CYP24A1 Is an Independent Prognostic Marker of Survival in Patients with Lung Adenocarcinoma

Running Title: CYP24A1 Prognostic Marker in Lung Adenocarcinoma

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Translational Relevance

1α,25-Dihydroxyvitamin D₃ (1,25-D₃, calcitriol) is the active form of vitamin D. 1,25-D₃ in supra-physiological doses, exhibits antiproliferative and differentiation-inducing effects in several cancers. Epidemiologic studies link low vitamin D levels both to increased incidence as well as poorer outcome in cancer. 1,25-D₃ role as an adjunct to therapy is being explored in lung adenocarcinoma (AC). Delineating factors predictive of the response of cancer cells to 1,25-D₃ is crucial. Here, we demonstrate that CYP24A1, the main metabolizing enzyme of 1,25-D₃, is independently prognostic of survival in lung AC. In parallel, we show that antiproliferative effects of 1,25-D₃ are proportional to mRNA levels of CYP24A1 in human lung cancer cell lines. Defining tumor-specific CYP24A1 expression levels may allow individualizing secondary prevention using exogenous 1,25-D₃ in human lung AC.
Abstract

Purpose: The active form of vitamin D, 1α,25-dihydroxyvitamin D₃ (1,25-D₃) exerts antiproliferative effects in cancers, including lung adenocarcinoma (AC). CYP24A1 is overexpressed in many cancers and catabolizes 1,25-D₃. The purpose of our study was to assess CYP24A1 as a prognostic marker and to study its relevance to antiproliferative activity of 1,25-D₃ in lung AC cells.

Experimental Design: Tumors and corresponding normal specimens from 86 patients with lung AC (stages I-III) were available. Affymetrix array data and subsequent confirmation by quantitative real time-PCR were used to determine CYP24A1 mRNA expression. A subsequent validation set of 101 lung AC was used to confirm CYP24A1 mRNA expression and its associations with clinical variables. The antiproliferative effects of 1,25-D₃ were examined using lung cancer cell lines with high as well as low expression of CYP24A1 mRNA.

Results: CYP24A1 mRNA was elevated 8-50 fold in lung AC (compared to normal non-neoplastic lung) and significantly higher in poorly-differentiated cancers. At 5 years of follow-up, the probability of survival was 42% (high CYP24A1, n = 29) versus 81% (low CYP24A1, n = 57) (P = 0.007). The validation set of 101 tumors showed that CYP24A1 was independently prognostic of survival (multivariate Cox model adjusted for age, gender and stage, P = 0.001). A549 cells (high CYP24A1) were more resistant to antiproliferative effects of 1,25-D₃ compared with SKLU-1 cells (low CYP24A1).

Conclusions: CYP24A1 overexpression is associated with poorer survival in lung AC. This may relate to abrogation of antiproliferative effects of 1,25-D₃ in high CYP24A1 expressing lung AC.
Introduction

Lung cancer is the leading cause of cancer-related death with over 100,000 deaths annually in the United States (1). The five-year survival for all stages is low at only 15%. Adenocarcinoma (AC) accounts for over 50% of all non-small cell lung cancers (NSCLC). At least 60% of these patients present with advanced stage III/IV metastatic disease. Current therapies are not curative and the rates of recurrence are very high after initial therapy. The only curative treatment is surgical resection in the earlier stages and even in these patients, the relapse rates, despite adjuvant chemotherapy, is high, varying from 20-50%. Consequently, it is important to focus research on early detection and secondary prevention strategies that are associated with less toxicity, such as the use of vaccines or natural compounds.

Recent epidemiological studies have shown that exposure to solar radiation (UVB) and vitamin D intake is associated with decreased incidence of many cancers including colon, breast, prostate and lung (2-6). There is an inverse correlation between cancer mortality rate and regional UVB irradiation for cancers such as the breast, colon, prostate, stomach, esophagus and lung cancers (2). Higher vitamin D levels are associated with improved survival in early-stage NSCLC patients (6) and circulating 25-hydroxyvitamin D₃ (25-D₃) levels predict survival in early-stage NSCLC patients (7).

