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Original Research

Collagen modifying enzyme P4HA1 is overexpressed and plays a role in lung adenocarcinoma

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ABSTRACT

Lung cancer is the leading cause of cancer-related deaths globally and is histologically defined as either small cell lung cancer (SCLC) or non-small cell lung cancer (NSCLC), with the latter accounting for 80% of all lung cancers. The 5-year overall survival rate for lung cancer patients is low as it is often discovered at advanced stages when potential cure by surgical resection is no longer an option. To identify a biomarker and target for lung cancer, we performed analysis of multiple datasets of lung cancer gene expression data. Our analyses indicated that the collagen-modifying enzyme Prolyl 4-Hydroxylase Subunit Alpha 1 (P4HA1) is overexpressed in NSCLC. Furthermore, our investigation found that overexpression of enzymes involved in this pathway predicts poor outcome for patients with lung adenocarcinoma. Our functional studies using knockdown strategies in lung cancer cell lines *in vitro* indicated that P4HA1 is critical for lung cancer growth, migration, and invasion. Additionally, diethyl pythiDC (PythiDC), a small molecule inhibitor, decreased the malignant phenotypes of lung cancer cells. Moreover, we found that miR-124 regulates and targets P4HA1 in lung cancer cells. Thus, our study suggests that collagen-modifying enzymes play an important role in lung cancer aggressiveness. Furthermore, our studies showed that P4HA1 is required for lung cancer cell growth and invasion, suggesting its potential as a valid therapeutic target in lung adenocarcinoma.

Introduction

The most common subtype of non-small cell lung cancer (NSCLC) is lung adenocarcinoma. The identification of specific molecular alterations present in a patient's lung cancer has changed the way clinicians treat this disease [1-4]. While multiple genomic and epigenomic events converge to trigger unregulated growth, invasion, and metastasis in lung cancer, the exact mechanisms of lung cancer initiation and progression are not fully understood. Thus, there is an urgent need for the identification of biomarkers that may aid in the early diagnosis or stratification of lung cancer as well as new therapeutic targets.

It is first critical to understand the molecular circuitry that plays a role in tumor initiation and progression to target cancer. Smoking has been positively correlated with lung tumor size during diagnosis suggesting an association between smoking and lung cancer [1, 3, 5-8]. *EGFR* (Epidermal Growth Factor Receptor) mutation status is now examined even if the patient is a former smoker because of the proven benefit these therapies have and the substantial improvement seen in patients over standard chemotherapy [5, 6, 9-11]. Those patients who present with localized disease represent the subset that undergo surgical resection and for which extensive molecular analyses have been performed. Tumors from stages 1B to 3B receive surgery followed by chemotherapy that often includes a platinum-based regiment [3, 12, 13]. Studies show EGFR mutations, such as L858R, are differentially responsive to small molecule inhibitors like gefitinib, suggesting differential sensitivity and clinical use of these inhibitors [14-17]. Importantly, high-risk early stage, as well as higher stage patients would benefit greatly from therapies that directly target the driver events present in their cancers. Recent advances in sequencing technology and high throughput data

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from lung adenocarcinoma have allowed for the identification of multiple overexpressed enzymes in lung adenocarcinoma, that may serve as excellent therapeutic targets. The current study focuses on one such enzyme P4HA1.

Collagen-modifying prolyl-4 hydroxylase (P4H) enzymes have been studied for their role in various cancers [18-23]. The most abundant subunit, P4HA1, catalyzes the 4-hydroxyproline formation necessary for proper collagen polypeptide folding. P4HA1 expression has been shown to play a significant role in colorectal, prostate, and breast cancers [18, 19, 24]. Based on these findings, we have conducted studies to assess the importance of P4HA1 in lung cancer growth and progression. The current study shows overexpression of P4HA1 in lung adenocarcinoma. Furthermore, we show the mechanism of regulation and functional role of P4HA1 in lung adenocarcinoma. Our study suggests that P4HA1 is an excellent therapeutic target.

Materials and methods

Cell Lines

Lung cancer cell lines, A549 and H1437, were obtained from ATCC (Manassas, VA, USA) and cultured in RPMI-1640 (Life Technologies, Carlsbad, CA, USA) supplemented with penicillin-streptomycin (100U/ml) and 10% FBS (Life Technologies). BEAS-2B cells were obtained from ATCC and cultured in BEBM (Lonza, Basel, Switzerland) supplemented with BEGM (Lonza, Basel, Switzerland). All cells were grown at 37 °C in 5% CO_2.

