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Differential response to 1α, 25-dihydroxyvitamin D_3 (1α, 25$(OH)_2D_3$) in non-small cell lung cancer cells with distinct oncogene mutations[1]

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Abstract

We previously demonstrated that non-small cell lung cancer (NSCLC) cells and primary human lung tumors aberrantly express the vitamin D_3-catabolizing enzyme, CYP24, and that CYP24 restricts transcriptional regulation and growth control by 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) in NSCLC cells. To ascertain the basis for CYP24 dysregulation, we assembled a panel of cell lines that represent distinct molecular classes of lung cancer: Cell lines were selected which harbored mutually exclusive mutations in either the *K-ras* or the *Epidermal Growth Factor Receptor* (EGFR) genes. We observed that *K-ras* mutant lines displayed a basal vitamin D receptor (VDR)^{low}CYP24^{high} phenotype, whereas *EGFR* mutant lines had a VDR^{high}CYP24^{low} phenotype. A mutation-associated difference in *CYP24* expression was also observed in clinical specimens. Specifically, *K-ras* mutation was associated with a median 4.2-fold increase in *CYP24* mRNA expression ($p = 4.8 \times 10^{-7}$) compared to *EGFR* mutation in a series of 147 primary lung adenocarcinoma cases. Because of their differential basal expression of VDR and CYP24, we hypothesized that NSCLC cells with an *EGFR* mutation would be more responsive to 1,25$(OH)_2D_3$ treatment than those with a *K-ras* mutation. To test this, we measured the ability of 1,25$(OH)_2D_3$ to increase reporter gene activity, induce transcription of endogenous target genes, and suppress colony formation. In each assay, the extent of 1,25$(OH)_2D_3$ response was greater in *EGFR* mutation-positive HCC827 and H1975 cells than in *K-ras* mutation-positive A549 and 128.88T cells. We subsequently examined the effect of combining 1,25$(OH)_2D_3$ with erlotinib, which is used clinically in the treatment of *EGFR* mutation-positive NSCLC. 1,25$(OH)_2D_3$/ erlotinib combination resulted in significantly greater growth inhibition than either single agent in both the erlotinib-sensitive HCC827 cell line and the erlotinib-resistant H1975 cell line. These

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data are the first to suggest that *EGFR* mutations may identify a lung cancer subset which remains responsive to and is likely to benefit from $1,25(OH)_2D_3$ administration.

Keywords

non-small cell lung cancer; vitamin D receptor; 1,25-dihydroxyvitamin D_3; epidermal growth factor receptor; K-ras

Introduction

Pre-clinical models support the idea that the active metabolite of vitamin D_3, 1,25-dihydroxyvitamin D_3($1,25(OH)_2D_3$) inhibits lung cancer growth [1, 2]. Anti-proliferative effects of $1,25(OH)_2D_3$ are mediated by binding to the vitamin D receptor (VDR) [3]. Upon ligand binding, VDR forms a heterodimer with the retinoid-X-receptor (RXR) and regulates the expression of genes whose promoters contain vitamin D response elements (VDREs). Transcriptional targets of $1,25(OH)_2D_3$ include genes that regulate cell cycle arrest and apoptosis [4–7]. To explore the potential role of vitamin D signaling in clinical disease control, the relationship between serum 25-hydroxyvitamin D_3 ($25(OH)D_3$) levels or tumor VDR expression and non-small cell lung cancer (NSCLC) survival was determined. Early-stage NSCLC patients who had $25(OH)D_3$ levels ≥ 21.6 ng/mL experienced a significant improvement in survival as compared to patients with $25(OH)D_3$ levels ≤ 10.2 ng/mL [8]. With regard to VDR status, 5 year overall survival rates were 59% for patients with high nuclear VDR expression versus 27% for low nuclear VDR expression [9]. In light of these results, mechanisms that decrease *VDR* expression and/or vitamin D levels in tumor cells would be predicted to adversely affect lung cancer outcomes.

1α,25-dihydroxyvitamin D_3 24-hydroxylase (CYP24) is the primary enzyme responsible for the catabolic inactivation of $1,25(OH)_2D_3$ and is considered a candidate oncogene [10, 11]. *CYP24* is frequently over-expressed in primary lung tumors [12–14], and its expression is independently prognostic of poor survival [15]. In prior mechanistic studies by us, the selective CYP24 inhibitor CTA091 suppressed $1,25(OH)_2D_3$ catabolism, preserved $1,25(OH)_2D_3$ regulation of gene expression through a VDR-dependent process, and reinforced its growth inhibitory effects in NSCLC cells [7]. These data support the hypothesis that *CYP24* expression promotes tumor growth by enabling NSCLC cells to bypass growth regulation by $1,25(OH)_2D_3$.