Vitamin D is hydroxylated by CYP27A1 to 25-D₃ in the liver followed by further metabolism by CYP27B1 to 1α,25-dihydroxyvitamin D₃ (1,25-D₃, also known as calcitriol). 1,25-D₃, the most active metabolite of vitamin D, not only plays a critical role in calcium homeostasis, but also has non-endocrine effects. At supraphysiologic doses, 1,25-D₃ is associated with antiproliferative activity (8-10), induction of cell differentiation (11), cell cycle arrest (12), apoptosis (13, 14), and inhibition of angiogenesis (15). This activity is mediated through the
vitamin D receptor (VDR). Previous studies have shown that 1,25-D$_3$ has significant antitumor activity both in vitro and in vivo in a variety of murine and human tumor models including lung (16, 17), squamous cell carcinoma (SCC) (10, 14, 18) and prostate (19) cancer model systems. The anti-cancer properties of vitamin D have been reviewed by Deeb et al (20). Vitamin D may be a useful adjuvant in patients who have had a surgical resection to prevent a recurrence. It is unclear whether all patients will benefit from this therapy. It is therefore imperative to find a marker that will assist in identifying patients who may benefit from vitamin D.

CYP24A1 catabolizes 1,25-D$_3$ to 1α,24,25-trihydroxyvitamin D$_3$. The strongest inducer of CYP24A1, 1,25-D$_3$ mediates this induction via an autocatalytic loop through vitamin D response elements (VDREs) located in the promoter region of the CYP24A1 gene (21). There is a differential induction of CYP24A1 by vitamin D in malignant and non-malignant cells (22). CYP24A1 is over-expressed in numerous human tumors, including breast, colon, prostate esophagus and lung (23, 24); however, it is not clear whether the high expression of CYP24A1 gene seen in cancer cells leads to a functional enzyme that abrogates vitamin D activity.

In an effort to find prognostic factors that could be targets for adjuvant therapy, we found several genes whose expression were “outliers” among the 50 survival related genes (25). One of these outlier genes was CYP24A1. In the current study we have evaluated CYP24A1 mRNA expression as a prognostic marker in a large cohort of lung AC patients. We have investigated the relationship between amplification and over expression of the CYP24A1 gene and have evaluated the functional role of CYP24A1 using high/low CYP24A1 expressing lung cancer cell lines in vitro.

Materials and Methods

Human samples

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Lung tumor samples were obtained from patients undergoing primary thoracic resection for lung cancer without preoperative radiation or chemotherapy, as previously described (25). Tissue specimens were banked with informed consent after approval from University of Michigan Institutional Review Board and Ethics Committee and were frozen in liquid nitrogen and stored in -80°C. Percentage of tumor purity in sections adjacent to regions used for RNA extraction was estimated during routine histopathologic analysis. Regions containing a minimum of 70% tumor cellularity were utilized for RNA isolation.

Cell culture

Human lung AC cancer cell lines including A549 and SKLU-1 were obtained from American Type Culture Collection (ATCC) and cultured with DMEM/F12 or DMEN medium with 10%FBS at 37°C in a humid atmosphere consisting of 5% CO₂/95% air.

RNA extraction and cDNA synthesis

Total RNA was isolated from tissue samples and cell lines followed by column purification using RNeasy Mini kit (Qiagen) according to the manufacturers' instructions. RNA was eluted from the spin column using RNase-free dH₂O. cDNA was prepared from RNA samples using High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to manufacturer's instructions.

