

Emerging role of nuclear phospholipase C- β 1 in lung cancer

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ABSTRACT

Non-small cell lung cancer (NSCLC) is a highly aggressive malignancy accounting for approximately 85% of all lung cancer cases. To better understand its pathology and prognosis, this study focused on the molecular profiling of tissues and clinical outcomes derived directly from lung cancer patients. Recently, the involvement of phospholipase enzymes in cancer has been highlighted, as they are involved in crucial cellular signaling pathways. The mRNA and protein expression levels of phospholipase C (PLC) isoforms were analyzed in biopsies from NSCLC patients. Utilizing Kaplan-Meier plotter database, the prognostic role of PLCs could be assessed. Researchers identified significant changes in mRNA and protein expression levels of various PLC isoforms in patient biopsies. Notably, PLC β 1 was found to be overexpressed in every tumor sample. In addition, the use of KM-Plotter hypothesized an association between elevated PLC β 1 levels and poorer survival outcomes, emphasizing the variations due to patient-specific factors, such as tumor stage and smoking history. Immunohistochemistry performed on patient-derived samples revealed a clear contrast in protein localization. In tumor tissues, PLC β 1 was predominantly found in the nucleus, whereas in adjacent healthy tissues, it remained almost exclusively in the cytoplasm. Nuclear localization is known to be linked to cell cycle modulation; therefore, it may represent a critical event in cancer development. The study highlighted critical alterations in PLC isoforms observed within the patient population. These findings demonstrate that PLC β 1 is significantly upregulated in malignant tissues and correlates with adverse clinical outcomes. Consequently, PLC β 1 may serve as a candidate biomarker, warranting further investigation into its potential role in the management and treatment of NSCLC patients.

Key words: non-small cell lung cancer; nucleus; phospholipase C.

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Introduction

Lung cancer (LC) is the leading cause of cancer morbidity and mortality worldwide, with 2.5 million new cases diagnosed globally (12.4% of all new cancer cases) and over 1.8 million deaths (18.7% of cancer-related deaths) in 2022.¹ Based on the type of cells in which cancer originates, primary lung cancer can be classified into two main forms: non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). SCLC is poorly differentiated, and it originates from neuroendocrine cells. It accounts for 10% of all lung cancer cases and it is characterized by faster growth and spread compared to NSCLC.² NSCLC represents almost 85% of all lung cancer cases and it is classified into three main subtypes: adenocarcinoma (AC), squamous cell carcinoma (SCC), and large cell carcinoma (LCC). These forms are categorized according to the distinct types of cells in which cancer originates.³ AC is the most common NSCLC subtype, and it typically occurs in non-smokers. It often arises in the external regions of the lungs, and it is associated with glandular cells that produce mucus. SCC originates from the flat cells lining the airways, and it is usually found in the central part of the lungs. Commonly, it is linked to smoking. LCC is a rare subtype that can occur in any part of the lungs, and it is characterized by large, abnormal-looking cells. LCC outcome is poor and very little is known about its clinical and biological characteristics, since its diagnosis is not common.⁴ Despite some progress in LC treatments, the benefits of surgery, radiotherapy, and chemotherapy remain limited. This is particularly true for late-stage lung cancer, which frequently has a low survival rate due to complications from distant metastases.⁵ As a matter of fact, approximately 40-50% of patients exhibit metastatic disease at the time of diagnosis.⁶ NSCLC is often diagnosed at advanced stages (III or IV), resulting in a poor prognosis. In fact, the 5-year relative survival rate is only 25% for all stages, and it decreases to 6% for the patients presenting distant metastases.⁷ Molecular alterations contributing to the aggressiveness of lung cancer led to the development of targeted therapies, that improved the landscape of LC treatment.⁸ In NSCLC, the main oncogenic alterations are Kirsten rat sarcoma virus (KRAS) and epidermal growth factor receptor (EGFR) driver mutations, together with anaplastic lymphoma kinase (ALK) and proto-oncogene tyrosine-protein kinase ROS (ROS1) rearrangements. On the other hand, in small cell lung cancer, tumor protein 53 (TP53), retinoblastoma (RB1) and phosphatase and tensin homolog (PTEN) were found to be the most frequent mutations in both smokers and non-smokers.⁹ Therefore, molecular testing may be used to select the best targeted treatment.⁵ However, despite the improved molecular knowledge, there are still many challenges. For instance, resistance to therapies is a critical issue, and it highlights the necessity for innovative therapeutic approaches. Recently, the interest in immunotherapies like programmed cell death protein 1/ligand (PD-1/PD-L1) inhibitors is increasing. Targeting both immune evasion via the PD-1/PD-L1 pathway and tumor blood supply through vascular endothelial growth factor (VEGF) inhibitors is a promising therapeutic strategy for patients with NSCLC, particularly those who have developed resistance to existing treatments.¹⁰ Nevertheless, the complex biology of lung cancer is still to be investigated, in order to understand its molecular aspects and to further improve treatments and survival of patients.

Recent research has highlighted the involvement of phospholipase enzymes in cancer, as they are integral to crucial cellular signaling pathways.^{11,12} Phospholipase C (PLC) family enzymes play a significant role in various cellular processes, particularly in the context of cancer, including LC.¹³ For instance, their deregulation

seems to be involved in tumorigenesis through the activation of pathways promoting inflammation, tumor growth, and invasion.¹⁴ PLCs hydrolyze phosphoinositol-4,5 bisphosphate to generate inositol 1,4,5-trisphosphate and diacylglycerol, which are important second messengers that elevate intracellular calcium levels and activate protein kinase C (PKC).¹⁵ Currently, 13 mammal PLC isozymes have been identified, and they can be divided into six groups: PLC β (1-4), PLC γ (1-2), PLC δ (1, 3 and 4), PLC ϵ (1), PLC ζ , PLC η (1-2).¹⁶