To dissect the mechanisms contributing to aberrant *CYP24* expression in lung cancer, we assembled a panel of NSCLC cell lines that harbored mutually exclusive mutations in either the epidermal growth factor receptor (*EGFR*) or *K-ras* genes. These were selected because they represent independent oncogenic pathways in lung cancer. Mutations within the *EGFR* gene occur in approximately 10% of all lung adenocarcinomas and are observed most commonly in the subset of patients who have never smoked [16]. Patients whose tumors harbor activating *EGFR* mutations show nearly 80% response rates to EGFR tyrosine kinase inhibitors (TKIs) [17, 18]. *K-ras* mutations occur in approximately 25% of lung adenocarcinomas and are associated with a history of cigarette use and resistance to EGFR TKIs [19]. Our analysis of NSCLC cell lines revealed that *K-ras* mutation-positive cells have a basal $VDR^{low}CYP24^{high}$ phenotype that is associated with limited response to $1,25(OH)_2D_3$. Conversely, NSCLC cells that harbor *EGFR* mutations have a $VDR^{high}CYP24^{low}$, $1,25(OH)_2D_3$-sensitive phenotype. Differential *CYP24* expression in the *K-ras* and *EGFR* mutation-positive subsets of lung adenocarcinomas was confirmed in a clinical case series. To the best of our knowledge, these data are the first to identify mutation-related differences in *CYP24* expression and the response of NSCLC cells to

$1,25(OH)_2D_3$ and to suggest that vitamin D supplementation may be most effective in the management of lung cancers that harbor *EGFR* mutations.

Materials and Methods

Cells

A549, HCC827, H1650, and H1975 cells were obtained from the American Type Culture Collection (Manassas, VA). 128.88T cells were generously provided by Dr. Jill Siegfried (University of Pittsburgh, Pittsburgh PA). HCC827, H1650, and H1975 cells were maintained in RPMI 1640 (Mediatech, Manassa, VA). A549 and 128.88T cells were maintained in BME (Life Technologies, Grand Island, NY). To prepare complete growth medium, RPMI or BME was supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Inc., Logan, UT), 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. The presence of a *K-ras* codon 12 mutation was confirmed in A549 and 128.88T cells using the method described by Mitchell *et al.* [20]. HCC827, H1650, and H1975 cells were authenticated by RADIL prior to use in these studies.

Chemicals

Erlotinib was purchased as a powder from ChemieTek (Indianapolis, IN). Stock solutions were prepared at a final concentration of 10 mM in dimethylsulfoxide (DMSO) and stored at −20°C. On the day of use, stocks were diluted in tissue culture medium to achieve the desired final concentration.

Preparation of whole cell extracts and immunoblot analysis

Procedures for preparation of whole cell extracts and immunoblot detection of VDR and CYP24 were the same as those described by us previously [7].

Construction of a *CYP24* promoter-luciferase reporter construct—Genomic DNA was isolated from 128.88T cells using the ChargeSwitch gDNA kit from Life Technologies. A 533 bp fragment of the *CYP24* promoter corresponding to nucleotides 725 to 1257 of GenBank entry HSU60669 was amplified from 100 ng of genomic DNA by 32 cycles of PCR using primers modified to contain either an Asp 718 or Bgl II site. The PCR product was gel-purified, digested with Asp 718 and Bgl II, and ligated into the Asp 718/Bgl II sites of the firefly luciferase reporter plasmid, pGL2 (Promega Corporation). Candidate clones were identified by restriction digestion and verified to contain human *CYP24* promoter sequences by automated DNA sequencing and nucleotide sequence alignment using NCBI BLAST.

Assay of CYP24 promoter activity—Cells were plated in six-well plates at 4×10^5 cells per well in complete growth medium lacking antibiotics. When cells achieved 50–70% confluence, they were transiently transfected using Fugene 6 (Roche Applied Science, Mannheim, Germany). Each reaction contained 100 ng of the *CYP24* promoter-luciferase reporter, 50 ng of CMV-β-galactosidase (β-gal), and pBluescript II (to adjust total DNA content to 1 μg per reaction). After 5 h, transfection medium was removed and cells were treated with either vehicle or 100 nM $1,25(OH)_2D_3$. Treatments were done in fresh medium containing 10% charcoal-stripped serum (CSS). Cells were harvested 24 h post-treatment. Firefly luciferase activity was assayed using the Promega Luciferase Assay System. Luminescence was read for 60 sec using an AutoLumat LB953 luminometer (Berthold, Pforzheim, Germany). Reporter activity was calculated as the ratio of firefly luciferase activity to β-gal activity. For each cell line, the ratio of luciferase/ β-gal activity for vehicle-treated cells was assigned a value of 1.0.

