

Overexpression of secretory phospholipase A2-IIa supports cancer stem cell phenotype via HER/ERBB-elicited signaling in lung and prostate cancer cells

SHAN LU and ZHONGYUN DONG

Department of Medicine, University of Cincinnati College of Medicine, Cincinnati, OH 45267, USA

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Abstract. Resistance to conventional chemotherapies remains a significant clinical challenge in treatment of cancer. The cancer stem cells (CSCs) have properties necessary for tumor initiation, resistance to therapy, and progression. HER/ERBB-elicited signaling supports CSC properties. Our previous studies revealed that secretory phospholipase A2 group IIa (sPLA2-IIa) is overexpressed in both prostate and lung cancer cells, leading to an aberrant high level in the interstitial fluid, i.e., tumor microenvironment and blood. HER/ERBB-PI3K-Akt-NF- κ B signaling stimulates sPLA2-IIa overexpression, and in turn, sPLA2-IIa activates EGFR family receptors and HER/ERBB-elicited signaling and stimulates sPLA2-IIa overexpression in a positive feedback manner. The present study determined the molecular mechanisms of sPLA2-IIa in stimulating HER/ERBB-elicited signaling and supporting CSC properties. We found that sPLA2-IIa binds both EGFR and HER3 demonstrated by co-immunoprecipitation experiments and also indirectly interacts with HER2, suggesting that sPLA2-IIa functions as a ligand for both EGFR and HER3. Furthermore, both side population CSCs from non-small cell lung cancer (NSCLC) A549 and H1975 cells and ALDH1-high CSCs from castration-resistant prostate cancer (CRPC) 22Rv1 cells overexpress sPLA2-IIa and produce tumors when inoculated into subcutis of nude mice. Given an aberrant high level of sPLA2-IIa in the tumor microenvironment that should be much higher than that in the blood, our findings support the notion that sPLA2-IIa functions as a ligand for EGFR family receptors and supports CSC properties via HER/ERBB-elicited signaling, which may contribute to resistance to therapy and cancer progression.

Introduction

The cancer stem cells (CSCs) are a rare population of tumor cells and enable them to simultaneously self-perpetuate with a consistently maintained CSC subpopulation and to generate differentiated progeny via asymmetrical cell division, giving rise to heterogenic tumors (1-3). CSCs are relatively quiescent and have the properties necessary for tumor initiation, resistance to therapy, and progression (4-7). The CSC population expands during periods of stress, such as radiation (8,9), chemotherapy (5), and castration (10), which are likely the initiating cells of chemoresistant cancer relapses and metastases. Based on CSC markers, a number of CSC populations have been identified. For instance, the side population (SP) cells, which express high levels of ABCG2 and ABCB1 multidrug efflux pumps that may contribute to chemoresistance, possess the stem cell-like properties (2,3,11). The aldehyde dehydrogenases (ALDH) are cytosolic isoenzymes responsible for oxidizing intracellular aldehydes. The ALDH1-high cell subpopulations show distinct stem-like characteristics and are highly resistant to chemotherapeutic agents commonly used as first-line therapy, such as cisplatin and docetaxel (2,3). The current anticancer therapies fail to destroy, but tend to favor the selection and expansion of resistant CSCs in tumors, resulting in poor responses and outcomes. The elimination of CSCs is of utmost importance at the time of therapeutic intervention in order to prevent CSC expansion and subsequent tumor recurrence, relapse, and metastasis.

Secretory phospholipase A2 group IIa (sPLA2-IIa) is distributed in trace amounts in a variety of normal mammalian tissues, but found at high levels in various inflamed tissues and some cancers. sPLA2-IIa, a NF- κ B target gene (12,13), has traditionally been associated with their enzymatic activity and participates in biosynthesis of potent biologically active lipid mediators, particularly arachidonic acid-derived eicosanoids, which promote inflammation, angiogenesis, and tumorigenesis (14,15). Independent of its enzymatic activity, sPLA2-IIa can also serve as a ligand for cell membrane receptors and stimulate integrin activation, COX-2 expression, and secretion of cytokines (16-18). sPLA2-IIa binds to integrin α v β 3 at K_D of 0.2 mM, and induces cell proliferation (16). sPLA2-IIa also weakly interacts with M-type receptor in human cells and induces pro-inflammatory signaling (15,19,20).

Correspondence to: Dr Zhongyun Dong, Division of Hematology-Oncology, Department of Medicine, Vontz Center for Molecular Studies, University of Cincinnati College of Medicine, Rm 1308, 3125 Eden Ave., Cincinnati, OH 45267, USA
E-mail: dongzu@ucmail.uc.edu

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sPLA2-IIa is associated with the pathology of several types of malignancies, including cancers of the colon, breast, stomach, esophagus, ovary, lung, and prostate (21,22). sPLA2-IIa is overexpressed in almost all human prostate cancer tissues and elevated levels are associated with advanced tumor grades (13,23-26). sPLA2-IIa remains elevated in androgen-independent prostate cancers (27) and is significantly increased in metastatic lesions (28). We are the first to demonstrate that cancer cells overexpress and secrete sPLA2-IIa into the interstitial fluids, i.e., tumor microenvironment and blood, in patients with both prostate and lung cancers (13,29,30). High levels of plasma sPLA2-IIa are associated with poor prognosis, advanced cancer stage, and short cancer survival. We confirmed that tumors secrete sPLA2-IIa into the circulation to a detectable level in the mouse model of human cancer (29). The most recent report by others supports our finding in that high levels of plasma sPLA2-IIa are associated with poor prognosis of cancers (31). More importantly, we revealed that elevated HER/ERBB-PI3K-Akt-NF- κ B signaling induces sPLA2-IIa overexpression and secretion in both lung and prostate cancer cells; in turn, sPLA2-IIa stimulates its overexpression via HER/ERBB-elicited signaling in the positive feedback regulation manner (26,32). sPLA2-IIa induces phosphorylation of HER2 and HER3 in a dose-dependent manner in NSCSC A549 and H1975 cells (32). However, the underlying molecular mechanisms of sPLA2-IIa at an aberrant high level in the tumor microenvironment in stimulating cancer progression and metastasis remains to be elucidated.

