

*Advances in Brief***Expression of Group IIa Secretory Phospholipase A2 Increases with Prostate Tumor Grade**

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Abstract

Purpose: Arachidonate release contributes to prostate tumor progression as arachidonate is metabolized into prostaglandins and leukotrienes, potent mediators of immune suppression, cellular proliferation, tumor motility, and invasion. The group IIa sPLA2 (sPLA2-IIa) can facilitate arachidonate release from cellular phospholipids. We therefore sought to determine whether sPLA2-IIa expression might be related to the development or progression of prostatic adenocarcinoma (CaP).

Experimental Design: sPLA2-IIa expression was examined by Western blot analyses of CaP cells and xenografts and by immunohistochemistry of benign prostatic hyperplasias and primary human CaPs ($n = 101$) using a sPLA2-IIa-specific polyclonal antibody.

Results: sPLA2-IIa expression was increased dramatically in the androgen-independent CWR-22R and LNAI CaP cells versus the androgen-dependent CWR-22 and LNCaP cells. Immunohistochemical analyses revealed that sPLA2-IIa expression was also significantly increased with CaP development and advancing disease (trend analysis; Pearson correlation coefficient, $P = 0.016$). High-grade CaPs showed intense, uniform staining for sPLA2-IIa that was significantly different from that in adjacent benign prostatic hyperplasias (Fisher's exact test, $P = 0.021$) or low-grade CaP ($P = 0.013$), both of which showed only focal or weak sPLA2-IIa staining. Further, uniform sPLA2-IIa expression was directly related to the increased proliferative index that typifies advancing disease ($P = 0.001$). Most significantly, enhanced sPLA2-IIa expression was inversely related to 5-year patient survival ($P = 0.015$).

Conclusions: These data show that sPLA2-IIa expression increases with progression to androgen-independence and is highest in the most poorly-differentiated, highest-grade primary human CaP samples.

Introduction

Increased dietary fat intake has been repeatedly linked with an increased risk for prostate malignancy (1, 2). The uptake of arachidonate and its metabolism into prostaglandins by the cyclooxygenases and into leukotrienes by the lipoxygenases is increased in prostate cancers (3). Prostaglandins and leukotrienes have been implicated in prostate tumor cell proliferation, motility, invasion, and metastasis (4–8). Furthermore, specific inhibitors of the lipoxygenase and cyclooxygenase pathways of arachidonate metabolism are potent inducers of apoptosis in human CaP² cells (9–11).

The release of arachidonate from the sn-2 position of cellular phospholipids is mediated by the family of phospholipase A2 enzymes. This family of enzymes includes the calcium-independent iPLA2, the high molecular weight cytosolic PLA2, and the low molecular weight secretory PLA2's designated group Ib, IIa, IIc, IIe, V, and X (12, 13). The group II sPLA2 enzymes are highly expressed in inflammatory tissues (12) and have been shown to be overexpressed in a variety of human breast, gastric, and hepatocellular carcinomas (14–21). Additionally, in a set of eight human prostate cancers (8), group II sPLA2 was shown to be overexpressed at both the mRNA and protein levels, particularly in the less-differentiated, high-grade tumors (8). Although derived from a small sample set, these data suggest that enhanced expression of the group II sPLA2 may be related to prostate tumor progression.

We therefore sought to determine whether expression of the group II isoform, sPLA2-IIa, might be up-regulated with progression to androgen-independence in experimental CaP models and whether sPLA2-IIa expression might be increased with advanced disease. Here, we show that sPLA2-IIa expression is increased with progression to androgen-independence in two distinct experimental models of CaP progression. Additionally, evaluation of sPLA2-IIa expression by immunohistochemistry in 101 primary human prostate tissue samples revealed that sPLA2-IIa expression increases significantly with prostate cancer development and is specifically up-regulated in the highest-grade CaP samples. Additionally, enhanced sPLA2-IIa expression was specifically related to the increased proliferative index that characterizes advanced CaP and was inversely related to 5-year patient survival. These data therefore suggest that en-

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² The abbreviations used are: CaP, prostate adenocarcinoma; BPH, benign prostatic hyperplasia; TUNEL, terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling.

