Clinical Cancer Research

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

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Abstract

Purpose: The active form of vitamin D, 1α,25-dihydroxyvitamin D_3 (1,25-D_3), exerts antiproliferative effects in cancers, including lung adenocarcinoma (AC). *CYP24A1* is overexpressed in many cancers and encodes the enzyme that catabolizes 1,25-D_3. The purpose of our study was to assess *CYP24A1* as a prognostic marker and to study its relevance to antiproliferative activity of 1,25-D_3 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-D_3 were examined using lung cancer cell lines with high as well as low expression of *CYP24A1* mRNA.

Results: *CYP24A1* mRNA was elevated 8- to 50-fold in lung AC (compared to normal nonneoplastic 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-D_3 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-D_3 in high *CYP24A1* expressing lung AC. *Clin Cancer Res; 17(4); 817–26. ©2010 AACR.*

Introduction

Lung cancer is the leading cause of cancer-related death with over 100,000 deaths annually in the United States (1). The 5-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% to 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 25-hydroxyvitamin D_3 levels are associated with improved survival in early-stage NSCLC patients (7).

Vitamin D is hydroxylated by CYP27A1 to 25-D_3 in the liver followed by further metabolism by CYP27B1 to 1α,25-dihydroxyvitamin D_3 (1,25-D_3, also known as calcitriol). 1,25-D_3, the most active metabolite of vitamin D, not only plays a critical role in calcium homeostasis, but also has nonendocrine effects. At supraphysiologic doses,

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

1α,25-Dihydroxyvitamin D_3 (1,25-D_3, calcitriol) is the active form of vitamin D. 1,25-D_3 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. The role of 1,25-D_3 as an adjunct to therapy is being explored in lung adenocarcinoma (AC). Delineating factors predictive of the response of cancer cells to 1,25-D_3 is crucial. Here, we demonstrate that *CYP24A1*, which encodes the main metabolizing enzyme of 1,25-D_3, is independently prognostic of survival in lung AC. In parallel, we show that antiproliferative effects of 1,25-D_3 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-D_3 in human lung AC.

1,25-D_3 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 anticancer properties of vitamin D have been reviewed by Deeb and colleagues (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 nonmalignant 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 this 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 overexpression 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

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% CO_2/95% 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 $d H_2O$. 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) 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′-ACCTGGGTATTTAGCATGAGCACTG-3′ (reverse). The primers for *VDR* (203 bp PCR product) were as follows: 5′-GCCCACCATAAGACCTACGA-3′ (forward) and 5′-AGATTGGAGAAGCTGGACGA-3′ (reverse). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β-actin expression was used to standardize the *CYP24A1* and *VDR* qRT-PCR results. Relative mRNA levels of were assessed using the $2^{-\Delta\Delta Ct}$ method.

Immunohistochemistry and tissue microarray

Tissue microarrays (TMA) were constructed, as previously described (26), with formalin-fixed, paraffin-embedded tissues from 86 patients. Immunohistochemical

Table 1. mRNA expression of *CYP24A1* in 86 lung adenocarcinomas

Variables	*N* (%)	*CYP24A1*	*P*[a]
Normal vs. tumor			
Normal	10	6.0	<0.0001
Tumor	86	296.5	
Stage			
Stage I	67 (77.9)	295.5	0.96
Stage III	19 (22.1)	300.2	
Classification			
Bronchioloaveolar	14 (16.3)	90.7	0.001
Bronchial-derived	53 (61.6)	351.1	
Others	19 (22.1)		
Degree of differentiation			
Poor	20 (23.5)	514.9	
Moderate	42 (49.4)	303.7	0.28[b]
Well	23 (27.1)	104.3	0.03[b]
Survival status[c]			
Dead	24 (27.9)	613.4	0.017
Alive	62 (72.1)	173.8	
Median overall survival (mo)	29.5		

[a]*P* values calculated using *t*-test.
[b]Compared to "Poor."
[c]Actual survival status.

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 minutes to 95°C. Deparaffinized and rehydrated sections of the TMA at 4-μm 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 2 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-D_3 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 to 3×10^3 (day 4) and 50 to 300 (day 12) cells per well in a 96-well microtiter plates (Corning) 6 wells per condition. Cells were treated 24 hours later with 0, 1, 10 and 100 nmol/L of 1,25-D_3 (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 to 4×10^4 (day 4) and 300 to 3×10^3 (day 12) cells per well in a 6-well microtiter 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 × 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 × 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 $Na_4P_2O_7$, 1 mmol/L β-glycerol 2-phosphate disodium salt hydrate, 1 mmol/L Na_3VO_4, 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 β-actin (1:10,000 dilution) (Abcam).

