Original Article

cPLA2α activates PI3K/AKT and inhibits Smad2/3 during epithelial–mesenchymal transition of hepatocellular carcinoma cells

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Cytosolic phospholipase A2α (cPLA2α), a key phospholipase that regulates lipid metabolism, plays an important role in tumor progression. In the present study of hepatocellular carcinoma (HCC), cPLA2α was overexpressed in highly metastatic HCC cell lines. Immunohistochemical staining showed increased levels of cPLA2α at the invasive edges of HCC, and a clinicopathological analysis of samples from 111 patients revealed that its expression level was linked with micro-vascular invasion and cirrhosis. Knockdown of cPLA2α inhibited migration, probably due to its role in actin polymerization. Overexpression of cPLA2α promoted cell migration and invasion. Based on the mechanistic analysis, our data suggested that cPLA2α mediated epithelial growth factor (EGF) induced epithelial–mesenchymal transition (EMT) through PI3K/AKT/ERK pathway. cPLA2α activity was required for the transforming growth factor-(TGF-β)–induced EMT. However, cPLA2α inhibited Smad2/3 activation and promoted the activation of the PI3K/AKT/ERK pathway. A xenograft tumor transplant model confirmed the role of cPLA2α in HCC invasion and metastasis. Based on the mechanistic analysis, cPLA2α mediated both EGF- and TGF-β–induced EMT, which are essential for HCC metastasis. cPLA2α is a potentially target for novel therapies of HCC.

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Introduction

Epithelial–mesenchymal transition (EMT) is an essential process in embryogenesis and organogenesis [1]. Accumulating evidence has demonstrated that EMT plays critical roles in pathological processes, including tumor invasion and tissue fibrosis [2,3]. Transforming growth factor-β (TGF-β) is a master inducer of EMT in cancer cells. Smad signaling elicited by TGF-β plays an important role in EMT. Moreover, non-Smad signaling pathways, such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) signals, are also activated in TGF-β–induced EMT [4]. Epidermal growth factor (EGF) is another well-characterized inducer of EMT in multiple human malignancies [5,6]. The EGF-induced PI3K/AKT, extracellular regulated kinase 1/2 (ERK1/2) and nuclear factor-κB (NF-κB) pathways also play important roles in EMT by up-regulating Snail, Slug, Twist and ZEB1 [6–8].

Bioactive lipid metabolites are prevalent in the tumor micro-environment and regulate EMT through several signaling pathways that are important in tumor growth and metastasis [9,10]. As shown in the study by Molina et al., overexpression of lipid metabolism genes induces EMT and increases the migratory and invasive properties of the cells [11]. However, the connection between EMT and lipid metabolism remains largely unknown.

Abbreviations: HCC, Hepatocellular carcinoma; EMT, epithelial–mesenchymal transition; shRNA, short hairpin RNA; SCR, scrambled; KD, knockdown; OE, over-expression; FBS, fetal bovine serum; EGF, epidermal growth factor; TGF-β, transforming growth factor-β; AA, arachidonic acid; LPI, l-α-lysophosphatidylinositol; H&E, Hematoxylin and Eosin staining.

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Cytosolic phospholipase A2α (cPLA2α) is a member of the phospholipase family that catalyzes the hydrolysis of the fatty acyl ester bond at the sn-2 position of phospholipids to produce bioactive lysophospholipids and arachidonic acid (AA) [12, 13]. AA is metabolized to biologically active eicosanoids by cyclooxygenases (COXs), lipooxygenases, and epoxygenases (cytochrome P450). These eicosanoids function as second messengers in the signaling pathways associated with tumor progression and cancer metastasis. Recent studies have indicated that increased cPLA2α expression is correlated with a poor prognosis and tumorigenesis in several human cancers, including hepatoma [14], non-small cell lung cancer [15], prostate cancer [16], and ovarian cancer [17]. However, the role of cPLA2α in EMT and metastasis of hepatocellular carcinoma (HCC) cells remains unknown, and the molecular mechanism requires further investigation.

In this study, we demonstrate that cPLA2α is expressed at high levels in HCC tissues compared with its expression in the associated nontumorous tissues. Moreover, cPLA2α is expressed at particularly high levels in mesenchymal-typed HCC cell lines and in the edges of clinical HCC tumor tissues. We also report the mechanism by which cPLA2α promotes EMT and the invasiveness of HCC cells.

