Journal of Steroid Biochemistry & Molecular Biology 136 (2013) 264–270

Contents lists available at SciVerse ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Review

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

Qiuhong Zhang[a], Beatriz Kanterewicz[a], Suzanne Shoemaker[b], Qiang Hu[c], Song Liu[c], Kristopher Atwood[d], Pamela Hershberger[b,*]

[a] University of Pittsburgh Cancer Institute, Pittsburgh, PA 15213, United States
[b] Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, NY 14263, United States
[c] Department of Biostatistics and Bioinformatics, Roswell Park Cancer Institute, Buffalo, NY 14263, United States
[d] Department of Biostatistics, Roswell Park Cancer Institute, Buffalo, NY 14263, United States

ARTICLE INFO

Article history:
Received 2 July 2012
Received in revised form
17 September 2012
Accepted 20 September 2012

Keywords:
Non-small cell lung cancer
Vitamin D receptor
1,25-Dihydroxyvitamin D_3
Epidermal growth factor receptor
K-ras

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 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.

This article is part of a Special Issue entitled 'Vitamin D Workshop'.

Contents

Abbreviations: 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.

☆ This work was supported by grant R01 CA132844.

* Corresponding author at: Department of Pharmacology and Therapeutics, CGP L4-317, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263, United States. Tel.: +1 716 845 1697; fax: +1 716 845 8857.
E-mail address: pamela.hershberger@roswellpark.org (P. Hershberger).

0960-0760/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jsbmb.2012.09.022

1. 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 $\geq$21.6 ng/mL experienced a significant improvement in survival as compared to patients with $25(OH)D_3$ levels $\leq$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.

2. Materials and methods

2.1. 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.

2.2. 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.

2.3. 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].

2.4. 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