Table 1 Summary of sPLA2-IIa staining patterns in human prostate tissues

Prostate Tissue Type	Mean Gleason score	Uniform	Focal	Negative
BPH from patients without cancer (Group E)		5 of 27 (19%)	19 of 27 (70%)	3 of 27 (11%)
BPH adjacent to low-grade CaP (Group D)		0 of 17 (0%)	12 of 17 (71%)	5 of 17 (29%)
BPH adjacent to high-grade CaP (Group C)		2 of 18 (11%)	8 of 18 (44%)	8 of 18 (44%)
Low-grade CaP (Group B)	4.6	1 of 15 (7%)	13 of 15 (87%)	1 of 15 (7%)
M1 & M1 + M2	3.8	1 of 9 (11%)	7 of 9 (78%)	1 of 9 (11%)
M2	5.2	0 of 6 (0%)	6 of 6 (100%)	0 of 6 (0%)
High-grade CaP (Group A)	7.0	11 of 24 (46%)	13 of 24 (54%)	0 of 24 (0%)
M2 + M3	6.8	5 of 16 (31%)	11 of 16 (69%)	0 of 16 (0%)
M3	7.5	6 of 8 (75%)	2 of 8 (25%)	0 of 8 (0%)

hanced sPLA2-IIa expression may be involved in the malignant progression of human CaP.

Materials and Methods

Prostate Tissue Collection. Archival, formalin-fixed, paraffin-embedded surgical specimens of BPH and prostatic carcinoma were obtained from the St. Elizabeth Medical Center, Covington/Edgewood, KY. Surgical consultation reports were available for all prostate specimens, and clinical follow-up information was available for all cancer patients. The St. Elizabeth Medical Center Institutional Review Board approved the use of the specimens in this study. A board-certified pathologist (H. W. C.) reviewed a H&E-stained section from each block of prostate tissue and graded each according to the Mostofi grading system (22). According to this grading system, M1 represents well-differentiated CaP, M2 represents moderately-differentiated CaP, and M3 represents poorly-differentiated CaP (22). Samples designated M1 + M2 and M2 + M3 were heterogeneous, with both tissue patterns present. The Mostofi grades assigned correspond to Gleason grades as follows: (a) the M1 grade for well-differentiated tumors corresponds to Gleason grades 1 and 2; (b) the M2 grade tumors correspond to tumors of Gleason grade 3; (c) and high-grade, poorly-differentiated M3 tumors correspond to the least-differentiated Gleason grade 4 and 5 tumors (23). The mean Gleason score for the samples analyzed in this study is noted in Table 1 following the Mostofi grade for each group of tumors.

Antibody Generation. Rabbit polyclonal antibody specific for sPLA2-IIa was generated by immunizing rabbits with purified, recombinant human sPLA2-IIa. Following affinity purification, the specificity of this antibody was verified by immunostaining and Western blotting of L cells that stably express human sPLA2-IIa. Chinese hamster ovary cells expressing sPLA2-V or sPLA2-X did not stain with this antibody, and no cross-reactivity was observed by Western blot of these cell lysates (data not shown).

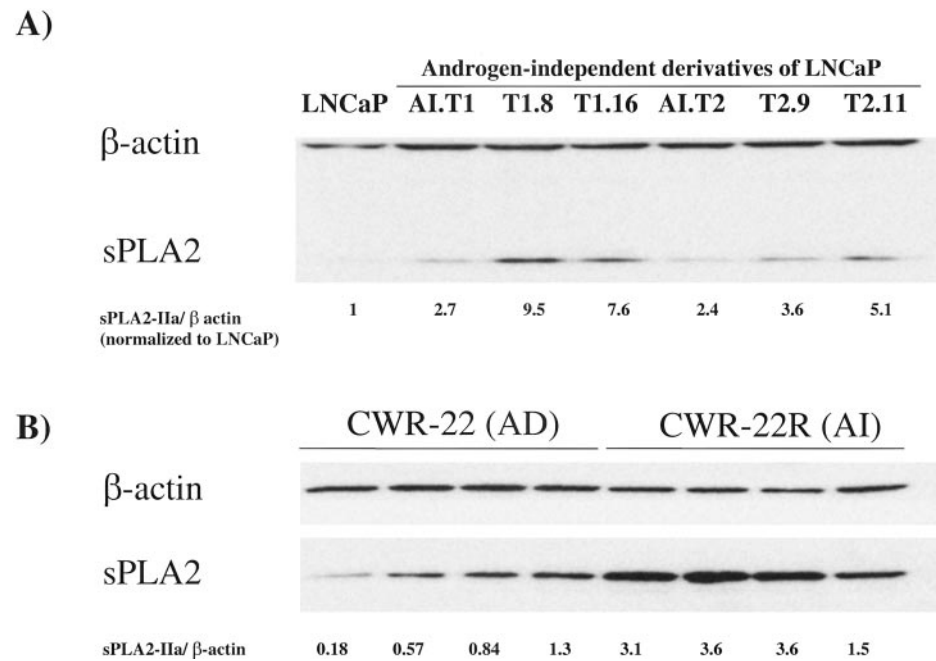
Western Blot Analyses. Western blot analyses were performed as described (24) using anti-sPLA2-IIA antibody at 10 μ g/ml overnight at 4°C with rotation. Protein extracts from xenograft tissue of the androgen-dependent prostate tumor xenograft line, CWR-22, and the androgen-independent derivative, CWR-22R (25), were harvested as described (24). Protein extracts from the androgen-dependent/sensitive human prostate cancer cell line LNCaP and its androgen-independent derivatives, LNAI cells, were harvested as described (24).

