Sphingosine kinase-1 Enhances Resistance to Apoptosis through Activation of PI3K/Akt/NF-κB Pathway in Human Non-small Cell Lung Cancer

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Translational Relevance

Clinical data have demonstrated that non-small cell lung cancer (NSCLC) is relatively insensitive to chemotherapy. Therefore, identification of minimally toxic targeting agents is urgent for the improvements of outcome of NSCLC. Herein, we report that upregulation of sphingosine kinase-1 (SPHK1) significantly inhibits Doxorubicin- or Docetaxel-induced apoptosis through activation of PI3K/Akt/NF-κB pathway. In contrast, ablation of SPHK1 expression or inhibition of SPHK1 kinase activity with specific inhibitor, SK1-I, significantly enhanced the sensitivity of NSCLC cells to apoptosis induced by chemotherapeutics both in vitro and in vivo. Taken together, our results suggested that SPHK1 targeting strategies might represent a new approach to sensitizing NSCLC to chemotherapy and improving the efficacy of anti-NSCLC treatment.
Abstract

Purpose: The present study was to examine the effect of sphingosine kinase-1 (SPHK1) on chemotherapeutics-induced apoptosis in non-small cell lung cancer (NSCLC) cells, which is relatively insensitive to chemotherapy, and its clinical significance in NSCLC progression.

Experimental Design: The correlation of SPHK1 expression and clinical features of NSCLC was analyzed in 218 paraffin-embedded archived NSCLC specimens by immunohistochemical analysis. The effect of SPHK1 on apoptosis induced by chemotherapeutics was examined both in vitro and in vivo, using Annexin V staining and TUNEL assays. Western blotting and luciferase analysis were performed to examine the impact of SPHK1 on the PI3K/Akt/NF-κB signaling.

Results: The expression of SPHK1 was markedly increased in NSCLC and correlated with tumor progression and poor survival of patients with NSCLC. Upregulation of SPHK1 significantly inhibited Doxorubicin- or Docetaxel-induced apoptosis, associated with induction of anti-apoptotic proteins Bcl-xl, c-IAP1, c-IAP2 and TRAF1. In contrast, silencing SPHK1 expression or inhibiting SPHK1 activity with specific inhibitor, SK1-I, significantly enhanced the sensitivity of NSCLC cells to apoptosis induced by chemotherapeutics both in vitro and in vivo. Moreover, we demonstrated that upregulation of SPHK1 activated the PI3K/Akt/NF-κB pathway, and that inhibition of the PI3K/Akt/NF-κB pathway abrogated the anti-apoptotic effect of SPHK1 on NSCLC cells.

Conclusions: Our results suggest that SPHK1 is a potential pharmacologic target for the treatment of NSCLC and inhibition of SPHK1 expression or its kinase activity might represent a novel strategy to sensitize NSCLC to chemotherapy.
Introduction

Lung cancer represents a leading cause of cancer-related mortality and morbidity worldwide, with more than 1.23 million new cases each year and the five-year survival rate lower than 15% (1). Two major types of lung cancer, small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC), account for 90 percent of cases (2). Of the NSCLC subtypes, the most common ones include squamous cell carcinoma, large cell carcinoma and adenocarcinoma, with other subtypes occurring at relatively lower frequencies (3). Currently available treatment strategies for NSCLC, including surgery, radiotherapy, chemotherapy and photodynamic therapy, remain generally unsuccessful (4). The five-year survival rate for patients with stage IV NSCLC is approximately 1%, with the median survival time of 7 months (5, 6). While SCLC usually reveals better responsiveness to chemotherapy and radiation, NSCLC is relatively insensitive to both therapeutic modalities (7). Hence, identification of novel targets for more effective anti-NSCLC strategies with minimal toxicity is urgent.

