Secretory phospholipase A2-IIa is involved in prostate cancer progression and may potentially serve as a biomarker for prostate cancer

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The majority of prostate cancers are indolent, whereas a significant portion of patients will require systemic treatment during the course of their disease. To date, only high Gleason scores are best associated with a poor prognosis in prostate cancer. No validated serum biomarker has been identified with prognostic power. Previous studies showed that secretory phospholipase A2-IIa (sPLA2-IIa) is overexpressed in almost all human prostate cancer specimens and its elevated levels are correlated with high tumor grade. Here, we found that sPLA2-IIa is overexpressed in androgen-independent prostate cancer LNCaP-AI cells relative to their androgen-dependent LNCaP cell counterparts. LNCaP-AI cells also secrete significantly higher levels of sPLA2-IIa. Blocking sPLA2-IIa function compromises androgen-independent cell growth. Inhibition of the ligand-induced signaling output of the HER network, by blocking PI3K-Akt signaling and the nuclear factor-kappaB (NF-kB)-mediated pathway, compromises both sPLA2-IIa protein expression and secretion, as a result of down-regulation of sPLA2-IIa promoter activity. More importantly, we demonstrated elevated serum sPLA2-IIa levels in prostate cancer patients. High serum sPLA2-IIa levels are associated significantly with high Gleason score and advanced disease stage. Increased sPLA2-IIa expression was confirmed in prostate cancer cells, but with high Gleason score and advanced disease stage. Increased regulation of sPLA2-IIa promoter activity. More importantly, we showed that sPLA2-IIa protein expression and secretion, as a result of down-regulation of their androgen-dependent LNCaP cells relative to their androgen-dependent LNCaP cell counterparts. LNCaP-AI cells also secrete significantly higher levels of sPLA2-IIa. Blocking sPLA2-IIa function compromises androgen-independent cell growth. Inhibition of the ligand-induced signaling output of the HER network, by blocking PI3K-Akt signaling and the nuclear factor-kappaB (NF-kB)-mediated pathway, compromises both sPLA2-IIa protein expression and secretion, as a result of down-regulation of sPLA2-IIa promoter activity. More importantly, we demonstrated elevated serum sPLA2-IIa levels in prostate cancer patients. High serum sPLA2-IIa levels are associated significantly with high Gleason score and advanced disease stage. Increased sPLA2-IIa expression was confirmed in prostate cancer cells, but with high Gleason score and advanced disease stage.

Introduction

Prostate cancer is the second leading cause of cancer death among men in the USA. Hormone ablation therapy, the therapeutic mainstay for advanced prostate cancer, only temporarily arrests prostate cancer progression and will ultimately fail in all patients. Standard diagnostic and prognostic modalities used in clinical decision making include PSA, tissue pathology, Gleason score and imaging. Although these factors provide meaningful clinical information, they all have their limitations. The PSA test lacks sensitivity and specificity and has not been validated in the setting of prostate cancer surveillance (1,2). Tissue biopsies can be subjected to sampling errors, which could result in misdiagnosis, whereas imaging frequently misses small tumors. Gleason score remains the sole modality, which has been confirmed to correlate with the presence of high-risk disease. Regrettably, to date, there are no validated prognostic biomarkers, which have been shown to correlate with tumor burden and disease progression.

Cancer is a complex disease caused by the progressive accumulation of multiple gene mutations in a variety of signaling pathways, including the cell cycle, cell growth, apoptosis, metabolism and the inflammatory response pathways. Prostate adenocarcinoma demonstrates significant heterogeneity and genetic instability and as such allow for the development of resistance to hormonal therapies and/or chemotherapy as alternate signaling pathways are established which circumvent those targeted with standard treatments. Elevated EGFR/HER2-Pi3K-Akt-NF-kB signaling and the inflammatory response have been shown to participate in the development and progression of many cancers, including prostate cancer. EGFR was found to be overexpressed in 41.4% of prostate cancer patients prior to treatment (3), whereas HER2 was also overexpressed in 30% of patients before androgen ablation therapy and in 65% of prostate patients failing hormone ablation therapy (4,5). Overexpression of HER2 in prostate cancer cells upregulates androgen receptor (AR) activity and stimulates androgen-independent growth (6). Elevated signaling of the PI3K-Akt pathway (7,8) and increased NF-kB expression was shown to correlate with disease progression (9,10). Given that PI3K-Akt signaling cross talks with the NF-kB-mediated pathway by Akt phosphorylation and activation of IKK, an upstream kinase of NF-kB (11–13), clinical efforts are now being directed toward studies involving inhibitors of EGFR/HER2-Pi3K-Akt-NF-kB signaling such as Pertuzumab, Erlotinib and Bortezomib (14–16).

