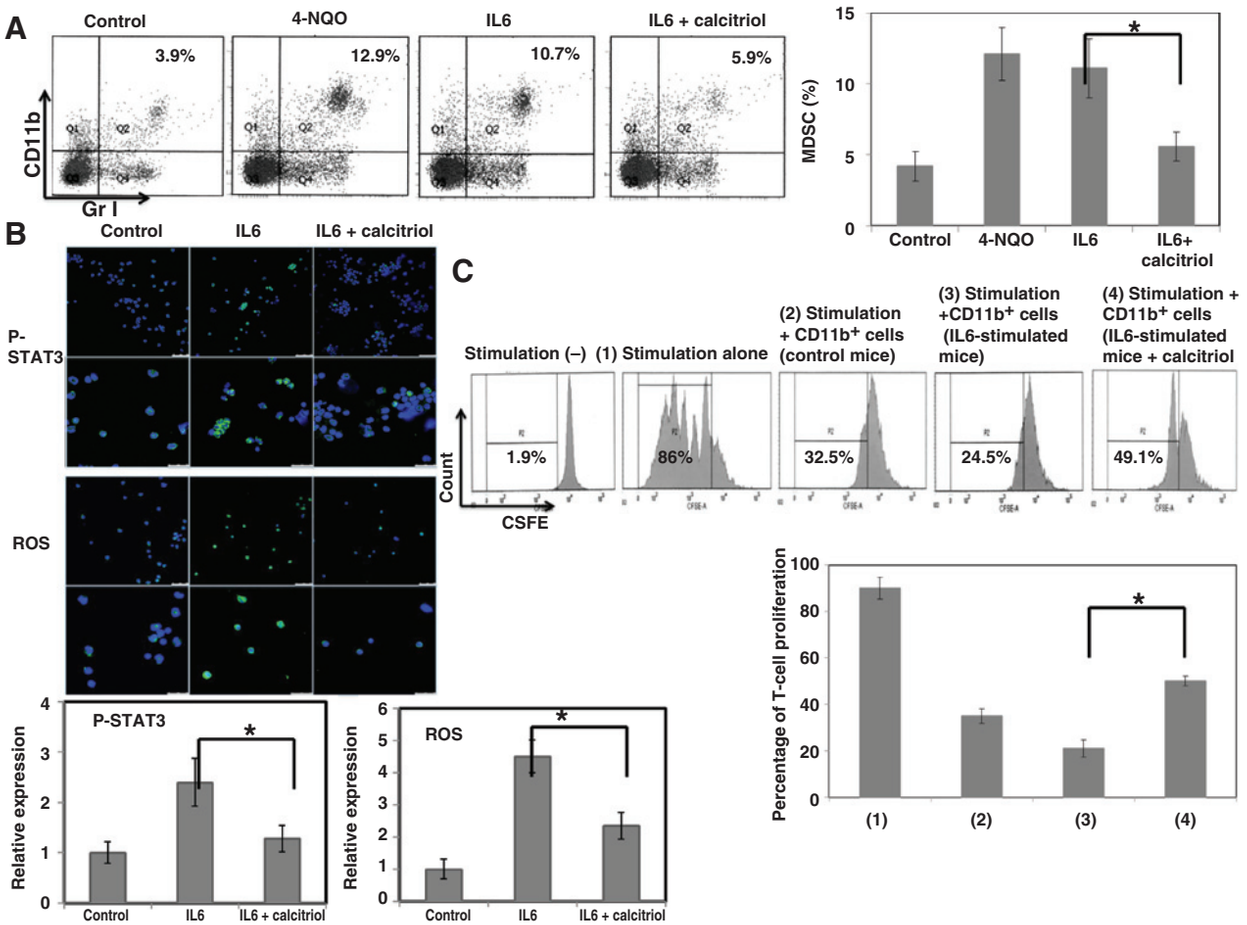

Figure 6.
Effect of calcitriol treatments on the function and recruitment of MDSC *in vivo*. A, effect of calcitriol on MDSC recruitment *in vivo* (control, C57 mice without treatment; 4NQO, 4-NQO–treated mice; IL6, IL6-stimulated mice; IL6 + calcitriol, IL6-stimulated mice with calcitriol injection) were evaluated by FACS. B, immunofluorescence staining revealed that calcitriol attenuated the IL6-induced increase in the levels of activated STAT3 and ROS in MDSC from murine spleen. (top row, low power field with scale bars, 50 μm; bottom row, high power field with scale bars, 25 μm). Representative images and quantitative data are shown. C, the suppressing ability of MDSC for T-cell proliferation was evaluated by FACS. Representative images and quantitative data are shown (stimulation alone, $CD8^+$ T cells with anti-CD3/CD28 stimulation beads without $CD11b^+$ cells; +CD11b cells, T cells with stimulation and coculture with $CD11b^+$ cells from control mice, or IL6-stimulated mice with or without calcitriol injection). *, $P < 0.05$.