Immunohistochemistry. Antibody staining was performed on 5- μ m histological sections of formalin-fixed, paraffin-embedded surgical specimens as detailed previously (26). Slides were rehydrated and placed in 1% hydrogen peroxide in methanol for 30 min. Antigen retrieval was performed on all sections using trypsin at 0.3 mg/ml for 8 min at room temperature. Sections were incubated with normal blocking serum, followed by anti-sPLA2-IIa antibody at 10 μ g/ml in 2% BSA/PBS at 4°C overnight. Sections were then washed and incubated in the antirabbit secondary antibody for 1 h, blocked again in 1% hydrogen peroxide in methanol for 10 min, and incubated with the avidin/biotin complex reagent (Vector Laboratories, Burlingame, CA) for 1 h. Diaminobenzidine detection of the antibody reaction was performed with the Vectastain ABC kit (Vector). All sections were counterstained with Gill's hematoxylin. Negative controls included human cancer sections stained without antibody (data not shown) and sections stained first with nonspecific rabbit IgG at the same protein concentration as the primary anti-sPLA2-IIA antibody, followed by horseradish peroxidase-linked antirabbit secondary antibody (see Ref. 26). Proliferative index was assessed by Ki-67 immunostaining (27). Apoptotic index was assessed by TUNEL staining (28). Apoptotic and proliferative indices were scored and calculated as described (26).

Evaluation of Immunohistochemical Stain. Staining patterns including tissue distribution (epithelial/stromal), extent of stain, and stain intensity were evaluated for each specimen by two experienced investigators (J. H. C. and H. W. C., a board-certified pathologist). In specimens with prostatic adenocarcinoma, the staining pattern was noted for both the neoplasm and the adjacent BPH tissue. The evaluation was categorical and assigned whether the stain was negative (<10% of the epithelium stained), focally positive (heterogeneous, positive typically in <50% of the epithelium), or intense, uniformly positive (involving >90% of the tumor epithelium).

Statistical Analyses. All statistical analyses were performed with the SAS program (SAS, Cary, NC). For statistical analyses, tissues were divided into five groups: (1) high-grade CaP, Mostofi grades 3 and 2 + 3 (group A); (2) low-grade CaP, Mostofi grades 2, 1 + 2, and 1 (Group B); (3) BPH adjacent to poorly-differentiated, high-grade CaP (Group C); (4) BPH adjacent to moderately well- and well-differentiated, low-grade CaP (Group D); and (5) BPH from patients without cancer (Group E). Multivariate trend analyses were performed to evaluate the relationships between staining patterns and tumor grade (M1 and M2, M2, M2 + 3,

Fig. 1 sPLA2-IIa is overexpressed in androgen-independent prostate cancer cells. Expression of sPLA2-IIa in human prostate cancer cells was assessed by Western blotting with rabbit anti-sPLA2-IIa at 1:2000 dilution. The prostate cancer cell lines LNCaP and CWR-22 are androgen-dependent, whereas the LNAI cells and CWR-22R are androgen-independent. The slower-growing first-generation LNAI cells (AI. T1 and AI. T2) show ~3-fold increased sPLA2-IIa expression relative to the parental LNCaP cells. The faster-growing, second-generation LNAI cells (T1.8, T1.16, T2.9, and T2.11; Ref. 24) show the highest sPLA2-IIa expression. To control for loading and transfer, sPLA2 blots were either simultaneously probed for β -actin (A) or reprobed for β -actin (B). Ratios for sPLA2-IIa/ β -actin expression are shown. B shows sPLA2-IIa expression in individual xenograft tumors of either CWR-22 or the hormone refractory CWR-22R.



and M3) or between staining patterns and tissue type (BPH from patients without CaP, BPH adjacent to low-grade CaP, BPH adjacent to high-grade CaP, low-grade CaP, and high-grade CaP). Fisher's exact test two-tailed analyses were also performed to assess the relationships between intense uniform staining and 5-year patient survival. Relationships between staining patterns and proliferative index, Ki-67 staining (26), or apoptotic fraction TUNEL staining (27) were calculated using the Student's *t* test.