Phospholipases A2 are phospholipid hydrolase enzymes that mediate the release of biologically active fatty acids and lysophospholipids such as arachidonic acid (AA) and lysophosphatidylcholine, which are the precursors of eicosanoids and platelet-activating factor, respectively (17,18). sPLA2-IIa is overexpressed in almost all human prostate cancer specimens and elevated levels correlate with tumor grade (19–21). sPLA2-IIa remains elevated in androgen-independent prostate cancers failing hormonal treatment (22). Recent studies show that sPLA2-IIa stimulates tumor cell growth (22,23). AA, the product of sPLA2-IIa, can activate PI3K-Akt signaling, resulting in NF-kB activation and expression of many pre-inflammatory NF-kB target genes, such as COX-2, IL-6 and IL-8 (24,25). AA also stimulates eicosanoid PGE2 synthesis resulting in proliferation of cultured prostatic tumor cells and promotes tumor vascularization and metastasis in animal models (26,27). Furthermore, elevated sPLA2-IIa expression is associated with androgen independence and a more aggressive cancer phenotype in the spontaneous TRAMP prostate cancer model (28). Finally, direct inhibition of eicosanoid signaling results in prostate cancer regression in mouse models (29,30).

The current study investigates the role of sPLA2-IIa in prostate cancer progression. We found that sPLA2-IIa is overexpressed in androgen-independent prostate cancer LNCaP-AI cells relative to their parental androgen-dependent cell line LNCaP. LNCaP-AI cells also secrete significantly higher levels of sPLA2-IIa into the medium as compared with LNCaP cells. These findings suggest that sPLA2-IIa may contribute to androgen-independent growth. We also showed that sPLA2-IIa overexpression may be a result of enhanced HER/HER2-Pi3K-Akt-NF-kB signaling. Finally, plasma specimens from...
all prostate cancer patients tested showed elevated sPLA2-IIa relative to plasma specimens obtained from cancer-free volunteers. High levels of serum sPLA2-IIa were found to be associated with increased tumor burdens in patients with advanced disease. These data suggest that serum sPLA2-IIa is a potential surrogate biomarker in prostate cancer and in the setting of therapy targeting the HER/HER2-P13K-Akt-NF-κB pathway.

Materials and methods

Reagents
RPPI 1640 medium was purchased from Invitrogen (Gaithersburg, MD). Fetal bovine serum (FBS) and charcoal/dextran-treated FBS were purchased from HyClone Laboratories (Logan, UT). Anti-sPLA2-IIa antibody for western blot and sPLA2-IIa ELISA kit were obtained from Cayman Chemical (Ann Arbor, MI). Anti-sPLA2-IIa antibody for immunohistochemical (IHC) staining was from LifeSpan BioSciences (Seattle, WA). Anti-P-Akt and Akt antibodies were from Cell Signaling Technology (Danvers, MA). Erbituvin, Gefitinib, Lapatinib, CI-1033 and Bortezomib are purchased from Selleck Chemicals LLC. Primers of sPLA2-IIa complementary DNA for real-time reverse transcription–polymerase chain reaction (PCR) are upper primer 5′-GATA-CACATCTGGAGTCTC-3′ and lower primer 5′-CTGAAGGCAACTC-GAG-3′ and real-time RT–PCR kit was from Qiagen. Heregulin-α is from Thermo Fisher Scientific (Fremont, CA).

Cell culture
The human prostate adenocarcinoma cell line LNCaP was obtained from ATCC (Rockville, MD) and maintained in RPMI-1640 medium supplemented with 10% FBS (complete medium) at 37°C in 5% CO2. LNCaP-AI cells were maintained in RPMI-1640 medium supplemented with 10% charcoal/dextran-treated FBS (stripped medium). LAPC-4 cells, which express wild-type AR, were maintained in RPMI-1640 medium supplemented with 10% bovine serum (FBS) and charcoal/dextran-treated FBS. RPMI 1640 medium was purchased from Invitrogen (Gaithersburg, MD). Fetal bovine serum (FBS) and charcoal/dextran-treated FBS were purchased from HyClone Laboratories (Logan, UT). Anti-sPLA2-IIa antibody for western blot and sPLA2-IIa ELISA kit were obtained from Cayman Chemical (Ann Arbor, MI). Anti-sPLA2-IIa antibody for immunohistochemical (IHC) staining was from LifeSpan BioSciences (Seattle, WA). Anti-P-Akt and Akt antibodies were from Cell Signaling Technology (Danvers, MA). Erbituvin, Gefitinib, Lapatinib, CI-1033 and Bortezomib are purchased from Selleck Chemicals LLC. Primers of sPLA2-IIa complementary DNA for real-time reverse transcription–polymerase chain reaction (PCR) are upper primer 5′-GATA-CACATCTGGAGTCTC-3′ and lower primer 5′-CTGAAGGCAACTC-GAG-3′ and real-time RT–PCR kit was from Qiagen. Heregulin-α is from Thermo Fisher Scientific (Fremont, CA).

