Group IIa secretory phospholipase expression correlates with group IIa secretory phospholipase inhibition–mediated cell death in K-ras mutant lung cancer cells

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Objective: There are currently no targeted therapies against lung tumors with oncogenic K-ras mutations that are found in 25% to ~40% of lung cancers and are characterized by their resistance to epidermal growth factor receptor inhibitors. The isozyme group IIa secretory phospholipase A₂ (sPLA₂IIa) is a potential biomarker and regulator of lung cancer cell invasion; however, the relationship between K-ras mutations and sPLA₂IIa has yet to be investigated. We hypothesize that sPLA₂IIa modulates lung cancer cell growth in K-ras mutant cells and that sPLA₂IIa expression in human lung tumors is increased in K-ras mutant tumors.

Methods: Baseline sPLA₂IIa expression in K-ras mutant lung cancer cell lines (A549, SW1573, H358, H2009) was assessed. Cells were treated with a specific sPLA₂IIa inhibitor and evaluated for apoptosis and cell viability. Nuclear factor kappa-b (NF-κB) and extracellular signal-regulated kinase 1/2 activity were detected by Western blot. Human tumor samples were evaluated for sPLA₂IIa mRNA expression by quantitative reverse-transcription polymerase chain reaction.

Results: Cytotoxicity of sPLA₂IIa inhibition correlates with sPLA₂IIa expression. Apoptosis in response to sPLA₂ inhibition parallels attenuation in NF-κB activity. In addition, sPLA₂IIa expression in human tumors correlates with squamous cell pathology and increasing stage of K-ras mutant lung tumors.

Conclusions: Baseline sPLA₂IIa expression predicts response to sPLA₂IIa inhibition in some K-ras mutant lung cancer cells. This finding is independent of p53 mutation status. Furthermore, squamous tumors and advanced-stage K-ras mutant tumors express more sPLA₂IIa. These data support a role for sPLA₂IIa as a potential global therapeutic target in the treatment of lung cancer. (*J Thorac Cardiovasc Surg* 2012;144:1479-85)

Lung cancer remains the most lethal cancer in the world. Gain-of-function mutations in K-ras are found in 25% to 40% of lung cancers and are thought to be significant drivers of oncologic virulence. There are currently no targeted therapies that effectively treat K-ras mutant lung tumors. In fact, these mutations are characterized by their resistance to epidermal growth factor receptor (EGFR) inhibitors.^{1,2}

Phospholipase A₂ (PLA₂) enzymes release arachidonic acid from cell membranes, initiating downstream production of tumor-promoting eicosanoids. Group IIa secretory phospholipase A₂ (sPLA₂IIa), a subgroup of PLA₂s, has recently been identified as a potential lung cancer biomarker.³

In a cohort of patients with solitary pulmonary nodules, patients with lung cancer had higher plasma levels of sPLA₂IIa than patients with a benign nodule. In addition, histologic analysis demonstrates overexpression of sPLA₂IIa in lung tumors compared with normal lung tissue.³ From a functional perspective, sPLA₂IIa has been shown to regulate lung cancer cell growth and invasion. RNA interference of sPLA₂IIa decreases tumor growth and nuclear factor kappa-b (NF-κB) activity in a xenograft model of lung cancer.⁴ In addition, sPLA₂IIa inhibition decreases intercellular adhesion molecule-1–associated invasion in vitro.⁵

Although cytosolic PLA₂ activity is increased in K-ras mutant lung tumors,⁶⁻⁸ the relationship between sPLA₂IIa and K-ras mutant lung cancer has not previously been explored. The aim of this study is to examine the potential of targeted sPLA₂IIa inhibition in K-ras mutant lung cancer. Our purpose was twofold: (1) to investigate the role of sPLA₂IIa in the growth of K-ras mutant lung cancer cells and (2) to examine sPLA₂IIa expression in human tumor specimens and correlate the results with K-ras mutation status. We hypothesized that sPLA₂IIa modulates lung cancer cell growth in K-ras mutant cells and that tumor expression of sPLA₂IIa would correlate with K-ras status and cancer stage.