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

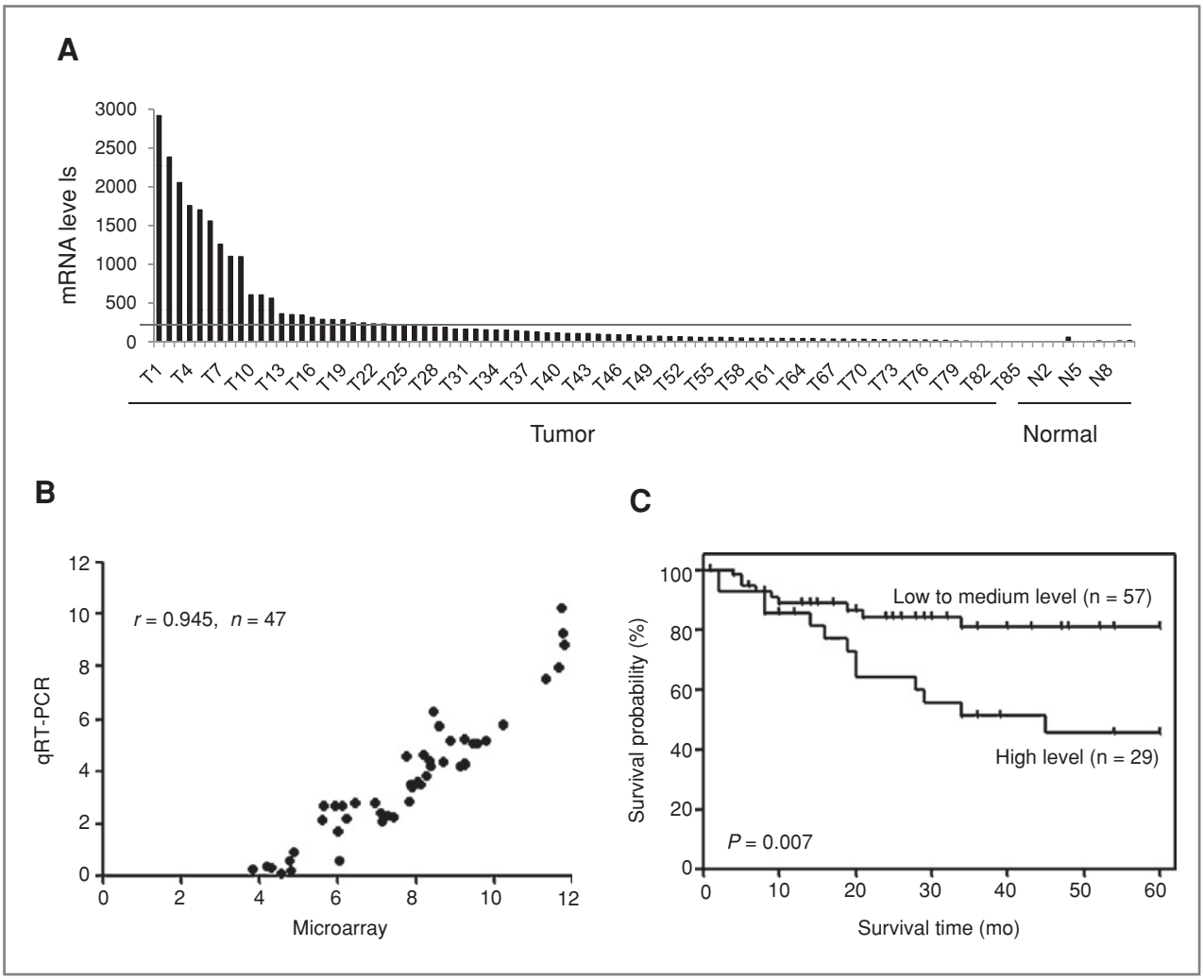

Fig. 1. *CYP24A1* mRNA expression in 86 lung adenocarcinoma (AC) patients. A, using Affymetrix microarray there was a differential expression of *CYP24A1* mRNA in 86 resected lung AC and 10 normal. One third of the patients had high *CYP24A1* mRNA levels (mRNA expression >170 relative units). B, strong 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. Log_2 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 to 3 NSCLC were divided into high (>170, $n = 29$) or low to medium (<170, $n = 57$).