The HER/ERBB-elicited signaling is essential for cell growth and survival. Recent studies further demonstrate that this signaling pathway is of critical importance in supporting CSC properties (33-35). We determined a role of sPLA2-IIa in CSCs and revealed that sPLA2-IIa is overexpressed in both side population (SP) CSCs from NSCSC cells and ALDH1-high CSCs from CRPC cells. Furthermore, sPLA2-IIa directly interacts with and activates EGFR family receptors, suggesting that sPLA2-IIa is a ligand for both EGFR and HER3. These findings, together with our previous data (13,29,30,32), support the notion that high levels of sPLA2-IIa in the tumor microenvironment support CSC phenotype via HER/ERBB-elicited signaling, and sPLA2-IIa is a novel therapeutic target against cancer and CSCs.

Materials and methods

Reagents. RPMI-1640 medium was purchased from Invitrogen (Gaithersburg, MD, USA). Fetal bovine serum (FBS) and charcoal/dextran-treated FBS were purchased from Hyclone Laboratories (Logan, UT, USA). sPLA2-IIa antibodies were obtained from Life Span BioSciences (Seattle, WA, USA) and Cayman Chemical (Ann Arbor, MI, USA). Recombinant human sPLA2-IIa was obtained from BioVendor (Candler, NC, USA). EGFR, HER2, and HER3 antibodies were from Cell Signaling Technology (Danvers, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). RT-PCR primers were customly synthesized by Genscript (Piscataway, NJ, USA). Plasmid sPLA2-IIa(-800)-Luc was constructed as we described previously (13).

Cell culture. The human prostate adenocarcinoma cell lines LNCaP and 22Rv1, and lung cancer adenocarcinoma H1975

were obtained from ATCC (Rockville, MD, USA) and maintained in RPMI-1640 medium supplemented with 10% FBS (complete medium) at 37°C in 5% CO₂. LNCaP-AI cells were generated by us previously (36) and maintained in RPMI-1640 medium supplemented with 10% charcoal/dextran-treated FBS (stripped medium). Lung cancer adenocarcinoma A549 cells were obtained from ATCC and maintained in MEM medium supplemented with 5% FBS (complete medium) at 37°C in 5% CO₂.

3D cell culture. A 96-well plate was coated with 100 μ l/well of medium containing 0.9% agarose. SP CSCs and non-SP control cells in medium with 10% FBS on ice were mixed with a same volume of cold (4°C) medium containing 10% FBS and 10% growth factor-reduced Matrigel matrix (BD Biosciences, Bedford, MA, USA) and seeded into the agarose plate at 100 μ l/well. The cells were cultured at 37°C for 14 days.

CSC sorting. The ALDH1-high CSCs from 22Rv1 cells was isolated using the Aldefluor assay kit (StemCell Technologies, Vancouver, Canada) and BD FACSAria II cell sorters. ALDH1 cleaves boron-dipyrromethene-aminoacetaldehyde (BAAA) to release fluorescent dye, which can be blocked by diethylaminobenzaldehyde (DEAB). After gating with DEAB control, the fluorescent cells were sorted out as ALDH1-high CSCs.

SP CSCs were isolated as previously described (37). Briefly, A549 and H1975 cells were resuspended at 1x10⁶/ml in prewarmed DMEM with 2% FCS and 10 mmol/l HEPES buffer. Hoechst 33342 dye was added at a final concentration of 5 μ g/ml in the absence or presence of reserpine (50 μ mol/l; Sigma) and the cells were incubated at 37°C for 90 min with intermittent shaking. At the end of the incubation, the cells were washed with ice-cold HBSS with 2% FCS and 10 mmol/l HEPES, centrifuged down at 4°C, and resuspended in ice-cold HBSS containing 2% FCS and 10 mmol/l HEPES. Propidium iodide at a final concentration of 2 μ g/ml was added to gate viable cells. Gated by control cells treated with reserpine that blocks Hoechst 33342 transporter, SP CSCs were sorted using BD FACSAria II cell sorters. SP CSCs were defined as the missing region in the presence of reserpine.

Tumorigenesis in mice. One thousand of CSCs or control cells in 50 μ l PBS were mixed with 50 μ l of cold Matrigel (Thermo Fisher Scientific) and inoculated subcutaneously into nude mice. Tumor incidence and sizes were measured using calipers and recorded twice a week. The mice were maintained in a facility approved by the American Association for Accreditation of Laboratory Animal Care (AAALAC) and in accordance with current regulations and standards of the U.S. Department of Agriculture, U.S. Department of Health and Human Services, and NIH. The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) and executed according to IACUC guidelines.