Enzyme sphingosine kinase-1 (SPHK1) has been demonstrated to play a role in oncogenesis, and its biological function is associated with maintaining the balance between pro-survival and apoptotic signaling (8). Accumulated evidence has associated SPHK1 upregulation with development of malignant phenotypes of various cancers, such as proliferation, anti-apoptosis, migration, angiogenesis and invasion (9-11). In 2000, Xia and colleagues first reported the oncogenic potential of SPHK1, namely, overexpressing SPHK1 induced malignant transformation of NIH3T3 cells (12). The biological function of SPHK1 in protecting cancer cells from apoptosis induced by TNF-α, ionizing radiation or anti-cancer drugs, due to increased ceramide levels, has been documented (11,13). Bonhoure and colleagues demonstrated that ectopic overexpression of SPHK1 conferred HL-60 leukemia cells resistance to doxorubicin and etoposide-induced cell death (14). Moreover, SPHK1 has been shown to act as a sensor during Imatinib-induced apoptosis of chronic myeloid leukemia (CML) cells...
and protect LAMA84 cells from death by blocking the mitochondrial release of cytochrome c and Smac/Diablo (15). The resistance to camptothecin or Docetaxel in prostate cancer cells was also associated with stimulation of SPHK1 activity (16). The above reports support a potential application of SPHK1 as a therapeutic anti-cancer target.

Our current study found that ablation of SPHK1 or inhibition of SPHK1 activity by specific inhibitor, SK1-I, significantly sensitized NSCLC cells to apoptosis induced by chemotherapeutics both in vitro and in vivo. We also demonstrated that the anti-apoptotic effect of SPHK1 on NSCLC cells was associated with the activation of the NF-κB and the PI3K/Akt pathways. Our findings suggest that inhibition of SPHK1 might represent a novel approach to the treatment of NSCLC.
Materials and Methods

Cell lines. Primary normal lung epithelial cells (NLEC) were established according to a previous report (17) and cultured in the Keratinocyte serum-free medium (Invitrogen, Carlsbad, CA) supplemented with epithelial growth factor and bovine pituitary extract. Lung cancer cell lines, namely, A549, H1299, PAa, 95D and HLAMP, were maintained in DMEM medium (Invitrogen Co., Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT).

Vectors and retroviral infection. An SPHK1 expression construct was generated by subcloning PCR-amplified full-length human SPHK1 cDNA into the pMSCV plasmid. For depletion of SPHK1, two human SPHK1-targeting siRNA sequences were cloned into pSuper-retro-puro to generate pSuper-retro-SPHK1-RNAi(s), respectively, and the sequences are RNAi#1: GGCTGAAAT CTCCTTCACG; RNAi#2: GGGCAAGGCCTTGCAGCTC. pNF-κB-luc and control plasmids (Clontech, Mountain View, CA) were used to quantify NF-κB activity. pBabe-Puro-IκBα-mut (plasmid 15291) expressing mutant IκBα was from Addgene (Cambridge, MA, USA). Retroviral production and infection were performed as previously described (18). Stable cell lines expressing SPHK1 or SPHK1 shRNAs were selected for 10 days with 0.5μg/ml puromycin.

Patients and tissue specimens. Paraffin-embedded, archived NSCLC samples were obtained from 218 patients diagnosed with NSCLC between January 2000 and October 2002 at the Department of Pathology and Cancer Center at Sun Yat-sen University. The histological characterization and clinicopathological staging of the samples were determined according to the WHO criteria (3) and current International Union Against Cancer (UICC) tumor–node–metastasis (TNM) classification (19). Detailed clinical information of all patients is summarized in Supplementary Table S1. Percentage tumor purity in sections adjacent to
regions used for RNA extraction was estimated during routine histopathologic analysis.

**Immunoblotting (IB).** Western blotting was performed according to standard methods as described previously (20), using anti-SPHK1 rabbit polyclonal antibody recognizing sphingosine kinase 1 isoform 2 (Q9NYA1, MW 42.5 kDa) (catalog # AP7237c, Abgent Inc., San Diego, CA); anti-Akt, anti-p-Akt, anti-IKK, anti-p-IKKα/β, anti-IκBα, anti-p-IκBα, anti-cleaved Caspase 3, anti-PARP, anti-Bcl-xL, anti-c-IAP1, anti-c-IAP2, anti-TRAFl, anti-BCL-2, anti-FLIP and anti-Bim antibodies (Cell Signaling, Danvers, MA); The membranes were stripped and re-probed with an anti-α-tubulin Ab (Sigma, Saint Louis, MI) as a loading control.