Gene expression analysis using TCGA data

P4HA1 gene expression levels of lung adenocarcinoma compared to normal samples were obtained via the UALCAN cancer analysis tool (http://ualcan.path.uab.edu) [25] which utilizes TCGA transcriptome sequencing datasets.

Gene expression analysis

We used 26 frozen primary tumors and associated non-malignant lung samples from lung adenocarcinoma patients who underwent resection at the University of Michigan Health System from 2000 to 2012. Informed consent was obtained for each subject and was approved by the Institutional Review Board. Tumor specimens were immediately frozen following resection and stored at −80 °C. Regions containing a minimum of 70% tumor cellularity were used for RNA isolation and RT-PCR. None of the patients included in this study received preoperative radiation or chemotherapy.

Affymetrix microarray U133Plus2 data set from Okayama [26] representing 226 lung adenocarcinomas was used in the survival analysis for genes P4HA1, P4HA2, and P4HA3. The CEL files of microarray data were normalized using Robust Multi-array Average (RMA) method. Overall survival is the outcome and censored at 5 years.

For *in vitro* assays, total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) and cDNA was generated via reverse transcription of the resulting RNA. Quantitative real-time polymerase chain reaction (q-RTPCR) was performed using Applied Biosystems QuantStudio™ 6 Flex Real-Time PCR (Polymerase Chain Reaction) (Thermofisher Scientific, Waltham, MA) System. SYBR green probes were used to determine mRNA expression levels. Primers used in the study were acquired from Integrated DNA Technologies (Coralville, IA). β-actin served as a normalized control. All q-RT-PCR reactions were performed in triplicate.

MicroRNA-124-3p analysis

Frozen lung tissues were obtained from the UAB tissue collection and banking facility and Vanderbilt Thoracic Biorepository with IRB approval. RNA was isolated from tissue using a Qiagen miRNeasy kit (Cat# 217004). 10ng RNA input was used for cDNA synthesis using Applied Biosystems TaqMan™ MicroRNA Reverse Transcription Kit (Cat#4366596). MiR-124-3p expression was quantified from cDNA using TaqMan qRT-PCR and normalized to endogenous control RNU6B. Mean miR-124-3p expression (Error bars ± SEM: $^*P = 0.048$, one-sided student's t-test with unequal variance). Precursor microRNAs were obtained from Ambion (Thermofisher, Austin, TX).

RNA Interference and miRNA transfection

P4HA1 small interfering RNA (siRNA) and non-targeting control duplexes were obtained from Dharmacon (GE Healthcare, Lafayette, CO). The P4HA1 sequences utilized for this assay are siRNA 1 (J-004275-07-0020) GGAAUUACAGGUAGCAAAU and siRNA 2 (J-004275-08-0020) GAUAAAGUCUCUGUUCUAG. Reverse transfection was accomplished using Lipofectamine RNAiMAX (Life Technologies) per manufacturer instructions. P4HA1 shRNAs were produced by System Biosciences (Mountain View, CA). Lentiviruses were generated by the University of Alabama at Birmingham Neuroscience NINDS Protein Core (P30 NS047466). P4HA1 shRNA expressing cells, along with scramble controls, were selected for by treatment with 1 μg/ml puromycin (Life Technologies).

Small molecule inhibition

The P4HA1-specific inhibitor, diethyl-PythiDC (PythiDC), was obtained from MedChemExpress (Monmouth Junction, NJ). Serial dilutions (10-100μM) were tested over 6 days to observe the effect of PythiDC on cell viability, colony formation, and invasion.

Immunoblot analyses

Protein lysates were collected using NP-40 lysis buffer (Boston BioProducts, Ashland, MA) with 1X Halt™ Phosphatase and Protease Inhibitor Cocktail (ThermoFisher Scientific, Waltham, MA). Samples were separated by SDS-PAGE and transferred onto Immobilon®-P PVDF membrane (MilliporeSigma, Billerica, MA). Membranes were blocked in a blocking buffer of 5% non-fat dry milk and TBS-T (Tris-buffered saline and 0.1% Tween) and then incubated in primary antibody (P4HA1, Cat#12658-1-AP, 1:1000) overnight at 4 °C. Blots were then washed with TBS-T and incubated with horseradish peroxidase-conjugated secondary antibody and developed with Luminata Crescendo Western HRP chemiluminescence substrate per manufacturer's protocol (MilliporeSigma, Billerica, MA). Images were taken using the Amersham™ Imager 600RGB (GE Healthcare Life Sciences, PA, USA). Antibody dilutions were optimized in house.