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

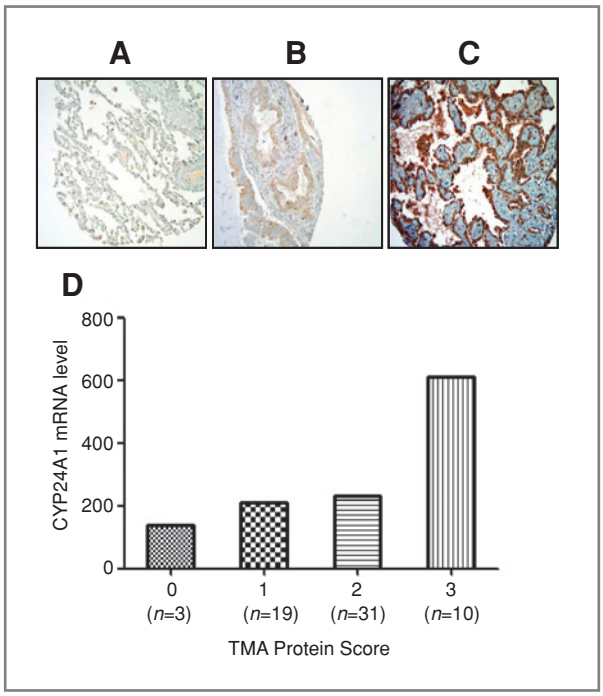

Fig. 2. Immunohistochemistry for CYP24A1 and correlation to mRNA expression. A, normal lung with weak staining (+1). B, moderate staining (+2). C, strong staining (+3). Magnification is 200×. D, correlation between mRNA (microarray data) and protein (TMA protein staining) expression of CYP24A1 in 63 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.

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 ($P < 0.0001$; 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 C). Of note, we did not find a correlation between *CYP24A1* mRNA and survival in a squamous lung cancer microarray data ($n = 130$) using 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). An increased copy number of 20q was detected in less than 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–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 (Supplementary 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 2 tumor samples with low *CYP24A1* mRNA showed strong staining (+3) (Supplementary 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 to 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. Fifty-six percent (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 $P = 0.001$) (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* overexpression is associated with poor prognosis in AC patients (Table 1 and Figs. 1B and 3B), we hypothesized that tumor-specific expression of *CYP24A1*

