

## Secretory Phospholipase A2 Digital Expression Analysis in Colon Adenocarcinoma

EVANGELOS FALIDAS<sup>1</sup>, EIRINI KITSIOULI<sup>2\*</sup>, DESPOINA SPYROPOULOU<sup>3\*</sup>,  
EVANGELOS TSIAMBAS<sup>4</sup>, ASIMINA KALOGIROU<sup>5</sup>, GEORGE TSOUVELAS<sup>6</sup>,  
STYLIANOS PAPAPOPOULOS<sup>2</sup>, MICHAEL MITSIS<sup>7</sup>, MARILENA LEKKA<sup>2</sup>, SOFIANIKI MASTRONIKOLI<sup>8</sup>,  
DIMITRIOS PESCHOS<sup>9</sup>, ODYSSEAS DIMAS<sup>10</sup> and KONSTANTINOS VLACHOS<sup>7</sup>

<sup>1</sup>Department of Surgery, Halkida General Hospital, Halkida, Greece;

<sup>2</sup>Laboratory of Biochemistry, Chemistry Department, University of Ioannina, Ioannina, Greece;

<sup>3</sup>Department of Radiation Oncology, Medical School, University of Patras, Patras, Greece;

<sup>4</sup>Department of Cytology, 417 VA (NIMTS) Hospital, Athens, Greece;

<sup>5</sup>Department of Pathology, Halkida General Hospital, Halkida, Greece;

<sup>6</sup>Department of Nursing, University of West Attica, Athens, Greece;

<sup>7</sup>Department of Surgery, University Hospital of Ioannina, University of Ioannina, Ioannina, Greece;

<sup>8</sup>Brighton and Sussex Medical School, Brighton, U.K.;

<sup>9</sup>Department of Physiology, Medical School, University of Ioannina, Ioannina, Greece;

<sup>10</sup>2<sup>nd</sup> Department of Internal Medicine, "Gennimatas" General Hospital, Athens, Greece

**Abstract.** *Background/Aim: Phospholipases A2 represent a family of enzymes that regulate the metabolism of phospholipids by hydrolyzing them into fatty acids. Secretory phospholipase A2 (SPLA2) catalyzes the calcium-dependent 2-acyl groups hydrolysis to produce 3-sn-phosphoglycerides. This study aimed to investigate SPLA2 expression in colon adenocarcinoma (CA). Materials and Methods: Thirty (n=30) formalin-fixed, paraffin-embedded primary CA tissue sections were used and analyzed. Immunohistochemistry was performed using an anti-SPLA2 antibody. Digital image analysis was also implemented for evaluating objectively the corresponding protein expression levels. Results: Increased SPLA2 protein expression (high & moderate immunostaining levels) was observed in 23/30 (76.6%) cases, whereas 7/30 (23.4%) CA tissues demonstrated low protein levels. High expression levels were detected in 9/30 (30%) cases. SPLA2 overall expression was strongly associated with tumor*

*diameter (p=0.004), whereas other statistically significant associations were not observed (stage: p=0.971, inflammatory infiltration: p=0.795; carcinoma location: p=0.340; differentiation grade: p=0.748; sex: p=0.369; ulceration: p=0.433). Conclusion: SPLA2 over-expression is observed in significant subsets of CAs correlating with advanced tumor growth progression (increased diameter). SPLA2 seems to influence endogenous cell responses by its crucial enzymatic activity and can potentially be a biomarker for monitoring CA patients.*

Among biochemical and metabolic deregulations that occur in the intra-cellular micro-environment during the carcinogenic process, fatty acid catabolism is a crucial procedure combined or not with oxidative stress (1, 2). Fatty acid hydrolysis is mediated by phospholipases, especially of type A2 (3). These enzymes catalytically hydrolyze the corresponding bonds and result in the release of arachidonic and lysophosphatidic acids (4). Molecules such as leukotrienes and prostaglandins demonstrate significant inflammatory activity (5). Concerning phospholipases A2, their pro-enzymes are activated by trypsin. Additionally, His-48/Asp-99/calcium complex is involved in significant biochemical reactions in the exocrine pancreatic region (6). Secretory phospholipase A2 (SPLA2) gene (PLA2G10 gene band: 16p13.12) encodes for the corresponding protein— a member of phospholipase enzymes family. It functions predominantly in insulin metabolism and resistance rates and it is also involved in atherosclerosis and response to

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\*These Authors equally contributed to this study.