Results and Discussion

sPLA2-IIa Expression Is Increased in Androgen-independent Human CaP Cells. To assess whether sPLA2-IIa expression may be involved in the progression of human CaP, we first examined sPLA2-IIa expression by Western blotting in two independent models of androgen-independent prostate cancer, the CWR-22/22R and LNCaP/LNAI models (Fig. 1). In both models, the androgen-independent cells (LNAI and CWR-22R, respectively) showed higher expression of sPLA2-IIa than the androgen-dependent cells (LNCaP and CWR-22, respectively). Moreover, sPLA2-IIa expression was increased most dramatically in the second-generation, aggressively-growing LNAI cell lines (T1.8, T1.16, T2.9, and T2.11; Ref. 24). These data indicate that sPLA2-IIa expression is enhanced with progression of prostate cancer to androgen-independence.

sPLA2-IIa Expression in Primary Human Prostate Tissues. To assess whether sPLA2-IIa expression might be related to CaP development and/or advancing disease, we examined sPLA2-IIa expression by immunohistochemistry in BPH samples and in CaP samples of increasing tumor grade. In BPH from patients without cancer, only a few samples showed uniform, intense immunostaining for sPLA2-IIa (19%; 5 of 27) whereas the majority of samples showed weak focal staining

(70%; 19 of 27; Fig. 2A; Table 1). Likewise, in BPH adjacent to cancer, uniform immunostaining of sPLA2-IIa was also infrequent (6%; 2 of 35; Fig. 2C, note the benign gland in the *bottom left quadrant*). Moreover, the vast majority of low-grade prostate cancers also failed to show uniform intense staining for sPLA2-IIa (1 of 15; Table 1). In fact, the majority of BPH and low-grade cancers showed only focal staining (Table 1; Fig. 2, B and D). In contrast, ~50% of the high-grade CaPs (M2 + 3 and M3; Gleason score ≥ 7) showed intense, uniform staining for sPLA2-IIa (Table 1; Fig. 2C). Most strikingly, 75% (6 of 8) of the highest-grade M3 tumors showed uniform sPLA2-IIa staining (Fig. 2C; Table 1).

To determine whether the staining patterns of these tissues were different, we performed trend analyses for tissue type (BPH from patients without CaP, BPH adjacent to low-grade tumor, BPH adjacent to high-grade tumor, low-grade tumor, and high-grade tumor) relative to staining pattern (negative *versus* focal *versus* uniform). These analyses revealed a significant difference in the staining patterns between these groups (Spearman correlation coefficient, $P = 0.016$). Similarly, trend analyses for staining patterns and tumors of different grades (M1 + 2, M2, M2 + 3, and M3) revealed a significant difference ($P = 0.0007$). Furthermore, Fisher's exact two-tailed analyses revealed that the staining patterns of the high-grade tumors were significantly different from that of the low-grade tumors ($P = 0.013$) as well as that from BPH adjacent to high-grade tumors ($P = 0.021$). There was only a marginal difference between the staining patterns of the high-grade cancers and the staining patterns of the BPH from patients without cancer ($P = 0.068$). In contrast, staining patterns for sPLA2-IIa did not differ significantly between low-grade tumors and adjacent BPH ($P = 0.469$) nor between low-grade tumors and BPH from patients without cancer ($P = 0.395$). Together, these data indicate that