**Immunohistochemistry (IHC).** Immunohistochemical analysis was performed to study altered protein expression in 218 human NSCLC tissues. The procedures were carried out similarly to previously described methods (21). The degree of immunostaining of formalin-fixed, paraffin-embedded sections was viewed and scored separately by two independent investigators, who were blinded to the histopathological features and patient data of the samples, and the scores were determined by combining the proportion of positively stained tumor cells and the intensity of staining. Scores given by the two independent investigators were averaged for further comparative evaluation of the SPHK1 expression. The proportion of positively stained tumor cells was graded as follows: 0 (no positive tumor cells), 1 (<10% positive tumor cells), 2 (10–50% positive tumor cells) and 3 (>50% positive tumor cells). The cells at each intensity of staining were recorded on a scale of 0 (no staining), 1 (weak staining, light yellow), 2 (moderate staining, yellowish brown) and 3 (strong staining, brown). The staining index (SI) was calculated as follows: staining index = staining intensity \times proportion of positively stained tumor cells. Using this method of assessment, we evaluated the expression of SPHK1 in NSCLC by SI (scored as 0, 1, 2, 3, 4, 6 or 9). Cutoff values to define the high- and low-expression of SPHK1 were chosen on the basis of a measure...
of heterogeneity with the log-rank test statistics with respect to overall survival. An optimal cutoff value was identified. The SI score of ≥ 6 was used to define tumors with high expression, and SI ≤ 4 as tumors with low expression of SPHK1.

**Luciferase reporter assay for NF-κB transcriptional activity.** Cells were seeded in triplicates in 6-well plates (50,000 cells/well) and allowed to settle for 12 h. One hundred nanogram of pNF-κB-luciferase plasmid or control-luciferase plasmid plus 10 ng pRL-TK renilla plasmid (Promega, Madison, WI) were transfected into NSLCL cells using the Lipofectamine 2000 reagent (Invitrogen Co., Carlsbad, CA). Medium was replaced after 6 h, and luciferase and renilla signals were measured 48 h after transfection using the Dual Luciferase Reporter Assay Kit (Promega, Madison, WI) according to a protocol provided by the manufacturer.

**Xenografted tumor model and anti-tumor effect of SK1-I in vivo.** Female BALB/c-nu mice (4–5 weeks of age, 18-20g) were purchased from the Center of Experimental Animal of Guangzhou University of Chinese Medicine, and were housed in barrier facilities on a 12 h light/dark cycle. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University. The BALB/c nude mice were randomly divided into three groups (n=5/group). One group of mice were inoculated subcutaneously with A549/vector cells (1x10^6, suspended in 100 μl sterile PBS) per mouse in the right oxter as control group. The other two groups were inoculated with A549/SPHK1 and A549/SPHK1 RNAi#1 cells (1x10^6, suspended in 100μl sterile PBS), respectively. Tumor volume was calculated using the equation (L*W^2)/2. In the experiment testing the anti-tumor effect of SK1-I, the BALB/c nude mice (4–5 weeks of age, 18-20 g) were implanted subcutaneously with A549 cells (2x10^6, suspended in 100 μl sterile PBS) in order to rapidly induce exponentially growing tumors. When tumors reached a volume of 50 to 100 mm^3, animals
were randomly assigned to three groups \((n=5/{\text{group}})\), followed by intraperitoneal injection of 100 μl vehicle (DMSO), SK1-I (50 mg/kg) or Docetaxel (10 mg/kg), respectively, on days 1, 5, 9, 11, 13 and 15. On day 17, animals were euthanized, and tumors were excised, weighed and subjected to pathological examination.

**Statistical analysis.** All statistical analyses were carried out using the SPSS 13.0 statistical software package. Comparisons between groups for Statistical Significance were performed with a two-tailed paired Student’s t test. The chi-square test was used to analyze the relationship between SPHK1 expression and clinicopathologic characteristics. Survival curves were plotted using the Kaplan-Meier method and compared by the log-rank test. Survival data were evaluated using univariate and multivariate Cox regression analyses. \(P<0.05\) was considered statistically significant in all cases.
Results