*Correspondence to:* Evangelos Tsiambas, MD, MSc, Ph.D., 17 Patriarchou Grigoriou E' Street, Ag. Paraskevi, 153 41 Athens, Greece. Tel: +30 2106526259, e-mail: tsiambasecyto@yahoo.gr

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inflammation (7). It is also well known that SPLA2 aberrant expression is correlated with deregulation of the autophagy mechanism and hydrolysis of surfactant phospholipids in neonates (8). In them, retreatment with surfactant is necessary. Alterations in gene and aberrant expression of the protein are implicated and detected in carcinomas of different origin (ovarian, esophageal) modifying clinicopathological parameters, even survival rates due to advanced stage and metastatic potential in the corresponding patients (9, 10). Especially in esophageal carcinoma, SPLA2 seems to act as a functional ligand for the epidermal growth factor receptors (type I and II) enhancing the corresponding receptor mediated signaling transduction to the nucleus. In the current study, we explored the role of SPLA2 protein expression in a series of CAs in order to reveal a potential association with specific clinic-histopathological features of the examined cases.

### Materials and Methods

**Study group.** For the purposes of our study, thirty (n=30) archival, formalin-fixed and paraffin-embedded tissue specimens of histologically confirmed primary CAs were used. The hospital ethics committee consented to the use of these tissues (Department of Pathology, Halkida General Hospital, Greece) for research purposes, according to World Medical Association Declaration of Helsinki guidelines. The tissue samples were fixed in 10% neutral-buffered formalin. Hematoxylin and eosin (H&E)-stained slides of the corresponding samples were reviewed for establishing the corresponding histopathological diagnoses. All lesions were classified according to the histological typing and staging criteria of World Health Organization (WHO) digestive system tumors classification (11). Clinicopathological data of the examined cases are presented in Table I.

**Antibodies and immunohistochemistry assay (IHC).** We used the rabbit polyclonal anti-SphA2 (ab23705, Abcam, Cambridge, UK; dilution/concentration at 5 µg/ml) antibody. The IHC protocol for the antigen detection was carried out on a 3 µm-thick paraffin sections. Tissue sections initially de-paraffinized in xylene and rehydrated via graded ethanol - were immunostained according to the EN Vision+ (DAKO, Glostrup, Denmark) assay using an automated staining system (I 6000 - Biogenex, Fremont, CA, USA) and according to the manufacturer's instructions. This assay is based on a soluble, dextran-polymer system preventing endogenous biotin reaction and increasing the quality of the stained slides. Briefly, the sections were pre-treated with sodium-based citrate buffer for 25 min as a heat mediated antigen retrieval. Then, they were incubated with the primary antibody for 15 min at room temperature followed by detection with a Horseradish peroxidase labeled polymer-HRP LP system. A wash with TBS was performed. The antigen-antibody reaction was visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB) as a chromogenic substrate (8 min at room temperature). Finally, the tissue sections were slightly counterstained with hematoxylin for 30 s, dehydrated and mounted with Dibutylphthalate Polyesterene Xylene (DPX: Thermo Shandon Limited, Cheshire, UK). For the negative control, the primary antibody was omitted. Focal or diffuse membranous and cytoplasmic/sub-membranous staining patterns were

Table I. Intraoperative details of the patients submitted to en bloc pancreatic and vascular resections.