Quantitative real time reverse transcription-polymerase chain reaction (qRT-PCR)

The qRT-PCR reaction was prepared using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), and qRT-PCR was performed with a Rotor-Gene 6000 Real Time Rotary Analyzer (Corbett Research, Sydney, Australia) or StepOne Real-Time PCR System (Applied
Biosystems). Each sample had a final volume of 15 µL containing approximately 100 ng of cDNA. The oligonucleotide primers for *CYP24A1* (144 bp PCR product) were as follows: 5'-GCCGTATTTAAAAGCCTGTCTGAA-3' (forward) and 5'-ACCTGGGTATTTAGCAGTGAGCACTG-3' (reverse). The primers for *VDR* (203 bp PCR product) were as follows: 5'-GCCACCATAAGACCTACGA-3' (forward) and 5'- AGATTGGAGAAGCTGGACGA-3' (reverse). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or beta actin expression was used to standardize the *CYP24A1* and *VDR* qRT-PCR results. Relative mRNA levels of were assessed using the 2−ΔΔCt method as described previously (55).

**Immunohistochemistry and tissue microarray**

Tissue microarrays (TMA) were constructed, as previously described (26), with formalin-fixed, paraffin-embedded tissues from 86 patients. Immunohistochemical staining was done on the DAKO Autostainer using DAKO LSAB+. Antigen retrieval was achieved with preheated 10 mmol/L (pH 6) citrate buffer for 20 min to 95°C. Deparaffinized and rehydrated sections of the TMA at 4-m thickness were labeled with CYP24A1 antibody (Santa Cruz Biotechnology, rabbit polyclonal antibody, 1:200 dilution). Staining was visualized with 3,3′-diaminobenzidine and sections were lightly counterstained with hematoxylin. Each sample was scored independently by two readers using a scale of 0 (no staining), 1+ (< 10% cells staining), 2+ (10-50% cells staining) or 3+ (≥ 50% cells staining).

**Cell proliferation assays**

The effect of 1,25-D₃ on proliferation of A549 and SKLU-1 cells was measured using WST-1 cell proliferation reagent (Roche) and cell counting using a hemocytometer. For WST-1 cell proliferation assay, cells were plated at 2~3×10³ (day 4) and 50~300 (day12) cells per well in a 96 well micro-titer plates (Corning), six wells per condition. Cells were treated 24 hours later
with 0, 1, 10 and 100 nmol/L of 1,25-D₃ (5-6 wells per each treatment). At day 4 and 12, cells were treated with WST-1 reagent (Roche) according to manufacturer's instructions. For cell counting assay, cells were plated at 3~4x10⁴ (day 4) and 300~3x10³ (day 12) cells per well in a 6 well micro-titer plates (Corning). At day 4 and 12, cells were collected, trypsinized and counted using a hemocytometer. For the WST assay, cell proliferation was estimated by dividing the mean absorbance of the treatment group divided by the mean absorbance of the vehicle-treated control x 100%. For cell counting, relative proliferation was measured by dividing the mean number of cells in each treatment group by the mean number of cells in vehicle-treated control x 100%.

**Protein isolation and immunoblot analysis**

Cells were plated and grown until 80% confluent. Cells were harvested with lysis buffer [150 mmol/L NaCl, 20 mmol/L Tris (pH 7.5), 1 mmol/L EDTA, 1 mmol/L EGTA, 2.5 mmol/L Na₄P₂O₇, 1 mmol/L β-glycerol 2-phosphate disodium salt hydrate, 1 mmol/L Na₃VO₄, 1% Triton X-100] supplemented with protease inhibitor cocktail (Sigma-Aldrich). Protein was quantified using Bio-Rad protein assay kit (Bio-Rad Laboratories) according to the manufacturer’s protocol. Proteins (20 μg) were resolved on 10% tris-glycine gels (Invitrogen) and transferred to Immobilon-P membranes (Millipore). The blots were probed with either anti-CYP24A1 (Santa Cruz Biotechnology) diluted 1:1,000, or beta actin (1:10,000 dilution) (Abcam). Secondary antibody used was anti-goat antibody.