Plasmid
sPLA2-IIa promoter fragment (800 bp) was amplified by PCR using LNCaP Plasmid performed in stripped medium. 10% FBS and 10 nmol/l DHT. Transient transfection experiments were maintained in Iscove’s modified Dulbecco’s medium supplemented with dimethylsulfoxide was added to each well, and the absorbance at 570 nm of MTT [2.5 mg/ml in phosphate-buffered saline (PBS)] was added to each well, and the cells were further incubated for 1 h at 37°C to allow complete reaction between the dye and the enzyme mitochondrial dehydrogenase in the viable cells. After removal of the residual dye and medium, 100 μl of dimethylsulfoxide was added to each well, and the absorbance at 570 nm was measured using BMG microplate Reader (BMG Labtech, Durham, NC).

Cell growth assay
Tumor cell growth was determined by the MTT assay as described previously (31). Briefly, LNCaP and LNCaP-AI cells were seeded into 96-well cell culture plates at a density of 5 × 103 cells per well in stripped medium. After incubation in 5% CO2 at 37°C overnight, the cells were treated with various concentrations of drugs in stripped medium for 5 days. At the end of incubation, 20 μl of MTT [2.5 mg/ml in phosphate-buffered saline (PBS)] was added to each well, and the cells were further incubated for 1 h at 37°C to allow complete reaction between the dye and the enzyme mitochondrial dehydrogenase in the viable cells. After removal of the residual dye and medium, 100 μl of dimethylsulfoxide was added to each well, and the absorbance at 570 nm was measured using BMG microplate Reader (BMG Labtech, Durham, NC).

Western blot analysis
Western blot analysis was performed as described previously (31). Briefly, aliquots of samples with the same amount of protein, determined using the Bradford assay (Bio-Rad, Hercules, CA), were mixed with loading buffer (final concentrations of 62.5 mM Tris–HCl, pH 6.8, 2.3% sodium dodecyl sulfate, 100 mM dithiothreitol and 0.005% bromphenol blue), boiled, fractionated in sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a 0.45 μm nitrocellulose membrane (Bio-Rad). The filters were blocked with 2% fat-free milk in PBS and probed with first antibody in phosphate-buffered saline containing 0.1% Tween 20 (PBS) and 1% fat-free milk. The membranes were then washed four times in PBST and incubated with horse-radish peroxidase-conjugated secondary antibody (Bio-Rad) in PBST containing 1% fat-free milk. After washing four times in PBST, the membranes were visualized using the ECL western blotting detection system (Amer sham Co., Arlington Height, IL).

Statistical analysis
Unpaired t-test was used to determine significance of serum sPLA2-IIa level between healthy donors versus prostate cancer patients and Gleason score 6–7 versus 8–10. Nonparametric test (Mann–Whitney U-test) was used to determine significance of staging T2 versus T3. Fisher’s exact test was used to determine whether elevated sPLA2-IIa (>1000 versus <1000 pg/ml) is significantly associated with Gleason score and cancer staging and sPLA2-IIa expression determined by IHC staining is associated with the stages of prostate cancer.

Results
Overexpression of sPLA2-IIa gene in androgen-independent prostate cancer LNCaP-AI cells
To study the molecular mechanisms controlling androgen-independent tumor growth, we established an androgen-independent subline LNCaP-AI from its parental androgen-dependent LNCaP counterpart (31). Since the genetic background of LNCaP and LNCaP-AI cells are identical, we compared expression levels of 16 000 genes using DNA oligonucleotide microarray analysis in order to identify genes contributing to androgen-independent growth. We found that the sPLA2-IIa gene is overexpressed in LNCaP-AI cells, in addition to Vav3 onco gene and the cell cycle inhibitor p21/WAF1 (31,32). sPLA2-IIa overexpression was confirmed by real-time reverse transcription–PCR at the messenger RNA level and by western blot analysis at the protein level (Figure 1A and B). Since sPLA2-IIa is a secretory protein, we investigated whether LNCaP cells secrete sPLA2-IIa into the cell culture medium at a detectable level. As expected, we found that LNCaP-AI cells secrete significantly higher levels of sPLA2-IIa compared with those by LNCaP cells, determined by ELISA analysis (Figure 1C). sPLA2-IIa is a potential NF-κB target gene and our previous studies have shown that LNCaP-AI cells express a constitutively active NF-κB, as demonstrated by an elevated NF-κB total protein level and nuclear NF-κB, an active form of NF-κB (33).
The role of sPLA2-IIa in growth of prostate cancer cells