calcitriol supplementation, MDSC recruitment, and esophageal tumor promotion in immunocompetent host, we induced esophageal tumors (24) and examined the effect of calcitriol on tumor progression in 4-NQO–treated mice. The model mimics many features observed in human esophageal SCC development; therefore, this model should be advantageous for studies of esophageal tumor development. In 4-NQO–induced esophageal tumor model, the levels of circulating IL6, SUVR in PET, and MDSC recruitment correlated with esophageal tumor formation. Calcitriol treatment was associated with a lower SUVR in esophageal lesions and a decreased incidence of invasive esophageal tumor formation compared with that in mice treated with 4-NQO alone. In addition, lower IL6 expression in esophageal tissue and serum combined with attenuated recruitment of MDSCs were noted in the calcitriol-treated group. Therefore, we suggested that calcitriol supplementation play a role in esophageal tumor prevention associated with inhibition of IL6 signaling.

We proposed that IL6 play an important role in esophageal tumor prevention induced by calcitriol treatment. Therefore, we examined the changes including MDSC recruitment and the incidence of esophageal carcinoma in 4-NQO–treated mice with the regulation of IL6. By direct regulation of IL6 expression in the 4-NQO–treated mice, we found that IL6 had a significant impact on the induction of MDSCs and the incidence of invasive tumors. Furthermore, the ability of MDSCs to suppress T-cell proliferation may be central to tumor formation (43–45). ARG-1–mediated depletion of L-arginine, ROS production, and STAT3 activation regulate the functions of MDSCs and have been reported to be the mechanisms responsible for the ability of MDSC to suppress T-cell functions (29–31). Through FACS analysis using murine spleen tissues, we demonstrated that calcitriol attenuated the increases in the levels of ROS, activated STAT3 and suppressive ability of T-cell proliferation in MDSCs obtained from IL6-stimulated or 4-NQO–treated mice. Therefore, decreased IL6 expression and subsequent inhibition of MDSC recruitment and suppression of their

function is suggested to be responsible for the inhibition of esophageal tumor progression induced by calcitriol.

These findings indicate that calcitriol may be a promising therapeutic agent for esophageal cancer by inhibiting tumor growth, attenuation of EMT and IL6 signaling. Moreover, we used a 4-NQO–induced tumor model to provide direct evidence of the linkage of vitamin D_3 with IL6, MDSC accumulation, and esophageal tumor formation in immunocompetent hosts. In the current study, the concentrations of calcitriol required for antineoplastic effects are significantly supraphysiologic, which are not achievable in patients when calcitriol is dosed daily due to predictable hypercalcemia. Therefore, further work is needed to elucidate the optimal amounts of calcitriol supplementation and the development of analogs of calcitriol (46). In summary, the application of vitamin D associated with targeting IL6 signaling could be a promising strategy for esophageal cancer prevention and the treatment of ESCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: P.-T. Chen, C.-C. Hsieh, M.-F. Chen
Development of methodology: C.-C. Hsieh, C.-T. Wu, T.-C. Yen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P.-T. Chen, C.-C. Hsieh, C.-T. Wu, T.-C. Yen, P.-Y. Lin, M.-F. Chen
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.-C. Hsieh, C.-T. Wu, P.-Y. Lin, M.-F. Chen
Writing, review, and/or revision of the manuscript: P.-T. Chen, M.-F. Chen
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): W.-C. Chen
Study supervision: W.-C. Chen, M.-F. Chen

Acknowledgments

The work was support by National Science Council, Taiwan by grant 102-2314-B-182-052-MY3 (to M.-F. Chen), and Chang Gung Memorial Hospital by grant CMRPG6A0523 (to C.-C. Hsieh).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received November 3, 2014; revised January 12, 2015; accepted March 9, 2015; published OnlineFirst March 30, 2015.