RT-PCR. RNAs from both CSCs and control cells were isolated using RNeasy Plus Universal Mini kit (Qiagen, Germany). RNA samples were then treated with DNase using DNA-free™ kit (Thermo Fisher Scientific) and subjected to reverse transcription reactions using High Capacity cDNA Reverse Transcription kits (Applied Biosystems, Thermo Fisher Scientific). Real-time

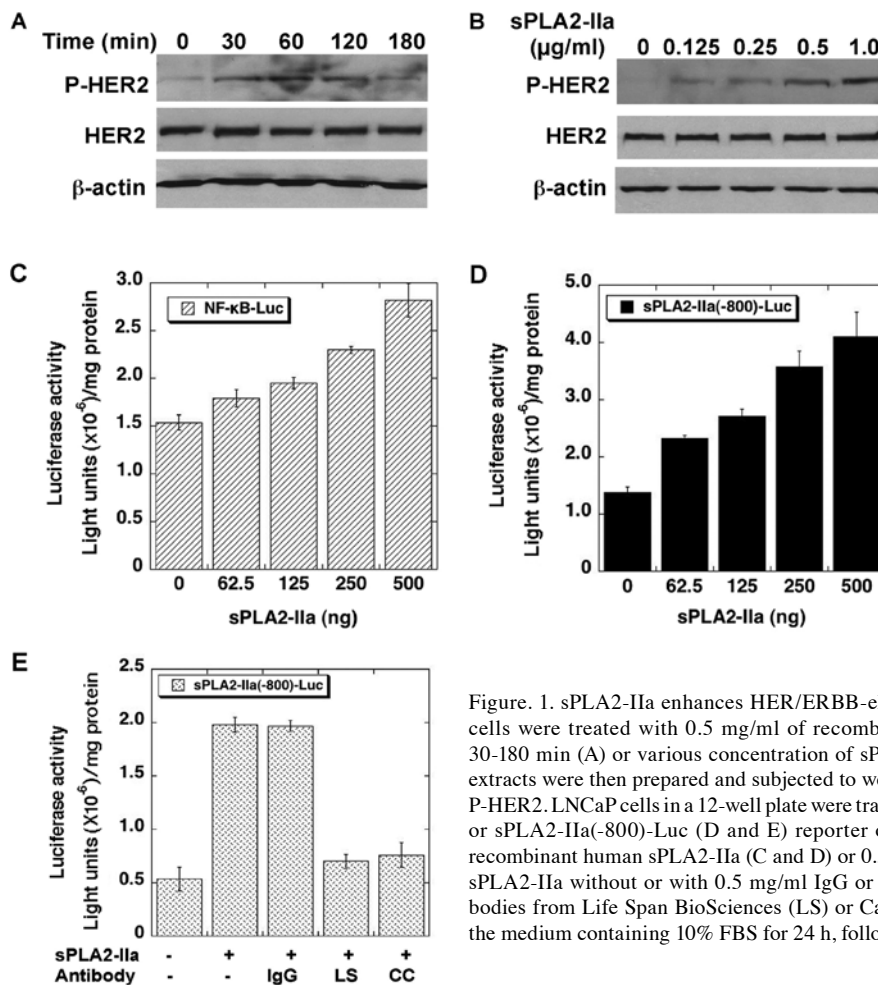


Figure 1. sPLA2-IIa enhances HER/ERBB-elicited signaling. LNCaP-AI cells were treated with 0.5 mg/ml of recombinant human sPLA2-IIa for 30-180 min (A) or various concentration of sPLA2-Ia for 2 h (B). The cell extracts were then prepared and subjected to western blotting for HER2 and P-HER2. LNCaP cells in a 12-well plate were transfected with NF-κB-Luc (C) or sPLA2-IIa(-800)-Luc (D and E) reporter overnight, then treated with recombinant human sPLA2-IIa (C and D) or 0.5 mg/ml recombinant human sPLA2-IIa without or with 0.5 mg/ml IgG or anti-human sPLA2-IIa antibodies from Life Span BioSciences (LS) or Cayman Chemical (CC) (E) in the medium containing 10% FBS for 24 h, followed by luciferase assay.

RT-PCR was performed using the Fast SYBR® Green Master Mix and 7300 Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific).

Co-immunoprecipitation assay. Cell extracts from LNCaP-AI cells were prepared. Co-immunoprecipitation was performed in a modified RIPA buffer (PBS, 0.1% NP-40, 0.1% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1X protease inhibitor cocktail). Antibody was first incubated with 1 mg of cell extract at 4°C on Rocker platform for 1-2 h of Protein G plus/protein A agarose beads (50 μl) (Calbiochem, Thermo Fisher Scientific) was then added, and the samples were incubated at 4°C on Rocker platform overnight. The beads were washed with the modified RIPA buffer four times and boiled in SDS loading buffer. The protein samples were subjected to SDS-PAGE and western blot analysis.

Western blot analysis. Western blot analysis was performed as previously described (13). Briefly, aliquots of samples with the same amount of protein, determined using the DC Protein assay kit (Bio-Rad, Hercules, CA, USA), were mixed with loading buffer (final concentrations of 62.5 mM Tris-HCl, pH 6.8, 2.3% SDS, 100 mM dithiothreitol, and 0.005% bromophenol blue), boiled, fractionated in an SDS-PAGE, and transferred onto a 0.45-mm nitrocellulose membrane (Bio-Rad). The membranes were blocked with 2% fat-free milk in PBS, and

probed with first antibody in PBS containing 0.01% Tween-20 (PBST) and 1% fat-free milk. The membranes were then washed four times in PBS and incubated with IRDye 800CW secondary antibody (LI-COR Biosciences, Lincoln, NE, USA) in PBST containing 1% fat-free milk for 30 min. After washing four times in PBS, the membranes were visualized using Odyssey imaging system (LI-COR).