The PLC β family comprises four distinct isoforms that play essential roles in various cellular signaling pathways, including cell proliferation, differentiation, motility, and apoptosis.¹¹ Certain isoforms of PLC β , such as PLC β 1, localize inside the nucleus and contribute to nuclear signaling pathways.¹⁷ Furthermore, PLC β 1 is essential for glucose-stimulated insulin release from pancreatic β -cells, highlighting its role in glucose homeostasis.¹⁸ Moreover, dysfunction in PLC β 1 signaling has been associated with various health conditions including epilepsy, since it can affect inhibitory neuronal circuits.¹⁹ Its deregulation may also promote certain tumors, where changes in its expression levels correlate with malignancy and survival rates. For instance, PLC β 1 was found to enhance the migratory and invasive potential of gastric cancer cells by regulating actin cytoskeletal dynamics and inducing the epithelial-mesenchymal transition process.²⁰ PLC β 1 is primarily known for its cytoplasmic activity, but many studies highlighted its presence in the nucleus. Actually, it exists in two splicing variants with different localization: PLC β 1a (150kDa) can be found in the cytoplasm, whereas PLC β 1b (140kDa) is almost exclusively present in the nucleus. This dual localization suggests that PLC β 1 may operate in different cellular compartments, affecting various biological processes. Nuclear localization is significant in the context of cell proliferation and differentiation.²¹ Indeed, PLC β 1 dysregulation may influence cell cycle and the transcription of genes crucial for tumor development and progression.²¹

The role of PLC β isozymes was also investigated in LC by Strassheim *et al.*,²² who examined the expression of PLC β 1 and PLC β 3 in both SCLC and NSCLC. Interestingly, PLC β 1 was found to be more expressed in SCLC compared to NSCLC, presumably due to the neuroendocrine nature of SCLC. In fact, PLC β 1 is mainly localized at the level of neuronal tissues, and its activity induces the stimulation of neuroendocrine growth factors, thus supporting SCLC progression.²² Recently, it was observed that the lncRNA Lnc1 exerts its effects by targeting PLC β 1 and regulating cellular processes associated with cancer progression. Moreover, the silencing of PLC β 1 was found to reverse the enhancing effects of Lnc1 on NSCLC cell proliferation and migration.²³ The correlation between lung cancer prognosis and PLC β genes expression was investigated by Zhang *et al.*²⁴ through the Kaplan-Meier Plotter database. They reported that increased mRNA levels of PLCB1, PLCB2 and PLCB3 were linked to a worse overall survival in NSCLC patients and to a poorer prognosis of AC. In contrast, increased mRNA levels of PLCB4 were linked to a better overall survival in AC patients.²⁴

Besides PLC β , also PLC δ enzymes are localized at nuclear level and they are implicated in the regulation of different phases of the cell cycle. Namely, PLC δ 4 was noticed for its role in controlling the G1/S phase transition, since its knockdown leads to cell cycle arrest, causing accumulation of cells in G1 phase.²⁵ In rhabdomyosarcoma, it was recently discovered that nuclear PLC δ 4 regulates cyclin B1 expression and impacts cell cycle progression by facilitating the transition into mitosis.²⁶ In lung cancer, PLC δ enzymes were found to exhibit tumor-suppressive properties. For example, overexpression of PLC δ 1 was associated with decreased cellular proliferation, invasion, and migration in lung adenocarci-

noma cells.²⁷ Concerning PLC ϵ , studies showed that its expression is higher in NSCLC tissues and cell lines, although PLC ϵ was also described to have tumor suppressor properties in certain cancer types.²⁸ Other studies found correlations between high levels of PLC γ 1 expression and poor prognosis in lung adenocarcinoma patients and demonstrated that PLC γ 1 positively drives tumorigenesis of the lung.²⁹ Moreover, PLC γ 1 has been associated with the regulatory mechanisms of immune checkpoint proteins, such as PD-L1.³⁰

Overall, the present study is framed around the investigation of PLC enzymes expression and their implications in lung cancer prognosis, aiming to provide valuable insights to the field of cancer research. After the investigation of all PLC isoforms, we restricted the study to PLC β 1, that appeared to be widely altered in the patient population and showed consistency throughout the different cohorts. To enhance reliability and to highlight the relevance of the findings, molecular and microscopical techniques were combined in an interdisciplinary approach.

Materials and Methods

Patients

This is an observational study on patients diagnosed with NSCLC that received a surgical resection of the tumor mass between 2022 and 2025 at the Morgagni-Pierantoni Hospital, Forlì, Italy. Inclusion criteria were the diagnosis of NSCLC, >18 years old and no treatment with chemotherapy. The study was approved by the Romagna Ethics Committee (CEROM), determination number 2100, on 11th July 2022. Informed consent was obtained before the enrollment. The study was conducted in accordance with the Declaration of Helsinki 1964 and later versions and privacy rights have been observed. For each patient, clinical and histopathological information was collected. A total of 12 patients satisfied the inclusion criteria (Table S1). For each resection, two samples were excised: tumoral tissue and non-cancerous adjacent tissue. Surgical specimens were immediately placed in ice and preserved in RNAlater-Stabilization Solution (Thermo-Fisher Scientific, Waltham, MA, USA) at -20°C for subsequent analyses.

RNA extraction and RT-qPCR

Total RNA was extracted by using the PureLink RNA Mini Kit (Thermo-Fisher Scientific); RNA quantification was carried out with Qubit 4 Fluorometer (Thermo-Fisher Scientific). Total RNA was subsequently reverse-transcribed into cDNA, employing the High-Capacity cDNA Reverse Transcription Kit (Thermo-Fisher Scientific). A qPCR analysis was conducted to assess the mRNA expression levels of all PLC isoforms in tumors and adjacent non-cancerous tissues from 12 patients. All PLC isoforms were analyzed, except for PLC ζ 1, which is restricted to sperm heads and is not expressed in the lungs.³¹ RT-qPCR was performed by using the TaqMan Fast Advanced Master Mix (Thermo-Fisher Scientific) and validated TaqMan probes (Thermo-Fisher Scientific) for the housekeeping genes and for all the PLC isoforms: Hs99999903_m1 (ACTB); Hs01591985_g1 (EEF1A1); Hs01001930_m1 (PLCB1); Hs01080541_m1 (PLCB2); Hs01100294_m1 (PLCB3); Hs00168658_m1 (PLCB4); Hs00979908_m1 (PLCD1); Hs00293536_m1 (PLCD3); Hs01110380_m1 (PLCD4); Hs06639798_m1 (PLCG1); Hs01101851_m1 (PLCG2); Hs00392783_m1 (PLCH1); Hs00206116_m1 (PLCH2); Hs01059040_g1 (PLCE1). PLCB1 probe was able to recognize both variants: PLCB1a and PLCB1b.

Three independent experiments were carried out.