Materials and methods

Clinical samples for cPLA2α survival correlation studies

The 111 archived paraffin blocks of normal liver tissue and invasive liver tumor tissues were obtained from the Department of Liver Cancer, Tianjin Medical University Cancer Institute and Hospital with the patients’ consent. The protocol which was conformed to the ethical guidelines of 1975 Declaration of Helsinki was approved by the ethics committee. High or low cPLA2α expression in liver cancer tissues and percentage of positive cells were scored in a semi-quantitative analysis using a standard immunohistochemical staining protocol.

Fresh human HCC tissue specimens and processing

Liver tumor and adjacent non-tumor liver tissue specimens were collected from nine patients who underwent hepatectomy for HCC between 2014 and 2015 in the Department of Liver Cancer, Tianjin Medical University Cancer Institute and Hospital with the patients’ consent. The protocol which was conformed to the ethical guidelines of 1975 Declaration of Helsinki was approved by the ethics committee.

Tumor tissue from xenografts and fresh tumors were minced into 1 mm cubes and lysed with Triton X-100 buffer. The tissue lysates were then centrifuged at 15,000 × g for 15 min at 4 °C and the pellets were removed. The total protein concentrations of the tissue extracts were measured using a bicinchoninic acid (BCA) assay. Then, Western blotting analyses were conducted on the samples.

Statistical analysis

All statistical analyses were performed using the statistical software SPSS 17 for Windows (SPSS Inc., Chicago, IL). All data are given as mean ± SD. Values of p < 0.05 were considered statistically significant.

The details about other assays are described in the Supplementary Materials and Methods.

Results

cPLA2α expression is associated with HCC tumorigenesis and invasion

A microarray was used to analyze the expression of 34 phospholipases in two HCC cell lines: Huh7, a cell line exhibiting low metastatic potential, and LM3, a highly metastatic cell line. The LM3 cells exhibited the greatest fold increase in cPLA2α expression (Supplementary Fig. 1A). The microarray results were further confirmed by quantitative real-time PCR analysis of 14 phospholipases. LM3 cells exhibited an approximately 2.5-fold increase in cPLA2α expression, which was much higher than the other phospholipases (Supplementary Fig. 1B). Western blotting assays were performed to further examine expression in Huh7 and LM3 cells; the cPLA2α levels were lower in the Huh7 cells than in LM3 cells (Supplementary Fig. 1C). The immunohistochemical analysis (IHC) of samples from 111 patients revealed low expression in adjacent nontumorous tissues and strong staining in tumor tissues (Fig. 1A). The clinicopathologic analysis revealed that cPLA2α expression was associated with histological differentiation, micro-vascular invasion and cirrhosis (Table 1). Western blotting of samples from seven patients showed a higher level of cPLA2α in tumor tissues than in tissues associated with the tumor (Fig. 1B). Interestingly, the expression levels of cPLA2α were higher at the edges than in the centers of the tumors (Fig. 1C and D). Western blotting of samples from two patients showed a higher level of cPLA2α in tissues from the tumor edges than the tumor centers. Furthermore, the staining in invasive foci and the tumor thrombus was also much higher than in the tumor tissues. Patients with high cPLA2α expression had lower survival rates (Fig. 1E). Multi-variate Cox regression analysis demonstrated that cPLA2α (HR = 2.116, 95% CI, 1.020–4.392; P = 0.044) is an independent prognostic indicator for HCC patients (Table 2). Taken together, our results suggest that cPLA2α expression is associated with tumor progression and metastasis. Moreover, cPLA2α expression levels correlate with patient survival.