Cell proliferation assay

Cells were seeded at 100–200 cells/well and proliferation was measured by cell counting using a Z2 Coulter particle counter (Beckman Coulter, Brea, CA). Proliferation was measured every 48 h. Media was changed every three days.

Colony formation assay

Cells were trypsinized and seeded into 6-well plates at a density of 1000–2,000 cells/well, depending on the cell line. Media was changed every three days and colony formation was measured after 9 days. Cells were washed with 1X PBS, fixed with 5% glutaraldehyde, stained with crystal violet, and quantified.

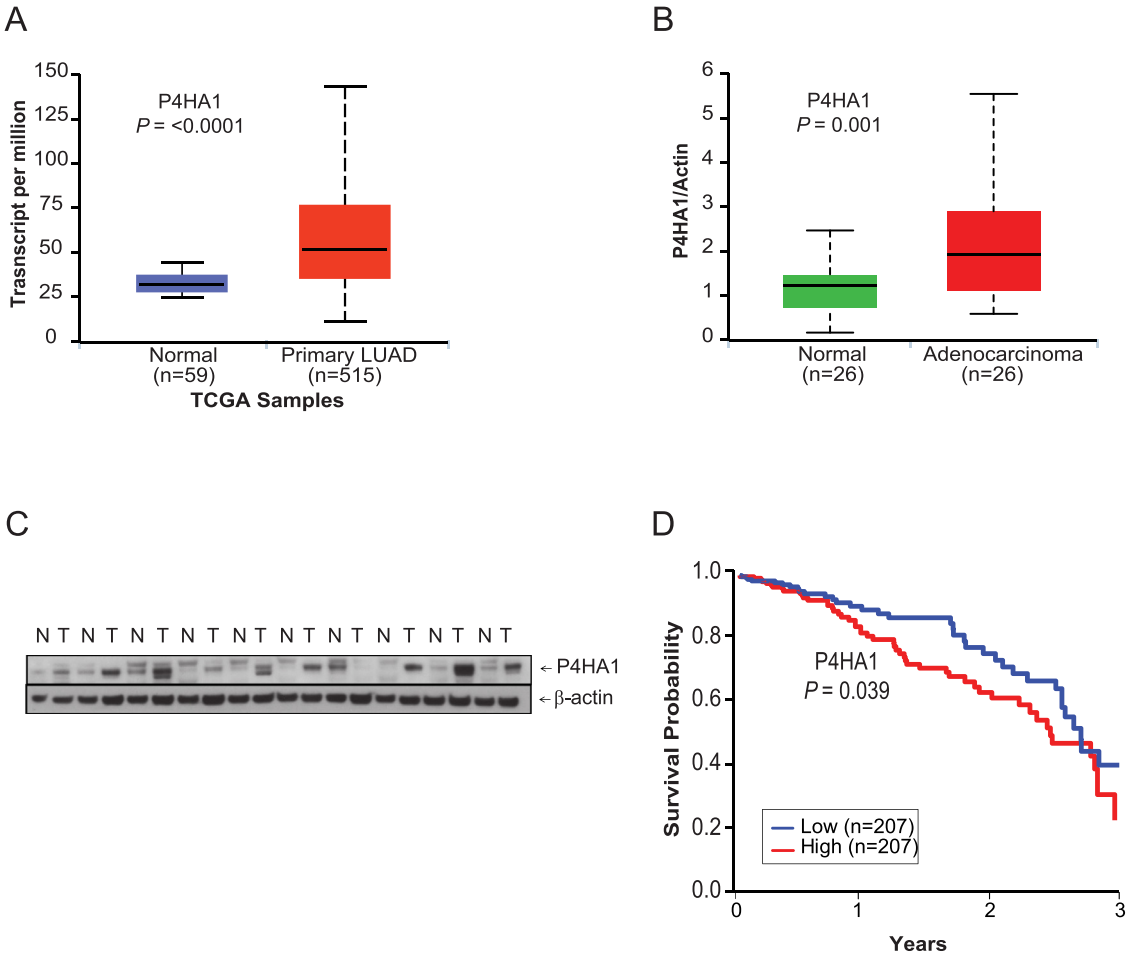

Fig. 1. P4HA1 is overexpressed in lung adenocarcinoma and significantly affects survival. (A) P4HA1 gene expression from TCGA data (P=<0.0001), comparing normal to primary tumors of lung adenocarcinoma patients, (B) quantitative real-time PCR of matched lung adenocarcinoma patient tissues (n=26; P=0.001), (C) P4HA1 protein expression in lung adenocarcinoma patient and matched normal tissues by immunoblot analysis using P4HA1 antibody, and (D) Kaplan-Meier plot showing overall survival probability based on P4HA1 expression (P=0.02).