**Statistical analysis**

The t-tests were used to identify statistically differences in mean gene-expression levels between different clinical variables. Survival curves were constructed using the method of
Kaplan-Meier and survival differences were assessed using the log-rank test. The univariate and multivariate (adjusted by sex, age and stage) Cox proportional hazards model with continuous value of CYP24A1 mRNA were used to assess survival results, censored at 5 years. Spearman correlation method was used to test the correlation of CYP24A1 expression between microarray and qRT-PCR data or protein and mRNA expression. P values < 0.05 were considered significant.

Results

CYP24A1 mRNA is differentially expressed in NSCLC and is correlated with survival

Clinical data including stage, age and survival information were available for 86 patients (Table 1 and supplementary Table S1). None of the patients received preoperative chemotherapy or radiation therapy. The information regarding adjuvant chemotherapy or radiation was collected in this study. Five out of 43 patients with stage 1A and 22/43 stage 1B or higher received adjuvant chemotherapy and or radiation therapy.

Our laboratory was one of the first to report on genomic profiling of resected lung AC and found that gene expression profile can predict patient survival in lung AC (25). CYP24A1 was one of few outlier genes with high expression in lung AC. CYP24A1 mRNA was several fold higher in lung AC compared with normal lung (Supplementary Fig. S1A and S1B). To confirm the Affymetrix data on relative differences in CYP24A1 mRNA levels in lung AC (Fig. 1A), we performed qRT-PCR on 47 of the 86 tumor samples. A significant correlation (r = 0.94, n = 47, P < 0.01) was observed between Affymetrix array and qRT-PCR for CYP24A1 mRNA expression (Fig. 1B). High level CYP24A1 mRNA expression (50-fold or greater) was found in a subset (~30%) of lung AC (Fig. 1A).
CYP24A1 gene expression was correlated with other tumor-related factors and survival. Tumor stage, differentiation and survival status are shown in Table 1. (More comprehensive information is shown in Supplementary Table S1). There was a significant correlation between poorly-differentiated tumors and high CYP24A1 mRNA expression ($P = 0.03$; Table 1). Mean expression level for CYP24A1 mRNA in uninvolved adjacent lung samples was 6.0 whereas the mean CYP24A1 mRNA of the tumors was 296.5 ($P < 0.001$; Table 1). Patients who had a high expression of CYP24A1 gene (top third) had a poorer 5-year survival than those with low to medium levels (bottom two-third) (log-rank test $P = 0.007$) (Fig. 1B and 1C). Of note, we did not find a correlation between CYP24A1 mRNA and survival in a squamous lung cancer microarray data, $n=130$, Cox model (adjusted by age, sex and stage) $p=0.9$, beta = 0.01. We did not have data for large cell lung cancer (27). To examine whether gene amplification might explain increased mRNA expression of CYP24A1, we used an Affymetrix single nucleotide polymorphism (SNP) array for gene copy number (data available for 71 out of 86 samples) (28)(28). An increased copy number of 20q was detected in one third of lung AC patients with increased CYP24A1 mRNA expression (Supplementary Fig. S2). Gene amplification alone did not explain increased mRNA expression.

We constructed TMAs with immunohistochemical staining to identify the relationship between mRNA and protein expression. We evaluated 63 tumors with TMA score (0 - 3) (Fig. 2A, B, and C). Higher mRNA expression correlated with higher TMA score ($r = 0.87$, $P < 0.05$, Fig. 2D). We also compared the top 22 high and bottom 22 low CYP24A1 mRNA tumor samples to TMA (Table S2). About 80% of samples with high CYP24A1 mRNA showed at least moderate (+2) or strong staining (+3) for CYP24A1. Ten out of 22 tumor samples with low CYP24A1 mRNA showed weak (+1) staining for CYP24A1 and only two tumor samples with low CYP24A1 mRNA showed strong staining (+3) (Table S2). Based on TMA availability on only 63
patients, we found that patients who demonstrated a 3+ (high, n=10) IHC staining for CYP24A1 had worse survival compared with the ones that exhibited absent –moderate (0 – 2+ n =53) IHC staining (Supplementary Fig. S3, HR=1.7, 0.48-6.14, p = 0.41).