cPLA2α promoted HCC cell migration, adhesion and proliferation in vitro

We then specifically downregulated cPLA2α expression in HepG2 cells by infecting them with a lentivirus expressing an shRNA to examine the role of cPLA2α in the invasion and metastasis of human HCC cells. The cells infected with cPLA2α shRNA were designated KD cells (Fig. 2A). Furthermore, full-length human cPLA2α was cloned into a lentiviral vector and then stably transfected into the HepG2 human HCC cell line; this line was designated the OE cell line. The HepG2 cells transfected with the lentiviral vectors expressing scrambled sequences were designated KD/SCR and OE/SCR cells. EGF-mediated migration and invasion of HCC cells is essential for HCC metastasis [18]. In vitro chemotaxis and Matrigel invasion assays indicated that cPLA2α overexpression efficiently increased the metastasis of HepG2 cells (Fig. 2A and B). In addition, cell invasion was strongly impaired in cPLA2α knockdown HepG2 cells compared with control cells. In a wound healing migration assay, the KD HepG2 cells showed delayed wound healing compared with KD/SCR HepG2 cells, whereas OE HepG2 cells showed enhanced wound healing ability (Fig. 2C), confirming the ability of cPLA2α to promote HCC cell migration in vitro. Cell adhesion plays an important role in cancer progression and metastasis [19]. Inhibition of cPLA2α expression significantly reduced HepG2 cell adhesion, whereas cPLA2α-overexpressing cells displayed increased adhesion (Fig. 2D). Reorganization of the actin cytoskeleton is a crucial event in cell migration [20]. As shown in Fig. 2E, EGF induced actin polymerization in HCC cells, whereas knockdown of cPLA2α expression inhibited actin polymerization, suggesting that cPLA2α has a role in cytoskeletal rearrangements. The growth analysis showed a significant reduction in the proliferation of cPLA2α knockdown cells at 6 days of observation (Fig. 2F). The colony formation in soft agar assay also confirmed that the proliferation rate was impaired by cPLA2α knockdown, and was increased by cPLA2α overexpression. These data clearly indicated that cPLA2α plays an important role in HCC cell migration, adhesion and proliferation in vitro.

cPLA2α mediated EGF-induced EMT in HCC cells

EMT of HCC is mediated by growth factors, particularly EGF and TGF-β [8]. We first examined the role of cPLA2α in EGF-induced EMT. As shown in Fig. 3A, cPLA2α knockdown...
increased E-cadherin expression and decreased N-cadherin and vimentin expression. Alterations in cell morphology were examined by microscopy. In both KD/SCR and OE/SCR cells, treatment with 15 ng/ml EGF for 48 h induced the formation of spindle-shaped cells, and cells lost their contacts, consistent with previous reports [6] (Fig. 3B). The cPLA2a KD cells maintained a cobblestone shape, whereas the cPLA2a OE cells displayed a spindle shape. These results suggest that cPLA2a regulates EMT in HCC cells.

As shown in the Western blotting, EGF elicited a marginal decrease in the E-cadherin levels and increases in the N-cadherin and vimentin levels, whereas cPLA2a overexpression increased E-cadherin expression and decreased the N-cadherin and vimentin expression levels (Fig. 3C). cPLA2a overexpression increased the N-cadherin and vimentin levels and significantly reduced the E-cadherin levels (Fig. 3D). The expression of these three biomarkers in cPLA2a knockdown and overexpressing cells was further confirmed by quantitative real-time PCR (Supplementary Fig. 2A). The fluorescent confocal microscopy analysis also revealed that cPLA2a played an important role in regulating the expression of the three biomarkers (Fig. 3E). In another HCC cell line, HLE cells, cPLA2a also increased N-cadherin and vimentin expression and reduced E-cadherin expression (Fig. 3F). These findings indicate that cPLA2a plays an important role in EGF-induced HCC EMT.

cPLA2a mediated EGF-induced EMT through the PI3K/AKT pathway

We next investigated the signal transduction pathways downstream of cPLA2a. We speculate that cPLA2a regulates PI3K/AKT activation in HCC cells. As shown in Fig. 3G, cPLA2a knockdown inhibited the phosphorylation of AKT/PKB and ERK1/2, two downstream signaling molecules of PI3K, suggesting that PI3K functioned downstream of cPLA2a. Western blotting analyses were performed to examine the expression of five transcription factors: Snail, Twist, Slug, and ZEB1 and 2. EGF induced a significant increase in Twist expression that was inhibited in cPLA2a knockdown cells (Fig. 3G). EGF also induced increases in Snail and ZEB1 expression that were blocked by cPLA2a knockdown. EGF did not induce detectable changes in Slug and ZEB2 expression (Supplementary Fig. 2B). In cPLA2a overexpressing cells, the basal levels of phosphorylated PKB/AKT and ERK1/2 were increased, and the EGF treatment further increased PKB/AKT and ERK1/2 activation (Fig. 3H). Meanwhile, the levels of Snail, Twist and ZEB1 were also increased in the absence of EGF, whereas the EGF treatment further increased the levels of these transcription factors.