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Abbreviations and Acronyms

EGFR	= epidermal growth factor receptor
ERK	= extracellular signal-regulated kinase
FBS	= fetal bovine serum
GAPDH	= glyceraldehyde 3-phosphate dehydrogenase
mRNA	= messenger RNA
MTT	= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF- κ B	= nuclear factor kappa-b
PLA ₂	= phospholipase A ₂
RT-PCR	= reverse-transcription polymerase chain reaction
sPLA ₂ Ila	= group Ila secretory phospholipase A ₂
TNF- α	= tumor necrosis factor-alpha

METHODS**Cells and Reagents**

All cell lines tested were K-ras mutant derived from non-small cell lung cancers (Table 1). A549 cells were a gift from Dr Anirban Banerjee (Aurora, Colo). H358 cells were a gift from Dr Kim O'Neill (Provo, Utah). SW1573 and H2009 were a gift from Dr Mary Reyland (Aurora, Colo). Cells were maintained in a humidified atmosphere with 5% carbon dioxide using Ham's F-12 (A549) or RPMI media (all other cell lines) with 10% fetal bovine serum (FBS). Tumor necrosis factor-alpha (TNF- α) (Sigma-Aldrich, St Louis, Mo) was used at a concentration of 20 ng/mL to mimic the inflammatory milieu of the tumor microenvironment. The sPLA₂Ila inhibitor S3319 (Sigma Aldrich) was dissolved in dimethyl sulfoxide. Dimethyl sulfoxide served as a vehicle control for all experiments with a maximum experimental concentration of 0.08%. Rabbit anti-human antibodies for phosphorylated and total NF- κ B p65, extracellular signal-regulated kinase (ERK) 1/2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtained from Cell Signaling Technology, Inc (Beverly, Mass). For Western blot, the rabbit anti-human-specific sPLA₂Ila antibody was from Abcam (Cambridge, Mass). For immunofluorescence, the rabbit anti-human sPLA₂Ila antibody was obtained from BioVendor, LLC (Candler, NC).

Immunofluorescence Microscopy

We cultured 5000 cells per chamber on Lab-Tek II 8-well glass chamber slides (Thermo Scientific, Rochester, NY) in full media for 24 hours and then serum-starved them (0.5% FBS) for 24 hours. Cells were pretreated with incrementally increasing concentrations of the sPLA₂ inhibitor in serum-starved media for 1 hour, then stimulated with 20 ng/mL TNF- α for 8 hours. Control cells were incubated with dimethyl sulfoxide in serum-starved media and stimulated with TNF- α for 8 hours. Immunofluorescence microscopy was performed to evaluate sPLA₂Ila expression in A549 cells as previously described.⁹

Apoptosis Assay

We cultured 1×10^6 cells per well in 12-well plates in full media for 24 hours and then serum-starved them (0.5% FBS) for 24 hours before treatment. Cells were treated with sPLA₂Ila inhibitor (20 or 40 μ mol/L) or vehicle control in serum-starved media for 8 hours. A time-response experiment using 4- and 8-hour time points was used to determine the 8-hour time point chosen for this experiment (data not shown). Apoptosis was assessed by flow cytometric detection of annexin V and propidium iodide (BD Biosciences, San Jose, Calif).

Cell Viability Assay

We cultured 5×10^3 cells per well in 96-well plates in full media for 24 hours and then serum-starved them (0.5% FBS) for 24 hours before treatment. Cells were exposed to sPLA₂Ila inhibitor (10-40 μ mol/L) or vehicle control in serum-starved media for 24 hours. A time-response experiment using 12-, 24-, and 48-hour time points was used to determine the 24-hour time point chosen for this experiment (data not shown). Cell viability was measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to manufacturer instructions (Roche Applied Science, Indianapolis, Ind).

Western Blot

Cells were plated on 6-well plates at a density of 3×10^6 in full media for 24 hours, then serum-starved (0.5% FBS) for 24 hours, and then treated. Time trials with TNF- α were performed to determine the time of maximal initial increase in phosphorylation of NF- κ B and ERK 1/2 in all cell lines. Cells were pretreated with sPLA₂Ila inhibitor (10-40 μ mol/L) for 1 hour, stimulated with TNF- α , then lysed with Laemmli buffer under reducing conditions. In separate experiments, untreated cells were lysed and analyzed for baseline sPLA₂Ila expression. Proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis techniques and transferred to a nitrocellulose membrane. Membranes were blocked in 5% nonfat milk in phosphate-buffered saline-Tween. Primary antibodies were dissolved in 5% bovine serum albumin in phosphate-buffered saline-Tween. Secondary antibodies were prepared in 5% nonfat milk in phosphate-buffered saline-Tween. The protein of interest was developed using Pierce ECL Chemiluminescent (Thermo Fisher Scientific, Inc, Rockford, Ill). Densitometric analysis was performed using ImageJ Software (National Institutes of Health, Bethesda, Md).