A validation data set confirmed shorter survival with high CYP24A1 mRNA expression

To confirm the expression of CYP24A1 gene from 86 lung AC patients, we used a prospective data set of 101 lung AC. None of the patients received preoperative chemotherapy or radiation therapy. The information regarding adjuvant chemotherapy or radiation was collected in this study. 56% (38/68 stage 1B or higher) of patients received adjuvant chemotherapy or radiation therapy. Among these 68 patients, no survival differences between patients receiving adjuvant therapy, or those not receiving adjuvant therapy were observed (log-rank test, \( P = 0.6 \)). No adjuvant therapy was provided to the 33 stage 1A patients. We performed qRT-PCR for tumors (n = 101) and non-neoplastic normal lung tissue (n = 12) to assess CYP24A1 mRNA expression levels. Compared to normal lung tissue, lung tumors had higher CYP24A1 mRNA expression (Fig. 3A and Table 2.) CYP24A1 mRNA expression was elevated in about 33% of the patients confirming our earlier observation (Fig. 3A). Patients who had high CYP24A1 mRNA expression had an overall poorer survival at 5-years compared with low to medium CYP24A1 mRNA (HR 2.1, 95% CI 1.14-3.75; log-rank test \( P = 0.01 \)) (Fig. 3B and Supplementary Table S3). The results of univariate and multivariate Cox regression survival analysis with continuous value indicate CYP24A1 mRNA was significantly related to survival independent of cancer stage, age, and sex (Supplementary Table S4). In addition, poorly differentiated tumors had a higher level of CYP24A1 expression compared to well differentiated tumors (Table 2). This also validates our previous data (Table 1).
High CYP24A1 mRNA expressing cells are more resistant to antiproliferative actions of 1,25-D$_3$

Because CYP24A1 over-expression is associated with poor prognosis in AC patients (Table 1 and Figs. 1B and 3B), we hypothesized that tumor-specific expression of CYP24A1 might determine the effects of 1,25-D$_3$ on lung cancer cells. We examined the CYP24A1 mRNA levels in 14 lung cancer cell lines. As shown in Fig 4A, CYP24A1 mRNA was variably expressed among 14 human lung cancer cell lines, with A549 demonstrating very high CYP24A1 expression and SKLU1, H1396 and H460 with extremely low CYP24A1 expression (Fig. 4A) relative to each other. We chose two representative cell lines, A549 (high CYP24A1) and SKLU-1 (low CYP24A1) to confirm the CYP24A1 protein levels by Western blot analysis. As shown in Fig. 4B, CYP24A1 protein was highly expressed in A549 cells whereas CYP24A1 protein expression was undetectable in SKLU-1 cells. To assess the functional consequences of CYP24A1 expression, we performed cell proliferation assays using both cell lines in the presence of varying doses of 1,25-D$_3$ for 4 or 12 days. On day four, A549 cells were more resistant to the antiproliferative effect of 1,25-D$_3$ when compared with SKLU-1 cells using both cell counting and the WST-1 assays (Fig. 4C). The antiproliferative effect of 1,25-D$_3$ was more pronounced in both cell lines at day 12 (Fig. 4C); again A549 cells were less sensitive compared with SKLU-1 cells.

Inverse relationship between CYP24A1 mRNA and VDR mRNA expression: High CYP24A1 mRNA expression in A549 cells was associated with low VDR mRNA expression and vice versa in SKLU-1 cells (Fig. 4B). A similar trend was noted across all 14 cell lines studied for CYP24A1 and VDR mRNA (Supplementary Fig. S4A). Similarly, a negative correlation trend was observed in 101 patients between CYP24A1 and VDR expression (Supplementary Fig. S4B).
Discussion