Reporter assay. Cells (10⁵/well) were seeded in 12-well tissue culture plates. The next day, Lipofectamine 3000 reagent was used for the transient transfection assay according to the protocol provided by Invitrogen/Life Technologies, Inc. The cells were then treated for 24 h. Subsequently, the cell extracts were prepared and luciferase activity was assessed in a Berthold Detection system (Titertek-Berthold, Pforzheim, Germany) using a Luciferase assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. For each assay, cell extract (20 μl) was used and the reaction was started by injection of 50 μl of luciferase substrate. Each reaction was measured for 10 sec in the luminometer. Luciferase activity was defined as light units/mg protein.

Results

sPLA2-IIa stimulates HER/ERBB-elicited signaling in cancer cells. We reported previously that treatment of NSCLC A549 and H1975 cells with recombinant human sPLA2-IIa

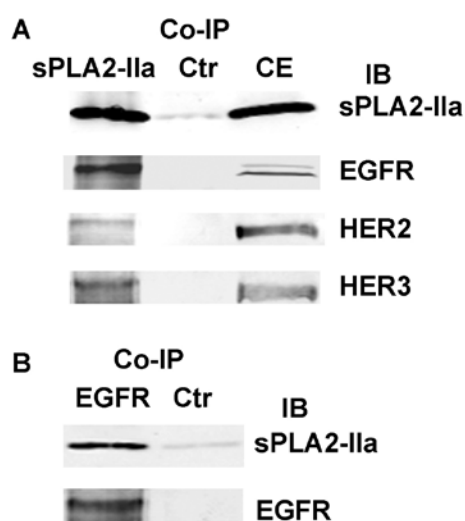


Figure 2. Determination of the interaction between sPLA2-IIa and EGFR family receptors by co-immunoprecipitation (co-IP) assay. Cell extract from LNCaP-AI cells were used for co-IP with either anti-sPLA2-IIa antibody (A) or anti-EGFR antibody (B). The samples without antibody were used as co-IP control (Ctr). Subsequently, the samples were fractionated by SDS-PAGE, followed by immunoblot (IB) analysis using antibodies to sPLA2-IIa, EGFR, HER2, or HER3, respectively. Cell extract (CE) from LNCaP-AI cells served as IB control.

induces phosphorylation of HER2 and HER3 within 2 h in a dose-dependent manner (32). The reporter assay revealed that sPLA2-IIa enhances the promoter activities of NF- κ B and sPLA2-IIa genes in a dose-dependent manner in lung cancer cells. For validation, we determined the effects of sPLA2-IIa in prostate cancer cells. As shown in Fig. 1A and B, recombinant human sPLA2-IIa induces phosphorylation of HER2 within 2 h in a dose- and time-dependent manner in CRPC LNCaP-AI cells (36). sPLA2-IIa also enhances the promoter activities of NF- κ B (Fig. 1C) and sPLA2-IIa (Fig. 1D) genes in a dose-dependent manner in the cells. The stimulatory effects of sPLA2-IIa on sPLA2-IIa promoter activity were abolished by antibodies against sPLA2-IIa (Fig. 1E). These data implicated that sPLA2-IIa enhances HER/ERBB-elicited signaling in prostate cancer cells.

sPLA2-IIa functions as a ligand for EGFR family receptors. A structural analysis we performed previously suggests that the sPLA2-IIa β hairpin shares significant similarity with the EGF hairpin and could be displaced to provide additional contacts with EGFR, and protein docking computation shows that sPLA2-IIa directly interacts with the extracellular domain (ECD) of EGFR in such a way as to stabilize EGFR in its active conformation (32). For validation of the interactions of sPLA2-IIa with EGFR family receptors, co-immunoprecipitation (co-IP) was performed using cell extracts from LNCaP-AI cells. As shown in Fig. 2A, EGFR, HER2, and HER3 were detected in the co-IP complex pulled down by an antibody to sPLA2-IIa. Reciprocally, sPLA2-IIa was detected in the co-IP complexes pulled down by an antibody to EGFR (Fig. 2B). These data reveal that sPLA2-IIa activates EGFR family receptors and complexes with EGFR, HER2, and HER3, strongly suggesting that sPLA2-IIa is a novel ligand for EGFR and HER3 (32).

