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## 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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### Abstract

**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 A<sub>2</sub> (sPLA<sub>2</sub>IIa) is a potential biomarker and regulator of lung cancer cell invasion; however, the relationship between K-ras mutations and sPLA<sub>2</sub>IIa has yet to be investigated. We hypothesize that sPLA<sub>2</sub>IIa modulates lung cancer cell growth in K-ras mutant cells and that sPLA<sub>2</sub>IIa expression in human lung tumors is increased in K-ras mutant tumors.

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

**Results**—Cytotoxicity of sPLA<sub>2</sub>IIa inhibition correlates with sPLA<sub>2</sub>IIa expression. Apoptosis in response to sPLA<sub>2</sub> inhibition parallels attenuation in NF- $\kappa$ B activity. In addition, sPLA<sub>2</sub>IIa expression in human tumors correlates with squamous cell pathology and increasing stage of K-ras mutant lung tumors.

**Conclusions**—Baseline sPLA<sub>2</sub>IIa expression predicts response to sPLA<sub>2</sub>IIa 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 sPLA<sub>2</sub>IIa. These data support a role for sPLA<sub>2</sub>IIa as a potential global therapeutic target in the treatment of lung cancer.

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.<sup>1,2</sup>

Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) enzymes release arachidonic acid from cell membranes, initiating downstream production of tumor-promoting eicosanoids. Group IIa secretory phospholipase A<sub>2</sub> (sPLA<sub>2</sub>IIa), a subgroup of PLA<sub>2</sub>s, has recently been identified as a potential lung cancer biomarker.<sup>3</sup> In a cohort of patients with solitary pulmonary nodules, patients with lung cancer had higher plasma levels of sPLA<sub>2</sub>IIa than patients with a benign nodule. In addition, histologic analysis demonstrates overexpression of sPLA<sub>2</sub>IIa in lung tumors compared with normal lung tissue.<sup>3</sup> From a functional perspective, sPLA<sub>2</sub>IIa has been shown to regulate lung cancer cell growth and invasion. RNA interference of sPLA<sub>2</sub>IIa decreases tumor growth and nuclear factor kappa-b (NF- $\kappa$ B) activity in a xenograft model of lung cancer.<sup>4</sup> In addition, sPLA<sub>2</sub>IIa inhibition decreases intercellular adhesion molecule-1-associated invasion in vitro.<sup>5</sup>

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

## 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- $\alpha$ ) (Sigma-Aldrich, St Louis, Mo) was used at a concentration of 20 ng/mL to mimic the inflammatory milieu of the tumor microenvironment. The sPLA<sub>2</sub>IIa inhibitor S3319 (Sigma Aldrich) was dissolved in dimethyl sulfoxide. Di-methyl 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- $\kappa$ 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 sPLA<sub>2</sub>IIa antibody was from Abcam (Cambridge, Mass). For immunofluorescence, the rabbit anti-human sPLA<sub>2</sub>IIa 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 sPLA<sub>2</sub> inhibitor in serum-starved media for 1 hour, then stimulated with 20 ng/mL TNF- $\alpha$  for 8 hours. Control cells were incubated with dimethyl sulfoxide in serum-starved media and stimulated with TNF- $\alpha$  for 8 hours. Immunofluorescence microscopy was performed to evaluate sPLA<sub>2</sub>IIa expression in A549 cells as previously described.<sup>9</sup>

### Apoptosis Assay

We cultured  $1 \times 10^6$  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 sPLA<sub>2</sub>Ia inhibitor (20 or 40  $\mu\text{mol/L}$ ) 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  $5 \times 10^3$  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 sPLA<sub>2</sub>Ia inhibitor (10–40  $\mu\text{mol/L}$ ) 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  $3 \times 10^6$  in full media for 24 hours, then serum-starved (0.5% FBS) for 24 hours, and then treated. Time trials with TNF- $\alpha$  were performed to determine the time of maximal initial increase in phosphorylation of NF- $\kappa$ B and ERK 1/2 in all cell lines. Cells were pretreated with sPLA<sub>2</sub>Ia inhibitor (10–40  $\mu\text{mol/L}$ ) for 1 hour, stimulated with TNF- $\alpha$ , then lysed with Laemmli buffer under reducing conditions. In separate experiments, untreated cells were lysed and analyzed for baseline sPLA<sub>2</sub>Ia 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  $1 \times 10^6$  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: sPLA<sub>2</sub>Ia (forward): CCC-TCC-TAC-TGT-TGG-CAG-TGA-T; sPLA<sub>2</sub>Ia (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  $\Delta\Delta\text{Ct}$  method.<sup>10</sup>