References

1. Fokas E, Weiss C, Rodel C. The role of radiotherapy in the multimodal management of esophageal cancer. Dig Dis 2013;31:30–7.
2. Enzinger PC, Mayer RJ. Esophageal cancer. N Engl J Med 2003;349:2241–52.
3. Siewert JR, Stein HJ, Feith M, Bruecher BL, Bartels H, Fink U. Histologic tumor type is an independent prognostic parameter in esophageal cancer: lessons from more than 1,000 consecutive resections at a single center in the Western world. Ann Surg 2001;234:360–7.
4. Bonavina L, Incarbone R, Saino G, Clesi P, Peracchia A. Clinical outcome and survival after esophagectomy for carcinoma in elderly patients. Dis Esophagus 2003;16:90–3.
5. Giovannucci E, Liu Y, Rimm EB, Hollis BW, Fuchs CS, Stampfer MJ, et al. Prospective study of predictors of vitamin D status and cancer incidence and mortality in men. J Natl Cancer Inst 2006;98:451–9.
6. Lipworth L, Rossi M, McLaughlin JK, Negri E, Talamini R, Levi F, et al. Dietary vitamin D and cancers of the oral cavity and esophagus. Ann Oncol 2009;20:1576–81.
7. Chiang KC, Yeh CN, Hsu JT, Chen LW, Kuo SF, Sun CC, et al. MART-10, a novel vitamin D analog, inhibits head and neck squamous carcinoma cells growth through cell cycle arrest at G0/G1 with upregulation of p21 and p27 and downregulation of telomerase. J Steroid Biochem Mol Biol 2013;138:427–34.
8. Colston KW, Chander SK, Mackay AG, Coombes RC. Effects of synthetic vitamin D analogues on breast cancer cell proliferation *in vivo* and *in vitro*. Biochem Pharmacol 1992;44:693–702.
9. Nakagawa K, Kawaura A, Kato S, Takeda E, Okano T. 1 alpha,25-Dihydroxyvitamin D(3) is a preventive factor in the metastasis of lung cancer. Carcinogenesis 2005;26:429–40.
10. Wang X, Studzinski GP. Activation of extracellular signal-regulated kinases (ERKs) defines the first phase of 1,25-dihydroxyvitamin D3-induced differentiation of HL60 cells. J Cell Biochem 2001;80:471–82.
11. Simboli-Campbell M, Narvaez CJ, Tenniswood M, Welsh J. 1,25-Dihydroxyvitamin D3 induces morphological and biochemical markers of apoptosis in MCF-7 breast cancer cells. J Steroid Biochem Mol Biol 1996;58:367–76.
12. Ma Y, Yu WD, Su B, Seshadri M, Luo W, Trump DL, et al. Regulation of motility, invasion, and metastatic potential of squamous cell carcinoma by 1alpha,25-dihydroxycholecalciferol. Cancer 2013;119:563–74.
13. Deeb KK, Trump DL, Johnson CS. Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. Nat Rev Cancer 2007;7:684–700.
14. Krishnan AV, Feldman D. Mechanisms of the anti-cancer and anti-inflammatory actions of vitamin D. Annu Rev Pharmacol Toxicol 2011;51:311–36.
15. Coussens LM, Werb Z. Inflammation and cancer. Nature 2002;420:860–7.
16. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? Lancet 2001;357:539–45.
17. Smyth MJ, Cretney E, Kershaw MH, Hayakawa Y. Cytokines in cancer immunity and immunotherapy. Immunol Rev 2004;202:275–93.
18. Abdel-Latif MM, Duggan S, Reynolds JV, Kelleher D. Inflammation and esophageal carcinogenesis. Curr Opin Pharmacol 2009;9:396–404.
19. Oshima H, Oshima M. The inflammatory network in the gastrointestinal tumor microenvironment: lessons from mouse models. J Gastroenterol 2012;47:97–106.
20. Chen MF, Chen PT, Lu MS, Lin PY, Chen WC, Lee KD. IL-6 expression predicts treatment response and outcome in squamous cell carcinoma of the esophagus. Mol Cancer 2013;12:26.
21. Chen MF, Kuan FC, Yen TC, Lu MS, Lin PY, Chung YH, et al. IL-6-stimulated $CD11b^+$ $CD14^+$ HLA-DR- myeloid-derived suppressor cells, are associated with progression and poor prognosis in squamous cell carcinoma of the esophagus. Oncotarget 2014;5:8716–28.
22. Hu CP, Hsieh HG, Chien KY, Wang PY, Wang CI, Chen CY, et al. Biologic properties of three newly established human esophageal carcinoma cell lines. J Natl Cancer Inst 1984;72:577–83.
23. Li SH, Huang EY, Lu HI, Huang WT, Yen CC, Huang WC, et al. Phosphorylated mammalian target of rapamycin expression is associated with the response to chemoradiotherapy in patients with esophageal squamous cell carcinoma. J Thorac Cardiovasc Surg 2012;144:1352–9.
24. Tang XH, Knudsen B, Bemis D, Tickoo S, Gudas LJ. Oral cavity and esophageal carcinogenesis modeled in carcinogen-treated mice. Clin Cancer Res 2004;10:301–13.
25. Li Y, Woodall C, Wo JM, Zheng H, Ng CK, Ray MB, et al. The use of dynamic positron emission tomography imaging for evaluating the carcinogenic progression of intestinal metaplasia to esophageal adenocarcinoma. Cancer Invest 2008;26:278–85.
26. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol 2009;9:162–74.
27. Gotzmann J, Mikula M, Eger A, Schulte-Hermann R, Foisner R, Beug H, et al. Molecular aspects of epithelial cell plasticity: implications for local tumor invasion and metastasis. Mutat Res 2004;566:9–20.
28. Keskinov AA, Shurin MR. Myeloid regulatory cells in tumor spreading and metastasis. Immunobiology 2015;220:236–42.
29. Vasquez-Dunddel D, Pan F, Zeng Q, Gorbounov M, Albesiano E, Fu J, et al. STAT3 regulates arginase-I in myeloid-derived suppressor cells from cancer patients. J Clin Invest 2013;123:1580–9.