**SPHK1 expression is associated with the clinical features of NSCLC**

Western blotting and real-time PCR analysis revealed that both protein and mRNA expressions of SPHK1 were markedly upregulated in multiple NSCLC cell lines, including A549, 95D, PAa, H-1299 and HLAMP, in comparison with those in two collections of NLEC (Fig. 1A and Supplementary Fig. S1A). In 8 NSCLC tissue samples (T), SPHK1 expression revealed >2-fold increases compared to that in the paired normal adjacent-nontumorous tissues (ANT), with each pair derived from a same patient, and SPHK1 upregulation in these clinical NSCLC samples was further confirmed by IHC analysis (Figs. 1B, 1C and Supplementary Fig. S1B and S1C). Taken together, our results indicated that SPHK1 was an enzyme upregulated in NSCLC.

Further evaluation of SPHK1 expression in paraffin-embedded, archived clinical tumor tissue specimens obtained from 218 cases of NSCLC, using IHC analysis with an antibody against human SPHK1, showed that the level of SPHK1 protein significantly increased in all types of NSCLC (Fig. 2A) and strongly correlated with clinical staging ($P = 0.019$), T classification ($P = 0.022$), N classification ($P = 0.010$) and M classification ($P = 0.027$) of NSCLC patients (Supplementary Table S2). Log-rank and Kaplan-Meier analysis tests demonstrated that overall survival time of patients with high expression of SPHK1 in tumors was significantly shorter than that of the low SPHK1 expression NSCLC group ($P <0.001$)(Fig. 2B). Furthermore, Multivariate survival analysis indicated that SPHK1 expression level was an independent prognostic factor for the assessment of patient outcomes (Supplementary Table S3 and S4). Moreover, the prognostic value of SPHK1 expression in different pathological types of NSCLC patients was examined. An adverse correlation between high SPHK1 expression in tumors and overall patient survival was clearly detected in all three NSCLC subtype groups, namely, squamous cell carcinoma ($n=82$; $P<0.001$, log-rank; Fig. 2C, left
panel), adneocarcinoma (n=91; \( P < 0.01 \), log-rank; Fig. 2C, middle panel) and adenosquamous carcinoma (n= 42; \( P < 0.05 \), log-rank; Fig. 2C, right panel). Collectively, these data demonstrated that SPHK1 expression was linked to the clinical progression of NSCLC and might represent a valuable prognostic marker generally for NSCLC patients and specifically for patients with major pathological types of NSCLC.

**SPHK1 plays an important anti-apoptotic role in NSCLC in vivo**

The above finding that SPHK1 expression was significantly associated with the progression of NSCLC prompted us to ask whether SPHK1 might play a role in NSCLC pathogenesis and therefore could be a novel therapeutic target. To this end, a panel of A549 NSCLC cell lines were constructed to stably express either SPHK1 cDNA (A549/SPHK1) or SPHK1 shRNAs (A549/SPHK1 shRNA) (Fig. 3A) and inoculated in nude mice. After the tumor-bearing mice were treated intraperitoneally with Docetaxel (10mg/kg), a clinically commonly used chemotherapeutic agent against NSCLC, as shown in Fig. 3B and Supplementary Fig. S2A, the volumes and weights of tumors formed by the A549/SPHK1 cells were significantly larger than those of vector-control cells-formed tumors (n=5, \( P < 0.01 \); \( P < 0.01 \)). Whereas, depletion of endogenous SPHK1 in A549 cells caused significant inhibition of tumor growth in terms of both tumor volume and weight (n=5, \( P < 0.01 \); \( P < 0.01 \); Fig. 3B and Supplementary Fig. S2A). Moreover, we also examined the effect of a specific SPHK1 inhibitor SK1-I on the growth of xenografted NSCLC tumors. As shown in Fig. 3C and Supplementary Fig. 2B, SK1-I treatment dramatically reduced the tumor size and weight as compared to those treated with vehicle control (n= 5, \( P < 0.01 \); \( P < 0.01 \)). It is noteworthy that no significant difference was detected in the body weights among nude mice in the three groups (Data not shown).

Collectively, our results suggest that SPHK1 may play an important role in the growth of NSCLC in vivo, and that targeting SPHK1 through silencing its expression or suppressing its enzyme activity may represent a potentially effective strategy against NSCLC, either alone or
in combination with chemotherapy.

