

## RESEARCH ARTICLE

# Overexpression of Phospholipase A<sub>2</sub> Group IIA in Esophageal Squamous Cell Carcinoma and Association with Cyclooxygenase-2 Expression

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### Abstract

**Background:** Esophageal cancer is one of the most frequently occurring malignancies and the seventh leading cause of cancer-related deaths in the world. The esophageal squamous cell carcinoma (ESCC) is the most common histological type of esophageal cancer worldwide. **Materials and Methods:** Our goal in this study was to detect phospholipase A<sub>2</sub> Group IIA (PLA2G2A) and cyclooxygenase-2 (COX-2) immuno-expression in ESCC in a high-risk population in China. **Results:** Positive expression of PLA2G2A protein was observed in 57.2% (166/290) of the cases, while COX-2 was found in 257 of 290 samples (88.6%), both PLA2G2A and COX-2 being expressed in 153 cases (52.8%), with a significant agreement ( $Kappa=0.091$ ,  $p=0.031$ ). Overexpression of PLA2G2A was significantly correlated with the depth of invasion ( $p=0.001$ ). Co-expression of PLA2G2A and COX-2 not only significantly correlated with the depth of invasion ( $p=0.004$ ) but also with TNM stage ( $p=0.04$ ). **Conclusions:** Our results showed that in patients with ESCC, PLA2G2A overexpression and PLA2G2A co-expression with COX-2 is significantly correlated with advanced stage. The biological role and pathophysiologic regulation of PLA2G2A and COX-2 overexpression in ESCC deserve further investigation.

**Keywords:** Esophageal SCC - phospholipase A<sub>2</sub> group IIA - cyclooxygenase-2 - immunohistochemistry

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### Introduction

Esophageal cancer is a highly aggressive malignant disease with a 5-year survival rate of 10% to 15% (Ferri et al., 2012). The incidence of esophageal cancer varies greatly between populations and across geographic regions. The Taihangshan Mountain area, including Linxian in Henan Province, a rural area of China, has the highest incidence of esophageal cancer, especially squamous cell carcinoma (ESCC), in the world (>100 per 100,000 population) (Zhang et al., 2011; Lin et al., 2013).

Inflammation has been recognized as a contributing factor for the pathogenesis of ESCC. There are results showing that two key inflammatory enzymes, Phospholipase A<sub>2</sub> Group IIA (PLA2G2A) and cyclooxygenase-2 (COX-2) are involved in the pathways of arachidonic acid (AA) biosynthesis, and both play an important role in inflammation and angiogenesis as well as cancer (Murakami et al., 2011). Recently, we reported that PLA2G2A and COX-2 gene single nucleotide polymorphisms (SNPs) may modify the risk of ESCC

development (Liu et al., 2014). However, the relationship between PLA2G2A and COX-2 protein expression and ESCC clinical features remains controversial in the literature (Li et al., 2009; Ren et al., 2013). Moreover, a correlation between the co-expression of PLA2G2A and COX-2 in ESCC has not yet been reported.

The present study aimed to evaluate the relationship between PLA2G2A and COX-2 expression in ESCC, and to determine if co-occur between these two proteins are correlated with a set of well-known clinical and pathological features of esophageal carcinoma in a high-risk Chinese population.

### Materials and Methods

#### *Patients and tissue samples*

This case-control study consecutively recruited a total of 290 primary esophageal squamous cell carcinoma patients, who underwent surgical resection of primary ESCC at the Yaocun County hospital in Linxian, Henan Province, China from October 2009 to September 2010.

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Lymph node status was determined through biopsy. Tumor stage was determined according to the AJCC TNM criteria (Edge et al., 2009), 38 cases were stage I, 137 cases were stage II, 97 cases were stage III and 18 cases were stage IV. The 290 cancerous and 30 paired noncancerous tissues from these patients were routinely fixed in 10% buffered formalin and blocked in paraffin, and embedded in paraffin blocks for histological procedures and immunohistochemical staining. None of the patients had received chemo cancer therapy before surgery.

The study was approved by the Institutional Review Board of Capital Medical University (Beijing, China), and all subjects gave written informed consent.