It has been reported that sPLA2-IIa stimulates prostate tumor growth and as such represents a potential therapeutic target in a mouse xenograft prostate cancer model (22,23). More importantly, elevated sPLA2-IIa expression is associated with androgen independence and a more aggressive cancer phenotype in the spontaneous TRAMP prostate cancer model (28). We confirmed the role of sPLA2-IIa in prostate cancer cell growth by MTT assay and found that blocking sPLA2-IIa activity by the peptide inhibitors cFLSYR or c(2NapA)LS(2NapA)R significantly blocked proliferation of LNCaP-AI cells. LNCaP-AI cells were cultured in 10% stripped medium in the presence of cFLSYR or c(2NapA)LS(2NapA)R for 4 days, followed by MTT assay.

Taking together, these findings suggest that the NF-κB-mediated pathway may contribute to elevated sPLA2-IIa expression and secretion in LNCaP-AI cells.

Regulation of sPLA2-IIa gene expression by the HER/HER2-Pi3K-Akt-NF-κB pathway

It has been well documented that elevated signaling in the EGFR/HER2-Pi3K-Akt and NF-κB-mediated pathways contributes to prostate cancer development and progression (6–10). Cross talking between these two pathways results from Akt phosphorylation and activation of IKK, an upstream kinase of NF-κB (11–13). Furthermore, NF-κB has been shown to activate human sPLA2-IIa promoter activity in liver cells (35). Therefore, we sought to determine whether elevated EGFR/HER2-Pi3K-Akt-NF-κB signaling regulates expression of sPLA2-IIa gene in prostate cancer cells. We determined whether epidermal growth factor (EGF) stimulates sPLA2-IIa expression and whether EGFR inhibitors Erlotinib and Gefitinib, EGFR and HER2 dual inhibitors Lapatinib and CI-1033, PI3K inhibitor LY294002 and NF-κB inhibitor Bortezomib could suppress basal and EGF-induced sPLA2-IIa gene expression.

LNCaP-AI cells were treated with inhibitors without or with EGF for 24 h and the resultant cell extracts were subjected to protein expression analysis. We found that EGF stimulated sPLA2-IIa expression (Figure 2B and C). Among the inhibitors examined, Lapatinib, LY294002 and Bortezomib dramatically downregulated sPLA2-IIa protein expression in both basal states and in the setting of EGF-induced expression, whereas Erlotinib, Gefitinib and CI-1033 had a moderate impact on sPLA2-IIa expression (Figure 2A–C). Consistently, Lapatinib and LY294002, but not Bortezomib (data not shown), significantly blocked PI3K-Akt signaling by inhibiting Akt phosphorylation since Bortezomib targets downstream PI3K-Akt signaling. sPLA2-IIa was also expressed in LAPC-4 (Figure 2D) and DU145 cells, but not PC-3 cells, which was inhibited by Lapatinib via blocking PI3K-Akt signaling. We then determined whether these inhibitors modulated sPLA2-IIa secretion in LNCaP-AI and LAPC-4 cells. We found that Lapatinib, LY294002 and Bortezomib significantly inhibited sPLA2-IIa secretion, whereas Erlotinib, Gefitinib and CI-1033 had a moderate effect in LNCaP-AI cells (Figure 2F) and in LAPC-4 cells (data not shown).

We then examined other HER family members in regulation of sPLA2-IIa expression. We found that Heregulin-α enhanced Akt phosphorylation and sPLA2-IIa expression via PI3K-Akt signaling in both LNCaP (Figure 2E) and LAPC-4 cells (data not shown). Secretion of sPLA2-IIa is also blocked by Lapatinib, LY294002 and Bortezomib in these cells (data not shown). Our findings suggest that the ligand-induced signaling output of the HER network contributes to regulation of sPLA2-IIa expression in response to both EGF and Heregulin-α stimulation in prostate cancer cells. Given that EGF preferentially binds to EGFR and Heregulin binds to HER3 or HER4, our data suggest that the ligand-induced signaling output of the HER network contributes to regulation of sPLA2-IIa expression via the PI3K-Akt-NF-κB pathway in prostate cancer cells. Lapatinib and Bortezomib are the most potent inhibitors of sPLA2-IIa protein expression and secretion.