AA and L-α-lysophosphatidylinositol (LPI) are two major physiological products of the cPLA2a-catalyzed reaction [21,22]. In cPLA2a knockdown cells, treatment with 50 μM AA partially rescued the phosphorylation of AKT (Ser473) within 48 h (Fig. 3I). Consequently, the EGF-induced expression of Snail, Twist, and ZEB1 was also elevated within 48 h. As shown in Fig. 3I, supplementation with 5 μM LPI for 48 h also partially rescued AKT/PKB phosphorylation and the expression levels of Snail, Twist, and ZEB1. These results suggest that the enzymatic activity of cPLA2a was important for EMT.

The role of the PI3K pathway in cPLA2a-mediated EMT was further examined using LY294002, a specific inhibitor of PI3K. In cPLA2a overexpressing cells without EGF, LY294002 significantly blocked the basal levels of phosphorylated AKT/PKB and ERK1/2 (Fig. 3J). The basal levels of Twist/Snail/ZEB1 were further decreased. In the presence of EGF, LY294002 blocked the EGF-induced phosphorylation of PKB/AKT and ERK1/2. The EGF-induced increase in Snail/Twist/ZEB1 expression was also

Fig. 1. cPLA2a expression is associated with HCC tumorigenesis and invasion. (A) Representative immunohistochemical staining for cPLA2a in HCC tumor tissues and adjacent normal tissues (original magnifications: 200×). (B) Western blotting analysis of the levels of the cPLA2a protein in 7 paired HCC tumor tissues and adjacent tumor edges. (C) Representative immunohistochemical staining for cPLA2a in HCC tumor centers, tumor edges, invasion foci, and a tumor thrombus. (E) The Kaplan-Meier analysis revealed a correlation between cPLA2a overexpression and a poorer prognosis in 111 patients with HCC ($\chi^2 = 3.865, p = 0.049$).
levels of Snail, Twist and ZEB1, which was blocked in the cPLA2α knockdown cells. In the cPLA2α overexpressing cells, the basal levels of Snail, Twist and ZEB1 were increased and treatment with TGF-β further increased their levels. Taken together, our results suggest that cPLA2α plays an important role in TGF-β-induced EMT.

cPLA2α mediated TGF-β-induced EMT

TGF-β has been described as both a key inducer and a factor that maintains EMT [23]. In HepG2 cells, treatment with 15 ng/ml TGF-β for 48 h induced the formation of elongated, spindle-shaped cells, and the cells lost their contacts, whereas cPLA2α knockdown led to cells maintaining a cobblestone shape with tight cell-cell contacts (Fig. 4A). The Western blotting analysis showed that TGF-β elicited an increase in the E-cadherin level and decreases in the N-cadherin and vimentin levels in the KD/SCR cells, whereas cPLA2α knockdown cells treated with TGF-β did not show any significant change in the expression levels of the three EMT biomarkers (Fig. 4B). The fluorescent confocal microscopy analysis also revealed that cPLA2α played an important role in regulating the expression of the three biomarkers (Fig. 4C).

As a critical step in the EMT process, the repression of E-cadherin expression is mainly due to up-regulation of the levels of Snail, Slug, Twist, ZEB1 and other transcription factors [24–27]. As shown in Fig. 4D, we examined the effect of cPLA2α on the TGF-β-induced expression of three EMT-related transcription factors, Snail, Twist and ZEB1, in HepG2 cells (KD/SCR, KD, OE/SCR, and OE). KD/SCR cells and OE/SCR cells treated with TGF-β (15 ng/ml, 48 h) exhibited a significant increase in the expression