RT-PCR Assay—Cells were seeded into 6-well plates in complete growth medium. When still sub-confluent, the cells were treated with vehicle (control) or 1,25$(OH)_2D_3$ (100 nM) for 17 h. Treatments were done in fresh medium containing 10% CSS. RNA was extracted using the PerfectPure RNA Cultured Cell Kit (5 Prime, Gaithersburg, MD, USA) in accordance with the manufacturer's instructions. The RNA concentrations were determined by NanoDrop. RNA (250–500 ng) was converted into cDNA using the First Strand cDNA Synthesis kit from Origene (Rockville, MD). A fixed volume of cDNA (2.0 μL) was used for each PCR reaction. Reactions were run using Hot Star Taq Plus Master Mix (Qiagen, Valencia, CA) on a BioRad C1000 Thermal Cycler. *VDR*, *CYP24*, *CAMP*, and *G3PDH* primer sequences were described previously [7]. PCR products were resolved on 1.2% agarose gels and visualized by staining with ethidium bromide. Gels were imaged using the BioRad Gel Doc XR instrument.

Clonogenic Assay

Cells were seeded in triplicate in 6-well plates in complete growth medium. The next day, cells were treated with fresh medium (controls) or medium containing the indicated concentrations of 1,25$(OH)_2D_3$ ± erlotinib. Treatments were repeated every 3 days. After 7 d, colonies were fixed with methanol and stained with crystal violet. Grids were scored onto the back of each plate. Colonies in each section of the grid were inspected using a microscope, and those containing ≥ 30 cells were counted. The percent remaining colonies was calculated using the equation: % Colonies Remaining = 100 × [number colonies for treatment group/average number colonies for control group].

Results

VDR and CYP24 are differentially expressed in *K-ras* and *EGFR* mutation-positive lung cancers

To identify factors that may contribute to aberrant basal expression of *CYP24* in NSCLC, we assembled a panel of cell lines that harbored distinct mutations in either the *K-ras* (A549, 128.88T) or *EGFR* genes (HCC827, H1650, and H1975). The *VDR* and *CYP24* expression profile of each line was established following growth in complete medium. Cells that harbor an activating *K-ras* mutation had lower levels of *VDR* mRNA and higher levels of *CYP24* mRNA than cells with an *EGFR* mutation (Fig. 1A). The differential expression profiles were also observed when VDR and CYP24 protein levels were evaluated by immunoblot (Fig. 1B). To determine whether the observed mutation-associated differences in *CYP24* expression had any clinical relevance, we performed a secondary analysis of a publically available microarray dataset that included 127 *EGFR* and 20 *K-ras* mutation–positive lung adenocarcinomas [21]. Consistent with the cell line data, we observed a statistically significant 4-fold increase in *CYP24* expression in the *K-ras* mutation-positive subset of lung cancers (Fig. 2).

NSCLC cells with an *EGFR* mutation respond preferentially to 1,25$(OH)_2D_3$ treatment

Given that VDR mediates transcriptional regulation by 1,25$(OH)_2D_3$, and CYP24 negatively regulates 1,25$(OH)_2D_3$ levels and activity, we predicted that NSCLC cells that have an *EGFR* mutation and a $VDR^{high}/CYP24^{low}$ phenotype would be more responsive to 1,25$(OH)_2D_3$ than cells with a *K-ras* mutation and a $VDR^{low}/CYP24^{high}$ phenotype. To test this, 1,25$(OH)_2D_3$ transcriptional activation was measured. Cells were transfected with a *CYP24* promoter-luciferase reporter vector and CMV-β-gal then treated 5h later with either vehicle (0.004% EtOH) or 100 nM 1,25$(OH)_2D_3$. When bound by 1,25$(OH)_2D_3$, the VDR increases *CYP24* transcription through a series of VDREs located in the *CYP24* promoter [22, 23]. HCC827 and H1975 cells displayed robust transcriptional responses to 1,25$(OH)_2D_3$ administration, with average increases in reporter activity of 10-fold (range