### 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).

## RESULTS

### sPLA<sub>2</sub>Ia Expressed in K-ras Mutant Lung Cancer Cells

Group Ia sPLA<sub>2</sub> expression varies in K-ras mutant lung cancer cells. SW1573 and H2009 express more sPLA<sub>2</sub>Ia 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 sPLA<sub>2</sub>Ia Expression

sPLA<sub>2</sub>Ia inhibition led to concentration-dependent apoptosis and cell death in all K-ras mutant lung cancer cells (Figure 2). Cells with higher baseline sPLA<sub>2</sub>Ia were more susceptible to cell death with sPLA<sub>2</sub>Ia inhibition. Early apoptosis detected by annexin V staining after 8 hours of sPLA<sub>2</sub>Ia 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 sPLA<sub>2</sub>Ia inhibition, again with increased vulnerability in cells with higher sPLA<sub>2</sub>Ia expression (Figure 2, B).

### sPLA<sub>2</sub>Ia Inhibition in K-ras Mutants Decreases NF- $\kappa$ B Phosphorylation

Previous work has demonstrated that sPLA<sub>2</sub>Ia inhibition-associated apoptosis is related predominantly to decreased NF- $\kappa$ B activity.<sup>4</sup> We confirmed that NF- $\kappa$ B phosphorylation is decreased with sPLA<sub>2</sub>Ia inhibition in 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- $\kappa$ B compared with SW1573 and A549 cells. H2009 and H358 cells are both p53 mutant.

### Human Lung Tumors Express sPLA<sub>2</sub>Ia

Fifteen lung tumors were examined for sPLA<sub>2</sub>Ia mRNA expression. One metastatic colon adenocarcinoma and 1 invasive esophageal adenocarcinoma were included in analysis for comparison. Lung tumors express detectable levels of sPLA<sub>2</sub>Ia mRNA (Figure 4). Although squamous and K-ras mutant tumors are less common in our cohort, there is a trend toward increased expression of sPLA<sub>2</sub>Ia in squamous tumors. In K-ras mutant tumors there is evidence of increased sPLA<sub>2</sub>Ia expression with increasing stage. Both large cell neuroendocrine tumors express low levels of sPLA<sub>2</sub>Ia.

## DISCUSSION

This is the first study to specifically explore the role of sPLA<sub>2</sub>Ia in K-ras mutant lung cancer. Consistent with our hypothesis, sPLA<sub>2</sub>Ia is a cytotoxic target in K-ras mutant lung cancer cells. Our finding that in vitro sensitivity to sPLA<sub>2</sub>Ia inhibition correlates with

baseline sPLA<sub>2</sub>Ia expression, regardless of p53 mutation status, suggests that sPLA<sub>2</sub>Ia expression may predict response to sPLA<sub>2</sub>Ia inhibition. Analysis of sPLA<sub>2</sub>Ia 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 sPLA<sub>2</sub>Ia may be a more sensitive biomarker for a subset of tumors, but small molecule inhibitors to sPLA<sub>2</sub>Ia have already been trialed for inflammatory disease,<sup>11</sup> thus underscoring the possibility for rapid translational study in lung cancer.