Quantitative Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

Cells were plated on 12-well plates at a density of 1×10^6 in full media for 24 hours and serum-starved (0.5% FBS) for 24 hours before lysis and RNA isolation. RNA was prepared using the Qiagen RNeasy Plus Mini Kit according to the manufacturer's protocol (Germantown, Md). Complementary DNA was synthesized using Bio-Rad iScript (Hercules, Calif). RT-PCR was performed using Applied Biosystems Sybr Green Master Mix (Carlsbad, Calif). The primer sequences used are as follows: sPLA₂Ila (forward): CCC-TCC-TAC-TGT-TGG-CAG-TGA-T; sPLA₂Ila (reverse): CCT-GTC-GTC-AAC-TTG-ATC-ATT-CTG; GAPDH (forward): CCT-GCA-CCA-CCA-ACT-GCT-TAG; GAPDH (reverse): TGA-GTC-CTT-CCA-CGA-TAC-CAA. Denaturation (95°C, 15 seconds), annealing (52°C, 30 seconds), and elongation (72°C, 30 seconds) steps were repeated for 40 cycles, flanked by a 10-minute denaturation and 5-minute elongation step. Data were analyzed by the $\Delta\Delta$ Ct method.¹⁰

Lung Tumor Tissue Collection

Human lung tissue and corresponding clinical data were collected under a tissue banking protocol approved by the University of Colorado Institutional Review Board. Informed consent was obtained from each participant. The stage reported is the pathologic stage after surgical resection according to American Joint Committee on Cancer guidelines. Tumor samples and normal-appearing tissue from resected lobes were collected in RNALater (Qiagen). Complementary DNA and subsequent quantitative RT-PCR were prepared as described above.

Statistical Analysis

All data distributions were inspected before statistical analysis. The Student *t* test was performed for 2-group comparisons, and the analysis of variance test was performed when comparing 3 or more groups. No multiple comparison was conducted. StatView version 5.0 was used for all statistical analysis (SAS Institute, Inc, Cary, NC).

TABLE 1. Cell line characteristics

Cell line	Tumor type	EGFR	K-ras	p53	NF-κB with sPLA ₂ inhibition	ERK 1/2 with sPLA ₂ inhibition	sPLA ₂
SW1573	BAC	WT	Mut	WT	Strong decrease	Strong increase	More
H2009	Adenoca	WT	Mut	Mut	Decrease	Strong increase	More
H358	BAC	WT	Mut	Mut	Decrease	Strong increase	Less
A549	Adenoca	WT	Mut	WT	Strong decrease	Decrease	Less

Strong attenuation of NF-κB activity was correlated with WT p53. *EGFR*, Epidermal growth factor receptor; *NF-κb*, nuclear factor kappa-b; *sPLA₂*, secretory phospholipase A₂; *ERK*, extracellular signal-regulated kinase; *BAC*, bronchoalveolar carcinoma; *WT*, wild-type; *Mut*, mutant; *Adenoca*, adenocarcinoma.

RESULTS
sPLA₂Ila Expressed in K-ras Mutant Lung Cancer Cells

Group Ila sPLA₂ expression varies in K-ras mutant lung cancer cells. SW1573 and H2009 express more sPLA₂Ila messenger RNA (mRNA) compared with H358 and A549 cells (Figure 1, A). Protein analysis by Western blot and immunofluorescence corroborate this finding (Figure 1, B and C).