5.7–14.5) and 9-fold (range 5.7–16.6), respectively (Fig. 3A). 128.88T and A549 cells also responded to treatment, but the average $1,25(OH)_2D_3$-mediated increase in reporter activity in these cells was 3-fold. When the reporter activity at 100 nM $1,25(OH)_2D_3$ was compared among the cell lines, significant differences were observed between HCC827 and A549 ($p = 0.009$); HCC827 and 128.88T ($p = 0.023$); and H1975 and A549 ($p=0.05$). To confirm these differential transcriptional responses, the effect of $1,25(OH)_2D_3$ treatment on endogenous gene expression was examined (Fig. 3B). To do this, we analyzed expression of the *CYP24*, *CD14*, and *CAMP* genes, whose promoters contain VDREs [24]. In both HCC827 and H1975 cells, $1,25(OH)_2D_3$ treatment was associated with a discernible increase in the expression of all 3 genes. In contrast, expression of the $1,25(OH)_2D_3$ target genes was essentially unchanged upon treatment in 128.88T and A549 cells. Cumulatively, these data indicate that transcriptional responses to $1,25(OH)_2D_3$ are preferentially retained in NSCLC cells that harbor an *EGFR* mutation.

To determine whether the observed differential transcriptional responses were indicative of differential sensitivity to $1,25(OH)_2D_3$ –mediated growth inhibition, clonogenic assays were conducted. Cells were allowed to attach and then were treated every 3 days with vehicle (control) or increasing concentrations of $1,25(OH)_2D_3$. Clonogenic survival was measured after 7 days. Although treatment resulted in dose-dependent growth inhibition in all 4 cell lines, the preferential sensitivity of *EGFR* mutation-positive cells to $1,25(OH)_2D_3$ was supported by two findings (Fig. 4). First, HCC827 and H1975 cells but not 128.88T or A549 cells displayed significant increases in growth inhibition between 1 and 10 nM $1,25(OH)_2D_3$. Second, at the highest concentration of $1,25(OH)_2D_3$ tested (100 nM), the average percent growth inhibition for HCC827 (86%) and H1975 (50%) cells was greater than that which was observed for 128.88T (39%) and A549 (30%) cells.

$1,25(OH)_2D_3$/erlotinib combination results in increased growth suppression of *EGFR* mutation-positive lung cancer cells

The EGFR tyrosine kinase inhibitor (EGFR TKI), erlotinib, is effective in the treatment of individuals with advanced NSCLC whose tumors harbor an activating mutation in the *EGFR* gene (reviewed in [17]). Because we found that NSCLC cells with an *EGFR* mutation are also likely to respond to $1,25(OH)_2D_3$, we sought to examine the effect of combining $1,25(OH)_2D_3$ with erlotinib. For this purpose, we used both HCC827 and H1975 cells. HCC827 cells have an exon 19 deletion (delE746-A750) in the *EGFR* gene and are sensitive to EGFR TKIs [7, 25]. H1975 cells are refractory to EGFR TKIs due to the presence of the T790M mutation in *EGFR* [26]. After overnight attachment, the cells were treated with vehicle, $1,25(OH)_2D_3$ alone, erlotinib alone, or $1,25(OH)_2D_3$ plus erlotinib. Consistent with the results presented above, $1,25(OH)_2D_3$ treatment resulted in significant inhibition of clonogenic survival in both cell lines (Fig. 5). Erlotinib inhibited the growth of both cell lines in a dose-dependent manner and, as expected, the concentration that significantly inhibited H1975 survival (5 μM) was greater than that required for HCC827 cells (10 nM). In both cell lines, a $1,25(OH)_2D_3$/erlotinib combination was identified which resulted in significantly greater growth inhibition than either single agent. These results suggest that $1,25(OH)_2D_3$ treatment may represent an effective approach to increase the responsiveness of erlotinib-sensitive and erlotinib-resistant *EGFR*-mutant NSCLCs to EGFR TKIs.

Discussion

Lung cancer remains the primary cause of cancer-related death in the United States. However, significant improvements in outcomes have been realized in recent years. Advances have resulted from the application of molecularly targeted therapies to specific subsets of lung cancer patients [17]. Based on the results presented in this manuscript, we suggest that *EGFR* mutations may identify a lung cancer subset which is likely to respond to

and benefit from $1,25(OH)_2D_3$ administration. Specifically, we find that *EGFR* mutation-positive lung cancer cells express relatively high levels of the VDR, have an intact $1,25(OH)_2D_3$ signaling axis, and are significantly inhibited by $1,25(OH)_2D_3$ administration. Because erlotinib is effective in the treatment of individuals with advanced NSCLC whose tumors harbor an activating mutation in the *EGFR* gene, we examined the potential impact of $1,25(OH)_2D_3$ on its activity. We discovered that combination of $1,25(OH)_2D_3$ with erlotinib resulted in significant inhibition of both HCC827 cells (a model of erlotinib-sensitive lung cancer) and H1975 cells (a model of acquired resistance to erlotinib). These results suggest that $1,25(OH)_2D_3$ may be useful in preventing the outgrowth of erlotinib-resistant disease. Pertinent to the potential clinical application of these findings, results from a prior Phase 1 clinical trial demonstrate that $1,25(OH)_2D_3$ (calcitriol) can be safely co-administered with an EGFR tyrosine kinase inhibitor in the oncology setting [27].