HER/HER2-Pi3K-Akt-NF-κB signaling regulation of sPLA2-IIa gene expression at the transcriptional level

With the identification of the NF-κB response element in the promoter of rat sPLA2-IIa gene, it has been confirmed that sPLA2-IIa is an NF-κB target gene in rats (36). It has also been reported that NF-κB activates the human sPLA2-IIa promoter via its interaction with the CCAAT/enhancer-binding protein (C/EBP) in the proximal promoter region of the human sPLA2-IIa gene in liver cells (35). When the human sPLA2-IIa promoter sequence was analyzed, a novel consensus sequence of the NF-κB-binding site was identified and located at −782 bp position upstream from the transcriptional start site (Figure 3A). In the next set of experiments, we sought to determine whether the HER/HER2-Vav3-Pi3K-Akt-NF-κB pathway regulates the promoter activity of the human sPLA2-IIa gene. As such, a sPLA2-IIa(-800)-Luc reporter construct was generated. We performed reporter assays by transiently transfecting sPLA2-IIa(-800)-Luc reporter into LNCaP and LNCaP-AI cells. Subsequently, the cells were treated with EGF without or with Erlotinib, Gefitinib, Lapatinib and CI-1033, LY294002 and Bortezomib for 24 h. Consistent with sPLA2-IIa protein expression data (Figure 2), we found that EGF significantly stimulates the promoter activity of sPLA2-IIa gene in both LNCaP and LNCaP-AI cells (shown in LNCaP-AI cells), whereas all the inhibitors tested downregulated the promoter activity both at the basal level (shown in LNCaP cells) and in response to EGF stimulation (shown in LNCaP-AI cells) (Figure 3B and C). Bortezomib is the most potent inhibitor of the promoter activity in sPLA2-IIa gene at the drug concentrations used. These data support our hypothesis that the elevated signaling of the HER/HER2-Pi3K-Akt-NF-κB pathway mediates sPLA2-IIa expression in prostate cancer cells. We
Expression analysis of sPLA2-IIa in prostate cancer cells. (A–C) LNCaP-AI cells were starved in 1% stripped medium for 24 h. The cells were then treated with Erlotinib (20 μM), Gefitinib (20 μM), Lapatinib (20 μM), CI-1033 (8 μM), LY294002 (20 μM) and Bortezomib (20 μM) without or with EGF (100 ng/ml) for 24 h. The cell extracts were prepared and subjected to western blot analysis for sPLA2-IIa, P-Akt, Akt and β-actin. (D–E) LAPC-4 cells (D) and LNCaP cells (E) were starved in 1% stripped medium for 24 h. The cells were then treated with Lapatinib (20 μM) or Heregulin-α (50 ng/ml) for 24 h. The cell extracts were prepared and subjected to western blot analysis for sPLA2-IIa, P-Akt, Akt, and β-actin. (F) LNCaP-AI cells were starved in 1% stripped medium for 24 h. The cells were then treated with Erlotinib (20 μM), Gefitinib (20 μM), Lapatinib (20 μM), CI-1033 (8 μM), LY294002 (20 μM) and Bortezomib (20 μM) for 24 h. Cell culture medium was collected from each sample and subjected to ELISA for sPLA2-IIa. The condition medium samples were diluted 10 times for ELISA. Average of duplicate samples was converted to nanogram per milliliter against standard curve. The data represent one of five repeated experiments.

HER/HER2-PI3K-Akt-NF-κB signaling regulates sPLA2-IIa promoter activity. (A) Human sPLA2-IIa promoter sequence (accession number NC_000001). The C/EBP-binding site in the core promoter region is indicated by bold letter. The consensus sequence of the NF-κB site located at −782 bp was indicated by underline. Most NF-κB sites appear to be 10 bp in length with the consensus sequence 5′-GGGRNWYYYCC-3′, where R denotes a purine base, N denotes any base, W denotes an adenine or thymine and Y denotes a pyrimidine base. (B–C) LNCaP (B) and LNCaP-AI (C) cells were transiently transfected with sPLA2-IIa(-800)-Luc (0.5 μg). The cells were then treated with Erlotinib (20 μM), Gefitinib (20 μM), Lapatinib (20 μM), CI-1033 (8 μM), LY294002 (20 μM) and Bortezomib (20 μM) without or with EGF (100 ng/ml) for 24 h. Luciferase assay was performed according to a standard protocol with Renilla luciferase as an internal control. Data are presented as the mean (±SD) of duplicate values of a representative experiment that was independently repeated for five times.
pathway upregulates expression of the sPLA2-IIa gene at the transcriptional level.