Although sPLA<sub>2</sub> inhibition significantly attenuated NF- $\kappa$ B activity in all lung cancer cell lines tested, cells with mutant p53 had a less robust decrease in NF- $\kappa$ B activity. Other investigators have demonstrated a relationship between mutant p53 and increased baseline NF- $\kappa$ B activity.<sup>12,13</sup> Our data corroborate this observation and further advance our understanding of the relationship between sPLA<sub>2</sub>Ia and NF- $\kappa$ B by demonstrating that sPLA<sub>2</sub>Ia inhibition has a more modest effect on the activity of NF- $\kappa$ B in cancer cells where baseline activity is high. Additional studies are needed to determine whether this observation has implications for the effect of sPLA<sub>2</sub>Ia inhibition on downstream NF- $\kappa$ B targets such as metastasis-associated adhesion molecules and expression of inducible growth factors.<sup>5,14-17</sup>

An interesting and unexpected finding from our tumor analysis was that sPLA<sub>2</sub>Ia expression was increased in squamous tumors. Kupert and associates<sup>3</sup> recently published strong sPLA<sub>2</sub>Ia 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.<sup>3</sup> 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 sPLA<sub>2</sub>Ia<sup>18</sup>.

The pathways most frequently implicated in phospholipase-related tumor growth are predominantly inflammatory, including NF- $\kappa$ B and the arachidonic acid-eicosanoid pathway.<sup>14,19,20</sup> Mechanistic investigation of how sPLA<sub>2</sub>Ia effects production of tumor-promoting eicosanoids, including its interactions with other phospholipase enzymes, is ongoing. In addition, sPLA<sub>2</sub>Ia is a secreted molecule with potential autocrine and paracrine signaling capability.<sup>21</sup> Ongoing studies aim to clarify the effect of sPLA<sub>2</sub>Ia inhibition on sPLA<sub>2</sub>Ia production and secretion with implications for understanding the role of sPLA<sub>2</sub>Ia 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 sPLA<sub>2</sub>Ia, resulting in decreased tumor growth in an animal model.<sup>4</sup> 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 sPLA<sub>2</sub>Ia mRNA transcript and sPLA<sub>2</sub>Ia protein expression. Future studies will need to include an analysis of protein expression of sPLA<sub>2</sub>Ia in tumors.

In summary, we have outlined the potential of sPLA<sub>2</sub>Ia as a global target for lung cancer therapy—sPLA<sub>2</sub> inhibition is cytotoxic and potency correlates with baseline sPLA<sub>2</sub> expression. Examination of human lung tumors suggests sPLA<sub>2</sub>Ia 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 sPLA<sub>2</sub>Ia in lung cancer that deserves further translational investigation.