Since clinical failure of chemotherapy is commonly and biologically attributable to the resistance of cancer cells to apoptosis, we further investigated whether SPHK1 enhances the anti-apoptotic activity of NSCLC cells \textit{in vivo}. As shown in Fig.3D, TUNEL assay demonstrated that Docetaxel induced far lower and higher degree of apoptosis in tumors derived from A549/SPHK1- and A549/SPHK1 shRNA-NSCLC cells, respectively, than in the A549/vector control tumors. Also noteworthy is that SK1-I treatment markedly elevated the number of apoptotic cells induced by Docetaxel treatment. Taken together, our data suggested that SPHK1 may confer NSCLC cells resistance to Docetaxel-induced apoptosis and thus might represent a target for improving the efficacy of anti-NSCLC chemotherapy.

**Dysregulation of SPHK1 alters the sensitivity of NSCLC cells to apoptosis**

To further understand and characterize the anti-apoptotic activity of SPHK1 in NSCLC cells, \textit{in vitro} studies were performed using NSCLC cell lines with SPHK1 overexpressed or silenced. As shown in Supplementary Fig. S3A, SPHK1-overexpressing 95D and A549 NSCLC cells displayed significantly higher survival than the vector-control cells after treatment of various doses of Doxorubicin or Docetaxel, while the number of dead cells markedly increased when SPHK1 expression was silenced by specific shRNA. Annexin V-binding and TUNEL assays showed that SPHK1 upregulation conferred NSCLC cells resistance, and SPHK1 downregulation drastically enhanced their sensitivities, to chemotherapeutics (Figs. 4A, 4B and Supplementary Fig.S3B,S3C). Furthermore, the noted effect of SPHK1 on the apoptosis was confirmed as decreased activating cleavages of PARP and caspase-3 induced by Doxorubicin or Docetaxel in SPHK1-overexpressing cells and a contrary effect in SPHK1-knocked down cells (Fig.4C and Supplementary Fig.S3D).

**Upregulation of SPHK1 activates NF-κB and PI3K/Akt pathways**
To further investigate the molecular mechanism mediating the anti-apoptotic effect of SPHK1, the levels of apoptosis relators Bcl-xl, CIAP1, CIAP2, FLIP, TRAF1, Bcl-2 and Bim were examined. Western blotting and real time-PCR analysis revealed that at both mRNA and protein levels, anti-apoptotic factors Bcl-xl, CIAP1, CIAP2 and TRAF1 were significantly upregulated in the SPHK1-transduced A549 and 95D NSCLC cells and downregulated in SPHK1-knocked down cells, as compared with those in control cells, respectively (Fig.5A and Supplementary Fig.S4). As the above identified upregulated anti-apoptotic genes are known downstream targets of NF-κB, we further tested whether the NF-κB activity was modulated by SPHK1. NF-κB reporter assay showed that SPHK1 upregulation significantly increased, and by contrast, downregulation of SPHK1 attenuated, NF-κB transcriptional transactivating activity in A549 and 95D cells (Fig.5B, left panel). Consistently, the transactivation activity of NF-κB decreased in SK1-I-treated cells as compared with that in control cells (Fig. 5B, right panel). Western blotting analysis revealed that phosphorylation of IκBα and IKK significantly increased in SPHK1-overexpressing NSCLC cells and reduced in SPHK1-knocked down cells (Fig.5C, left panel) and in SK1-I-treated cells dose-dependently (Fig.5C, right panel), indicating that the effect of SPHK1 on apoptosis resistance mainly due to the SPHK1 activity. Meanwhile, we found that overexpressing SPHK1 drastically increased and knockdown SPHK1 reduced the cellular S1P, which produced by SPHK1, in both A549 and 95D NSCLC cells (Supplementary Fig. S5). Moreover, Annexin V-binding and TUNEL assays showed that Doxorubicin- or Docetaxel-induced apoptosis of SPHK1-overexpressing cells dramatically increased when the activity of NF-κB was blocked by IκBα super-repressor (IκBα mu), by treatment of NF-κB inhibitor (JSH-23), or by IKK inhibitor (Wedelolactone), indicating that the IKK/IκBα/NF-κB pathway was essential for the anti-apoptotic function of SPHK1 in NSCLC cells (Fig.5D).
As the IKK/IκBα/NF-κB pathway could be activated by Akt (22), we then examined whether SPHK1 activated PI3K/Akt signaling. As shown in Fig.6A, the level of phosphorylated Akt was indeed elevated in SPHK1-overpressing NSCLC cells in comparison with that in the control cells. Whereas, SPHK1 downregulation or SPHK1 inhibition by SK1-I significantly decreased the phosphorylation level of Akt (Fig.6, A and B). Furthermore, Western blotting analysis showed that the observed increases of phosphorylated IκBα and IKK by overexpressing SPHK1 could be reversed by treatment of PI3K inhibitor LY294002 or a specific Akt inhibitor (Akt inhibitor X), indicating that SPHK1-mediated IKK/IκBα/NF-κB activation might be through PI3K/Akt pathway (Fig.6C). Moreover, SK1-I inhibition of SPHK1 significantly enhanced apoptosis of NSCLC cells induced by Doxorubicin or Docetaxel. However, apoptosis only slightly increased when the NSCLC cells were treated with SK1-I combined with PI3K inhibitor or Akt inhibitor, suggesting that activation of PI3K/Akt was essential for the SPHK1-mediated protection against apoptosis (Fig.6D). Taken together, our results indicated that the PI3K/Akt pathway may play a role in SPHK1-mediated activation of NF-κB and prevention of apoptosis in NSCLC cells.
Discussion