Cell Survival in K-ras Mutant Lung Cancer Cells Depends on sPLA₂Ila Expression

sPLA₂Ila inhibition led to concentration-dependent apoptosis and cell death in all K-ras mutant lung cancer cells (Figure 2). Cells with higher baseline sPLA₂Ila were

more susceptible to cell death with sPLA₂Ila inhibition. Early apoptosis detected by annexin V staining after 8 hours of sPLA₂Ila inhibition was increased in SW1573 and H2009 cells compared with H358 and A549 cells (Figure 2, A). The MTT assay confirmed concentration-dependent cell death after 24 hours of sPLA₂Ila inhibition, again with increased vulnerability in cells with higher sPLA₂Ila expression (Figure 2, B).

sPLA₂Ila Inhibition in K-ras Mutants Decreases NF-κB Phosphorylation

Previous work has demonstrated that sPLA₂Ila inhibition-associated apoptosis is related predominantly to decreased NF-κB activity.⁴ We confirmed that NF-κB phosphorylation is decreased with sPLA₂Ila inhibition in

FIGURE 1. K-ras mutant lung cancer cell lines analyzed for baseline group Ila secretory phospholipase A₂ (sPLA₂Ila) expression. Expression varies with SW1573 and H2009 cells expressing more sPLA₂Ila compared with H358 and A549 cells. Messenger RNA (mRNA) was evaluated by quantitative reverse-transcription polymerase chain reaction (RT-PCR), mean ± SEM, *P < .05, n = 5, run in triplicate (A). sPLA₂Ila protein expression was evaluated by Western blot. Graph of densitometry, mean ± SEM, **P < .005, n = 4 (B). Immunofluorescence for sPLA₂Ila was used for additional protein, n = 3 run in duplicate, sPLA₂Ila = red, cell membrane = green, nuclei = blue (C). IgG, Immunoglobulin G; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

FIGURE 2. Group IIa secretory phospholipase A₂ (*sPLA₂IIa*) expression inhibition promotes concentration-dependent apoptosis and cell death in lung cancer cell lines, and cytotoxicity of *sPLA₂IIa* inhibition parallels the level of *sPLA₂IIa* expression. SW1573 and H2009 cells experience more apoptosis at 20 and 40 μmol/L compared with H358 and A549 cells. Percent of cells with annexin V staining were normalized to staining of the respective vehicle control, mean ± SEM, **P* < .03 compared with SW1573, H2009. ***P* < .01 compared with vehicle control, n = 3-4 (A). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (*MTT*) assay was used to assay cell viability after *sPLA₂IIa* inhibition. LD50 at 24 hours increases as *sPLA₂IIa* expression decreases, mean ± SEM, **P* < .003, n = 3 run in triplicate (B).

all K-ras mutant cells, whereas ERK 1/2 phosphorylation increases in 3 cell lines but decreases in A549 cells (Figure 3). H2009 and H358 cells have a more modest decrease in NF-κB compared with SW1573 and A549 cells. H2009 and H358 cells are both p53 mutant.

Human Lung Tumors Express *sPLA₂IIa*

Fifteen lung tumors were examined for *sPLA₂IIa* mRNA expression. One metastatic colon adenocarcinoma and 1 invasive esophageal adenocarcinoma were included in analysis for comparison. Lung tumors express detectable levels of *sPLA₂IIa* mRNA (Figure 4). Although squamous and K-ras mutant tumors are less common in our cohort, there is a trend toward increased expression of *sPLA₂IIa* in squamous tumors. In K-ras mutant tumors there is evidence of increased *sPLA₂IIa* expression with increasing stage. Both large cell neuroendocrine tumors express low levels of *sPLA₂IIa*.

DISCUSSION

This is the first study to specifically explore the role of *sPLA₂IIa* in K-ras mutant lung cancer. Consistent with

our hypothesis, *sPLA₂IIa* is a cytotoxic target in K-ras mutant lung cancer cells. Our finding that in vitro sensitivity to *sPLA₂IIa* inhibition correlates with baseline *sPLA₂IIa* expression, regardless of p53 mutation status, suggests that *sPLA₂IIa* expression may predict response to *sPLA₂IIa* inhibition. Analysis of *sPLA₂IIa* expression in human lung tumors revealed some trends that we did not anticipate. Expression increases with cancer stage in K-ras mutant tumors, but it does not necessarily increase with stage of non-small cell lung cancers overall. In addition, we found a trend of relatively high expression in squamous cell tumors, another subset of lung tumors that presently lacks targeted therapy. These findings are intriguing. Not only do they suggest that *sPLA₂IIa* may be a more sensitive biomarker for a subset of tumors, but small molecule inhibitors to *sPLA₂IIa* have already been trialed for inflammatory disease,¹¹ thus underscoring the possibility for rapid translational study in lung cancer.