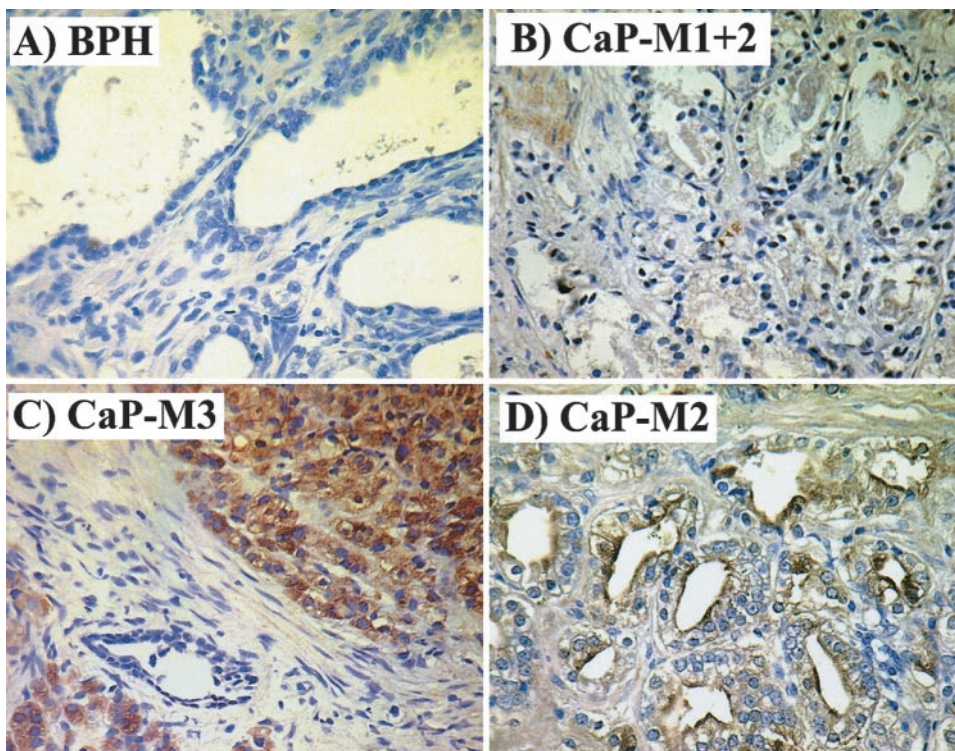


Fig. 2 sPLA2-IIa immunostaining of human prostate tissues. Expression of sPLA2-IIa was assessed by immunohistochemistry. **A** depicts the typical staining pattern for BPH from a patient without cancer. **B** shows a low-grade (M1+M2) prostate adenocarcinoma with light staining in both the epithelium and stroma. **C** shows the intense uniform staining typical of an M3 prostate tumor (*top right quadrant*) juxtaposed to the adjacent normal stroma and a normal glandular structure (*bottom left quadrant*). **D** depicts the focal staining for sPLA2-IIa typical of mid-grade (M2) prostate adenocarcinomas.

Table 2 Relationship between sPLA2-IIa staining and proliferation or apoptosis

Staining pattern	Proliferative index	Apoptotic index
Focal or no staining	1.66 ± 0.23	0.88 ± 0.07
Uniform staining	3.68 ± 0.73	0.995 ± 0.19
Student's <i>t</i> test	<i>P</i> = 0.001	<i>P</i> = 0.502

sPLA2-IIa expression is specifically up-regulated in the least-differentiated, highest-grade prostate cancers.

Because expression of sPLA2-IIa was specifically increased in the highest-grade tumors, we next assessed whether expression may be related to the disproportionate increase in proliferation relative to apoptosis that characterizes advanced CaPs (29, 30). Indeed, the proliferative index was increased >2-fold in the tumors with intense, homogeneous staining for sPLA2-IIa *versus* those with “focal” or “no” staining (*P* = 0.001; Table 2). In contrast, sPLA2-IIa staining patterns were unrelated to apoptotic index as measured by TUNEL staining (*P* = 0.502).

We next evaluated whether sPLA2-IIa staining patterns might be related to patient survival. Indeed, only 2 of 12 patients with tumors showing intense, uniform sPLA2-IIa staining survived 5 years, whereas 16 of 26 patients with tumors showing only “focal” or “no” staining survived (*P* = 0.015). As such, intense, uniform expression of sPLA2-IIa (as exemplified in Fig. 2C) may be related to poor prognosis in CaP patients.

Taken together, these data indicate that sPLA2-IIa expression increases with progression to androgen independence and is specifically increased in the most advanced CaPs. These data

therefore suggest that sPLA2-IIa may contribute to CaP progression, perhaps by enhancing arachidonate release from membrane phospholipids and thereby fueling the formation of the prostaglandins and leukotrienes, potent mediators of tumor motility, invasion, cellular proliferation, and immune suppression (4–8). It is also conceivable that enhanced sPLA2-IIa expression may play a role in CaP progression by triggering a mitogenic signal cascade. Indeed, sPLA2-IIa can activate the MAP kinases and cPLA2 (31). Activation of the MAP kinase cascade has recently been related specifically to human prostate cancer progression (32).

In summary, we have shown that the expression of the group IIa sPLA2 is specifically increased with progression of human prostate cancer cells to androgen independence. Furthermore, sPLA2-IIa expression is dramatically increased in primary, high-grade prostate cancers. This increase is related to the increased proliferative index that typifies the more advanced CaPs (26, 29). More importantly, our data show that sPLA2-IIa expression is inversely related to 5-year patient survival. This report therefore provides compelling evidence that enhanced sPLA2-IIa expression may be involved in the malignant progression of human prostate cancer and suggests that specific inhibitors of the group IIa sPLA2 may be useful for prostate cancer chemotherapy.

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