### Immunohistochemistry

Immunohistochemistry (IHC) was performed using the streptavidin-biotin method. Four-micrometer-thick sections of representative blocks from each case were deparaffinized in xylene, rehydrated, and treated with 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity. All sections were subjected to heat-induced epitope retrieval by autoclaving sections in a 10mM citrate buffer (pH6.0) for 10 minutes. After cooling to room temperature, the sections were treated with 3% hydrogen peroxide for 5 minutes followed by application of the primary antibody and then incubated at 4°C overnight. The antibodies used were rabbit anti-human PLA2G2A polyclonal antibody (ab23705, 1:200; Abcam, Cambridge, MA, USA) and mouse anti-human COX-2 monoclonal antibody (1:100; Cayman Chemical Co., Ann Arbor, MI). The sections that were tested for PLA2G2A and COX-2 IHC were incubated with polyperoxidase-anti-mouse/rabbit immunoglobulin (GBI) for 30 minutes at 37°C, and antibody-binding sites were visualized by DAB kit (Zhongshan Golden Bridge Co., Beijing, China). Non-immune serum instead of the primary antibody was used as negative control.

### Evaluation of immunohistochemistry

The immunohistochemical expression of PLA2G2A and COX-2 was examined independently by 2 pathologists. The percentage of positive cells was graded semiquantitatively, and each sample was assigned to one of the following categories: grade 1, <5% cells showed immunoreactivity; grade 2, 5%-30% positive cells; and grade 3, >30% positive cells. For final statistical analysis, positive expression was defined as cells with grade 2 or above ( $\geq 5\%$ ) expression levels.

### Statistical analysis

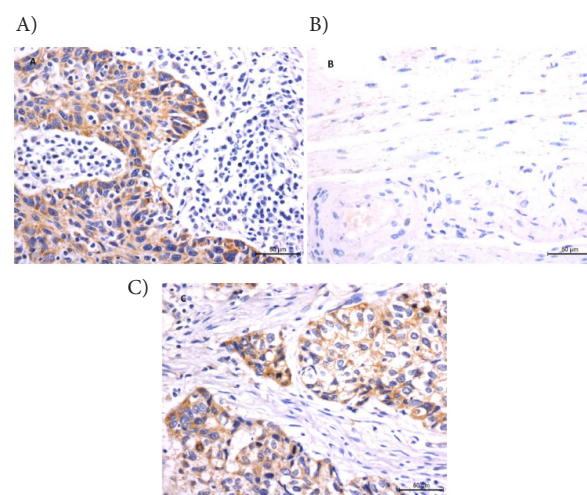
All statistical analyses were performed using SPSS software version 13.0 (SPSS Inc., Chicago, IL, USA). The distribution between the expression of COX-2 or PLA2G2A and the clinical and pathological features of ESCC patients was evaluated using Chi-squared or Fisher's exact test. The agreement between expression of COX-2 and PLA2G2A was assessed by consistency test. The relationship between the two biomarkers co-expression and the clinical and pathological features of patients was determined using Spearman's rank order correlation coefficients. Two sided significance tests were used throughout,  $p \leq 0.05$  was considered statistically significant.

## Results

Background of all patients is shown in Table 1. A total of 290 patients, including 202 (69.7%) males and 88 (30.3%) females, were included in this study. The mean age of patients was  $59.46 \pm 7.95$  years old.

### PLA2G2A protein expression in primary esophageal cancer

PLA2G2A protein expression in esophageal cancer tumor specimens was determined by immunohistochemistry.



**Figure 1. Representative Immunostaining of COX-2 and PLA2G2A in Esophageal Cancerous and Noncancerous Tissues.** A) Positive staining for PLA2G2A in esophageal tumor tissues. B) Negative staining in normal esophageal mucosa. C) Positive staining for COX-2 in esophageal tumor tissues. Original magnifications  $\times 400$  (A, B and C)