In summary, we found that elevated signaling of the HER/HER2-PI3K-Akt-NF-κB pathway is responsible for upregulation of sPLA2-IIa promoter activity, expression and secretion. Lapatinib and Bortezomib are the most potent inhibitors of sPLA2-IIa promoter activity, expression and secretion, implicating these as potential effective therapies in prostate cancers associated with elevated HER/HER2-PI3K-Akt-NF-κB signaling.

Serum sPLA2-IIa as a potential prognostic and predictive biomarkers for prostate cancer

It has been reported that almost all prostate cancers overexpress sPLA2-IIa by IHC analysis (19–21). Expression levels of sPLA2-IIa are increased with tumor grade and androgen independence, with the highest levels found in the most poorly differentiated, highest grade primary tumors. It was reported that serum levels of sPLA2-IIa can be detected and increased in response to bacteria, viral infection or IL-2 infusion (37,38). Since serum sPLA2-IIa has yet to be identified and quantified in cancer patients, we investigated a possible association between elevated serum sPLA2-IIa levels and the presence of prostate cancer and its progression. As such, we examined serum sPLA2-IIa levels in prostate cancer patients and compared them with those measured in cancer-free volunteers. Forty-three plasma specimens were obtained from the Cancer Center Tumor Bank and 20 plasma samples from healthy donors were acquired from The Cincinnati Hoxworth Blood Center. Serum levels of sPLA2-IIa were determined by ELISA. We found that all prostate cancer patients showed elevated serum sPLA2-IIa levels, ranging from 400 to 18 000 pg/ml (Figure 4; supplementary Table 1 is available at Carcinogenesis Online). Serum levels of sPLA2-IIa in healthy donors were zero in 15 samples, whereas those of the remaining 5 samples were <275 pg/ml (supplementary Table 2 is available at Carcinogenesis Online). A statistical analysis revealed that serum sPLA2-IIa correlated significantly with the presence of prostate cancer (P = 0.0024). Since among the 20 healthy donors, 6 donors were >60 and 4 donors were >50, we postulate that age is not a major factor contributing to elevated serum sPLA2-IIa levels. In addition, serum sPLA2-IIa is very stable and the protein levels do not change by storing the serum at 4°C for 5 days.

At present, the Gleason score is the best prognostic marker for prostate cancer. We compared serum sPLA2-IIa levels in intermediate Gleason score (6–7) and high Gleason score (8–10) specimens and found that levels of serum sPLA2-IIa were significantly elevated in high Gleason score specimens relative to those of intermediate Gleason score (P = 0.0252) (supplementary Table 1 is available at Carcinogenesis Online). Focusing on the stage of prostate cancer, we found that the serum levels of sPLA2-IIa in advanced stage T3 patients significantly were higher than those of T2 patients (P = 0.0298). Remarkably, two samples with the highest serum sPLA2 levels, 15 209 and 18 003 pg/ml, respectively, originated from patients with T3 disease and Gleason 9 and 10 prostate cancers (supplementary Table 1 is available at Carcinogenesis Online). We did not find any association between sPLA2 and PSA, probably due to the small sample size and dramatic variation in serum levels of both sPLA2-IIa and PSA.

Next, we confirmed the expression levels of sPLA2-IIa in prostate cancer tissues relative to normal prostate glands by IHC analysis. Immunostaining of sPLA2-IIa showed a cytoplasmic granular pattern in prostatic adenocarcinoma cells (Figure 5). Benign prostatic glands adjacent to the cancerous glands in the same sections serving as the internal control showed negative staining with sPLA2-IIa. The intensity of the staining appeared correlated with Gleason scores: moderate in Gleason 6 (Figure 5A), stronger in Gleason 7 (Figure 5B) and strongest in Gleason 8 (Figure 5C). sPLA2-IIa overexpression was not observed in normal prostate epithelial cells and stroma, but found in prostate cancer cells. Furthermore, among 41 prostate cancer specimens analyzed, high levels of sPLA2-IIa expression are significantly associated with the advanced pathological stages of prostate cancer (P = 0.0476) (supplementary Table 3 is available at Carcinogenesis Online). This study confirmed the findings reported by others in that elevated levels of sPLA2-IIa are associated with high Gleason score and advanced stages of prostate cancer (19–21).