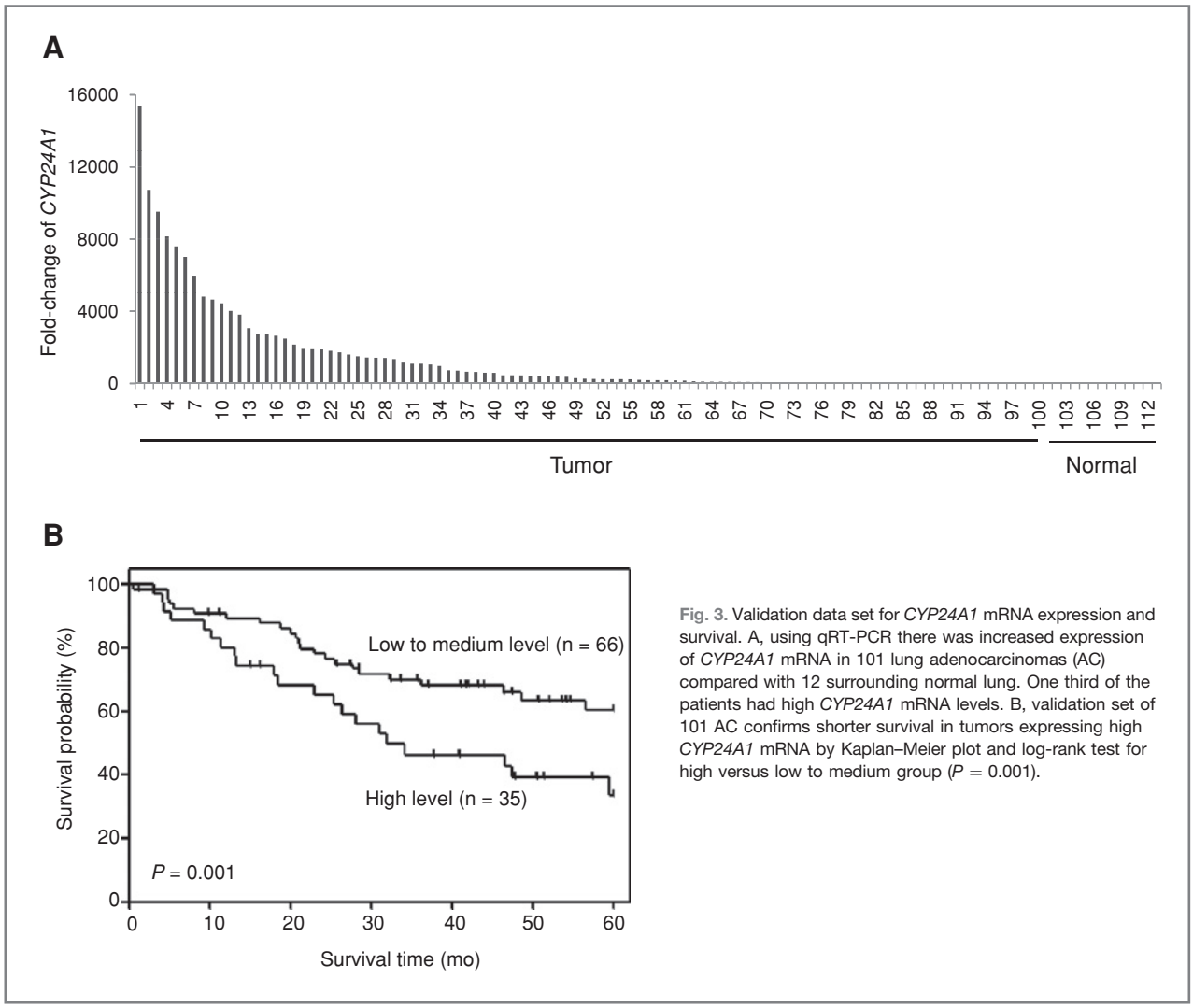

Fig. 3. Validation data set for *CYP24A1* mRNA expression and survival. A, using qRT-PCR there was increased expression of *CYP24A1* mRNA in 101 lung adenocarcinomas (AC) compared with 12 surrounding normal lung. One third of the patients had high *CYP24A1* mRNA levels. B, validation set of 101 AC confirms shorter survival in tumors expressing high *CYP24A1* mRNA by Kaplan–Meier plot and log-rank test for high versus low to medium group ($P = 0.001$).

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 2 representative cell lines, A549 (high *CYP24A1*) and SKLU-1 (low *CYP24A1*) to confirm the CYP24A1 protein levels by immunoblot 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 4, 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 *CYPA1* 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, an inverse 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.

Table 2. Verification of *CYP24A1* in validation set of 101 lung adenocarcinomas

Variables	*N* (%)	*CYP24A1*	*P*[a]
Normal vs. tumor			
Normal	12	1.3	<0.001
Tumor	101	9.1	
Stage			
Stage I-II	75 (74.3)	9.2	0.4
Stage III-IV	26 (25.7)	8.7	
Degree of differentiation			
Poor	34 (23.7)	10.3	
Moderate-well	39 (38.6)	8.5	0.01[b]
Well	28 (27.7)	8.5	0.01[b]

[a]*P* values calculated using *t*-test.
[b]Compared to "Poor."

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 (28), we found that lung AC demonstrated a higher expression of *CYP24A1* compared with normal nonneoplastic lung. Differential overexpression of *CYP24A1* gene has been observed not only in lung (28) 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 antitumor effects of 1,25-D_3. Other groups have also verified the overexpression of *CYP24A1* in non–small cell lung cancer (28). Kim and colleagues, 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 and colleagues confirmed that *CYP24A1* mRNA expression was higher in lung cancer compared to normal bronchial epithelium (30). 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 antitumor activity and reduces the antiproliferative activity of 1,25-D_3 (30). Anderson and colleagues 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 (31), colon (32), breast (33), prostate (34), head and neck (35) as well as lung tumors (28). 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 (36). 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 protein. This result has been also confirmed using immunoblot analysis lung AC cell lines with high *CYP24A1* (A549) and low *CYP24A1* (SKLU-1).