The 2^{- $\Delta\Delta$ Ct} method was applied to calculate the results, which were expressed as fold change. Values higher than 1 correspond to the overexpression of the gene compared to a physiological state, whereas values lower than 1 are associated with a decreased gene expression. Each tumor sample was normalized on its corresponding peritumoral tissue; ACTB (β -actin) and EEF1A1 (eukaryotic translation elongation factor 1 alpha 1) were used as housekeeping genes. Statistical comparisons between tumor and matched normal tissues were performed using a paired Student's t-test applied directly to the Δ Ct values, ensuring an appropriate linear scale for parametric analysis. Given the sample size (n=12), the assumption of normality was formally verified for each isoform dataset using the Shapiro-Wilk normality test prior to parametric testing. To account for multiple hypothesis testing across the 12 distinct PLC isoforms, raw p-values were adjusted using the Benjamini-Hochberg False Discovery Rate (FDR) procedure. An adjusted p-value of <0.05 was considered statistically significant (*).

Protein extraction and Western blotting of PLC β 1 protein

Protein analysis was conducted on four available samples. Tissue samples were fragmented with a scalpel and then lysed in RIPA buffer with Halt protease and phosphatase inhibitors (Thermo-Fisher Scientific). Lysates were cleared by centrifugation, and protein concentrations were determined using the BCA protein assay kit (Thermo-Fisher Scientific). Proteins were resolved by SDS-PAGE on 4-12% gradient gels and immunoblotted. Primary antibodies used were as follows: Santa Cruz Biotechnology (Dallas, TX, USA): anti- β -actin (sc-1616; 1:1000); anti-PLC β 1 (sc-205; 1:500). This anti-PLC β 1 antibody recognizes both PLC β 1a (150kDa) and PLC β 1b (140kDa) variants. Membranes were incubated with anti-rabbit peroxidase-conjugated secondary antibody (1:10,000, 1 h, room temperature) (Thermo-Fisher Scientific) and washed. Both primary and secondary antibodies were diluted in 1X PBS-Tween 20. Bands were visualized using Electrochemiluminescence (ECL) and imaged with iBright CL1500 Imaging System (Thermo-Fisher Scientific).

Kaplan-Meier plotter survival analysis

The impact of PLCs on the prognosis of lung cancer was assessed by using the Kaplan-Meier Plotter database (<https://kmplot.com/analysis/>). All available datasets were included in the analysis. Patients were split into two groups, according to the mRNA expression level of the selected gene. A univariate analysis was performed for each gene separately. At first, it was necessary to establish a cut-off value, that permits the designation of the patients to one of the two cohorts. All cut-off values between the lower and upper quartiles were computed and the best performing threshold was automatically selected as the best cut-off value. Afterwards, it was created a curve representing the probability of survival (y-axis) in a certain time interval (x-axis). Then, the two divisions were compared using the Cox-Mantel (Log Rank) test; the hazard ratio (HR) with 95% confidence intervals and the p-value were calculated. An HR higher than 1 correspond to a significant decrease in overall survival (OS) in high-expression cohort, whereas values lower than 1 are associated with a protective effect of the gene. When possible, the best probe was selected, as defined by the platform, the Affymetrix IDs used for the analyses are as follows: 215687_x_at (PLCB1); 210388_at (PLCB2); 213384_x_at (PLCB3); 203896_s_at (PLCB4); 205125_at (PLCD1); 234971_x_at (PLCD3); 224505_s_at (PLCD4); 216551_x_at (PLCG1); 204613_at (PLCG2); 214745_s_at (PLCH1); 206080_at (PLCH2); 205111_s_at (PLCE1).

Immunohistochemistry (IHC)

To investigate PLCβ1 expression in tumor samples, a human paraffin-embedded NSCLC Tissue Microarray (TMA) was employed (2019-2023 TissueArray.com). The array contained samples from 50 patients with a NSCLC diagnosis, with matched lung cancer adjacent tissue (Figure 1). To further explore PLCβ1 behavior in early-stage NSCLC, IHC was employed on 56 paraffin-embedded samples obtained from a local tissue biobank containing surgical resections of NSCLC. The biobank is located at the IRCCS IRST institute (IRCCS Istituto Romagnolo per lo Studio dei Tumori), Meldola (FC), Italy. Specimens were obtained between 2019 and 2020. For each patient, clinical and histopathological information was collected (Table S2). IHC was carried out with a primary antibody against PLCβ1 (dilution 1:1000, Invitrogen #PA5-78439) using the OptiView DAB IHC Detection Kit (Roche, Basel, Switzerland) on an automated immunostainer (Ventana Benchmark Ultra, Roche), using a brown chromogen (diaminobenzidine, DAB). Antibody dilution buffer (Roche) was employed for antibody dilution. Hematoxylin was used as nuclear counterstain. To ensure reaction specificity, a negative reagent control was implemented. As positive tissue control, a kidney section was used. Negative elements were considered as internal tissue controls. The evaluation of PLCβ1 expression levels was based on the observation of the distribution and localization of the brown precipitate under optical microscope (Nikon eclipse Ci, Tokyo, Japan, objectives: 4X, 20X, 40X). The microscopic fields were selected by avoiding areas with tissue damage, necrosis, or artifacts. A total of 5 fields were examined for each section. Only nucleated cells within the selected areas were considered for quantitative analysis, while inflammatory infiltrates were excluded. IHC staining was independently evaluated by three expert clinical pathologists, who were completely blinded to all clinicopathological data and patient outcomes. Subcellular localization of PLCβ1 was strictly defined: nuclear staining was considered positive only when a distinct signal was observed within the nuclear boundary, whereas cytoplasmic staining was defined as diffuse or granular signal confined to the cytoplasm, excluding the nucleus. Immunohistochemical stains were scored by pathologists as negative (0), weak (1+), moderate (2+), strong (3+), and very strong (4+), according to the intensity of the stained sections. Subsequently, the scores were translated into a percentage ranging

from 0% to 100%, respectively: 0% (negative), 25% (weak), 50% (moderate), 75% (strong), 100% (very strong), then the values were graphically visualized. The focus of the analysis was to evaluate the difference of percentage in healthy and tumor tissues within the same patient. This approach accounted for inter-individual variability. Sample normality was not formally assessed prior to the analysis. Nevertheless, the relatively large number of paired observations (n=50 for the first analysis; n=56 for the second) and the paired design were considered appropriate for evaluating the mean difference within the patient. Statistical analyses were conducted through paired *t*-test and results were considered significant at *p*<0.05; very significant at *p*<0.01; highly significant at *p*<0.001.