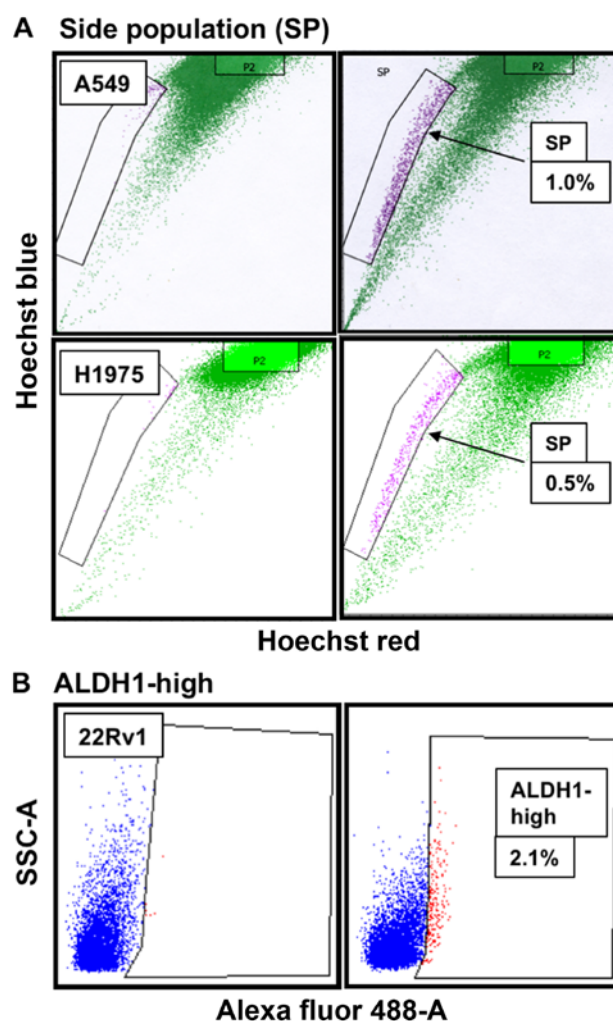


Figure 3. Isolation of CSCs. (A) SP CSCs from A549 and H1975 cells. The SP gate was defined as the missing region (in purple) in the presence of reserpine that blocks the activity of Hoechst 33342 transporter and sorted using BD FACSAria II cell sorters. The cells collected from P2 region (in green) were used as non-SP control. (B) ALDH1-high CSCs from 22RV1 cells. ALDH1-high cells (in red) were isolated using the ALDEFluor assay kit and BD FACSAria II cell sorters.

Overexpression of sPLA2-IIa in CSCs. SP CSCs express high levels of ABCG2 and/or ABCB1 multidrug efflux pump proteins and possess stem cell-like properties (37). We isolated SP CSCs from NSCLC cells using flow cytometry and Hoechst 33342 dye efflux assay (37) and found ~1% of SP CSCs in A549 cells and 0.5% of SP CSCs in H1975 cells (Fig. 3A). ALDH1 is a universal functional marker for CSCs (38-44) and is involved in cellular responses to oxidative stress (42) and drug resistance (41). Overexpression of ALDH1 correlates with poor prognosis in prostate cancer (38). ALDH1-based approach has been used to successfully isolate CSCs from many cell lines of diverse cancer types (45,46), including CRPC 22Rv1 and PC-3 cells (39,44). Consistent with data reported in the literature (44), we found ~2.1% of ALDH1-high CSCs in 22Rv1 cells (Fig. 3B).

The real-time RT-PCR was performed to determine expression of CSC marker genes in the CSCs. As shown in (Tables I and II), the CSC markers, including ABCG2, ALDH1, CD133, CD44, CD56, Focx2, MDR1, Nanog,

Table I. Primers used for RT-PCR.

ABCG2	Upper primer	5'-AAACCTGGTCTCAACGCCATCC-3'
	Lower primer	5'-TGCCCATCACAACATCATCTTG-3'
ALDH1	Upper primer	5'-CTTACCTGTCTACTCACCATTG-3'
	Lower primer	5'-CCTTGTCAACATCCTCCTTATCTCC-3'
AR	Upper primer	5'-GTCTTCGGAAATGTTATGAAGCA-3'
	Lower primer	5'-ACGATCGAGTTCCTTGATGTAG-3'
CD133	Upper primer	5'-TGAGACCCAAGACTCCCATAAAGC-3'
	Lower primer	5'-GGACACAGCATAGAATAATCCCTGC-3'
CD44	Upper primer	5'-CGTGGAGAAAAATGGTCGCTAC-3'
	Lower primer	5'-TACTGGGAGGTGTTGGATGTGAGG-3'
CD56	Upper primer	5'-CGGCATTTACAAGTGTGTGG-3'
	Lower primer	5'-GACATCTCGGCCTTTGTGTT-3'
Focx2	Upper primer	5'-AGAATTACTACCGGGCTGCG-3'
	Lower primer	5'-TGAGCGCGATGTAGCTGTAG-3'
HER2	Upper primer	5'-AATGGAGACCCGCTGAACAATAC-3'
	Lower primer	5'-CACAAAATCGTGTCTGCTGGTAGCAG-3'
HER3	Upper primer	5'-TACGAGAGGTGTGAGGTGGTGATG-3'
	Lower primer	5'-GGAGGTTGGGCAATGGTAGAGTAG-3'
MDR1	Upper primer	5'-TGGGAAGAGCACAAACAGTCCAG-3'
	Lower primer	5'-CGTGGTGGCAAACAATACAGGTTC-3'
Nanog	Upper primer	5'-CCAGTCCCAAAGGCAAACAAC-3'
	Lower primer	5'-TGGAGGCTGAGGTATTTCTGTCTC-3'
NKX3.1	Upper primer	5'-AAAGGCACTTGGGGTCTTATCTG-3'
	Lower primer	5'-CTTCTGATGGCTGAACTTCCTCTC-3'
NSE	Upper primer	5'-GTCCACGTGTCTTCCACTT-3'
	Lower primer	5'-CCCAAGTCAGGCCAGTTTTA-3'
Slug	Upper primer	5'-GAGCATTTGCAGACAGGTCA-3'
	Lower primer	5'-GCTTCGGAGTGAAGAAATGC-3'
Snail	Upper primer	5'-ACCCACATCCTTCTCACTG-3'
	Lower primer	5'-TACAAAACCCACGCAGACA-3'
SOX2	Upper primer	5'-GCACCGCTACGACGTGA-3'
	Lower primer	5'-TGCGAGTAGGACATGCTGTAGG-3'
sPLA2-IIa	Upper primer	5'-TTGACGACAGGAAAGGAAGCCG-3'
	Lower primer	5'-TCTGCTCCCCGAGTTGCTAAAC-3'
Twist	Upper primer	5'-GGAGTCCGCAGTCTTACGAG-3'
	Lower primer	5'-CCAGCTTGAGGGTCTGAATC-3'
Vimentin	Upper primer	5'-AATCCAAGTTTGCTGACCTCTCTG-3'
	Lower primer	5'-CTCTTCCATTTCACGCATCTGG-3'