Matrigel invasion assay

Cells were trypsinized and seeded at 15K-30K cells/well into Matrigel® invasion chambers (Corning, Corning, NY) in serum-free media in the upper chamber. Media containing 10% FBS was added to the lower chamber as a chemoattractant. After 18–24 h, non-invading cells were removed along with the Matrigel using a cotton swab. Invaded cells were fixed with 5% glutaraldehyde and stained with crystal violet. Wells were then imaged using an inverted scope (4X) and the number of invaded cells was quantified.

Statistical analysis

To determine significant differences between two groups, Student's two-tail t-test was used for all experiments. P-values <0.05 were considered significant. Kaplan-Meier survival curve with log-rank test was used for overall survival analysis based on gene expression value.

Results

P4H genes are overexpressed in lung adenocarcinoma and are markers of survival

We have previously demonstrated a role for P4HA1 in prostate cancer growth and progression [19]. For this reason, we decided to pursue deciphering the role P4HA1 in lung adenocarcinoma. Upregulation of prolyl-4-hydroxylase (P4H) genes (P4HA1, P4HA2, and P4HA3) in primary LUAD were observed via transcriptome analysis of TCGA datasets and gene expression microarray profiling (**Fig. 1A; Supplementary Figure 1A**). To validate these findings, qRT-PCR was performed using RNA isolated from both lung adenocarcinoma and associated normal lung tissues. This analysis confirmed significant P4HA1 overexpression in lung adenocarcinoma (**Fig. 1B**). Immunoblot analysis also showed overexpression of P4HA1 in lung adenocarcinoma compared to matched normal tissues (**Fig. 1C**). The presence of various P4HA1 isoforms around 50 and 57 KD accounts for the multiple bands observed in the western blots. Transcriptome analysis also conveyed the association of P4HA1 with poor overall patient survival (**Fig. 1D).** Interestingly, other enzymes belonging to the same family, P4HA2 and P4HA3 expression also showed poor patient survival (**Supplemental Figure 1B**).

P4HA1 plays a role in cell proliferation and invasion in lung adenocarcinoma

The importance of P4HA1's role in lung adenocarcinoma was determined by altering its expression levels in the lung adenocarcinoma cell lines, A549 and H1437. Knockdown was confirmed via immunoblot analysis. Transient and stable knockdown of P4HA1 resulted in a significant decrease in cell proliferation when compared to non-targeting