Clinicopathological parameters CAs (n=30)	SPLA2		p-Value
	OE	LE	
	23/30 (76.6%)	7/30 (23.4%)	
	n (%)		
Gender			0.369
Male	15 (50%)	13 (42.9%)	2 (6.6%)
Female	15 (50%)	10 (33.3%)	5 (16.4%)
Grade			0.748
I	1 (3.3%)	1 (3.3%)	0 (0%)
II	22 (73.3%)	16 (52.8%)	6 (19.9%)
III	7 (23.3%)	6 (19.9%)	1 (3.3%)
Stage			0.971
I	3 (10%)	3 (10%)	0 (0%)
II	13 (43.3%)	10 (33.3%)	3 (10%)
III	10 (33.3%)	7 (23.3%)	3 (10%)
IV	4 (13.3%)	3 (10%)	1 (3.3%)
Anatomical location			0.340
Sigmoid	13 (43.3%)	11 (36.3%)	2 (6.6%)
Rectum	8 (13.3%)	6 (19.8%)	2 (6.6%)
Ascending-Caecum	9 (23.3%)	6 (19.8%)	3 (10%)
Inflammatory infiltration			0.795
Yes	25 (83.5%)	20 (66%)	5 (16.5%)
No	5 (16.7%)	3 (10%)	2 (6.7%)
Ulceration			0.433
Yes	5 (16.5%)	4 (13.2%)	1 (3.3%)
No	25 (83.5%)	19 (62.7%)	6 (19.8%)
Diameter max (cm)			<b>0.040</b>
<4	14/30 (47.2%)	12 (39.6%)	2 (6.6%)
≥4	16/30 (52.8%)	11 (36.3%)	5 (16.5%)

CAs: Colon adenocarcinomas; SPLA2: secretory phospholipase A2; OE: over-expression (high/moderate expression), staining intensity values ≤137; LE: low expression, staining intensity values >141. Bold p-Value shows statistical significance.

acceptable for the marker's expression (Figure 1). Normal colon epithelial tissue sections were used as positive markers for its immunostaining pattern, according to the antibody's manufacturer instructions.

**Digital image analysis assay (DIA).** SPLA2 protein expression levels were evaluated quantitatively by estimating the corresponding staining intensity levels (densitometry evaluation) in the stained malignant cells. We performed DIA using a semi-automated system [hardware: Microscope CX-31, (Olympus, Melville, NY, USA) equipped with a Digital camera (Sony, Tokyo, Japan); Windows XP/NIS-Elements Software AR v3.0, (Nikon Corp, Tokyo, Japan)]. Areas of interest per tissue section were identified (5 optical fields at ×400 magnification) and filed in a digital database as snapshots. Measurements were performed by implementing a specific macro (diffuse membranous and cytoplasmic/sub-membranous expression

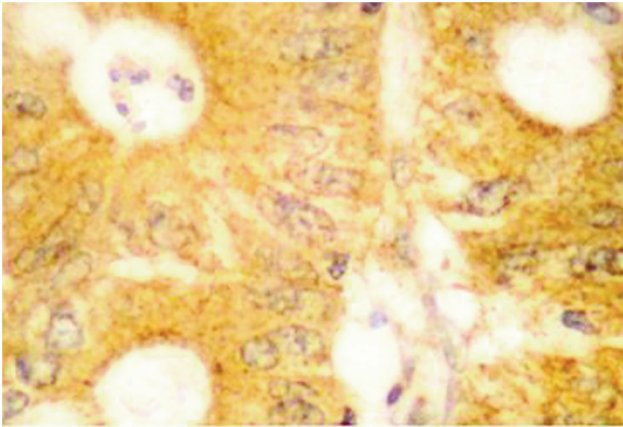


Figure 1. *SPLA2* protein expression in colon adenocarcinoma. High *SPLA2* protein expression presented as strong diffuse membrane/cytoplasmic brown staining pattern; anti-secretory phospholipase A2 (*SPLA2*), diaminobenzidine tetrahydrochloride (DAB) staining, original magnification: 100×.

for tumor cells, according to manufacturer’s datasheet). Based on an algorithm, intensity of staining of normal tissue sections (control) was measured independently and compared to the corresponding values in malignant tissue sections. A broad spectrum of continuous grey scale values (0-255) at the RedGreenBlue (RGB) analysis was available for discriminating different protein expression levels. In this digitized process, immunostaining intensity values decreasing to 0 are considered as a progressive over-expression of the marker, whereas values increasing to 255 show a progressive loss of the staining intensity. All results and DIA values are shown in Table I.