The key findings made in our present study are that progression of human NSCLC is related to an increase of SPHK1 expression, and that SPHK1 upregulation sustains NSCLC cell survival and inhibits their sensitivity to apoptosis inducers. We also demonstrated that inhibiting SPHK1 activity with specific SPHK1 inhibitor or downregulating SPHK1 with RNAi might represent a novel strategy for the treatment of NSCLC.

Increased resistance to apoptosis is a hallmark alteration in most types of cancers (23). Abrogation of pro-apoptotic pathways has been demonstrated to be one of the events key to tumor development and progression, and impairments in apoptotic programming are tightly linked to the commonly seen failure of anti-cancer chemotherapy and radiotherapy. Thus, clarification of the mechanisms modulating the apoptosis/survival process in a particular cancer type will bring new insights in developing more effective therapeutic strategies (24-26). Notably, in the current study, we found that SPHK1 plays an important role in anti-apoptosis of NSCLC that is relatively insensitive to chemotherapy, both in vivo and in vitro. Ectopic expression of SPHK1 in NSCLC cells dramatically enhances their resistance to apoptosis induced by Doxorubicin or Docetaxel, two commonly used chemotherapeutics. Whereas, suppressing SPHK1 expression with shRNAs or inhibiting SPHK1 activity with a specific SPHK1 inhibitor, SK1-I, markedly abrogated the ability of NSCLC cells to resist cytotoxic reagent-induced cell death, suggesting that SPHK1 activity contributes to sustaining the unwanted survival of NSCLC cells under the treatment of chemotherapeutics.

The balance between lipid mediators, such as Sphingosine-1-phosphate (S1P), sphingosine and ceramide, has been considered as a cellular converter determining cell fate (27). Thus, key enzymes in this context, such as SPHK1 and SPHK2, which regulate the S1P/ceramide conversion that contributes to determining whether a cell proliferates or undergoes apoptosis, could be potential targets for new anticancer drugs. Recently, Kevin and colleagues screened
a library of synthetic compounds using recombinant human SK1 as a bait and identified a panel of inhibitors of SPHK1, including SK1-I (28). Prompted by the concern that NSCLC is generally insensitive to currently available chemotherapeutic agents, we asked whether SPHK1 plays a role in conferring NSCLC cells resistance to chemotherapeutics-induced apoptosis during the treatment of NSCLC and thereby could be a target for the treatment of NSCLC. Strikingly, inhibition of SPHK1 activity by SK1-I markedly sensitizes NSCLC cells to the pro-apoptotic effect of chemotherapeutics. Robustly, intraperitoneal injection of SK1-I potently enhanced the tumor suppression effect of Docetaxel, a clinically well established pro-apoptotic chemotherapy drug against breast, ovarian and non-small cell lung cancers (29, 30). Notably, the unwanted toxicity of SK1-I appeared to be low as the body weight of treated mice was well kept and other signs of toxicity seemed to be absent in our study, despite that the exact toxicity profiles of SPHK1 targeting strategies are yet to be pharmaceutically and clinically determined.