NKX3.1, NSE, Slug, Snail, Sox2, and Twist (2,3,47), are heterogeneously overexpressed in SP CSCs relative to non-SP control cells or in ALDH1-high CSCs relative to ALDH1-low control cells. In addition, HER2, HER3, or AR gene is also moderately increased in these CSCs. Interestingly, sPLA2-IIa levels in SP and ALDH1-high CSCs are elevated 41-fold

(H1975), 4.3-fold (A549), and 6-fold (22Rv1), respectively, suggesting that sPLA2-IIa is a novel CSC marker and supports CSC properties (Table II).

Characterization of CSCs in 3D cell culture and in mice. We performed 3D cell culture to validate CSCs properties and

Table II. CSCs express a high level of *sPLA2-IIa*.

H1975 cells	None-SP control	SP CSCs	Fold increase
ABCG2	2.26E-04	3.05E-03	13.53
ALDH1	2.01E-06	4.00E-05	19.92
CD133	1.01E-06	4.53E-02	45033.82
Focx2	4.95E-05	2.46E-04	4.97
HER3	1.20E-04	5.07E-04	4.21
MDR1	3.43E-07	7.19E-05	209.32
Nanog	4.59E-05	7.80E-05	1.7
NKX3.1	4.31E-03	2.83E-02	6.57
Slug	1.26E-02	6.91E-02	5.5
Snail	5.60E-02	1.83E-01	3.27
<i>sPLA2-IIa</i>	7.66E-08	3.15E-06	41.1
Twist	7.66E-08	3.33E-05	435.24
A549 cells			
SOX2	5.05E-04	2.22E-03	4.39
<i>sPLA2-IIa</i>	1.83E-04	7.91E-04	4.31
Twist	3.64E-04	1.15E-03	3.16

22RV1 cells	ALDH1-low control	ALDH1-high CSCs	Fold increase
ALDH1	2.80E-04	1.81E-02	64.63
AR	3.72E-05	9.44E-05	2.54
CD133	1.26E-07	3.56E-05	283.24
CD44	3.40E-03	3.11E-02	9.16
CD56	5.99E-06	2.33E-05	3.89
Focx2	5.17E-05	1.79E-04	3.47
HER2	1.01E-02	1.87E-02	1.85
HER3	1.08E-02	2.95E-02	1.56
Nanog	4.77E-04	7.85E-04	1.6
NSE	1.24E-02	8.63E-02	6.44
Slug	3.31E-06	1.09E-05	2.25
Snail	2.19E-04	2.23E-03	10.2
SOX2	1.93E-05	6.20E-05	3.21
<i>sPLA2-IIa</i>	3.93E-06	2.34E-05	5.95

The mRNA expression levels are determined relative to that of β -actin.

found that SP CSCs from A549 cells generate more and much larger spheroids than those by non-SP control cells (Fig. 4). Similar observation was also made in ALDH1-high CSCs from 22RV1 cells and SP CSCs from H1975 cells (data not shown).

To further characterize the tumor initiating properties of CSCs in mice, a subcutaneous inoculation of 1,000 cells/mouse of SP CSCs, but not non-SP control cells, into nude mice produced visible tumors on day 20. The tumors reached $\sim 100 \text{ mm}^3$ on day 30. A subcutaneous inoculation of 1,000 cells/mouse of ALDH1-high CSCs, but not ALDH1-low

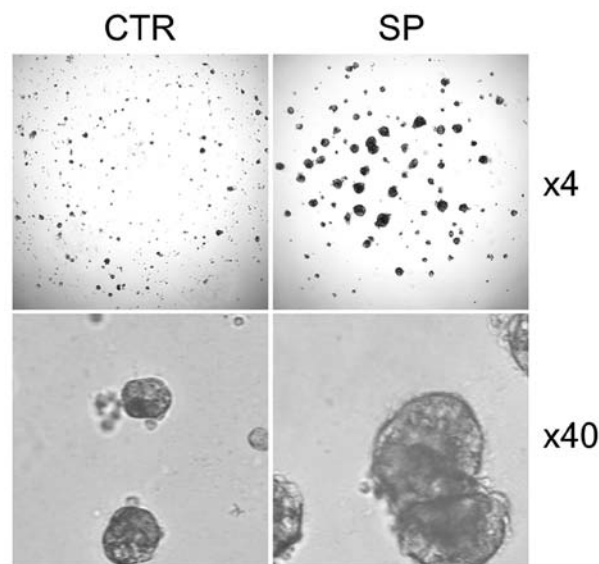


Figure 4. 3D culture of A549 cells. A 96-well plate was coated with $100 \mu\text{l}$ /well of medium containing 0.9% agarose. SP CSCs and non-SP control cells in medium with 10% FBS on ice were mixed with the same volume of cold (4°C) medium containing 10% FBS and 10% growth factor-reduced Matrigel matrix and seeded into the 3D culture plate at $100 \mu\text{l}$ /well. The cells were cultured at 37°C for 14 days.

Table III. Establishment of taxane-resistant 22Rv1-R cell line.