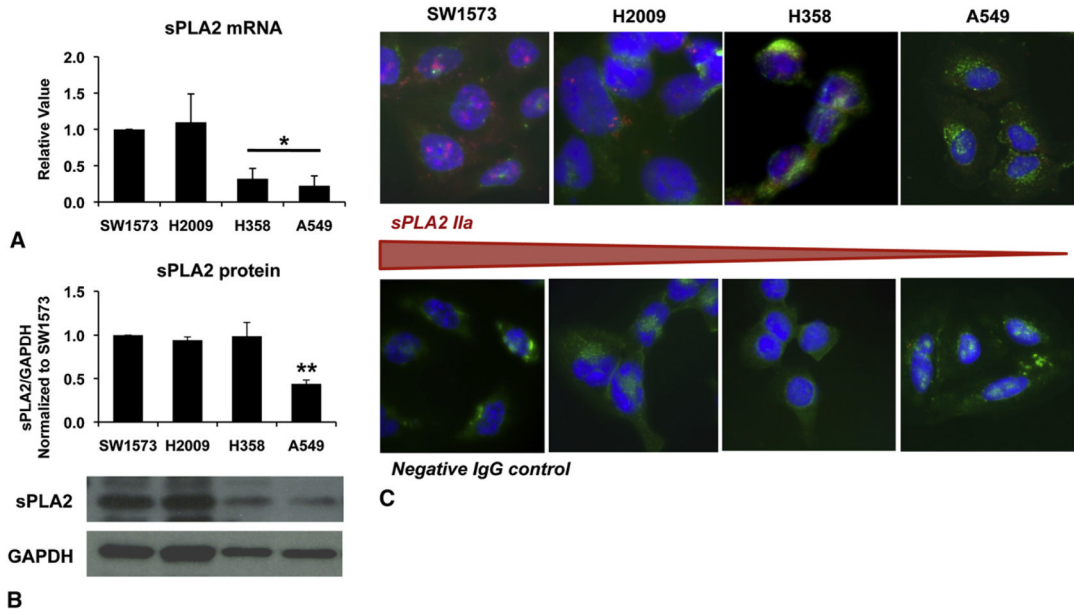
## Abbreviations and Acronyms

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

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