*Statistical analysis.* For statistical analyses, descriptive and inferential tests were applied. Quantitative variables are presented as mean±standard deviation, while the qualitative variables are presented in frequency tables. To evaluate the relationship between qualitative and quantitative variables, because of the small number of subjects in each group, the non-parametric Mann-Whitney, Kruskal-Wallis, and also Fisher tests were applied. Statistical significance (*p*) was evaluated in pairs and differences <0.05 were considered statistically significant. All IHC results and differences (*p*-values) are shown in Table I.

**Results**

According to digital expression analysis, the examined immunostained CA tissue sections demonstrated different expression levels of the *SPLA2* marker. *SPLA2* protein over expression (increased immunostaining levels, including high & moderate) was observed in 23/30 (76.6%) cases, whereas 7/30 (23.4%) CA tissues demonstrated low protein levels. High expression levels were detected in 9/30 (30%) of the analyzed cases. *SPLA2* overall expression was strongly associated with the tumor diameter (*p*=0.004), whereas no other statistically significant associations were observed

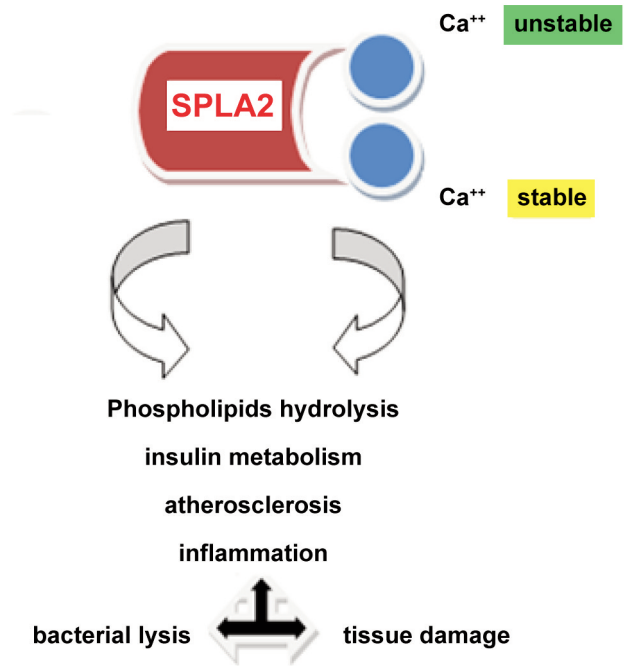


Figure 2. Schematic representation of anti-secretory phospholipase A2 (*SPLA2*) functions. Phospholipase enzymes’ family is involved in crucial intracellular metabolic activities. *SPLA2* protein – as a calcium dependent enzyme- is implicated in various biological reactions affecting lipid metabolism and inflammation. Interestingly at the biochemical level, its second calcium binding site is unstable, affecting the whole catalytic mechanism of the molecule.

(stage: *p*=0.971; inflammatory infiltration: *p*=0.795; carcinoma location: *p*=0.340; differentiation grade: *p*=0.748; sex: *p*=0.369; ulceration: *p*=0.433). In fact, *SPLA2* over-expression is involved in the volume progression of the neoplasm.