Gene	pTx	dTx	cTx
22Rv1	2	1	1
22Rv1-R	327	305	15
R index	164	305	15

IC₅₀ (nM) of the cells to pTx, dTx, and cTx in a 4-day MTT assay. Resistance (R) index = IC₅₀ of 22Rv1-R/IC₅₀ of 22Rv1.

control cells, into nude mice produced tumors on day 25. The tumors reached $\sim 100 \text{ mm}^3$ on day 35.

Overexpression of CSC markers in CRPC LNCaP-AI cells and taxane-resistant 22Rv1-R cells. CRPC LNCaP-AI cells were established by culturing androgen-dependent LNCaP cells in the stripped medium as we described previously (36). CRPC 22Rv1 cells express constitutively active androgen receptor (48,49). We cultured 22Rv1 cells in the presence of increasing concentrations of paclitaxel to derive 22Rv1-R cells. As shown in Table III, 22Rv1-R cells are resistant to all taxanes.

We found that CSC markers, including ABCG2, ALDH1, CD44, CD56, Focx2, MDR1, Nanog, NSE, Snail, Sox2, and Vimentin, were heterogeneously overexpressed in LNCaP-AI cells relative to parental LNCaP cells (Table IV) and in taxane-resistant 22Rv1-R cells relative to parental 22Rv1 cells (Table V). Strikingly, MDR1 level is increased by 1,380-fold in 22Rv1-R cells and 66-fold in LNCaP-AI cells. The expression of *sPLA2-IIa* in LNCaP-AI cells relative to LNCaP cells is increased 60-fold (Table IV), confirming the finding reported

Table IV. Overexpression of CSC markers in CRPC LNCaP-AI cells.

Gene	LNCaP cells	LNCaP-AI cells	Fold increase
ALDH1	5.60E-05	2.77E-04	4.94
CD44	8.96E-05	1.19E-04	2.44
CD56	1.20E-06	8.10E-05	67.74
MDR1	4.49E-06	2.97E-04	66.2
Nanog	2.95E-04	5.54E-04	1.88
NSE	2.64E-03	8.11E-03	3.07
SOX2	2.40E-07	1.26E-06	8
sPLA2-IIa	9.19E-03	5.50E-01	59.9
Vimentin	2.35E-03	8.55E-02	36.4

The mRNA expression levels are determined relative to that of β -actin.

Table V. Overexpression of CSC markers in taxane-resistant CRPC 22RV1-R cells.

Gene	22RV1 cells	22RV1-R cells	Fold increase
ABCG2	1.98E-03	1.17E-02	5.89
ALDH1	1.35E-03	5.63E-03	4.18
CD44	2.30E-03	6.56E-03	2.85
Foxc2	6.70E-06	3.29E-05	4.91
MDR1	1.56E-04	2.15E-01	1,380.42
Nanog	3.02E-04	1.03E-03	3.4
NSE	3.19E-02	9.42E-02	3
Snail	5.56E-04	3.80E-03	9.9
SOX2	1.04E-04	2.52E-04	2.57

The mRNA expression levels are determined relative to that of β -actin.

in our previous study (13). sPLA2-IIa is not overexpressed in 22Rv1-R cells relative to 22Rv1 cells, but is overexpressed in CSCs from 22Rv1 cells (Table II), indicating the heterogeneity of gene expression in cancer cells. These findings suggest that CSC population is elevated in LNCaP-AI and 22Rv1-R cells, supporting the notion that the current anticancer therapies favor the selection and expansion of CSCs.

Discussion

We demonstrated that cancer cells overexpress and secrete sPLA2-IIa into the interstitial fluid, i.e., tumor microenvironment and blood (13,29,30). Plasma sPLA2-IIa continuously increased with prostate cancer progression and reached as high as 18 ng/ml at the late stage in metastatic prostate cancer (29). High levels of plasma sPLA2-IIa, based on the optimum cutoff value of 2.0 ng/ml, significantly predicted advanced stage and high Gleason score in prostate cancer (13,29). We

further showed that sPLA2-IIa is overexpressed in almost all lung cancers and is significantly elevated in the blood of lung cancer patients (30). High levels of plasma sPLA2-IIa, at the optimum cutoff value of 2.4 ng/ml, are significantly associated with advanced lung cancer stage and decreased overall cancer survival (30). The most recent report by others supports our finding in that high levels of plasma sPLA2-IIa are associated with poor prognosis of cancers (31).

The ultimate cause of cancer treatment failure is that tumor cells evolve and develop multiple mechanisms to escape the cytotoxic effects of anticancer drugs, including enhancement of cell survival pathway, impaired apoptotic machinery, increased DNA repair mechanisms, and multidrug resistance phenotype by overexpression of drug-efflux pump proteins P-glycoprotein (MDR1) and ABCG-2 (50-52). These mechanisms may also drive CSC properties and tumor progression. ATP-binding cassette (ABC) transporters, such as P-glycoprotein (MDR1), multidrug resistant associated protein (MRP1), and ATP-binding cassette membrane transporter G2 [ABCG2, also known as breast cancer resistance protein 1 (BCRP1)], are membrane transporters that can pump cytotoxic chemotherapeutic drugs out of the cell. CSCs express high levels of ABC transporters leading to low intracellular drug concentrations and conferring multidrug resistance to many anticancer drugs. The efflux capacity of SP CSCs, determined by high ABCG2 activity, is associated with tumor growth, progression, and metastasis. The aldehyde dehydrogenase (ALDH) family members are cytosolic isoenzymes responsible for oxidizing intracellular aldehydes. ALDH1-high CSCs are highly resistant to chemotherapeutic agents commonly used as first-line therapy in the clinical setting, such as cisplatin, gemcitabine, doxorubicin, vinorelbine and docetaxel, whereas ALDH1-low cells are sensitive to the cytotoxic activity of these drugs. In the present study, we found that both SP CSCs from NSCLC cells and ALDH1-high CSCs from CRPC cells overexpress a number of CSC markers, supporting the notion that multiple mechanisms contribute to CSC phenotype (Table II). More importantly, we found that both SP CSCs and ALDH1-high CSCs overexpress sPLA2-IIa. It was reported that ALDH1-high CSCs from lung cancer cells also overexpress sPLA2-IIa (53). These findings strongly suggest that sPLA2-IIa is a marker for CSCs and may support CSC properties.