In addition, 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

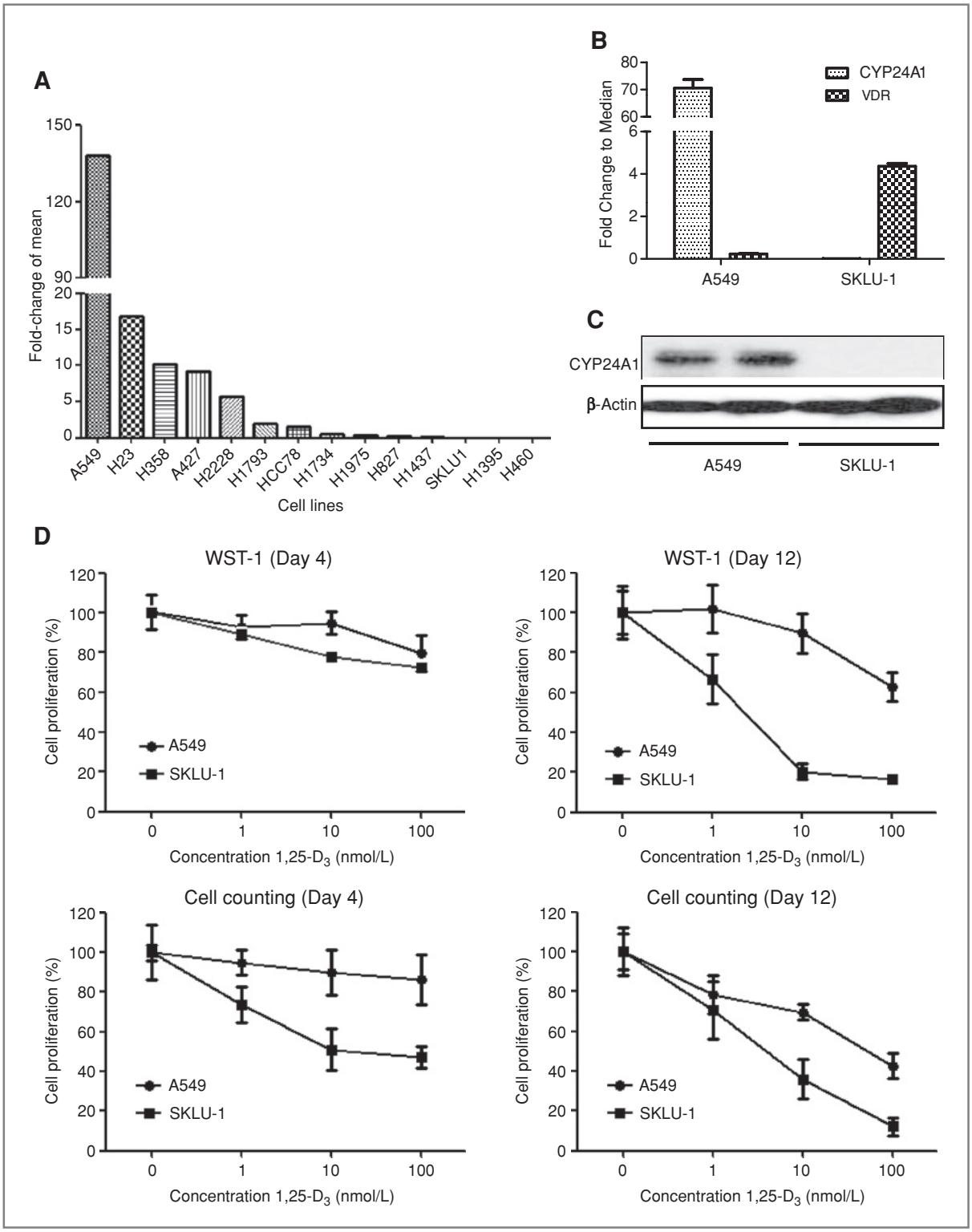

Fig. 4. mRNA expression of *CYP24A1* and the effect of 1,25-D_3 in human lung cancer cell lines. A, qRT-PCR results from 14 human lung cancer cell lines showed wide variation from high to low *CYP24A1* mRNA expression. Number indicates fold change to median. B, qRT-PCR results of *CYP24A1* and *VDR* 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.

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, noncalcemic 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 (37, 38).

In summary, we correlate *CYP24A1* mRNA expression with survival in resected lung AC. Our data demonstrate that overexpression 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 were disclosed.

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