It has been demonstrated that NF-κB plays important roles in the development of malignant phenotypes through multiple signaling pathways, and aberrant activation of NF-κB has been observed in variety of cancer types (31). A prominent mechanism linking NF-κB signaling to cancer progression is the abrogation of apoptosis (32). Numerous anti-apoptotic proteins, such as Bcl-xL, c-IAPs, TRAFs and c-FLIP6, are transcriptionally regulated by the NF-κB (32, 33). Our current study found that several NF-κB regulated anti-apoptotic proteins were upregulated in SPHK1-overexpressing NSCLC cells and downregulated in SPHK1-knocked down or -inhibited NSCLC cells, and that the transactivating activity of NF-κB could be stimulated by SPHK1 upregulation and suppressed by SPHK1 inhibition. On the other hand, while the IKK/IκB/NF-κB axis is subjected to activation by a variety of distinct upstream signals, the data we obtained thus far suggests an involvement of the PI3K/Akt pathway, the activation of which requires contribution of lipid mediators. Indeed, suppression of PI3K/Akt...
signaling in NSCLC cells had little, if any, enhancing effect on SK1-I caused apoptosis, and such a lack of additive effect between SPHK1 inhibition and PI3K inhibition suggests a seemingly linear relation of the two signaling molecules along the action axis. Apparently, the molecular mechanism underlying SPHK1 mediated-activation of PI3K/Akt pathways, as well as its biological outcome, needs to be further delineated. In addition, several other issues also remain to be addressed. For example, it would be of great interest to know whether other pathways are also involved in mediating the anti-apoptotic effect of SPHK1 in NSCLC cells and what other malignant phenotypes of NSCLC cells could also be modulated by upregulated SPHK1. These issues are under further investigation in the laboratory. Nevertheless, understanding the role of SPHK1 in NSCLC progression will not only advance our knowledge of the mechanisms underlying NSCLC survival, but also will help establish SPHK1 as a potential therapeutic target for the treatment of NSCLC.

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Conflict of Interest
The authors declare that they have no conflict of interest related to this work.
References


Figure Legends

Figure 1. Expression of SPHK1 is elevated in NSCLC. (A) Expression of SPHK1 protein in two NLEC collections and cultured NSCLC cell lines (A549, HLAMP, PAa, H-1299 and 95D), using α-tubulin as loading control. (B and C) Western blotting (B) and IHC (C) analysis of SPHK1 protein in primary NSCLC (T) and paired adjacent non-cancerous lung tissues (ANT). Expression levels were normalized with α-tubulin.

Figure 2. Overexpression of SPHK1 in archived NSCLC specimens. (A) Representative IHC images of SPHK1 expression in normal lung tissue and NSCLC specimens of different subtypes. (B) Survival curves of NSCLC patients with low- versus high-expression of SPHK1 (n=218; P<0.001, log-rank test). (C) Statistical significance of the difference between curves for patients with high- and low-SPHK1 expression within subgroups of squamous cell carcinoma (n= 82, P<0.001; left panel), adenocarcinoma (n =91, P<0.01; middle panel) and adenosquamous carcinoma (n=42, P<0.05; right panel).

Figure 3. The impact of SPHK1 expression on tumor growth in vivo. (A) Expression of SPHK1 was examined in indicated cells. α-Tubulin was used as a loading control. (B) Tumor volumes measured on the indicated days. Indicated cells (2x10^6) were injected s.c. in nude mice. When the mean tumor volume reached 50 mm^3, mice were injected i.p. with Docetaxel (10mg/kg) every 2 days for 21 days. Data points are presented as the mean tumor volume ± SD. (C) Tumor volumes were measured on the indicated days. A549-vector cells (2x10^6) were injected in the right oxter of 15 nude mice. When the mean tumor volume reached 50mm^3, mice were randomized into three groups (n= 5) and injected i.p. with vehicle (DMSO), Docetaxel (10mg/kg) or SK1-I (50mg/kg) every 2 days for 17 days. Data points are presented as the mean tumor volume ± SD. (D) Representative immunofluorescent images (left panel) and quantification of (right panel) of TUNEL-stained cells in indicated tumors. The numbers of TUNEL-positive cells were counted from 10 random fields and presented as...
percentages of total cell numbers. *, $P < 0.05$.