Discussion

Given the heterogeneous nature of malignant neoplasms, successful future treatments will probably be ‘personalized’ to a tumor’s unique genotypic and phenotypic characteristics. Tumor biomarkers with prognostic and predictive (theragnostic) power will aid in the selection of optimal pharmacologic therapies. sPLA2-IIa is a potential NF-κB target gene and regulates the provision of AA to the eicosanoid biosynthesis pathway. It functions as a growth factor for prostate cancer cells and is overexpressed in almost all prostate tumors. sPLA2-IIa expression levels are further increased in patients with high tumor burdens (19–21). In the current study, we demonstrated that sPLA2-IIa is overexpressed in androgen-independent prostate cancer LNCaP-AI cells relative to their parental androgen-dependent counterparts. LNCaP-AI cells also secrete significant higher level of sPLA2-IIa. Furthermore, we found that inhibition of the HER/HER2-elicited pathway, by blocking HER2, PI3K-Akt signaling and the NF-κB-mediated pathway inhibits sPLA2-IIa promoter activity, expression and secretion (Figure 6). More importantly, we demonstrated elevated serum sPLA2-IIa levels in all prostate cancer patients. High serum sPLA2-IIa, but not PSA, associated significantly with high Gleason score and advanced disease stage. These findings strongly suggest that elevated HER/HER2-PI3K-Akt-NF-κB signaling contributes to sPLA2-IIa overexpression and secretion, suggesting a mechanism leading to prostate cancer progression. Enhanced serum sPLA2-IIa could represent a powerful surrogate biomarker in patients.
with prostate cancer. A more rational and intelligent selection of therapeutic agents, based on biomarker expression in the context of activated signaling pathways such as HER/HER2-P13K-Akt-NF-κB, could result in improved anticancer therapy.

Eicosanoids are products of both sPLA2-IIa and cyclooxygenase-2, synthesized from AA, and these signaling molecules exert control over many physiologic processes such as inflammation and immunity (Figure 6). Recent findings implicate elevated eicosanoid signaling in the pathogenesis of prostate cancer and its progression. Multiple key genes in the eicosanoid biosynthetic pathway, e.g. NF-κB (9,10), cyclooxygenase-2 (39,40) and sPLA2-IIa (19–21), are overexpressed in prostate cancer and associated with disease progression. Most sPLA2-IIa is stored in cytoplasmic granules and released into the extracellular environment upon cell activation under normal physiologic conditions. Gene expression can be induced by inflammatory cytokines under pathological conditions, which explains their presence in the plasma and biologic fluids of patients with systemic inflammatory, autoimmune or allergic diseases (37,38). Overexpression of sPLA2-IIa in LNCaP-AI cells suggests that an elevated alternative cell signaling pathway stimulates sPLA2-IIa gene expression and supports androgen-independent growth in prostate tumors, which is unrelated to autoimmune disease or pathogen infection. That almost all prostate cancers overexpress sPLA2-IIa, strongly supports the notion that secretion of sPLA2-IIa by tumor cells results in elevated serum sPLA2-IIa levels. Increased signaling of the HER/HER2-P13K-Akt-NF-κB pathway during cancer development and progression is probably to represent an important mechanism resulting in enhanced serum sPLA2-IIa levels.

The EGF family of ligands, such as EGF and Heregulin, stimulate the formation of HER (ErbB) receptor homodimers and heterodimers and tyrosine kinase activity. EGF preferentially binds to EGFR and induces EGFR homodimers or EGFR/HER2 heterodimers. Herespin binding to HER3 or HER4 induces the formation of HER2/HER3 or HER2/HER4 heterodimers (41). HER2 does not bind to any ligand with high affinity, but preferentially forms heterodimers with other HER family members for activation. In addition to the role of EGF and HER2, it was reported recently that HER3 and Herespin-α were overexpressed in prostate cancer and associated with a poor prognosis (42). Furthermore, AR transactivation and cell proliferation induced by Heregulin were more potently inhibited by the EGFR/HER2 dual tyrosine kinase inhibitor Lapatinib than the EGFR-specific inhibitor Gefitinib, suggesting that HER2/HER3 activation perhaps by an autocrine pathway contributes to the proliferation signal (43,44). In the current study of EGF-induced sPLA2-IIa expression observed, EGFR inhibitors Erlotinib and Gefitinib had limited effects on the level of P-Akt and sPLA2-IIa, whereas EGFR/HER2 dual inhibitor Lapatinib had more potent inhibitory effects. We also found that Heregulin-α enhanced Akt phosphorylation and sPLA2-IIa expression in prostate cancer cells and sPLA2-IIa secretion was blocked by Lapatinib, LY294002 and Bortezomib. Our findings suggest that the ligand-induced signaling output of the HER network contributes to regulation of sPLA2-IIa expression and secretion in prostate cancer cells.