Results

Analysis of PLCs gene expression in LC patients

Fold changes of all PLC genes are displayed in Figure 2. *PLCB1* gene was overexpressed in all patients enrolled. In contrast, *PLCB2* mRNA levels showed a noticeable reduction, even if not statistically significant. *PLCH1* was overexpressed in most patients, and the remaining PLC isoforms exhibited a fluctuating trend. Individual results are gathered in Table S3. Relative expression of *PLCB1* in single patients is represented in Figure 3. Notably, two patients outstand the others for their massive expression of *PLCB1*: T_06 and T_10. Both of them were diagnosed with LCC, suggesting a remarkable function of *PLCB1* in this very rare and aggressive histotype. The fact that *PLCB1* overexpression is observed in all the patients enrolled is a very powerful finding. Consequently, we decided to further explore the functional role of PLCβ1 in lung cancer.

Analysis of PLCβ1 protein expression in LC patients

Considering the significant increase of PLCβ1 at transcriptional level in all patients, we investigated whether this increment was reflected at protein level. Protein analysis was conducted on four available samples, confirming an elevated expression of PLCβ1 in lung cancer tissue compared to their corresponding control specimens (Figure 4). Both variants, i.e. PLCβ1a (150kDa) and PLCβ1b

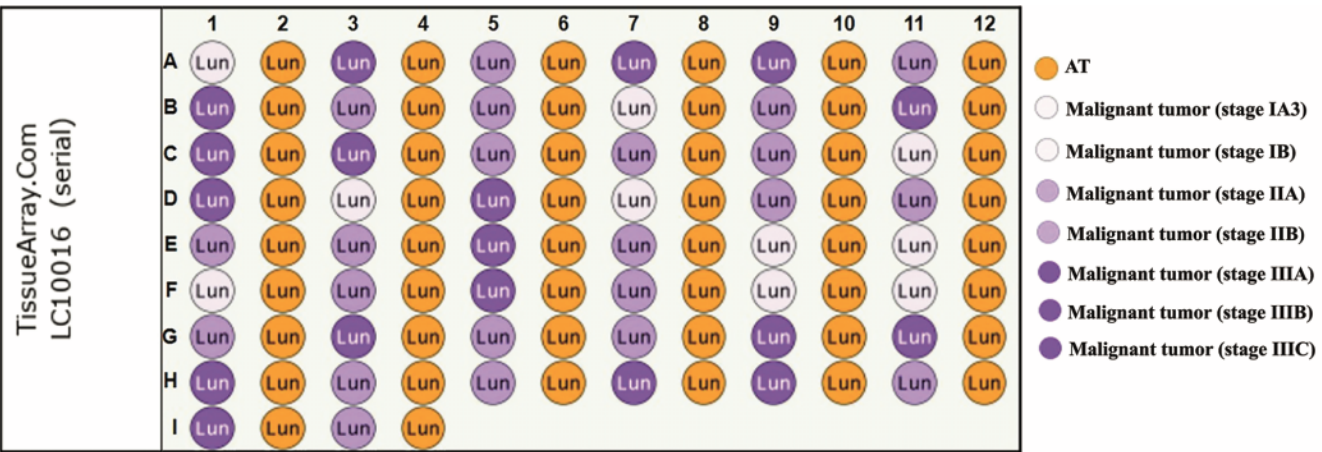


Figure 1. Tissue microarray legend (<https://www.tissuearray.com/tissue-arrays/Lung/LC10016>).

(140kDa), appeared with more intense bands. Nuclear fractionation was not performed due to the limited amount of patient biopsy material available. Therefore, we cannot biochemically confirm the exclusive isolation of PLC β 1b within the nuclear compartment.

PLCB1 has a prognostic role in LC

The Kaplan-Meier plotter database was used to assess the prognostic value of PLC family enzymes in lung cancer, as well as to correlate their expression with tumor histotype, sex, and smoking status of the patients. All PLC isoforms were investigated except for *PLCZ1*. In Figure 5, the results are gathered in a forest plot graph. *PLCB1*, *PLCB2*, *PLCB3*, *PLCD3*, *PLCG1* and *PLCH2* had an HR higher than 1. On the contrary, *PLCB4*, *PLCD1*, *PLCD4*, *PLCG2*, *PLCH1* and *PLCE1* showed an HR lower than 1.

Table S4 contains all the information concerning *PLCB1* analysis, i.e. the number of patients analyzed, and the statistical parameters involved in plot design. There is a statistically signifi-

cant correlation between *PLCB1* expression and OS. In fact, high mRNA expression was associated with a worse OS in all lung cancer histotypes, with a marked difference when analysis was conducted on distinct histotypes. The same behavior was observed when the examination was restricted to the female sex and to the male sex. By investigating the relation with smoke, it could be observed that non-smokers with an elevated expression of *PLCB1* mRNA had the worst prognosis.

PLC β 1 increases in the nucleus of tumor tissue

To establish PLC β 1 localization, we focused on the examination of PLC β 1 expression in tissue samples of lung cancer patients. A NSCLC TMA was used to facilitate the analysis of multiple samples simultaneously. IHC was performed on tissue samples to assess the expression of PLC β 1 within the tumor and control specimens. The evaluation of PLC β 1 amount revealed an increase in PLC β 1 expression across the majority of the examined tumor sam-

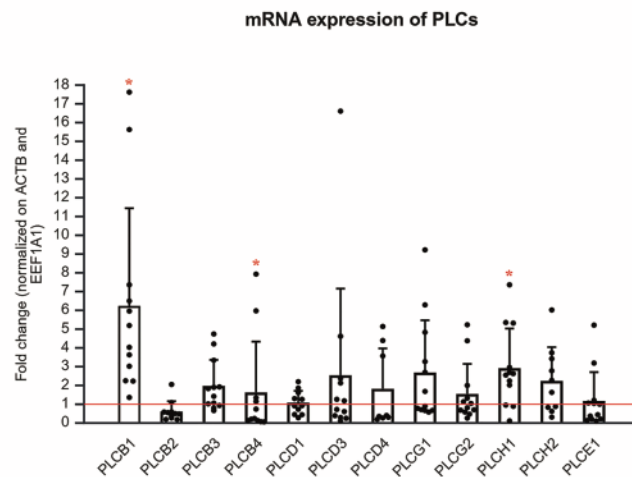


Figure 2. mRNA expression of PLC isoforms in patients. Results are expressed as fold change, normalized on ACTB and EEF1A1 housekeeping genes. Each tumor sample was normalized on the corresponding non-cancerous adjacent tissue. The red line defines the threshold. * $p < 0.05$.