CYP24A1 is a member of the cytochrome p450 enzyme family that is primarily responsible for catabolizing the active form of vitamin D (1,25-D$_3$) to inactive calcitroic acid. CYP24A1 is normally found in high levels in the kidney, playing a crucial role in vitamin D homeostasis. The level and biological activity of 1,25-D$_3$ in tissues is normally controlled by maintaining a precise balance between the rates of its synthesis by CYP27B1 and degradation by CYP24A1. We have determined that CYP24A1 is overexpressed in a subset of lung AC. Here we show that that high CYP24A1 mRNA in lung AC patients is prognostic for survival. Patients whose tumors demonstrated high CYP24A1 mRNA levels were associated with both poor differentiation and a poorer survival than those with low levels of CYP24A1 mRNA, independent of other clinical and pathologic prognostic parameters of survival. We hypothesize that CYP24A1 overexpression facilitates lung cancer growth by abrogating the antiproliferative effects of locally-produced 1,25-D$_3$.

Consistent with previous reports(29), we found that lung AC demonstrated a higher expression of CYP24A1 compared with normal non-neoplastic lung. Differential over-expression of CYP24A1 gene has been observed not only in lung (28, 29) but also in colon, cervical, ovarian, cutaneous squamous cell and esophageal carcinomas (23, 24) which suggests CYP24A1 overexpression may be involved in the carcinogenesis process, possibly related to the abrogation of the anti-tumor effects of 1,25-D$_3$. Other groups have also verified the over-expression of CYP24A1 in non-small cell lung cancer (29). Kim et al, selected 20 genes for experimental validation using semi-quantitative RT-PCR. They used clinical specimens from patients with benign lung disease and NSCLC. Two genes (CBLC and CYP24A1) qualified as highly probable novel biomarkers and potential value as drug targets (29).
More recently Parise et al confirmed that CYP24A1 mRNA expression was higher in lung cancer compared to normal bronchial epithelium (28). Analysis of NSCLC cell cultures revealed time-dependent loss of 1,25-D$_3$ coincident with the appearance of CYP24A1-generated metabolites. Specific inhibition of CYP24A1 slowed the loss of 1,25-D$_3$ and increased the half-life of 1,25-D$_3$. These data suggest that increased CYP24A1 gene expression in lung tumors inhibits 1,25-D$_3$ anti-tumor activity and reduces the antiproliferative activity of 1,25-D$_3$ (35).

Anderson et al, demonstrated in various cancer cell lines that the antiproliferative activity of 1,25-D$_3$ is inversely proportional to CYP24A1 mRNA expression (23).

The CYP24A1 gene is located on chromosome 20q13.2. One of the possible reasons for high CYP24A1 mRNA expression might be related to gene amplification. Several studies have already examined the gain of 20q in gastro-esophageal junction (30), colon (30), breast (32), prostate (31), head and neck (32) as well as lung tumors (35, 36). Our data showed that only a third of patients with high CYP24A1 mRNA have increased copy number of 20q suggesting that amplification of the gene alone could not explain the increased expression of CYP24A1 mRNA and that other reasons for increased transcription were likely.