Gene amplification, overexpression, and mutations in EGFR family receptors have been well described in various cancers, including breast, head and neck, prostate, and NSCLC, and contribute to resistance to therapy and cancer progression (54-59). EGF is a preferable ligand for EGFR/EGFR homodimer or EGFR/HER2 heterodimer, while heregulin-a is a preferable ligand for HER2/HER3 heterodimer (34,60,61). HER2 has no ligand and HER3 has no tyrosine kinase activity, and they function by forming heterodimer with other HER receptor. Because HER3 has six tyrosine containing binding sites for p85, the regulatory subunit of PI3K, HER2/HER3 complex is much more effective than EGFR/HER2 in activation of the PI3K/Akt pathway. HER3, which signaling function cannot be inhibited by tyrosine kinase inhibitor (TKI), provides a focal point in resistance to TKI therapy (62). Increasing body of evidence highlight the role of HER3 in lung cancer, which has not been successfully addressed in the targeted therapy to date. By coupling to numerous signaling pathways, such as

the RAS-ERK and PI3K-Akt pathways, and multiple feedback regulatory loops, HER/ERBB-elicited signaling propels the clonal expansion of CSCs (33-35). One such positive feedback loop is stimulation of sPLA2-IIa overexpression via HER/ERBB-PI3K-Akt-NF- κ B signaling (13,29,30). We uncovered that elevated HER/ERBB-PI3K-Akt-NF- κ B signaling induces sPLA2-IIa overexpression and secretion in both lung and prostate cancer cells, and in turn, sPLA2-IIa activates EGFR family receptors and HER/ERBB-elicited signaling and stimulates sPLA2-IIa overexpression in a positive feedback manner (13,29,30).

We further investigated the molecular action of sPLA2-IIa. Given the potential ligand activity of sPLA2-IIa, we hypothesized that sPLA2-IIa functions as a ligand for EGFR family receptors, leading to an enhanced HER/ERBB-elicited signaling. Indeed, we found that sPLA2-IIa enhances HER/ERBB-PI3K-Akt-NF- κ B signaling in both prostate cancer cells (Figs. 1 and 2) and lung cancer cells (32). Our protein docking analysis and co-immunoprecipitation experiments revealed that sPLA2-IIa interacts with EGFR family receptors (Fig. 2) (32). sPLA2-IIa directly or indirectly interacts with EGFR, HER2, and HER3, suggesting that it may be a ligand for both EGFR and HER3. Given that both SP and ALDH1-high CSCs overexpress sPLA2-IIa (Table II), sPLA2-IIa in the tumor microenvironment may function as a ligand for EGFR family receptors, stimulates HER/ERBB-elicited signaling, and promotes the clonal expansion of CSCs and cancer progression (33-35).

sPLA2-IIa stimulates growth of prostate cancer cells (13,27,63), colon cancer cells (64), and brain tumor cells (65,66), which may be via the EGFR-, MAPK-, PI3K/Akt-, NF- κ B-mediated cell growth and survival signaling pathways (67-70). sPLA2-IIa abrogates TNF- α -induced apoptosis and compromises immune surveillance function (71). In the transgenic adenocarcinoma of the mouse prostate (TRAMP) model, sPLA2-IIa contributes to aggressive phenotypes, androgen-independent growth, and metastasis (72). sPLA2-IIa binds to integrins and induces proliferation of monocytic cells in an integrin-dependent manner (16). We showed that sPLA2-IIa is overexpressed in CSCs, which may support CSC properties. On the other hand, knocking down expression of sPLA2-IIa reduces lung cancer growth (73). Our previous and current studies revealed the underlying mechanisms of sPLA2-IIa action, in which sPLA2-IIa functions as a ligand for EGFR family receptors, leading to sPLA2-IIa overexpression via HER/ERBB-PI3K-Akt-NF- κ B signaling in a positive feedback manner. Aberrant high levels of sPLA2-IIa in the tumor microenvironment support CSC properties and contribute to cancer progression and metastasis. sPLA2-IIa is a novel therapeutic target against cancer.

It has been shown that the treatment with taxol and cisplatin may not affect growth, but can even stimulate growth in CSCs (74,75). Similarly, castration has been shown to induce epithelial-mesenchymal transition, promote growth of CSCs in prostate cancer, and lead to castration-resistance and metastasis (10). Consistent with these observations, we found that CRPC LNCaP-AI cells, selected in the stripped medium (36), and multidrug resistant 22Rv1-R cells, derived from CRPC 22Rv1 cells selected in the medium containing paclitaxel, overexpress several CSC markers and drug efflux

pump proteins (Tables IV and V). Therefore, LNCaP-AI and 22Rv1-R cells provide valuable tools for studying CSCs and determine the roles of sPLA2-IIa in tumor progression.

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