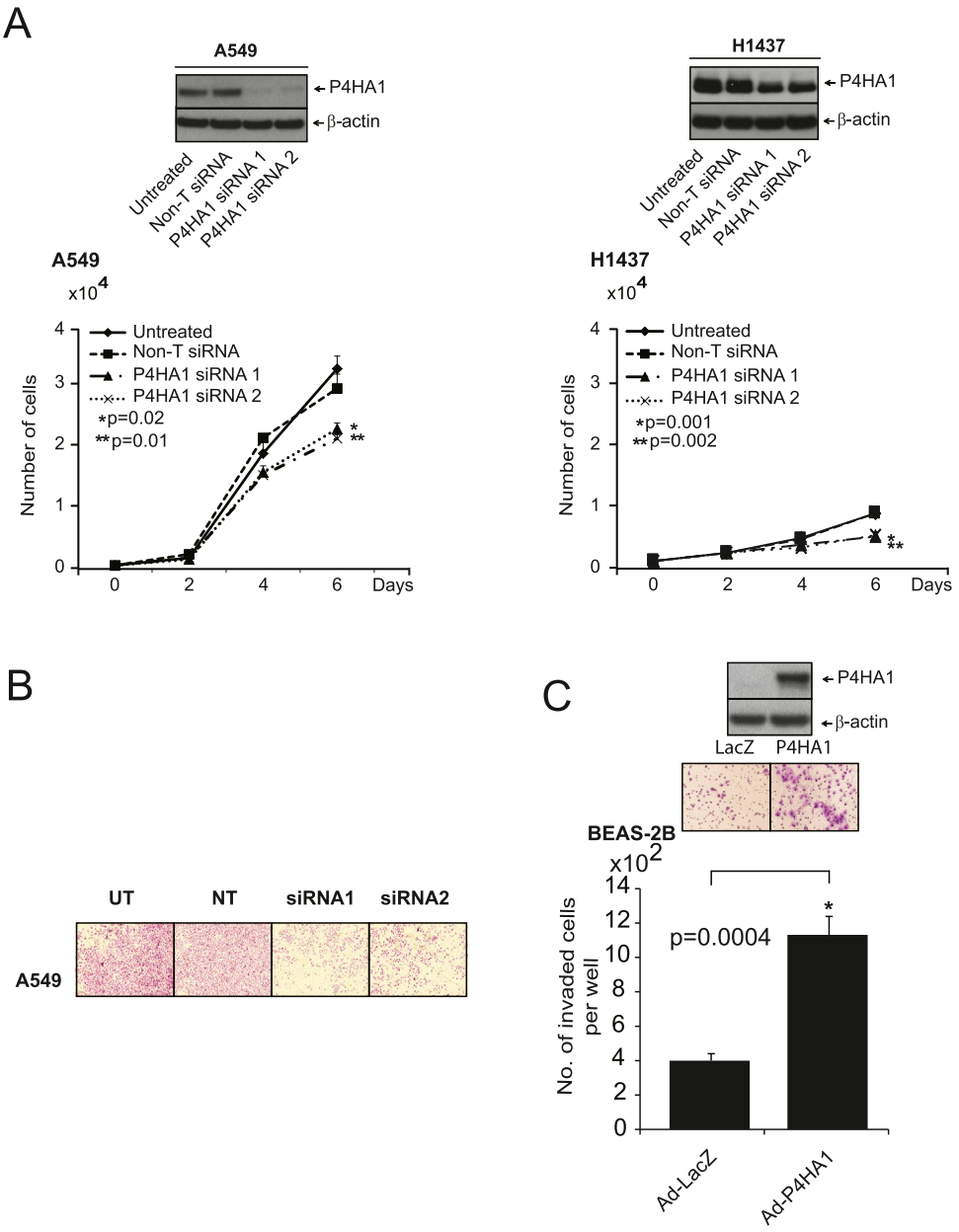

Fig. 2. P4HA1 plays a significant role in cell proliferation and invasion for lung adenocarcinoma. (A) Immunoblot analysis of protein lysates from A549 and H1437 cells treated with independent P4HA1 siRNA duplexes. *β*-actin serves as a loading control. Transient knockdown of P4HA1 in lung adenocarcinoma cell lines (A549 and H1437) shows reduction in cell proliferation. Cell proliferation assay of P4HA1 siRNA treated A549 and H1437 compared to non-targeting siRNA (Non-T siRNA) controls. (B) Boyden chamber Matrigel invasion assay of P4HA1 siRNA treated A549 cells compared to Non-T siRNA controls. Invaded cells were stained and imaged. (C) Overexpression of P4HA1 via adenovirus transduction in normal human lung cells (BEAS-2B). Invaded cells were quantified.

control cells (Fig. 2**A; Supplemental Figure 2A**). To test the effect of P4HA1 on invasion, the Boyden Matrigel invasion assay was used. Transient and stable P4HA1 knockdown effectively reduced the invasive potential of lung adenocarcinoma cell lines (Fig. 2**B; Supplemental Figure 2B**). To support the importance of P4HA1 in lung adenocarcinoma, P4HA1 was stably expressed in BEAS-2B cells via adenovirus transduction. Overexpression of P4HA1 in BEAS-2B cells resulted in a significant increase in invasion (Fig. 2**C**). These findings suggest a role for P4HA1 in lung adenocarcinoma proliferation and invasion *in vitro*.