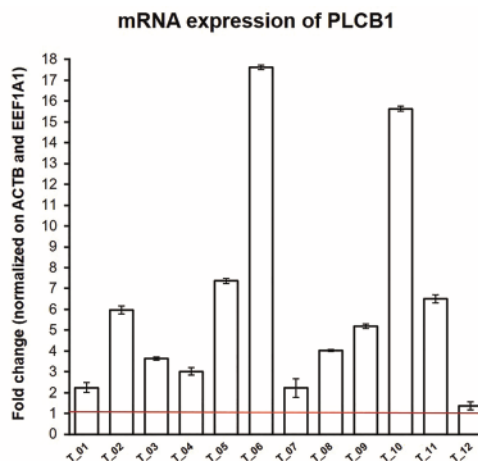


Figure 3. Individual mRNA expression of PLCB1 in patients. Results are expressed as fold change, normalized on ACTB and EEF1A1 housekeeping genes. Each tumor sample was normalized on the corresponding non-cancerous adjacent tissue. The red line defines the threshold. Results represent the average of three independent experiments.

ples, compared to their matched adjacent control tissues. This trend was observed in both SCC (Figure 6) and AC (Figure 7). Concerning the localization, in tumoral tissue PLCβ1 was found to be localized at nuclear level (Figure 6 J,L and Figure 7 J,L), whereas in the adjacent normal tissue its localization was almost completely cytoplasmic (Figure 6 I,K and Figure 7 I,K). All in all, a consistent increase in PLCβ1 expression was observed in almost all the examined tumor samples when compared to their corresponding adjacent healthy tissues. This trend was seen in both major subtypes of NSCLC: SCC and AC.

A score ranging from 0% to 100% was assigned to the color intensity of the brown precipitate indicating PLCβ1 (Figure 8), then samples were sorted by tumor histotype and tumor stage (Figure 9). Remarkably, both SCC and AC displayed a statistically significant increase in PLCβ1 expression compared to the healthy counterpart (Figure 9A). A similar behavior was observed for the samples divided by tumor stage (Figure 9B). However, only stages IB, IIB, and IIIB were statistically significant.

IHC performed on 56 paraffin-embedded tissues is showed in Figure 10 A-D. PLCβ1 expression can be visualized by tumor histotype and stage in Figure 10 E,F. In general, we observed a lower enzymatic expression compared to the TMA. However, the variation between control and tumor tissues was considerable and it resembled the one seen in the TMA. When sorted by tumor stage, also initial stages (i.e., IA and IB) showed a statistical significance.

Discussion

Globally, cancer is a major cause of mortality and morbidity, generating social and economic challenges for the governments and their healthcare systems.³²⁻³⁴ Platinum-based chemotherapy is the standard treatment for NSCLC patients and several targeted therapies have been developed, based on distinct biomarkers, e.g. epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK), proto-oncogene tyrosine-protein kinase ROS (ROS1), human epidermal growth factor receptor 2 (HER2).³⁵ Recently, targeted therapies have improved patient survival, but drug resistance is a major concern.¹⁰ Immunotherapy has demonstrated favorable evidence in late-stage or metastatic NSCLC. For example, programmed cell death protein 1/ligand (PD-1/PD-L1) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) immune checkpoint inhibitors have been widely studied.¹⁰ Nevertheless, there are limitations concerning the identification and validation of immune markers and the immunosuppressive ability of tumors, causing primary or acquired resistance.¹⁰

Phospholipase C (PLC) enzymes are involved in many signaling pathways associated with cell cycle, proliferation, differentiation, and motility,¹¹ therefore facilitating processes related to tumor onset and progression.¹³ Moreover, certain PLC isoforms display nuclear localization, an indicator of their contribution in the regulation of neoplastic transformation.¹³ For these reasons, PLC

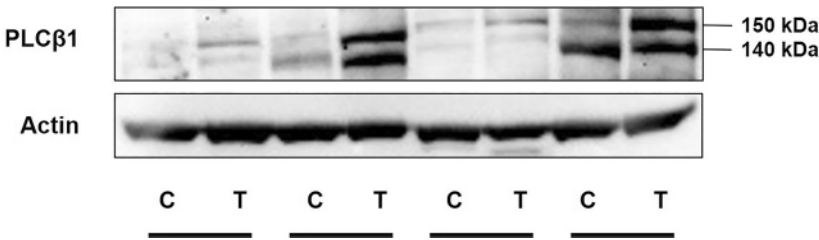


Figure 4. Protein expression of PLCβ1 in four patients. C, control; T, tumor.

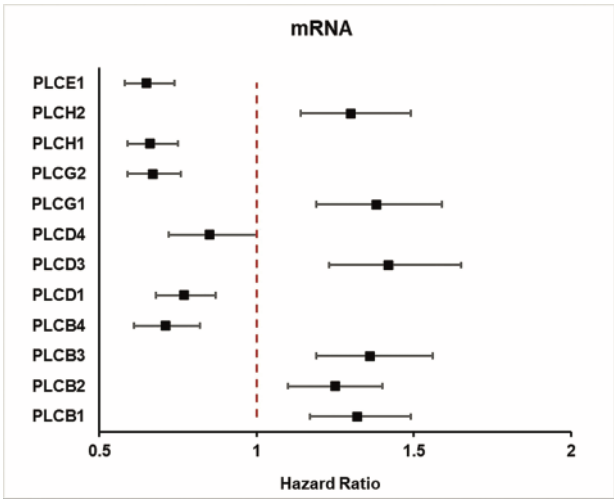


Figure 5. Hazard ratio for each PLC gene. Squared markers indicate the HR for the specific enzyme, and the confidence intervals are shown as bars.