We also examined whether the increased expression of CYP24A1 mRNA translates into higher amount of CYP24A1 enzyme, which eventually leads to more metabolism of 1,25-D$_3$ to 1,24,25-D$_3$. The relationship between mRNA and protein expression is not linear in all cancers. In fact, reports in breast cancer suggest a discordance between both mRNA and protein hypothesizing a potential role of miRNA in modifying gene translation (33). We however, have noted that high CYP24A1 mRNA expression showed moderate or strong expression for CYP24A1 and low CYP24A1 mRNA expression showed weak or low expression for CYP24A1. This result has been also confirmed using immunoblot analysis lung AC cell lines with high CYP24A1 (A549) and low CYP24A1 (SKLU-1).
Additionally, we have demonstrated a dose response effect of 1,25-D$_3$, inversely proportional to CYP24A1 mRNA expression. A549 cells showed more resistance to 1,25-D$_3$ than SKLU-1 and this phenomenon was more marked when the cells were treated for longer period of time. Interestingly, the cell lines with high expression of CYP24A1 (example A549) exhibited the lowest expression of VDR mRNA; the converse was observed with SKLU-1. This was noted in the 101 patient samples as well. This suggests that low VDR and high CYP24A1 will have the lowest amount of substrate that will drive the reaction to the right and lead to the least amount of active calcitriol in the milieu. CYP24A1 is a member of the cytochrome P450 system with only one major substrate, 1,25-D$_3$. Cells having high CYP24A1 mRNA expression have a functional enzyme that leads to increased catabolism of 1,25-D$_3$ and therefore lower substrate available for antiproliferative effects. Taken together with in vivo and in vitro, the differential expression of CYP24A1 mRNA in lung AC cancer presents a potential target for the development of compounds that can block CYP24A1 at the tumor site or vitamin D compounds that retain their genomic, non-calcemic functions, and are not substrates to CYP24A1 enzyme. This has led to the development of new vitamin D analogues and specific CYP24A1 inhibitors in various phases of drug development (34, 35).

In summary, we correlate CYP24A1 mRNA expression with survival in resected lung AC. Our data demonstrate that over-expression of CYP24A1 mRNA is associated with poorer survival of lung AC patients and an inverse relationship between CYP24A1 mRNA expression and differentiation status of the lung cancer. Increased CYP24A1 mRNA counteracts the antiproliferative effect of 1,25-D$_3$ in lung AC cancer cell lines, suggesting that high CYP24A1 mRNA expression in NSCLC leads to abrogation of antiproliferative effects of 1,25-D$_3$ and ultimately poorer survival. CYP24A1 is not only a prognostic biomarker for lung AC but tumor levels may allow individualized secondary prevention strategies using either 1,25-D$_3$ alone or in
combination with a CYP24A1 inhibitor. Further studies assessing pulmonary vitamin D metabolism in lung AC are underway in our laboratory.

**Disclosure of Potential conflicts of Interest**

No potential conflicts of interest

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Reference

Legends for figures

**Fig. 1.** *CYP24A1* mRNA expression in 86 lung adenocarcinoma (AC) patients. A, using Affymetrix microarray there was a differential expression of *CYP24A1* mRNA in 86 resected lung AC and 10 normal. One third of the patients had high *CYP24A1* mRNA levels. (mRNA expression >170 relative units). B, Strong correlation between Affymetrix array and qRT-PCR for *CYP24A1* mRNA expression using the Spearman correlation method (r = 0.94, P <0.01). qRT-PCR for *CYP24A1* mRNA expression was conducted for 47 samples of the 86 tumor set. Log2 transformed value was shown. C, Kaplan-Meier survival curve showing poor survival in patients with lung tumors expressing high *CYP24A1* mRNA. Surgically resected patients with stages 1-3 NSCLC was divided into high (>170, n = 29) or low to medium (<170, n = 57).

**Fig. 2.** Immunohistochemistry for *CYP24A1* and correlation to mRNA expression. A, shows normal lung with weak staining (+1) for *CYP24A1*. B, shows moderate staining (+2) for *CYP24A1* and C, shows strong staining (+3) for *CYP24A1*. Magnification is 200X. D, correlation between mRNA (microarray data) and protein (TMA protein staining) expression of *CYP24A1* in 63 lung tumors. Higher TMA score corresponded to higher mRNA level (r = 0.87, P < 0.05). Mean value of mRNA in each scoring group was used.

**Fig. 3.** Validation data set for *CYP24A1* mRNA expression and survival. A, Using qRT-PCR there was increased expression of *CYP24A1* mRNA in 101 lung adenocarcinomas (AC) compared with 12 surrounding normal. One third of the patients had high *CYP24A1* mRNA levels B, validation set of 101 AC confirms shorter survival in tumors expressing high *CYP24A1* mRNA by Kaplan-Meier plot and log-rank test for high vs. low to medium group (P = 0.01).