PythiDC: target-specific small molecule inhibition

PythiDC is a small molecule inhibitor of P4HA1 [27]. In order to evaluate the effect of P4HA1 inhibition on lung cancer cells, serial dilutions of PythiDC were added to lung cancer cell lines and evaluated. Cell viability saw a significant reduction at 75 and 100 μM (Fig. 3**A**). This trend was also observed with Boyden Matrigel invasion and colony formation assays (Fig. 3**B and** 3**C**).

miR-124 regulates P4HA1 expression in lung cancer

MicroRNAs have been shown to act as tumor suppressors targeting oncogenes, causing their repression, and vice versa. We have previously demonstrated miR-124's tumor-suppressive role in regulating P4HA1 in aggressive prostate cancer, and so sought to investigate the consistency of these findings within lung adenocarcinoma [19]. To determine miR-124's regulation of P4HA1 expression in lung adenocarcinoma, lung cancer cells were treated with precursor miRs for miR-122, 124, and 499a and P4HA1 protein expression levels were measured via immunoblot analysis. Both precursor miR-122 and 124 resulted in a significant reduction in P4HA1 protein expression, compared to control non-targeting precursor microRNA (Fig. 4**A**). This data supports the role of miR-124 as a regulator of P4HA1 in lung adenocarcinoma. Lung cancer cell line A549 was treated with precursor miR-124 and showed a significant decrease in invasion as determined by Matrigel invasion assays (Fig. 4**B**).

The significance of miR-124 in lung adenocarcinoma patients was determined by ascertaining the microRNA levels in lung adenocarcinomas. RNA was isolated from frozen lung and tumor tissues and qPCR