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### Table 1
Correlation between cPLA2α and clinicopathologic characteristics of patients with hepatocellular carcinoma.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Total</th>
<th>cPLA2α expression</th>
<th>χ²</th>
<th>P Value</th>
</tr>
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<tr>
<td></td>
<td>Weakly (%)</td>
<td>Strongly (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age ≤60</td>
<td>75</td>
<td>44 (58.7)</td>
<td></td>
<td>0.741</td>
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<tr>
<td>Age &gt;60</td>
<td>36</td>
<td>18 (50.0)</td>
<td></td>
<td>0.170</td>
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<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>91</td>
<td>50 (54.9)</td>
<td></td>
<td>0.170</td>
</tr>
<tr>
<td>Female</td>
<td>20</td>
<td>12 (60.0)</td>
<td></td>
<td>0.170</td>
</tr>
<tr>
<td>Tumor size ≤5 cm</td>
<td>65</td>
<td>39 (60.0)</td>
<td></td>
<td>1.092</td>
</tr>
<tr>
<td>Tumor size &gt;5 cm</td>
<td>46</td>
<td>23 (50.0)</td>
<td></td>
<td>0.335</td>
</tr>
<tr>
<td>Histological differentiation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well differentiated</td>
<td>15</td>
<td>7 (46.7)</td>
<td></td>
<td>9.114</td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>90</td>
<td>55 (61.1)</td>
<td></td>
<td>35.89</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>6</td>
<td>0 (0.0)</td>
<td></td>
<td>100.0</td>
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<td>Microvascular invasion</td>
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<td></td>
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<tr>
<td>Present</td>
<td>62</td>
<td>29 (46.8)</td>
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<tr>
<td>Absent</td>
<td>49</td>
<td>33 (67.3)</td>
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<td>0.036*</td>
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<tr>
<td>TNM</td>
<td>32</td>
<td>20 (62.5)</td>
<td></td>
<td>2.532</td>
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<tr>
<td>TNMII</td>
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<td>TNMIII</td>
<td>41</td>
<td>19 (46.3)</td>
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<td>52.37</td>
</tr>
<tr>
<td>TNMIV</td>
<td>9</td>
<td>5 (55.6)</td>
<td></td>
<td>4 (44.4)</td>
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<tr>
<td>AFP level</td>
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</tr>
<tr>
<td>≤400</td>
<td>82</td>
<td>45 (54.9)</td>
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<tr>
<td>&gt;400</td>
<td>29</td>
<td>17 (58.6)</td>
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<td>HBV</td>
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</tr>
<tr>
<td>+</td>
<td>87</td>
<td>46 (52.9)</td>
<td></td>
<td>1.451</td>
</tr>
<tr>
<td>–</td>
<td>24</td>
<td>16 (66.7)</td>
<td></td>
<td>0.255</td>
</tr>
<tr>
<td>HCV</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
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<td>7 (87.5)</td>
<td></td>
<td>3.501</td>
</tr>
<tr>
<td>Absent</td>
<td>103</td>
<td>55 (53.4)</td>
<td></td>
<td>0.075</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>60</td>
<td>25 (41.7)</td>
<td></td>
<td>10.663</td>
</tr>
<tr>
<td>Absent</td>
<td>51</td>
<td>37 (72.5)</td>
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<td>0.001*</td>
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<tr>
<td>Satellite</td>
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<tr>
<td>Present</td>
<td>40</td>
<td>19 (47.5)</td>
<td></td>
<td>1.771</td>
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<tr>
<td>Absent</td>
<td>71</td>
<td>43 (60.6)</td>
<td></td>
<td>0.233</td>
</tr>
</tbody>
</table>

**Note:** *Statistical significance (P < 0.05) is shown in bold.

**Abbreviations:** AFP, alpha fetal protein; HBV, hepatitis B virus; HCV, hepatitis C virus.

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### Table 2
Univariate and multivariate analysis of the prognostic factors for overall survival.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
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</thead>
<tbody>
<tr>
<td>Prognostic factors</td>
<td>HR (95% CI)</td>
<td>p Value</td>
</tr>
<tr>
<td>Age (&lt;60/≥60)</td>
<td>1.026 (0.472–2.30)</td>
<td>0.948</td>
</tr>
<tr>
<td>Sex</td>
<td>0.794 (0.520–1.21)</td>
<td>0.286</td>
</tr>
<tr>
<td>Histological differentiation</td>
<td>0.785 (0.476–1.29)</td>
<td>0.343</td>
</tr>
<tr>
<td>Micro-vascular invasion</td>
<td>0.732 (0.430–1.245)</td>
<td>0.493</td>
</tr>
<tr>
<td>Macro-vascular invasion</td>
<td>1.066 (0.752–1.511)</td>
<td>0.719</td>
</tr>
<tr>
<td>AFP</td>
<td>0.950 (0.621–1.452)</td>
<td>0.812</td>
</tr>
<tr>
<td>HBV</td>
<td>0.769 (0.475–1.245)</td>
<td>0.285</td>
</tr>
<tr>
<td>HCV</td>
<td>1.641 (0.656–4.449)</td>
<td>0.330</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>0.578 (0.388–0.861)</td>
<td>0.007*</td>
</tr>
<tr>
<td>Satellite</td>
<td>0.733 (0.516–1.042)</td>
<td>0.083</td>
</tr>
<tr>
<td>cPLA2α</td>
<td>2.394 (1.15–4.961)</td>
<td>0.019*</td>
</tr>
</tbody>
</table>

**Notes:** *P-value < 0.05 was considered to be significant. Univariate and multivariate analyses: Cox proportional hazards regression model.