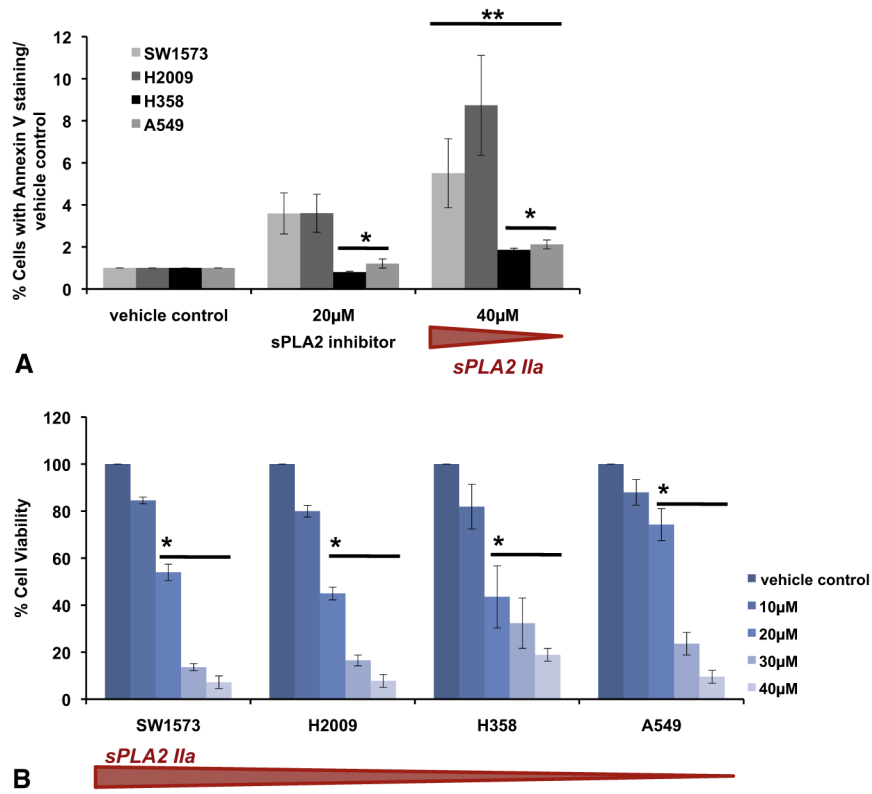
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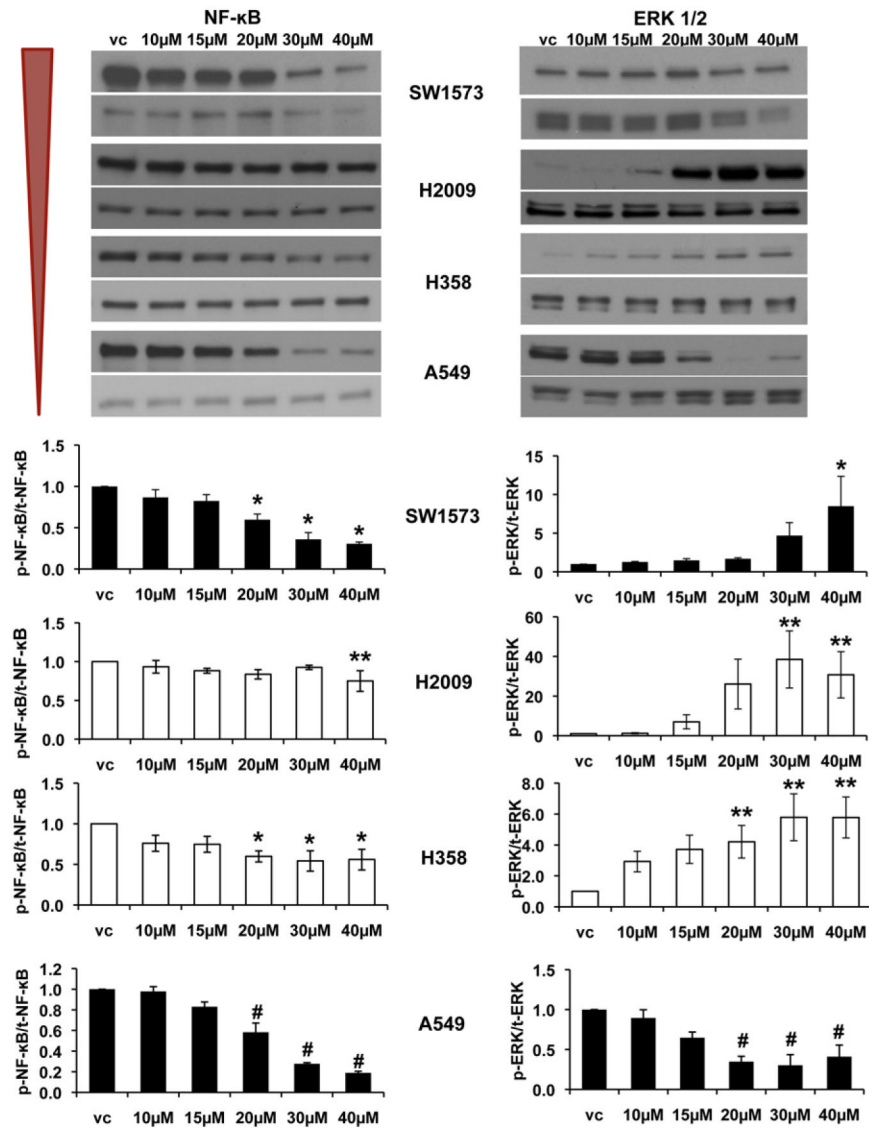
**FIGURE 1.**