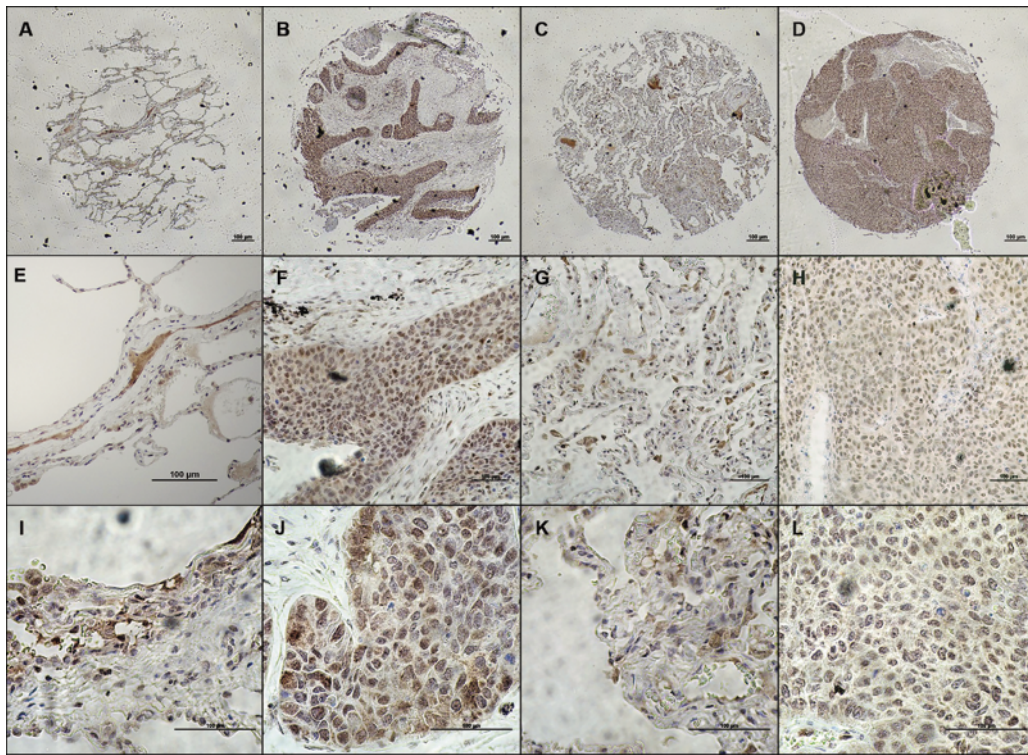


Figure 6. TMA optical microscopy images of representative squamous cell carcinoma samples after immunohistochemistry. Keratinizing (**B,F,J**) and non-keratinizing (**D,H,L**) squamous cell carcinomas display higher levels of PLC β 1 compared to their corresponding adjacent control tissues (**A,E,I,C,G,K**). PLC β 1 is located in the nucleus in tumor samples (**F,J,H,L**), but it is cytoplasmic in control specimens (**E,I,G,K**).

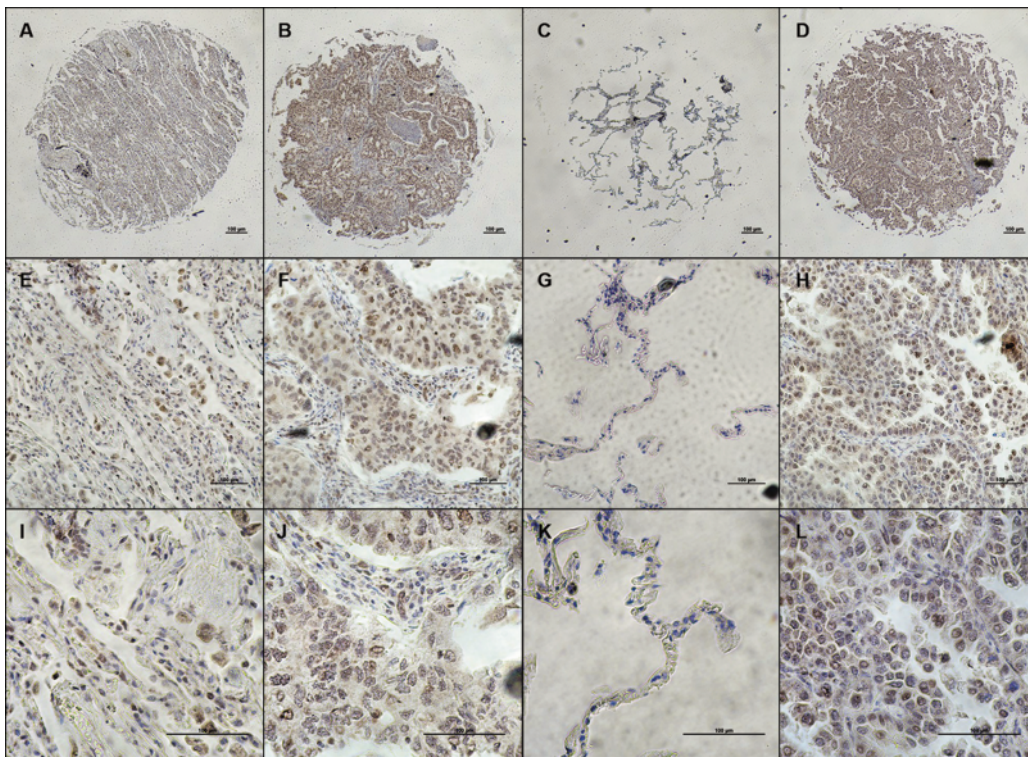


Figure 7. TMA optical microscopy images of representative adenocarcinoma samples after immunohistochemistry. Invasive (**B,F,J**) and papillary (**D,H,L**) adenocarcinomas display higher levels of PLC β 1 compared to their corresponding adjacent control tissues (**A,E,I,C,G,K**). PLC β 1 is located in the nucleus in tumor samples (**F,J,H,L**), but it is cytoplasmic in control specimens (**E,I,G,K**).