Although *sPLA₂* inhibition significantly attenuated NF-κB activity in all lung cancer cell lines tested, cells

FIGURE 3. Group IIA secretory phospholipase A₂ (sPLA₂IIa) inhibition attenuates nuclear factor kappa-b (NF-κB) activity in all cell lines, with a variable effect on extracellular signal-regulated kinase (ERK) 1/2 activity. NF-κB attenuation does not correlate with sPLA₂IIa expression level. Both H2009 and H358 (white bar graphs) are mutant p53 and demonstrate a more modest decrease in NF-κB activity (vc, vehicle control). Representative Western blots with corresponding densitometry, mean ± SEM, **P* < .05, ***P* < .01, #*P* < .001, n = 3-5.

with mutant p53 had a less robust decrease in NF-κB activity. Other investigators have demonstrated a relationship between mutant p53 and increased baseline NF-κB activity.^{12,13} Our data corroborate this observation and further advance our understanding of the relationship between sPLA₂IIa and NF-κB by demonstrating that sPLA₂IIa inhibition has a more modest effect on the activity of NF-κB in cancer cells where baseline activity is high. Additional studies are needed to determine whether this observation has implications for the effect of sPLA₂IIa inhibition on downstream NF-κB targets such as metastasis-associated adhesion molecules and expression of inducible growth factors.^{5,14-17}

An interesting and unexpected finding from our tumor analysis was that sPLA₂IIa expression was increased in squamous tumors. Kupert and associates³ recently published strong sPLA₂IIa protein staining in non-small cell lung tumor cores compared with normal lung tissue, including 100% of squamous cell tumor cores, although they did not quantify differences between histologic subtypes.³ This trend requires focused investigation of more squamous tumors inasmuch as there are currently no targeted therapies for this subset of non-small cell lung cancers. Furthermore, there is potential to examine the relationship between specific genetic alterations in squamous tumors and the tumor promoting activity of sPLA₂IIa.¹⁸

FIGURE 4. Human lung tumors express group IIa secretory phospholipase A₂ (sPLA₂IIa). Primary lung tumors (n = 15), invasive esophageal adenocarcinoma (n = 1), lung metastasis from colon adenocarcinoma primary (n = 1). K-ras mutant adenocarcinomas (gray) express increasing levels of sPLA₂IIa with increasing stage. Squamous cell carcinomas (black) express higher levels of sPLA₂IIa. Samples that did not include K-ras, ALK, or epidermal growth factor receptor (EGFR) mutant status indicate patients that did not consent to mutation analysis. Messenger RNA evaluated by quantitative reverse-transcription polymerase chain reaction (RT-PCR), samples run in triplicate. LCNEC, Large cell neuroendocrine carcinoma; mut, mutant; wt, wild-type; s/p, status post; XRT, radiation therapy.

The pathways most frequently implicated in phospholipase-related tumor growth are predominantly inflammatory, including NF- κ B and the arachidonic acid-eicosanoid pathway.^{14,19,20} Mechanistic investigation of how sPLA₂IIa effects production of tumor-promoting eicosanoids, including its interactions with other phospholipase enzymes, is ongoing. In addition, sPLA₂IIa is a secreted molecule with potential autocrine and paracrine signaling capability.²¹ Ongoing studies aim to clarify the effect of sPLA₂IIa inhibition on sPLA₂IIa production and secretion with implications for understanding

the role of sPLA₂IIa in the tumor microenvironment to promote tumor progression and metastasis.

A limitation of this study is the use of a single small molecule inhibitor. We have previously presented comparable results with RNA interference of sPLA₂IIa, resulting in decreased tumor growth in an animal model.⁴ In addition, our tumor tissue analysis was limited owing to small sample size. Furthermore, we were only able to analyze mRNA secondary to the limited amount of tissue available to us. The analysis of RNA and protein in the lung cancer cell

lines mostly demonstrates a parallel relationship between sPLA₂ IIA mRNA transcript and sPLA₂IIa protein expression. Future studies will need to include an analysis of protein expression of sPLA₂IIa in tumors.

In summary, we have outlined the potential of sPLA₂IIa as a global target for lung cancer therapy—sPLA₂ inhibition is cytotoxic and potency correlates with baseline sPLA₂ expression. Examination of human lung tumors suggests sPLA₂IIa has particular relevance for K-ras mutant and squamous cell tumors, 2 lung cancer subsets that presently pose therapeutic challenges. These data contribute to growing evidence of a role for sPLA₂IIa in lung cancer that deserves further translational investigation.

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