**Table 1. Comparison of Characteristics in ESCC Patients from High-Risk Linxian County, China**

Characteristics	N(%)	PLA2G2A n(%)		$p^*$	COX-2 n(%)		$p^*$	
		Negative	Positive		Negative	Positive		
Gender	Male	202(69.7)	90(72.6)	112(67.5)	0.35	23(69.7)	179(69.6)	0.99
	Female	88(30.3)	34(27.4)	54(32.5)		10(30.3)	78(30.4)	
Age(years)	<60	143(49.3)	64(51.6)	79(47.6)	0.49	13(39.4)	130(50.6)	0.23
	$\geq 60$	147(50.7)	60(48.4)	87(52.4)		20(60.6)	127(49.4)	
Smoking	Yes	181(62.4)	78(62.9)	103(62.0)	0.88	20(60.6)	161(62.6)	0.82
	No	109(37.6)	46(37.1)	63(38.0)		13(39.4)	96(37.4)	
Drinking	Yes	107(36.9)	50(40.3)	57(34.3)	0.29	17(51.5)	90(35.0)	0.06
	No	183(63.1)	74(59.7)	109(65.7)		16(48.5)	167(65.0)	

\*Chi-squared test

**Table 2. Association of PLA2G2A and COX-2 Expression with Pathological Features in ESCC**

Diagnosis	N(%)	PLA2G2A n (%)		p*	COX-2 n (%)		p*	
		Negative	Positive		Negative	Positive		
Differentiation	Well	52(17.9)	18(14.5)	34(20.5)	0.42	7(21.2)	45(17.5)	0.56
	Moderated	140(48.3)	62(50.0)	78(47.0)		13(39.4)	127(49.4)	
	Poorly	98(33.8)	44(35.5)	54(32.5)		13(39.4)	85(33.1)	
Histological types	Intramucosal cancer	10(3.4)	6(4.8)	4(2.4)	0.26	2(6.1)	8(3.1)	0.38
	Invasive cancer	280(96.6)	118(95.2)	162(97.6)		31(93.9)	249(96.9)	
Depth of invasion	T1	47(16.2)	30(24.2)	17(10.2)	0.001#	9(27.3)	38(14.8)	0.13
	T2	44(15.2)	12(9.7)	32(19.3)		6(18.2)	38(14.8)	
	T3+ T4	199(68.6)	82(66.1)	117(70.5)		18(54.5)	181(70.4)	
Lymph node metastasis	Negative	155(53.4)	71(57.3)	84(50.6)	0.26	17(51.5)	138(53.7)	0.81
	Positive	135(46.6)	53(42.7)	82(49.4)		16(48.5)	119(46.3)	
Metastasis	Negative	272(93.8)	115(92.7)	157(94.6)	0.52	30(90.9)	242(94.2)	0.47
	Positive	18(6.2)	9(7.3)	9(5.4)		3(9.1)	15(5.8)	
TNM stages	I	38(13.1)	23(18.5)	15(9.0)	0.08	7(21.2)	31(12.1)	0.38
	II	137(47.2)	56(45.2)	81(48.8)		12(36.4)	125(48.6)	
	III	97(33.4)	36(29.0)	61(36.7)		11(33.3)	86(33.5)	
	IV	18(6.3)	9(7.3)	9(5.4)		3(9.1)	15(5.8)	

\*Chi-squared or Fisher's exact test.#Significant at the level of p&lt;0.05

**Table 3. Relation between the Protein Expression of COX-2 and PLA2G2A in ESCC**

COX-2 expression	PLA2G2A expression n(%)		Kappa	p*
	Positive n=166	Negative n=124		
Positive	153(59.5)	104(40.5)	0.091	0.03
Negative	13(39.4)	20(60.6)		

\*Consistency test, Significant at the level of p&lt;0.05

**Table 4. Comparison of Characteristics in ESCC Patients between the COX-2(+)/PLA2G2A(+) and all other Groups Combined**

Characteristics	COX-2 (+)/PLA2G2A (+) group (n=153), n (%)		Other groups (n=137), n (%)	p*
	Gender	Male		
	Female	52 (34.0)	36 (26.2)	
Age	<60	72 (47.1)	71 (51.8)	0.42
	≥60	81 (52.9)	66 (48.2)	
Smoking	Yes	92(60.1)	89(65.0)	0.39
	No	61(39.9)	48(35.0)	
Drinking	Yes	49(32.0)	58(42.3)	0.07
	No	104(68.0)	79(57.7)	

\*Chi-squared test

As shown in Figure 1A, PLA2G2A immunoreactivity was localized mainly in the cytoplasm. The intensity of PLA2G2A staining was remarkably higher in primary esophageal carcinoma than in matched normal mucosa (Figure 1B), which nearly had absent staining. Prevalence of PLA2G2A expression positivity was found in 166 of 290 (57.2%) cancers and 1 of 30 (3.3%) non-cancerous tissues (p<0.001).