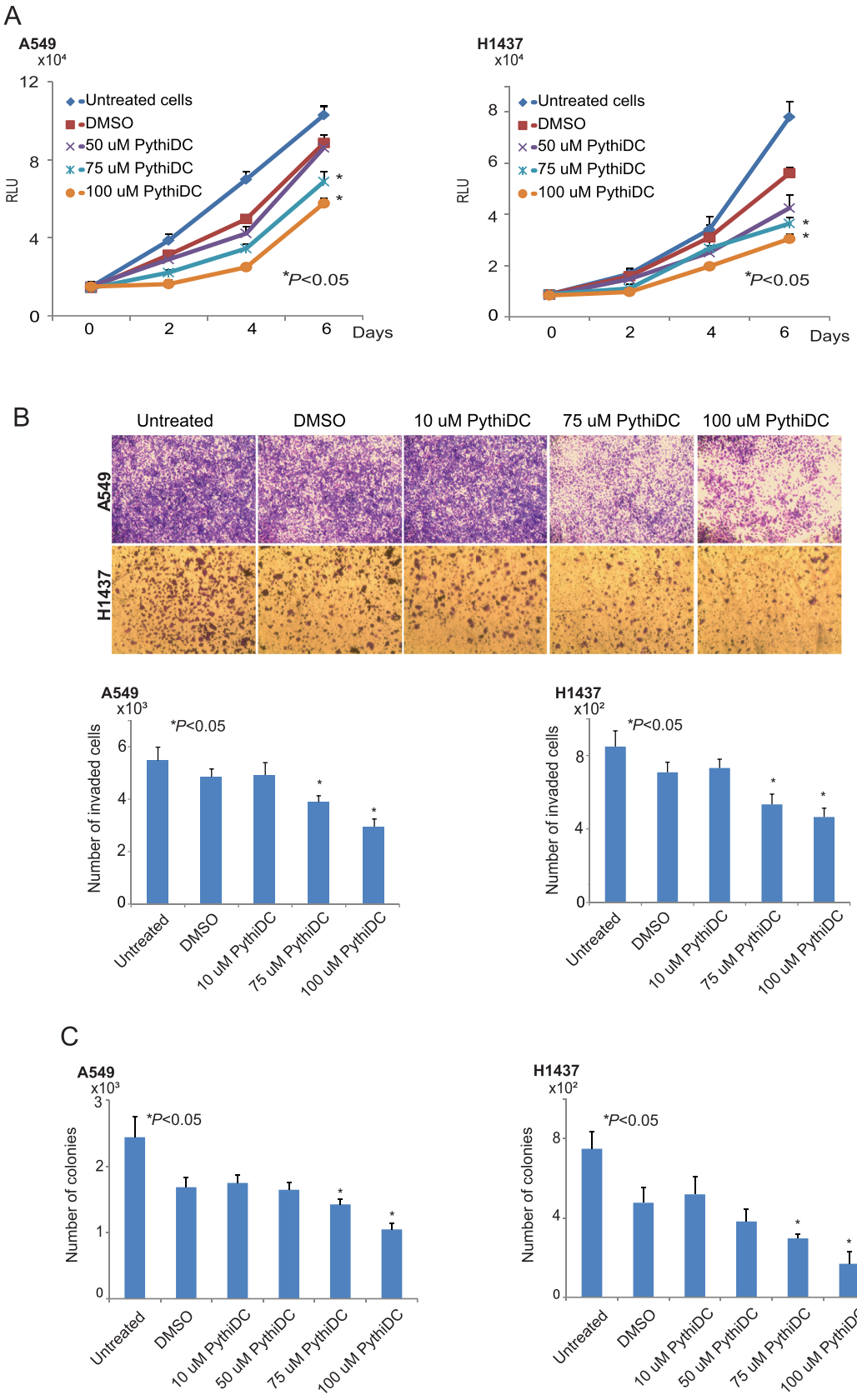

Fig. 3. Small molecule inhibition of P4HA1 by PythiDC inhibits invasion and colony formation. (A) A549 and H1437 lung adenocarcinoma cell lines show reduced cell proliferation (A), after treatment with different doses of PythiDC over a time. Treatment with PythiDC also affected lung cancer cell invasion (B), and colony formation (C).

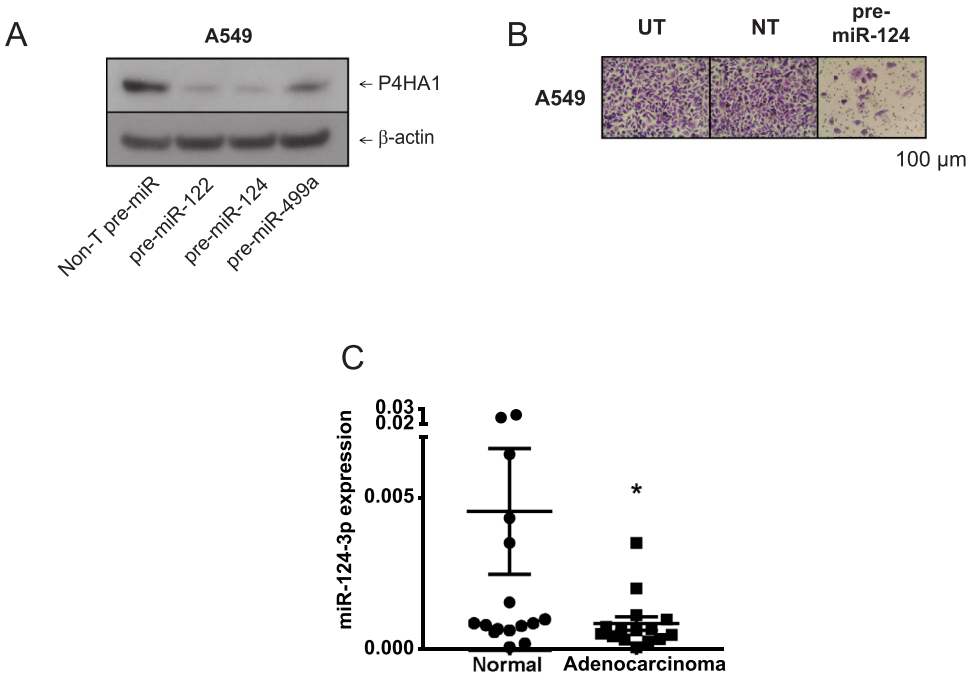

Fig. 4. MiR-124-3p targets and downregulates P4HA1 and is reduced in lung adenocarcinoma patients. (A) Immunoblot analysis of protein lysates from A549 cells treated with independent pre-miR duplexes. *β*-actin serves as a loading control. Pre-miR- 124 show reduced P4HA1 expression. (B) Boyden Matrigel invasion assay of A549 treated with pre-Mir-124. Non-targeting microRNA serves as a control. (C) RNA isolated from frozen lung tissue shows decreased levels of miR-124-3p in lung adenocarcinoma patients compared to normal lung. Expression normalized to endogenous RNU6B control. Mean miR-124-3p expression (Error bars ± SEM: $^{*}P$= 0.048, one-sided student's *t*-test with unequal variance). Clinicopathologic characteristics of lung cancer patients are mentioned in the associated table.

	Normal (n=16)	Adenocarcinoma (n=15)
Age	60.25 ± 3.16	70.20 ± 2.96
Female	9	6
Male	7	9
Stage I	N/A	12
Stage II	N/A	1
Stage IV	N/A	2

performed using RNU6B as an endogenous control. Results showed a significant decrease in miR-124 levels in lung adenocarcinoma when compared to normal lung tissues (**Fig. 4C**).