30. Filipazzi P, Huber V, Rivoltini L. Phenotype, function and clinical implications of myeloid-derived suppressor cells in cancer patients. Cancer Immunol Immunother 2012;61:255–63.
31. Kusmartsev S, Nefedova Y, Yoder D, Gabrilovich DI. Antigen-specific inhibition of CD8+ T cell response by immature myeloid cells in cancer is mediated by reactive oxygen species. J Immunol 2004;172:989–99.
32. Cheung FS, Lovicu FJ, Reichardt JK. Current progress in using vitamin D and its analogs for cancer prevention and treatment. Expert Rev Anticancer Ther 2012;12:811–37.
33. Feldman D, Krishnan AV, Swami S, Giovannucci E, Feldman BJ. The role of vitamin D in reducing cancer risk and progression. Nat Rev Cancer 2014;14:342–57.
34. Ruggeri BA, Camp F, Miknyoczki S. Animal models of disease: pre-clinical animal models of cancer and their applications and utility in drug discovery. Biochem Pharmacol 2014;87:150–61.
35. Knox JD, Mack CF, Powell WC, Bowden GT, Nagle RB. Prostate tumor cell invasion: a comparison of orthotopic and ectopic models. Invasion Metastasis 1993;13:325–31.
36. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2002;2:442–54.
37. Li N, Grivennikov SI, Karin M. The unholy trinity: inflammation, cytokines, and STAT3 shape the cancer microenvironment. Cancer Cell 2011;19:429–31.
38. Schafer ZT, Brugge JS. IL-6 involvement in epithelial cancers. J Clin Invest 2007;117:3660–3.
39. Heikkila K, Ebrahim S, Lawlor DA. Systematic review of the association between circulating interleukin-6 (IL-6) and cancer. Eur J Cancer 2008;44:937–45.
40. Koul HK, Pal M, Koul S. Role of p38 MAP kinase signal transduction in solid tumors. Genes Cancer 2013;4:342–59.
41. Min L, He B, Hui L. Mitogen-activated protein kinases in hepatocellular carcinoma development. Semin Cancer Biol 2011;21:10–20.
42. Nonn L, Peng L, Feldman D, Peehl DM. Inhibition of p38 by vitamin D reduces interleukin-6 production in normal prostate cells via mitogen-activated protein kinase phosphatase 5: implications for prostate cancer prevention by vitamin D. Cancer Res 2006;66:4516–24.
43. Ostrand-Rosenberg S. Myeloid-derived suppressor cells: more mechanisms for inhibiting antitumor immunity. Cancer Immunol Immunother 2010;59:1593–600.
44. Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. J Immunol 2009;182:4499–506.
45. Najjar YG, Finke JH. Clinical perspectives on targeting of myeloid derived suppressor cells in the treatment of cancer. Front Oncol 2013;3:49.
46. Beer TM, Myrthue A. Calcitriol in cancer treatment: from the lab to the clinic. Mol Cancer Ther 2004;3:373–81.