**Fig. 4.** mRNA expression of *CYP24A1* and the effect of 1,25-D$_3$ in human lung cancer cell lines. A, qRT-PCR results from 14 human lung cancer cell lines showed wide variation from high to low.
low CYP24A1 mRNA expression. Number indicates fold change to median. B, qRT-PCR results of CYP24A1 and VDR mRNA expression in A549 and SKLU-1 cells. High CYPA1 mRNA expression in A549 cells was associated with low VDR mRNA expression and vice versa in SKLU-1 cells. C, immunoblot analysis of CYP24A1 protein in low (SKLU-1) and high (A549) CYP24A1 mRNA expressing lung AC cell lines. The protein expression of CYP24A1 was significantly higher in A549 cells. D, the effect of 1,25-D$_3$ on cell proliferation in SKLU-1 and A549 cell lines by WST-1 reading and cell counting. Both assays showed that SKLU-1 demonstrated more marked decrease in cell proliferation in response to 1,25-D$_3$ compared to A529 at both days 4 and 12.
Table 1. mRNA expression of CYP24A1 in 86 lung adenocarcinomas

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</tr>
</tbody>
</table>

NOTE; *P values calculated using t-test
**Compared to ‘Poor’
*** Actual survival status.
Table 2. Verification of CYP24A1 in validation set of 101 lung adenocarcinomas

<table>
<thead>
<tr>
<th>Variables</th>
<th>N (%)</th>
<th>CYP24A1</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal vs. Tumor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>12</td>
<td>1.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tumor</td>
<td>101</td>
<td>9.1</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I-II</td>
<td>75 (74.3)</td>
<td>9.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Stage III-IV</td>
<td>26 (25.7)</td>
<td>8.7</td>
<td></td>
</tr>
<tr>
<td>Degree of differentiation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>34 (23.7)</td>
<td>10.3</td>
<td></td>
</tr>
<tr>
<td>Moderate-well</td>
<td>39 (38.6)</td>
<td>8.5</td>
<td>0.01**</td>
</tr>
<tr>
<td>Well</td>
<td>28 (27.7)</td>
<td>8.5</td>
<td>0.01**</td>
</tr>
</tbody>
</table>

NOTE: *P values calculated using t-test
**Compared to ‘Poor’
Fig. 1

A

![Histogram showing mRNA levels in tumor and normal samples.](A)

<table>
<thead>
<tr>
<th>mRNA levels (l)</th>
<th>Tumor</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>3000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

![Scatter plot showing correlation between qRT-PCR and microarray data.](B)

$r = 0.945, n = 47$

C

![Kaplan-Meier survival curve showing survival probability.](C)

Low to medium level ($n = 57$)

High level ($n = 29$)

$P = 0.007$
Fig. 2

A

B

C

D

TMA Protein Score

CYP24A1 mRNA level

(n=3) (n=19) (n=31) (n=10)
Fold Change of CYP24A1

Tumor

Normal

Fig. 3
Fig. 4A, 4B and 4C

A

Fold Change of Mean

B

Fold Change to Median

C

CYP24A1

β-Actin

A549      SKLU-1

Research.

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Fig. 4D

**WST-1 (Day 4)**

- **Concentration 1,25-D$_3$ (nmol/L)**
  - A549
  - SKLU-1

**WST-1 (Day 12)**

- **Concentration 1,25-D$_3$ (nmol/L)**
  - A549
  - SKLU-1

**Cell Counting (Day 4)**

- **Concentration 1,25-D$_3$ (nmol/L)**
  - A549
  - SKLU-1

**Cell Counting (Day 12)**

- **Concentration 1,25-D$_3$ (nmol/L)**
  - A549
  - SKLU-1
Clinical Cancer Research

CYP24A1 Is an Independent Prognostic Marker of Survival in Patients with Lung Adenocarcinoma

Guoan Chen, So Hee Kim, Amanda N. King, et al.

Clin Cancer Res Published OnlineFirst December 17, 2010.

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