Discussion

Lung cancer is one of the most common cancers affecting both men and women. Lung adenocarcinoma is the common histological subtype of aggressive lung cancers [28]. Multiple molecular alterations have been identified that initiates and drives lung cancer progression. Effective therapeutic modalities are available targeting specific subtypes of lung adenocarcinoma [13]. Recently, immunotherapy has emerged as an effective method to treat some lung cancers [4, 29], however, there is still an urgent need for additional effective and novel means of targeting lung adenocarcinoma. We have previously shown that P4HA1 can serve as a biomarker and potential therapeutic target in prostate cancer [19]. The current study suggest that P4HA1 is overexpressed and plays a critical role in lung adenocarcinoma.

Prolyl-4-hydroxylase catalyzes the formation of 4-hydroxyproline (Hyp) from proline, which plays a number of roles, one being the stabilization of collagen's triple helix [30]. Post-translational modifications, like proline hydroxylation, have awide range of effects on various proteins, altering both interactions with other proteins and protein conformation. P4HA1 is also known to effect argonaute 2 (Ago2) [31]. With such widespread activities, targeting P4HA1 is a promising therapeutic strategy. Li et al. showed that high levels of P4HA1 were important for the clinical characteristics of the patients and were regulated in the tumor tissue. Adverse clinical outcomes were seen in patients who had elevated P4HA1 levels. High P4HA1 expression is an independent prognostic factor that is poor for relapse-free survival and overall survival in lung cancer patients [32]. Our studies showed microRNA-124 can target P4HA1. Different miRs have been shown to target P4HA1. It has been shown repeatedly that genes can be targeted by multiple microRNAs in an intricate flow of target gene expression [33]. MiR-122 inhibition has been shown to result in P4HA1 upregulation [34, 35]. MiR-124-3p targets P4HA1 and inhibits collagen synthesis in smooth muscle cells [20]. As shown in the present study, hsa-miR-124-3p is significantly downregulated in primary lung adenocarcinoma and that the exogenous addition of pre-miR-124-3p is able to affect the ability of lung cancer cell lines to invade. Thus, reintroduction of miR-124 can be an effective strategy to target P4HA1.

We have demonstrated significant upregulation of P4HA1 in lung adenocarcinoma and shown its correlation with patient survival. Prolyl-4-hydroxylases are tetrameric proteins made up of alpha subunits (P4HA1, P4HA2, and P4HA3) and a beta subunit (P4HB) [36]; all of which have been shown to be upregulated and predict poor patient survival in lung adenocarcinoma. P4HA2 upregulation can promote cancer progression in breast cancer [37], while low expression of P4HA2 is detrimental for pancreatic cancer patients [21]. P4HB being the only beta subunit is also upregulated as shown in gastric cancer and hepatocellular carcinoma [22, 23, 38]. P4HA3 is upregulated and associated with metastasis and poor survival in gastric cancer and silenced in a subset of melanomas [22]. Therefore, we examined P4HA1 as it is the most abundant of the alpha subunits. The study by Gilkes et al. showed that

P4HA1enhanced invasion and metastasis via alignment of cancer cells along the collagen fibers it helps to stabilize [39]. Our studies have also shown that inhibition of P4HA1 using PythiDC, reduces cell viability, invasion, and colony formation. In summary, our study highlights the essential role of P4HA1 in lung cancer progression and suggests P4HA1 as a viable therapeutic target in lung adenocarcinoma patients.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Alyncia D. Robinson: Investigation, Writing – original draft, Writing – review & editing, Visualization. **Balabhadrapatruni V.S.K. Chakravarthi:** Conceptualization, Investigation, Writing – review & editing, Formal analysis. **Sumit Agarwal:** Investigation, Writing – review & editing, Formal analysis. **Darshan Shimoga Chandrashekar:** Visualization, Data curtion, Software, Writing – review & editing. **Mackenzie L. Davenport:** Investigation, Visualization, Formal analysis, Writing – review & editing. **Guoan Chen:** Investigation, Visualization, Formal analysis, Writing – review & editing. **Upender Manne:** Investigation, Writing – review & editing, Formal analysis. **David G. Beer:** Investigation, Visualization, Formal analysis, Writing – review & editing. **Mick D. Edmonds:** Investigation, Formal analysis, Writing – review & editing. **Sooryanarayana Varambally:** Conceptualization, Resources, Investigation, Funding acquisition, Writing – review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2021.101128.

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