Elevated HER/HER2-P13K-Akt-NF-κB signaling contributes to the development of many cancers including prostate. NF-κB activation results in the expression of inflammatory cytokines and growth factors, which block apoptosis and promote tumor proliferation, angiogenesis and tumor invasion. Studies from our laboratory as well as others demonstrated that elevated P13K-Akt signaling is required for growth of androgen-independent prostate cancer (45). The Vav3 oncogene is a signal molecule in the HER/HER2-elicited pathway and our prostate-specific Vav3 transgenic mice have been shown to develop non-bacterial chronic prostate inflammation and prostatic tumors (46). Overexpression of Vav3 leads to enhanced signaling in both AR- and NF-κB-mediated pathways, whereas NF-κB may function as an intermediate in Vav3-induced inflammation associated with prostate cancer development. Therefore, overexpression and aberrant activation of the genes HER/HER2, Vav3, PI3KCA and NF-κB, as well as reduced or loss of PTEN function, result in enhanced HER/HER2-P13K-Akt-NF-κB signaling (3–5,9,10,47–49). Altered activities of these genes probably participate in sPLA2-IIa overexpression in prostate cancer.

Many new drugs targeting HER/HER2-P13K-Akt-NF-κB signaling are currently being tested in clinical trials for cancer therapy and include Erlotinib, Gefitinib, Lapatinib and CI-1033. Bortezomib (Velcade) is the first therapeutic proteasome inhibitor of NF-κB activity. At present, the efficacy of Erlotinib and Bortezomib in prostate cancer is under clinical investigation. We found that Lapatinib and Bortezomib, but not Erlotinib, Gefitinib and CI-1033, strongly inhibit sPLA2-IIa promoter activity, expression and secretion in LNCaP-AI cells, suggesting that these drugs may be good candidates for treatment in patients with elevated serum sPLA2-IIa. With a mechanistic understanding of the oncogenic pathways associated with a given prostate tumor, an evidence-based approach could yield therapies with optimal likelihood of therapeutic efficacy as opposed to the ‘trial and error’ approach typically utilized in clinical medicine, which may contribute to failed clinical trials including new drug Bortezomib (50).

While prostate cancer is epithelial cell origin, benign prostatic hyperplasia is characterized by hyperplasia of both stroma and epithelial cells resulting in nodules of glands and intervening stroma. Expression level of sPLA2-IIa in benign prostatic hyperplasia has not been determined. Therefore, clinical cutoff values for serum sPLA2-IIa level with prognostic and predicative power for prostate cancer need to be determined in clinical trial. sPLA2 gene expression is neither prostate tissue specific nor prostate tumor specific. Histo-pathology still remains the gold standard of cancer diagnosis. We also found that cancers other than prostate cancer overexpress sPLA2-IIa and show associated elevated serum levels (data not shown), implicating a potential use for other cancer therapy. Since sPLA2-IIa could be elevated in the setting of infection or inflammation, it should not be considered a replacement to current diagnostic and prognostic modalities, but rather an aid to them.

In summary, multiple lines of evidence support the notion that serum sPLA2-IIa may represent a surrogate biomarker in prostate cancer: (i) sPLA2-IIa is overexpressed in androgen-independent prostate cancer cells and in almost all human prostate cancers tested; (ii) all prostate cancer patients show elevated serum sPLA2-IIa levels;
(iii) high serum sPLA-II levels associate with high Gleason score and advanced tumor stages; (iv) increased sPLA-II expression and secretion implicate elevated HER/HER2-Pi3K-Akt-NF-kB signaling in tumor development and associated inflammation; (v) new anticancer drugs, such as Lapatinib (Tykerb/Tyverb) and Bortezomib (Velcade), significantly inhibit sPLA-IIa promoter activity, expression and secretion. Potential applications of the findings reported herein include the use of sPLA-IIa to direct early treatment in patients with high serum sPLA-II levels irrespective of stage. Additionally, information provided by sPLA-IIa levels could assist with the management of intermediate Gleason score tumors. Finally, sPLA-IIa levels could guide sound and informed selection of pharmacologic agents based on tumor biology as evidenced by their associated biomarkers. These potential applications with significant clinical cutoff values for serum sPLA-IIa level with prognostic and predictive power will require validation in clinical trials.

Supplementary material

Supplementary Tables 1–3 can be found at http://carcin.oxfordjournals.org/

Funding

Cancer Center Foundation, University of Cincinnati College of Medicine; National Institutes of Health (R01 CA119935, CA131137); New South Wales Cancer Council, Australia (RG07/17 to K.F.S.).

Acknowledgements


Conflict of Interest Statement

None declared.

References


Received April 16, 2010; revised August 19, 2010; accepted September 5, 2010