**Abbreviations:** HR, hazard ratio; CI, confidence interval.
cPLA2 overexpression promoted HCC metastasis in vivo

We then evaluated the contribution of cPLA2a to the metastasis of HCC cells in vivo using a mouse xenograft model. cPLA2a OE cells were injected into the groins of nu/nu mice, and OE/SCR cells were used as a control. First, we assessed the survival rates of tumor-bearing mice. Twenty-one days after the injections, mice injected with the OE cells started to die, which continued for 84 days after injection. Mice injected with OE/SCR cells had a significantly shorter median overall survival (approximately 46 days) than mice injected with the OE/SCR cells (median overall survival, 84 days) (Fig. 6A). All tumors were collected. No significant difference in the primary tumor sizes was observed between the two groups (Fig. 6B). We did not detect tumor formation in mice injected with KD cells within three weeks (data not shown). The correlation between cPLA2a and EMT-related markers in primary tumors was
Fig. 3. cPLA2α regulates the EGF-induced EMT in HCC cells through the PI3K pathway. (A) Western blotting analysis of the expression of cPLA2α, E-cadherin, N-cadherin and vimentin in KD/SCR and KD cells. (B) Morphological changes in the KD/SCR, KD, OE/SCR and OE cells that had been treated with 15 ng/ml EGF for 48 h. Western blotting analysis of the expression of E-cadherin, N-cadherin and vimentin in KD/SCR and KD cells (C) and OE/SCR and OE cells (D) cultured with or without EGF. GAPDH was used as a control. (E) Immunofluorescence staining for E-cadherin, N-cadherin and vimentin in KD/SCR and KD cells cultured with or without EGF. (F) (Upper panel) Morphological changes in HLE cells after transfection with the cPLA2α-overexpressing lentivirus (HLE/OE). (Lower panel) Western blotting analysis of the expression of cPLA2α, E-cadherin, N-cadherin and vimentin in HLE/SCR and HLE/OE cells. (G) and (H) Western blotting analysis of EGF-induced phosphorylation of AKT Ser473, ERK1/2 and the EMT-related transcription factors Snail, Twist and ZEB1 in total cell lysates from KD/SCR, KD, OE/SCR and OE cells. Total AKT, ERK1/2 and GAPDH were used as controls. (I) KD cells were treated with 20 μM AA and 5 μM LPI in the...
verified by using H&E and IHC. Epithelial marker E-cadherin was downregulated in tumors of mice injected with OE cells, whereas mesenchymal marker vimentin was upregulated (Fig. 6C). The results were consistent with results in vitro. Next, we examined the metastasis potential by sacrificing mice four weeks after the xenograft transplant. As shown in Fig. 6D, we observed significant metastasis to the livers, intestines, diaphragm, and peritoneal cavity in 10 mice that were transplanted with OE cells (100%), whereas only 1 mouse injected with the OE/SCR cells showed distant metastasis (10%, p < 0.001). In the third set of experiments, we focused on lung metastasis using histochemical staining with hematoxylin and eosin (H&E). In four weeks, extensive tumor foci were observed in five out of six mice (83.3%) injected with the OE cells, whereas only one out of nine mice injected with the OE/SCR cells displayed lung metastasis (11.1%, p < 0.005) (Fig. 6E). Thus, our results clearly indicated that cPLA2α overexpression promotes the metastasis of human HCC in vivo.