K-ras mutant lung cancer cell lines analyzed for baseline group IIa secretory phospholipase A<sub>2</sub> (*sPLA<sub>2</sub>IIa*) expression. Expression varies with SW1573 and H2009 cells expressing more *sPLA<sub>2</sub>IIa* 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). *sPLA<sub>2</sub>IIa* protein expression was evaluated by Western blot. Graph of densitometry, mean ± SEM, \*\**P* < .005, n = 4 (B). Immunofluorescence for *sPLA<sub>2</sub>IIa* was used for additional protein, n = 3 run in duplicate, *sPLA<sub>2</sub>IIa* = red, cell membrane = green, nuclei = blue (C). *IgG*, Immunoglobulin G; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.



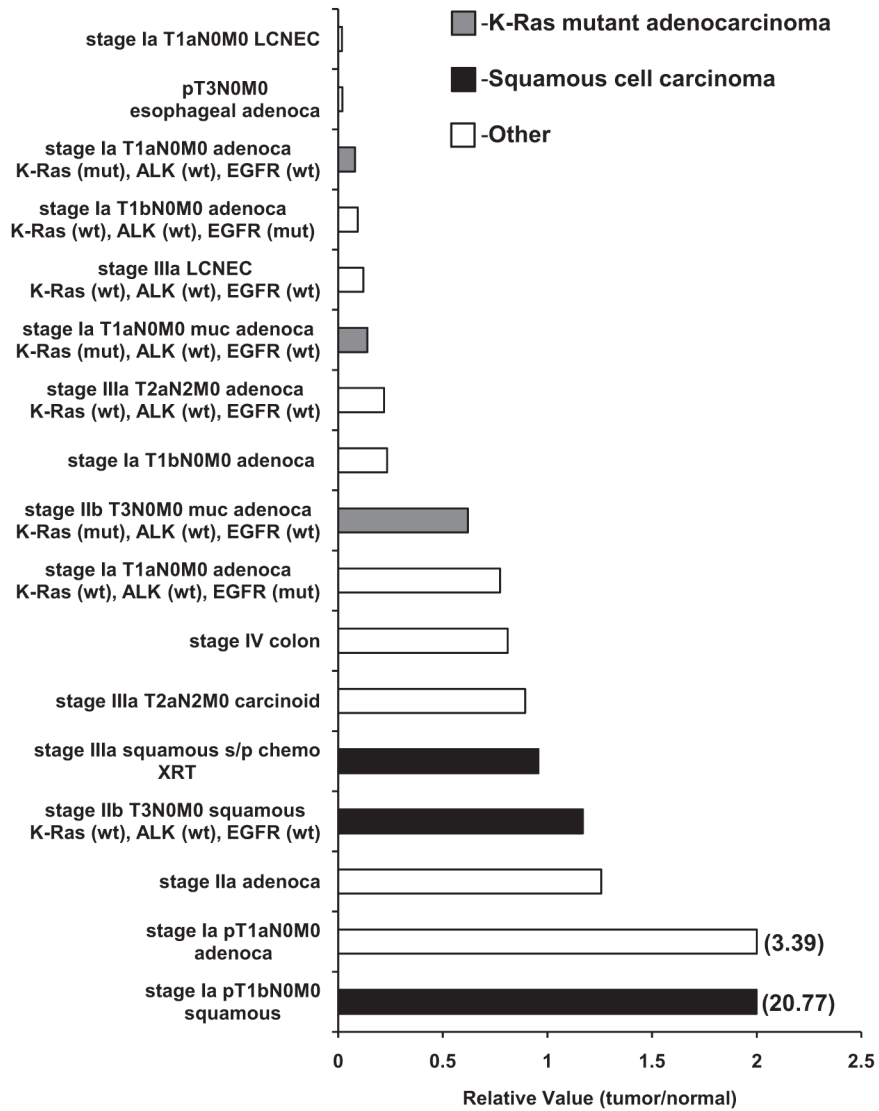


**FIGURE 2.** Group IIa secretory phospholipase A<sub>2</sub> (*sPLA<sub>2</sub>IIa*) expression inhibition promotes concentration-dependent apoptosis and cell death in lung cancer cell lines, and cytotoxicity of *sPLA<sub>2</sub>IIa* inhibition parallels the level of *sPLA<sub>2</sub>IIa* expression. 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 *sPLA<sub>2</sub>IIa* inhibition. LD50 at 24 hours increases as *sPLA<sub>2</sub>IIa* expression decreases, mean ± SEM, \**P*<.003, n =3 run in triplicate (B).



**FIGURE 3.** Group IIa secretory phospholipase A<sub>2</sub> (*sPLA<sub>2</sub>Ia*) 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 *sPLA<sub>2</sub>Ia* 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.

sPLA<sub>2</sub> IIa mRNA Expression in Lung Tumors



**FIGURE 4.** Human lung tumors express group IIa secretory phospholipase A<sub>2</sub> (sPLA<sub>2</sub>IIa). 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 sPLA<sub>2</sub>IIa with increasing stage. Squamous cell carcinomas (black) express higher levels of sPLA<sub>2</sub>IIa. 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.

**TABLE 1**

Cell line characteristics

Cell line	Tumor type	EGFR	K-ras	p53	NF-κB with sPLA <sub>2</sub> inhibition	ERK 1/2 with sPLA <sub>2</sub> inhibition	sPLA <sub>2</sub>
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
A-549	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; *sPLA<sub>2</sub>*, secretory phospholipase A<sub>2</sub>; *ERK*, extracellular signal-regulated kinase; *BAC*, bronchoalveolar carcinoma; *WT*, wild-type; *Mut*, mutant; *Adenoca*, adenocarcinoma.