We observe an inverse relationship between *VDR* and *CYP24* expression in NSCLC cells (Fig 1). A statistically significant inverse association between these molecules was also observed in a large series of primary lung adenocarcinomas [15]. One possible explanation for these findings is that VDR functions as a repressor of basal *CYP24* transcription in NSCLC. The existence of such a regulatory mechanism is supported by prior studies in kidney and breast cancer cells [28, 29]. Using VDR over-expression and VDR siRNA approaches, Alimirah et al. were able to demonstrate the repressive action of unliganded VDR on *CYP24* expression in multiple breast cancer cell lines [29]. It was hypothesized by these investigators that *CYP24* repression occurs when VDR recruits the nuclear co-repressors, NCoR and SMRT, to the *CYP24* promoter. Once bound, the VDR/corepressor complex recruits other proteins that mediate transcriptional repression via epigenetic processes. If this model also applies to lung cancer, then it is expected that in *K-ras* mutation-positive NSCLC cells that have low VDR levels, NCoR/SMRT are no longer recruited to the promoter, *CYP24* repression is relieved and basal transcription increases. In constructing such models, it is important to consider the possibility that VDR/NCoR complexes may also mediate basal repression of *CYP24* through elements other than the promoter proximal VDREs. VDR and NCoR both bind to a VDRE located 50 kb downstream of the *CYP24* gene in human colonic LS180 cells in the absence of $1,25(OH)_2D_3$ [30]. In 3-dimensional space, this region comes into proximity with the *CYP24* transcription start site [30]. In this configuration, VDR/NCoR complexes bound to the +50 kb element may contribute to basal repression of the *CYP24* promoter.

A549 and 128.88T cells, which harbor a *K-ras* mutation, display a $VDR^{low}/CYP24^{high}$, vitamin D-resistant phenotype. Might the *K-ras* mutation itself contribute to *CYP24* upregulation? Ras signaling activates Ets transcription factors, and there is a functional Ets-binding site (EBS) located adjacent to the proximal VDRE within the *CYP24* promoter [31]. Ras-activated Ets transcription factors cooperate with VDR in the $1,25(OH)_2D_3$-mediated induction of *CYP24* transcription [31]. Ets-1 also increases *CYP24* transcription in the absence of $1,25(OH)_2D_3$, but only when repressive VDR:RXR heterodimers cannot form at the VDRE within the *CYP24* promoter [31]. Based on these data, we speculate that the low VDR levels observed in *K-ras* mutant NSCLC cells preclude the formation of repressive VDR:RXR complexes under basal growth conditions. Consequently, K-ras activated Ets-1 transcription factors are able to bind to the EBS, where they stimulate *CYP24* transcription. Consistent with this idea, we find that mutation of the EBS within a *CYP24* promoter-luciferase construct significantly decreases basal reporter activity in A549 and 128.88T cells (data not shown).

Although *K-ras* mutation may be a determinant of the $VDR^{low}/CYP24^{high}$ phenotype in NSCLC, it is clearly not sufficient for phenotype generation. Some *K-ras* mutation-positive lung adenocarcinomas express low levels of *CYP24* mRNA (Fig. 2), and some *K-ras*

positive NSCLC cells (SK-LU-1) are $VDR^{high}/CYP24^{low}$ and $1,25(OH)_2D_3$-responsive [15, 32]. An alternative hypothesis is that: (1) The mutation-related differences in $1,25(OH)_2D_3$ sensitivity that we observe occur because *K-ras* and *EGFR* mutations are differentially associated with smoking; and (2) Smoking drives epigenetic changes in *K-ras* mutation-positive cells that contribute to inactivation of the vitamin D pathway. This idea is supported by prior reports showing that *VDR* and *CYP24* are subject to epigenetic regulation [33, 34], and that specific epigenetic alterations are differentially observed in *K-ras* and *EGFR* mutant lung cancers [35]. Ongoing studies in our laboratory are designed to establish the relationship between smoking, *K-ras* mutation, and acquisition of the $VDR^{low}/CYP24^{high}$, vitamin D-resistant phenotype.