**Discussion**

Phospholipase enzymes’ family modifies crucial intracellular metabolic activities. Especially, *SPLA2* protein – as a calcium dependent enzyme- is implicated in various biological reactions affecting lipid metabolism and inflammation (Figure 2). Interestingly - at the biochemical level- one of the two calcium binding sites is unstable, affecting the catalytic mechanism of the molecule (12). In the current study, we explored *SPLA2* expression in a series of CA tissues. We observed that high levels of *SPLA2* correlated strongly to tumor growth (increased diameter). Very limited data are available concerning *SPLA2* gene deregulation and the impact of aberrant expression of *SPLA2* on CA biological behavior compared to normal epithelia. Differences in *SPLA2* expression have been detected in a study examining its expression by IHC in the gastrointestinal tract, gall bladder, and pancreatic acinar cells (13). Over-expression of *SPLA2* was observed in Crohn’s

disease and ulcerative colitis, whereas colonic adenomas and carcinomas demonstrated varying intensity levels. Similarly, another study group revealed elevated SPLA2 expression analyzing a set of sporadic CAs tissues by implementing quantitative polymerase chain reaction (PCR) (14). They also focused on the SPLA2-III variant and its potential role in CA onset and progression for both left and right colon anatomical locations. Besides these molecular observations, another study detected increased levels of SPLA2-IIA expression by western blotting in peripheral compared to core regions of malignant tissues. This unique pattern of expression in peripheral and central sites of the examined malignancies, may affect tumor micro-environment and immunology response status in the corresponding patients (15). Furthermore, studies in cell lines (such as human colon carcinoma cell line HCT-116/murine colon tumor cell line AJ02nm0) revealed a potential relation of specific SPLA2 allelic variants in different tissue substrates (16). Because the enzyme acts as a key regulator of lipid droplets formation, another study explored its role in lipid droplet accumulation in colon cancer cell lines. They detected differences in extracellular/intracellular phospholipases, but the exact mechanism of their implication in CA development and progression is still under investigation (17). The involvement of SPLA2 in CA neo-angiogenesis, invasion/metastasis, and deregulated cell proliferation/apoptosis pathways is another target for research. A study group focused on its role in the synthesis and supply of arachidonic acid (AA) and its impact on COX-2-dependent prostaglandin signaling pathway. They reported that hypertonic stress increased SPLA2 activity leading to COX-2 induction (18). Similarly, another experimental study based on mouse colon carcinoma cell lines showed an inverse relation between COX-2 and cytosolic SPLA2. Involvement of TNF- $\alpha$ -mediated apoptosis in tumor progression was also observed (19, 20). A study group analyzed an expanded set of polymorphisms in order to detect specific SPLA2-related genetic signatures in a series of CAs combined with other genes. They concluded that the genetic variability of the PLA2G3 CT haplotype was significantly correlated to a higher risk for CA in the corresponding carriers (21). Similar SPLA2 gene and protein imbalances that affect crucial metabolic pathways have already been detected in lung and gynecological carcinomas including breast and ovarian cancer. Interestingly, aberrant SPLA2 protein expression affects epithelial to mesenchymal transition in these cancers (22-26).

In conclusion, SPLA2 overexpression is observed in a significant subset of CAs correlating with aggressive biological behavior (progressive growth of malignant tumor). It is a crucial enzyme for cell response and metabolic homeostasis and also an interesting protein that can potentially be a biomarker and therapeutic target (27, 28). Although the current study included a relatively limited number of specimens for SPLA2 protein analysis, it seems that different grade and stage intensity levels may provide

some information regarding the relationship between SPLA2 expression and cancer progression. Further genetic and protein-based studies on the molecule's deregulation mechanisms in cancerous and non-cancerous colon epithelia could reveal unexplored intra-cellular pathways regarding its interactions with other metabolic regulators.

### Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

### Authors' Contributions

Evangelos Falidas, Eirini Kitsioulis, Evangelos Tsiambas, Asimina Kalogirou: research, article writing-formatting, George Tsouvelas: statistics, Stylianos Papadopoulos, Michail Mitsis, Marilena Lekka, Sofianiki Mastronikoli, Odysseas Dimas: specimen collection, clinic-pathologic feature stratification, reference detection, Dimitrios Peschos, Despoina Spyropoulou Konstantinos Vlachos: academic advisor, article review.

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