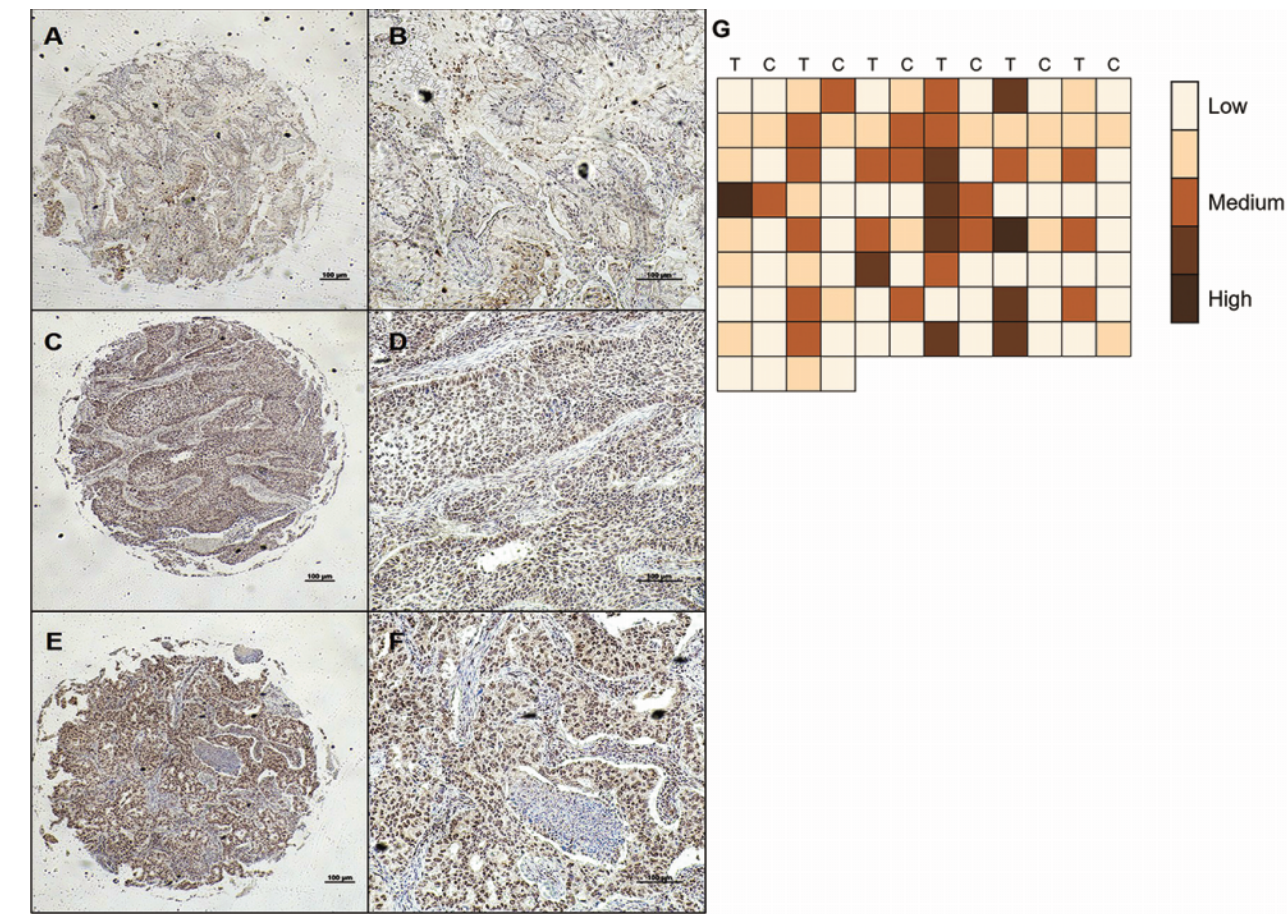


Figure 8. Optical microscopy images of representative samples after immunohistochemistry. Low (A,B), medium (C,D) and high (E,F) expression of PLCβ1. Expression levels of PLCβ1 of each TMA sample (G).

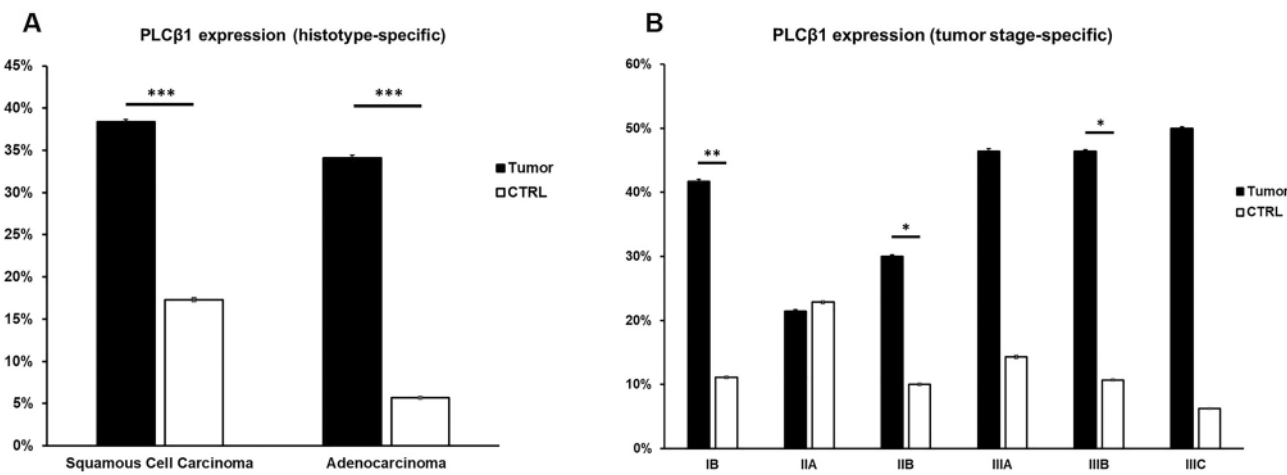


Figure 9. Analysis of PLCβ1 expression in TMA samples sorted by tumor histotype (A) and tumor stage (B). * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

enzymes might assume promising significance as biomarkers in the context of cancer.

The present study highlighted the significant role of PLC β 1 in lung cancer. Specifically, its overexpression at nuclear level was observed in tumor tissues, correlating with poor patient prognosis. The findings suggest that PLC β 1 could serve as a potential biomarker for lung cancer diagnosis and prognosis, particularly in NSCLC. Here we found that PLC β 1 was overexpressed in lung cancer samples compared to adjacent normal tissue. To support the results, protein expression was assessed in four patients, that exhibited a substantial increase. In addition, the KM-plotter database enabled us to rapidly screen for potential biomarkers and therapeutic targets, suggesting complex and isoform-specific involvement of PLC enzymes in lung cancer prognosis. Indeed, the genes with HR>1 might be oncogenic or play a role in promoting tumor

progression. On the other hand, genes with HR<1 might have tumor-suppressive roles, or their increased expression may be indicative of a less aggressive disease. All in all, the results obtained by KM-plotter database suggest that PLC enzymes, particularly PLCB1, have a prognostic role in lung cancer. A limitation of this study is the absence of an appropriate survival analysis based on our patient cohort. Although clinical data were collected and presented in Tables S1 and S2, the follow-up information was incomplete due to relatively short observation windows, rendering a reliable internal outcome analysis unfeasible. Consequently, we relied exclusively on the external KM-plotter database to evaluate the prognostic value of PLC β 1. While the KM-plotter provides a robust sample size, we are aware that using an automated best cut-off approach to stratify patients can potentially inflate statistical significance. Future prospective studies with well-annotated, long-