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Abbreviations used

CSS	Charcoal-stripped serum
DMSO	Dimethylsulfoxide
EGFR	Epidermal growth factor receptor
FBS	Fetal bovine serum
1, 25$(OH)_2D_3$	1,25-dihydroxyvitamin D_3
NSCLC	non-small cell lung cancer
TKI	tyrosine kinase inhibitor
VDRE	vitamin D response element

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Highlights

- Aberrant CYP24 expression limits $1,25(OH)_2D_3$ activity in NSCLC cells
- CYP24 levels are increased in NSCLC cells and lung tumors with K-ras mutations
- NSCLC cells and lung tumors with EGFR mutations have low basal CYP24 levels
- K-ras and EGFR mutation-positive NSCLC cells respond differentially to $1,25(OH)_2D_3$
- EGFR mutations may identify a NSCLC subset likely to benefit from $1,25(OH)_2D_3$

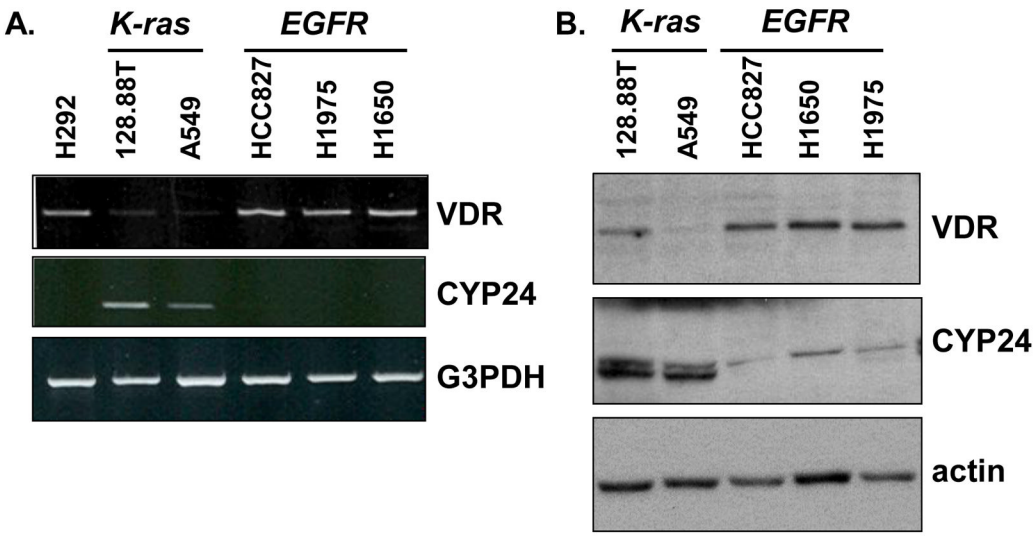

Figure 1. *EGFR* and *K-ras* mutation positive NSCLC cells differ in their basal expression of VDR and CYP24

(A) mRNA expression levels of VDR and CYP24 were evaluated in the indicated cell lines by semi-quantitative RT-PCR. **(B)** Whole cell extracts were prepared from the indicated cell lines. Equivalent amounts of protein were analyzed by immunoblot for VDR and CYP24. Blots were reprobed for actin as a control for protein quantitation and loading. Results are representative of 3 independent experiments.

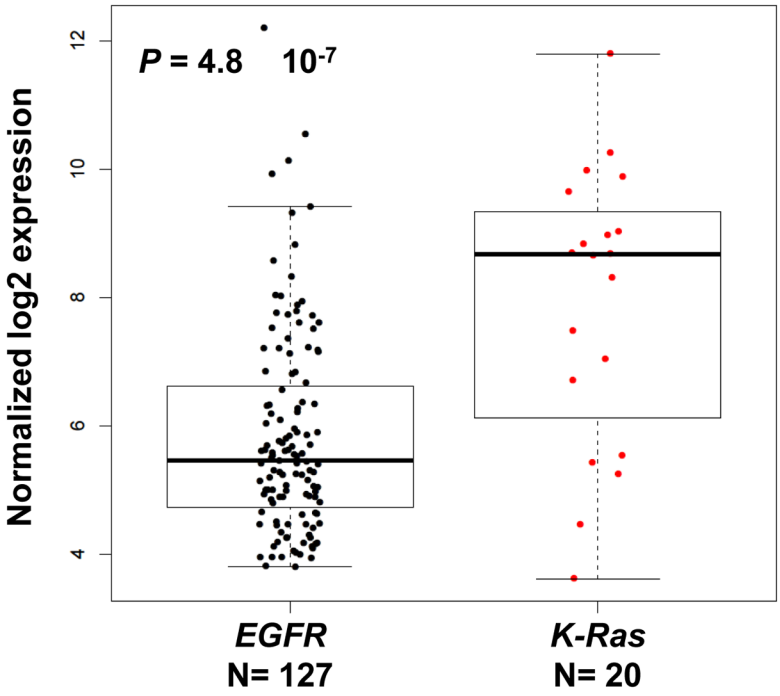

Figure 2. *CYP24* is differentially expressed in primary human lung adenocarcinomas that harbor *EGFR* or *K-ras* mutations