**Discussion**

cPLA2α plays an important role in lipid metabolism, the inflammatory response, and cell migration [16,29]. Our results showed that the aberrant expression of cPLA2α was associated with HCC progression and invasion. High cPLA2α expression was detected in samples from patients with cirrhosis, consistent with its pro-inflammatory role. Moreover, cPLA2α expression was linked to the differentiation status and microvascular invasion of the tumor. More interestingly, cPLA2α expression appeared to follow a spatial distribution pattern within tumors, which was high at the edges and invasive foci and low at the centers. Based on these clinical results, an aberrant increase in cPLA2α expression is linked to HCC invasion. According to functional studies, cPLA2α regulates a plethora of cellular responses in HCC exacerbation. Knockdown of cPLA2α reduced cell proliferation and inhibited tumor formation in absence or presence of 15 ng/ml EGF for 48 h. Western blotting analysis of phosphorylation of AKT Ser473 and the EMT-related transcription factors Snail, Twist and ZEB1 in OE cells treated with 50 μM LY294002 in the absence or presence of 15 ng/ml EGF for 48 h. LY294002 is a PI3K inhibitor. Western blotting analysis of EGF-induced phosphorylation of AKT Ser473, ERK1/2 and the EMT-related transcription factors Snail, Twist and ZEB1 in total cell lysates from KD cells. Total AKT and GAPDH were used as controls. (J) OE cells were treated with 50 μM LY294002 for 12 h and 5 μM LPI in the absence or presence of 15 ng/ml EGF for 48 h. Western blotting analysis of EGF-induced phosphorylation of AKT Ser473, ERK1/2 and the EMT-related transcription factors Snail, Twist and ZEB1 in total cell lysates from OE cells. Total AKT, ERK1/2 and GAPDH were used as controls. (K) OE/SCR cells were treated with 50 μM LY294002 for 12 h and 5 μM LPI in the absence or presence of 15 ng/ml EGF for 48 h.
xenograft transplant tumor models, suggesting a role for cPLA2α in proliferation, consistent with a previous report showing that cPLA2α regulates the proliferation of colorectal cancer cells and HCC cells [30,31]. cPLA2α appeared to regulate cell adhesion and EGF-induced actin polymerization, which might account for its role in HCC cell migration and invasion. Furthermore, high cPLA2α levels clearly promoted mesenchymal phenotypes. Knockdown of cPLA2α inhibited both EGF- and TGF-β-induced EMT. Animal experiments further confirmed the role of cPLA2α in HCC metastasis. Thus, high levels of cPLA2α can promote HCC progression and metastasis.

The tumor microenvironment has been consistently shown to instruct cancer cells to undergo EMT to obtain motility, invasive-ness and higher resistance to environmental stress in the circula-tory system [32,33]. A plethora of biochemical and biophysical factors in the microenvironment directly promote EMT, including pro-inflammatory cytokines, hypoxia, extracellular matrix components, and mechanical properties. According to our mechanistic studies, cPLA2α mediated EGF-induced AKT/ERK phosphorylation during EMT. Knockdown of cPLA2α inhibited EGF-induced AKT/ERK phosphorylation, whereas cPLA2α overexpression increased the basal levels of phosphorylated AKT/ERK, and treatment with EGF further increased the levels of the phosphorylated proteins. The expression levels of three EMT-related transcription factors, Snail, Twist and ZEB1, were increased concomitantly with AKT/ERK phosphorylation. The observation that LY294002, a PI3K inhibitor, inhibited the expression of the three EMT-related transcription factors in the cPLA2α overexpressing cells further indicated that cPLA2α-induced EMT was mediated by the PI3K/AKT/ERK pathway. We attempted to determine which cPLA2α products promoted EMT. The Western blotting results indicated that both AA and LPI could rescue AKT phosphorylation and the expression of the three

Fig. 5. cPLA2α regulates the TGF-β-induced EMT in HCC cells by activating the PI3K pathway and inhibiting the Smad pathway. (A) Western blotting analysis of the p-Smad2/3 levels in KD/SCR, KD, OE/SCR and OE cells treated with or without TGF-β (10 ng/ml, 30 min). The total Smad2/3 and GAPDH levels were used as controls. (B) Immunoblot analysis of the p-Smad2/3 and total Smad2/3 levels in the cytoplasm and nuclei of KD/SCR and KD cells treated with or without TGF-β (10 ng/ml, 30 min). (C) The nuclear localization of p-Smad2/3 in KD/SCR and KD cells treated with or without TGF-β (10 ng/ml, 30 min) was determined by immunofluorescence staining. (D) Immunoblot analysis of the levels of p-AKT (Ser473), p-ERK (Thr202/Tyr204), and the EMT-related transcription factors Snail, Twist and ZEB1 in OE/SCR cells treated with or without TGF-β (15 ng/ml, 48 h), the PI3K inhibitor LY294002 (50 μM) or the ERK inhibitor PD98059 (25 μM). Total AKT, total ERK and GAPDH were used as controls. (E) Immunoblot analysis of the levels of p-AKT (Ser473), p-ERK (Thr202/Tyr204), and the EMT-related transcription factors Snail, Twist and ZEB1 in KD cells treated with or without TGF-β (15 ng/ml, 48 h), AA (20 μM) or LPI (5 μM). Total AKT, total ERK and GAPDH were used as controls.