Moreover, increased immunoreactivity of PLA2G2A in advanced tumor invasion was also observed (p=0.001). No significant correlation between PLA2G2A expression was observed for the degree of tumor differentiation, histological types, T class, lymph node metastasis or tumor stage in this case-control study (Table 2).

**COX-2 protein expression in primary esophageal cancer**

COX-2 protein expression in esophageal cancer tumour specimens was also determined by immunohistochemistry. As shown in Figure 1C, COX-2 immunoreactivity was also localized mainly in the cytoplasm. The intensity of COX-2 staining was remarkably higher in primary

**Table 5. Comparison of Pathological Features between the COX-2(+)/PLA2G2A(+) and all other Groups Combined**

Diagnosis	COX-2(+)/PLA2G2A(+) group (n=153), n(%)	Other groups (n=137), n(%)	p*
Differentiation			0.86
Well	29 (19.0)	23 (16.8)	
Moderated	74 (48.4)	66 (48.2)	
Poorly	50 (32.6)	48 (35.0)	
Histological types			0.41
Intramucosal cancer	4 (2.6)	6 (4.4)	
Invasive cancer	149 (97.4)	131 (95.6)	
Depth of invasion			0.004#
T1	16 (10.5)	31 (22.6)	
T2	30 (19.6)	14 (10.2)	
T3+T4	107 (69.9)	92 (67.2)	
Lymph node metastasis			0.37
Negative	78 (51.0)	77 (56.2)	
Positive	75 (49.0)	60 (43.8)	
Metastasis			0.09
Negative	147(54.0)	125(46.0)	
Positive	6(33.3)	12(66.7)	
TNM stages			0.04#
I	14 (9.2)	24 (17.5)	
II	77 (50.3)	60 (43.7)	
III	56 (36.6)	41 (30.0)	
IV	6 (3.9)	12 (8.8)	

\*Chi-squared or Fisher's exact test.#Significant at the level of p&lt;0.05.

esophageal carcinoma than in matched normal mucosa, high levels of COX-2 expression were found in 257 of 290 (88.6%) cancers and 3 of 30 (10.0%) non-cancerous tissues (p<0.001).

There was no significant correlation between the expression rate of COX-2 with the clinical and pathological features in ESCC (Table 2).

**Co-expression of PLA2G2A and COX-2 in ESCC**

To determine the degree of co-expression of PLA2G2A and COX-2 in ESCC, we divided the 290 patients into 4 groups: (1) PLA2G2A and COX-2 positive group [PLA2G2A (+)/COX-2 (+) group], n=153 (52.7%); (2) PLA2G2A positive only group [PLA2G2A (+)/COX-2 (-) group], n=13 (4.5%); (3) COX-2 positive only group [PLA2G2A (-)/COX-2 (+) group], n=104 (35.9%); and (4)

both PLA2G2A and COX-2 negative group [PLA2G2A (-)/COX-2 (-) group], n=20 (6.9%). We found a significant co-expression of PLA2G2A and COX-2 ( $Kappa=0.09$ ,  $p=0.03$ ) (Table 3).

Further, we divided the 290 patients into another 2 sub-groups: the (PLA2G2A (+)/COX-2 (+) group and the other three groups combined. The clinical background of these 2 groups is provided in Table 4. We found that the PLA2G2A (+)/COX-2 (+) group was significantly more advanced with regard to the depth of invasion ( $p=0.004$ ) and TNM stage ( $p=0.04$ ) (Table 5), compared with the other three combined groups.