Gene expression values were abstracted for the 20 *K-ras* mutation-positive lung adenocarcinomas and 127 *EGFR* mutation-positive lung adenocarcinomas included in the whole genome, gene expression profiling study of Okayama et al., (GSE31210) [21]. Each point represents an individual case. Data were analyzed using the Bioconductor packages in the R statistical computing environment microarray data processing. Specifically, the RMA function was used to generate expression summary values through convolution background correction, quantile normalization, and a summarization based on a multi-array model fit using the medial polish algorithm. The Limma program was used to calculate the level of differential expression based on the log2 transformed gene expression value. Multiple testing was corrected using Benjamin and Hochberg's algorithm. Box edges indicate the 25%-ile and 75%-ile of *CYP24* expression for each population. Horizontal bars indicate the median *CYP24* expression value.

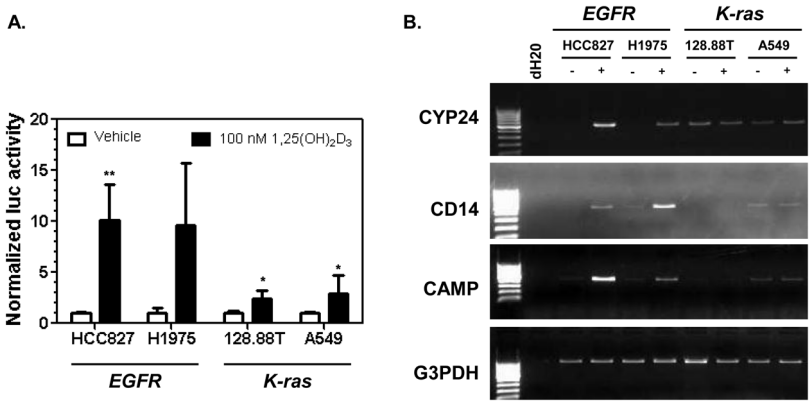

Figure 3. *EGFR* and *K-ras* mutation-positive NSCLC cells differ in their transcriptional responses to $1,25(OH)_2D_3$

(A) Cells were co-transfected with a CYP24 promoter-luciferase reporter plasmid and CMV β-galactosidase (to control for transfection efficiency). Transfected cells were treated with vehicle or 100 nM $1,25(OH)_2D_3$. Luciferase and β-galactosidase activities were measured after 17 h. Normalized activity values (luc/β-gal) were calculated for each treatment group. For each cell line, the control (vehicle) value was set to a value of 1.0. Values represent the mean ± SD for 3–6 determinations. The difference between the reporter activity in the control group versus the 100 nM $1,25(OH)_2D_3$ treatment group was assessed using one-sided t-tests. * $p < 0.05$; ** $p < 0.001$. **(B)** The indicated cell lines were treated with vehicle (-) or 100 nM $1,25(OH)_2D_3$ (+). RNA was isolated 17h later, and the expression of endogenous $1,25(OH)_2D_3$ target genes *CYP24, CD14,* and *CAMP* was measured by semi-quantitative PCR. *G3PDH* was used as a control for RNA quantitation and cDNA synthesis. Similar results were obtained in a second, independent study.

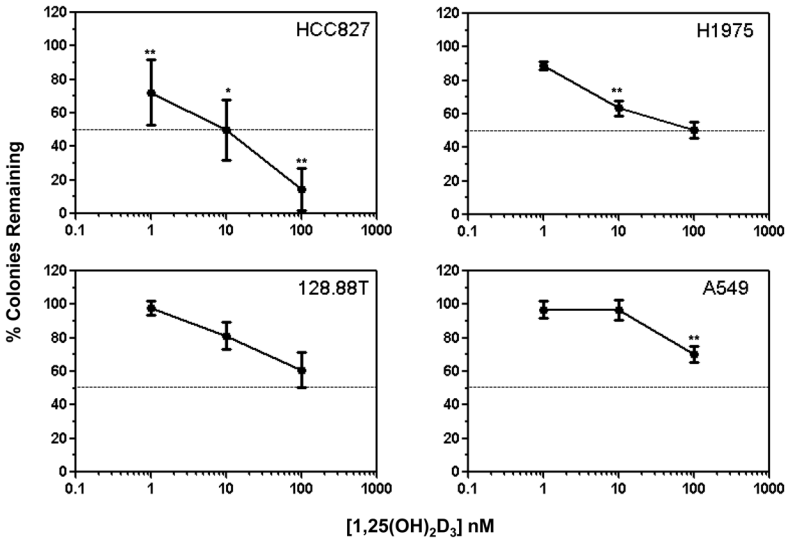

Figure 4. *EGFR* and *K-ras* mutation-positive NSCLC cells are differentially sensitive to $1,25(OH)_2D_3$-mediated growth inhibition