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transcription factors in cPLA2α knockdown cells. In cPLA2α overexpressing cells, LPI failed to rescue the inhibitory effects of the LY294002 treatment, suggesting that LPI functioned upstream of PI3K. However, the detailed molecular mechanism requires further investigation. Based on a large body of evidence, biologically active lipids, including AA, lysophosphatidic acid (LPA), and LPI, orchestrate the complex interactions between tumor cells and their microenvironment [9,34]. AA induced migration and invasion of breast cancer cells by promoting PI3K/AKT activation [35]. COX-2 is the inducible isoform of the rate-limiting enzyme that converts AA to pro-inflammatory prostaglandins [36]. Accumulating clinical and epidemiological evidence has demonstrated that the nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, which inhibits COX-2 activity, reduce risk of cancer initiation, progression and tumor metastasis [37,38]. Therefore, bioactive lipids from the microenvironment might promote EMT through a paracrine interaction. Qihan Dong etc. demonstrated that cPLA2α was required for PI3K/AKT signaling by phosphorylate AKT at Ser473 in colorectal cancer cells [31]. However, the detailed molecular mechanism of the association between cPLA2α and PI3K/AKT/ERK signaling in HCC requires further investigation. Taken together, our results clearly showed that the bioactive lipid

![Image of Fig. 6](image_url)

**Fig. 6. cPLA2α plays a role in HCC metastasis in vivo.** (A) Comparison of the survival of nu/nu mice implanted with OE/SCR or OE cells. (B) Comparison of the tumor size in nu/nu mice implanted with OE/SCR or OE cells. (C) Comparison of cPLA2α and EMT-related markers expressions in tumors of nu/nu mice implanted with OE/SCR or OE cells. (D) Comparison of spontaneous metastasis and images of representative metastatic foci in the liver, intestines, diaphragm and peritoneum of nu/nu mice implanted with OE/SCR or OE cells. (E) Comparison of human tumor foci in mouse lungs visualized using H&E staining.
products of cPLA2, AA and LPI, promoted EMT in HCC cells through the PI3K/AKT/ERK pathway.

TGF-β-induced Smad2/3 activation is a classical pathway that induces EMT [39]. TGF-β has recently been shown to activate the PI3K pathway [40]. Based on our results, cPLA2α is able to distinguish between the biological effects of these two pathways. Surprisingly, cPLA2α overexpression inhibited Smad2/3 phosphorylation, whereas cPLA2α knockdown increased Smad2/3 phosphorylation. Furthermore, cPLA2α overexpression significantly inhibited the nuclear localization of Smad2/3. These results argue against a role for Smad2/3 in cPLA2α-mediated EMT. In a previous study, Tong Wu revealed that cPLA2α regulated the antiproliferative actions of TGF-β by activating PPAR-γ and thus counteracting Smad2/3 in HCC cells [30]. Interestingly, cPLA2α appeared to mediate TGF-β-induced AKT/ERK phosphorylation. Moreover, both AA and LPI played important roles in the TGF-β-induced EMT. Therefore, our results indicated that cPLA2α played opposing roles in two TGF-β-induced signaling pathways by inhibiting Smad2/3 phosphorylation and promoting the activation of PI3K/AKT/ERK pathways.

In summary, our studies of both clinical samples and HCC cell lines showed that cPLA2α plays an important role in HCC proliferation, migration, adhesion, EMT, and metastasis. Among several oncogenic pathways, cPLA2α appeared to inhibit Smad2/3 phosphorylation and promote PI3K/AKT/ERK phosphorylation. Both products of cPLA2α, AA and LPI, stimulated EMT (Supplementary Fig. 3). These data highlight the importance of bioactive lipids in EMT and revealed a novel cPLA2α-mediated mechanism that may be used as a target for novel therapies.

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Conflicts of interest
The authors declare no potential conflicts of interest.

Appendix A. Supplementary data
Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.canlet.2017.06.022.

References


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