## Discussion

Previous studies have shown that the incidence of esophageal chronic inflammation is very high in high-risk populations for esophageal cancer, and that the mortality of ESCC is closely related with the high incidence of chronic inflammation (Savage et al., 2004; Liu et al., 2014). Thus, it suggested that chronic inflammation may be an important precancerous esophageal lesion. In the present study, we investigated the expression of the inflammatory proteins PLA2G2A and COX-2 in ESCC patients; in particular we examined the correlation of these two biomarkers with clinical and pathological data, in order to determine if the dysregulation of the proteins could be associated with ESCC.

Our results showed a moderate increase (57.2%) in PLA2G2A expression in ESCC, and PLA2G2A overexpression in ESCC correlated with greater mucosa invasion potential ( $p=0.001$ ), which is contrary to at least one other report (Ren et al., 2013). Although the reasons for this difference remain to be elucidated, dysregulation of the PLA2G2A protein might cause carcinogenesis through multiple mechanisms (Jiang et al., 2002; Fijneman et al., 2008; Wang et al., 2013). The pro-tumorigenic (e.g., prostate cancer (Jiang et al., 2002; Graham et al., 2008)) or anti-tumorigenic effects (e.g., gastric cancer (Leung et al., 2002; Ganesan et al., 2008; Xing et al., 2011; Wang et al., 2013)) of PLA2G2A appear to be tissue specific, as well as in human intestinal cancer, the role of PLA2G2A remains elusive. Several studies have reported both up- and down-regulation of PLA2G2A expression in human colorectal cancers (MacPhee et al., 1995; Cormier et al., 1997; Cormier et al., 2000).

A few in vitro studies further demonstrated that PLA2G2A expression induces the proliferation of human astrocytoma and esophageal adenocarcinoma cells, and treatment with specific PLA2G2A inhibitors or gene knockdown attenuates tumor growth (Martin et al., 2009; Mauchley et al., 2010). Consequently, PLA2G2A has been proposed as a target for the treatment of human cancer (Cummings, 2007; Scott et al., 2010).

COX-2 enzyme is one of the two isoforms of cyclooxygenase (COX) producing prostaglandins from arachidonic acid. COX-2 levels have been found to be high in various tumors (Liu et al., 2006; Gomes et al., 2013; Peng et al., 2013; Tabriz et al., 2013). This protein has an important role in carcinogenesis by promoting increased cell proliferation, reducing apoptosis and

acting in angiogenesis (Takedo, 1998). Our results show a significant increase (88.6%) in COX-2 expression in ESCC, as previously reported (Gomes et al., 2013). We found no significant correlation of COX-2 with tumor data. However, both PLA2G2A (+)/COX-2 (+) group was significantly more advanced with regard to the depth of invasion ( $P=0.004$ ) and TNM stage ( $p=0.04$ ) (Table 5), PLA2G2A and COX-2 co-occur with ESCC and that are characterized by inflammation. These results suggested that the activation of eicosanoid metabolic pathway is one of the important predictive factors of esophageal cancer. It has been known that disruption of the mouse PLA2G2A, a potential source of AA for COX-2, increases tumor number despite the fact that the mutation has been predicted to decrease prostaglandin production (Hong KH, et al., 2001). Some studies have suggested that PLA2G2A and COX-2 play a role in other diseases (Galecki et al., 2012).

Previously we have shown that functional SNPs (rs11644) in the genes encoding PLA2G2A and COX-2 (rs12042763) confer susceptibility to develop ESCC (Liu et al., 2014). Therefore, one might conclude that the increased expression for PLA2G2A and COX-2 established in the present study might be genotype dependent. There is no evidence for a relation between the PLA2G2A and COX-2 gene SNPs and expression (data not shown). The variant might be in linkage disequilibrium with other variants that affect expression. The expression might also be related to another completely different variant and increased expression might be circumstances. The increased level of PLA2G2A and COX-2 in the ESCC tissue might explain their effectiveness of non-steroid anti-inflammatory drugs (NSAID) as prevention and therapies for ESCC.

In summary, PLA2G2A and COX-2 might play an important role in the progression of ESCC, as co-expression of PLA2G2A and COX-2 correlates with not only the depth of invasion but also TNM stage. The precise mechanisms underlying the risk effect of PLA2G2A and COX-2 interaction on depth of invasion remain to be elucidated in further cell- and population-based studies.

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