The indicated cells were seeded into 6 well plates (3 wells per treatment group). The next day, cells were treated with fresh medium containing vehicle (control) or varying concentrations of $1,25(OH)_2D_3$. Treatments were repeated every 3 days. Crystal violet staining was used to assess colony formation as described in Methods. Values represent the mean ± SD for 6–9 determinations, pooled from 2–3 independent experiments per cell line. The association between cell growth inhibition and treatment was assessed using a one-way ANOVA. The ordered pair-wise comparisons between successive dose-levels were made using t-tests (* $p<0.05$, ** $p\leq 0.001$).

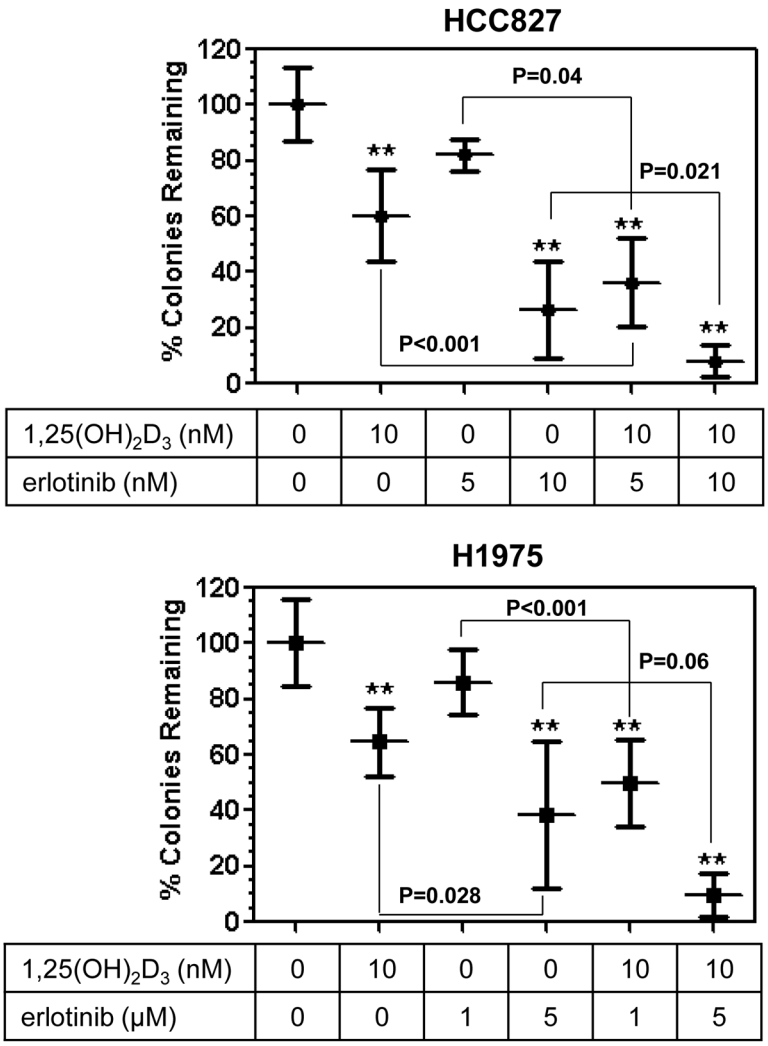

Figure 5. Combination of $1,25(OH)_2D_3$ with erotinib results in increased growth inhibition of *EGFR* mutation-positive NSCLC cells

(A) HCC827 and H1975 cells were seeded into 6 well plates (3 wells per treatment group). The next day, the cells were treated with vehicle (control), $1,25(OH)_2D_3$ alone, erlotinib alone, or the combination. Treatments were repeated every 3 days. Colony formation was assessed as described in Fig. 4. Values represent the mean ± SD for 3 pooled experiments per cell line. The association between treatment and cell growth for each cell line was assessed using a one-way ANOVA test. One-sided post-hoc t-tests, conducted with Dunnett's adjustment for multiple tests, were used to compare the control group to all other treatments (** $p<0.001$). Within each cell line, the combination treatments were subsequently compared to the individual treatments using one-sided t-tests with a Bonferroni adjustment for multiple tests (p values for each comparison are shown adjacent to brackets).