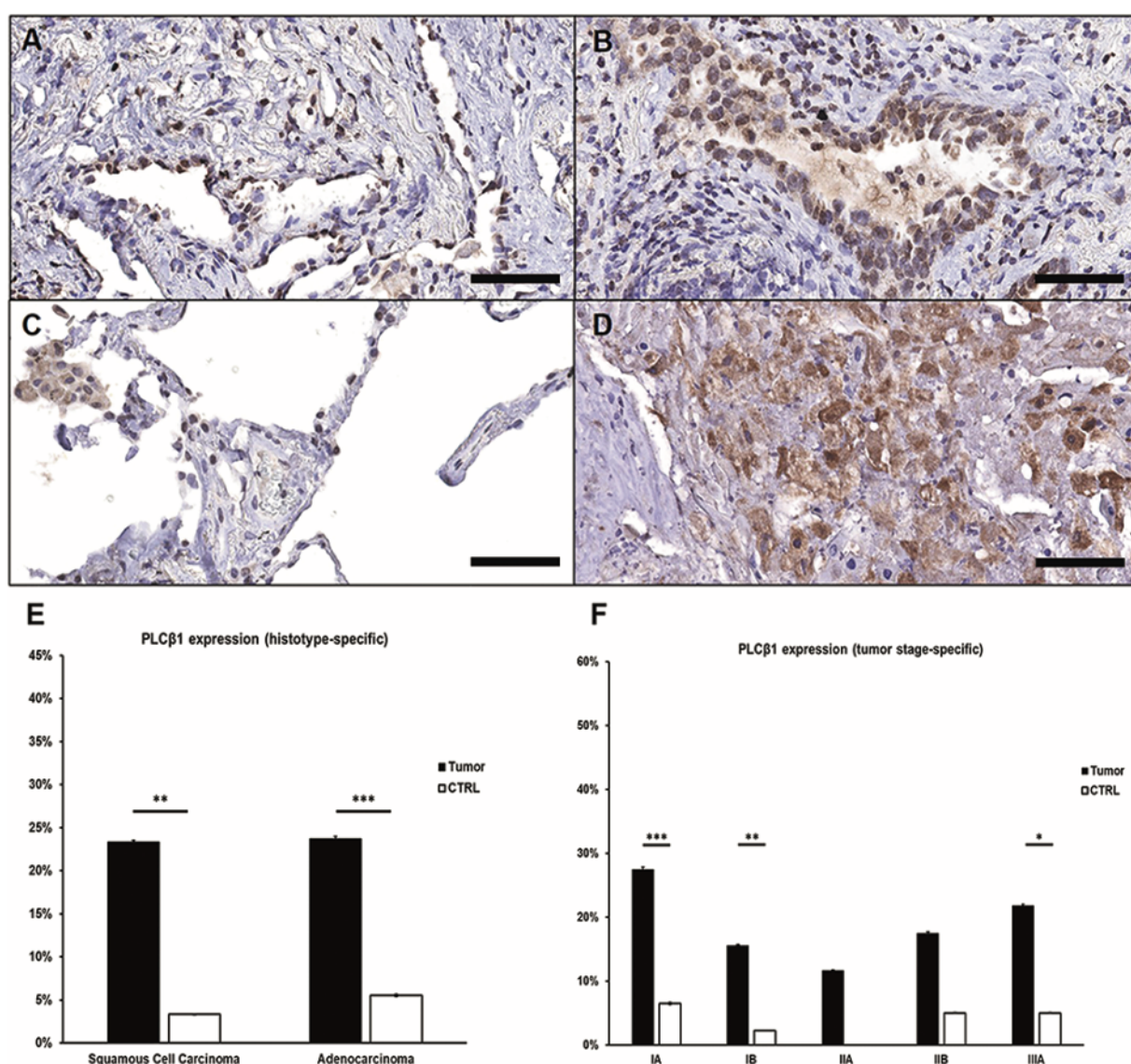


Figure 10. Optical microscopy images of representative adenocarcinoma (A,B) and squamous cell carcinoma (C,D) samples after immunohistochemistry. Tumor tissues (B,D) display higher levels of PLC β 1 compared to their corresponding adjacent control tissues (A,C); scale bars: 60 μ m. Analysis of PLC β 1 expression in biobank tissues sorted by tumor histotype (E) and tumor stage (F). * p <0.05; ** p <0.01; *** p <0.001.

term internal clinical follow-up are warranted to definitively validate these findings.

By looking at multiple clinical features, we established a link between PLC β 1 expression and patient survival outcomes. Tissue microarray analysis revealed that PLC β 1 is predominantly confined in the nucleus of tumor tissues. On the other hand, in adjacent normal tissues PLC β 1 exhibited cytoplasmic localization. Due to its specific nuclear localization in tumoral tissue we hypothesize that PLC β 1 may have critical functions in nuclear signaling pathways that influence malignant transformation and tumor progression.¹⁷ In particular, PLC β 1 modulates the activity of transcription factors, influencing the expression of genes involved in cell proliferation, differentiation, and apoptosis.¹⁷ It participates in nuclear signaling pathways that regulate cellular responses to growth factors or stress signals, impacting tumor progression.¹⁷ We must note a biochemical limitation regarding the *in situ* characterization of PLC β 1 signaling in our cohort: the commercial antibody employed for IHC targets a conserved epitope present in both the PLC β 1a and PLC β 1b splice variants. Consequently, visual immunohistochemical scoring reflects total PLC β 1 expression and cannot definitively discriminate between the two isoforms within the cell. Interestingly, our total Western blot analyses successfully resolved two distinct bands corresponding to the predicted molecular weights of both PLC β 1a and PLC β 1b, confirming the co-expression of both variants in these tissues. Since nuclear fractionation was not performed, due to the limited amount of patient biopsy material available, we cannot biochemically confirm the exclusive isolation of PLC β 1b within the nuclear compartment. The prominent nuclear accumulation observed *via* IHC in malignant tissues may therefore be driven by two non-mutually exclusive mechanisms: a selective upregulation or stabilization of the nuclear-resident PLC β 1b variant, or an active nuclear translocation of the natively cytoplasmic PLC β 1a variant under oncogenic stimuli. Future prospective studies using isoform-specific knockdown models or sub-cellular fractionation will be required to fully dissect the precise individual contributions of these two variants to NSCLC progression.

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Online Supplementary Material

Table S1. Baseline patients characteristics and histopathology of tumors (qPCR and WB analyses).

Table S2. Baseline patients characteristics and histopathology of tumors (immunohistochemistry).

Table S3. Summary of PLCs expression for each patient.

Table S4. Summary of Kaplan-Meier plotter analysis.

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