**Figure 4.** Anti-apoptotic effect of SPHK1 in NSCLC in vitro. (A) Representative immunofluorescent images (left panel) and quantification (right panel) of TUNEL staining in vector-, SPHK1- or SPHK1 shRNA-transduced A549 and 95D cells after Docetaxel treatment (5 nM) for 24 hr. Numbers are cells counted from 10 random fields. **, $P < 0.01$. (B) Immunofluorescent images (left panel) and quantification of (right panel) of Annexin V/PI staining of vector-, SPHK1- or SPHK1 shRNA-transduced A549 and 95D cells after Docetaxel treatment (5nM) for 6 hr. Numbers are cells counted from 10 random fields. **, $P < 0.01$. (C) Western blotting for proteolytic cleavage of pro-caspase-3 and PARP in vector-, SPHK1- or SPHK1 shRNA-transduced A549 and 95D cells after Docetaxel treatment (5 nM) for 24 hr, using $\alpha$-tubulin as loading control.

**Figure 5.** SPHK1-mediated protection of apoptosis through activation of NF-κB and PI3K/Akt pathways. (A) Western blotting of Bcl-xL, c-IAP1, c-IAP2, FLIP, TRAF1, Bcl-2 and Bim in indicated cells, using $\alpha$-tubulin as loading control. (B) The effect of SPHK1 expression (left panel) and activity (right panel) on NF-κB activity in NSCLC cells, analyzed by luciferase reporter assay. *, $P < 0.05$. (C) Western blotting for phosphorylated IKK (p-IKK), total IKK, phosphorylated IκBα (p-IκBα) and total IκBα in vector-, SPHK1- or SPHK1 shRNA-transduced A549 and 95D cells (left panel) or in the SK1-I treated-A549/SPHK1 and -95D/SPHK1 cells (right panel), using $\alpha$-tubulin as a loading control. The numbers under panel of the p-IκBα expression are quantification analyses of p-IκBα / IκBα ratio. (D) Quantification of TUNEL-positive (right panel) and Annexin V+/PI− (left panel) A549/SPHK1 and -95D/SPHK1 cells treated with Doxorubicin or Docetaxel combined with an IκB mutant (IκBmu), NFκB inhibitor JSH-23 (30 μM, NF-κB in), IKK inhibitor Wedelolactone (100 μM, IKK in). *, $P < 0.05$. Error bars represent SD from three
independent experiments

**Figure. 6. Upregulation of SPHK1 activates the PI3K/Akt pathway.**  (A and B) Western blotting for phosphorylated Akt (p-Akt), total Akt, phosphorylated GSK-3β (p-GSK-3β) and total GSK-3β levels in vector-, SPHK1- or SPHK1 shRNAi-transduced A549 and 95D cells (A) or in SK1-I treated-A549/SPHK1 or -95D/SPHK1 cells (B), using α-tubulin as loading control.  (C) Western blotting for phosphorylated IKK (p-IKK), total IKK, phosphorylated IκBα (p-IκBα) and total IκBα in A549/SPHK1 and-95D/SPHK1 cells treated by vehicle (DMSO), PI3K inhibitor LY294002, or a specific Akt inhibitor (Akt inhibitor X), using α-tubulin as loading control.  (D) Quantification of Annexin V+/PI- (right panel) or TUNEL-positive (left panel) A549/SPHK1 or 95D/SPHK1 cells treated with chemotherapeutics agent Doxorubicin or Docetaxel in combination with vehicle (DMSO), LY294002, Akt inhibitor X, Akt inhibitor X plus SK1-I, or LY294002 plus SK1-I.  *, $P < 0.05$.  Error bars represent SD from three independent experiments.
Figure 1

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C

[Images of tissue sections for each patient]
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Libing Song, Huaping Xiong, Mengfeng Li, et al.

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