Pathology - Research and Practice 215 (2019) 152398

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

cPLA$_2$a correlates with metastasis and poor prognosis of osteosarcoma by facilitating epithelial-mesenchymal transition

Xumei Pang[a,1], Peng Yin[b,1], Jiliang Han[c], Zhiqian Wang[a], Feng Zheng[d], Xuanhuang Chen[d,*]

[a] Department of Oncology, Yidu Central Hospital of Weifang City, Weifang, China
[b] Department of Orthopedic Surgery, Yidu Central Hospital of Weifang City, Weifang, China
[c] Department of Radiotherapy, Yidu Central Hospital of Weifang City, Weifang, China
[d] Department of Orthopedics, The Affiliated Hospital of Putian University, Putian, Fujian, China

ARTICLE INFO

Keywords:
cPLA$_2$a
Osteosarcoma
Invasion
Metastasis
Prognosis
Epithelial-mesenchymal transition

ABSTRACT

Background: Osteosarcoma (OS) patients with metastasis have very dismal prognoses, and lack effective target therapies. Overexpression of cytosolic phospholipase A2 (cPLA$_2$) has been shown to promote progression in several types of cancers, but its functions in OS have not been investigated.

Materials and methods: In our study, the expression of cPLA$_2$a was detected with immunohistochemistry in 102 cases of OS. The clinical significance of cPLA$_2$a was evaluated by analyzing its correlation with clinicopathological factors. The prognostic significance of cPLA$_2$a was estimated by univariate and multivariate analysis. The oncogenic functions of cPLA$_2$a on cell proliferation and invasion were investigated by MTT assay and tranwell assay respectively. Western blotting was applied to detect the markers of epithelial-mesenchymal transition (EMT) after silencing cPLA$_2$a expression or inhibiting its activity by a specific antagonist.

Results: In our study, high expression of cPLA$_2$a was significantly associated with metastasis and advanced Enneking stage. High cPLA$_2$a expression was significantly associated with poor prognosis and it was an independent prognostic biomarker of OS. By silencing cPLA$_2$a or inhibiting its activity by a specific antagonist, we demonstrated that cPLA$_2$a promoted cell invasion of OS cells via inducing the EMT process.

Conclusions: High cPLA$_2$a expression was an independent prognostic biomarker of OS, and cPLA$_2$a could promote OS cell invasion via inducing the EMT process, indicating that cPLA$_2$a was an independent prognostic biomarker and may be an effective drug target for OS.

1. Introduction

Osteosarcoma (OS) is the most common malignant bone cancer, with a peak incidence from 10 to 20 years old [1]. It is highly aggressive, with high potency to metastasize to other organs, especially lungs [2]. The treatment principles to OS contain surgical resection, followed by chemotherapy or other adjuvant therapies [3]. The overall survival rates of OS increased dramatically in the past decades because of the development of chemotherapy and surgical methods, but the 5-year overall survival rates of patients with metastasis remain very poor, ranging from 20% to 25% [4]. However, the systemic treatment options remain stagnant and the 5-year overall survival rates for OS patients make little progresses, keeping approximately 60%–65% for the last 30 years [5]. The predictive or prognostic biomarkers of OS are in urgent need for the improvement of individual treatment and target therapy. However, the identification of novel biomarkers of OS lagged behind other cancer types because of its low incidence.

Phospholipase A2 (PLA2) is a family of enzymes that cleave the phospholipids to free fatty acid especially arachidonic acid and lysophospholipid [6]. As an important immune modulator, the arachidonic acid can be catalyzed by cyclooxygenases (COX) and transferred into eicosanoids including prostaglandins (PG) and leukotrienes, playing the anti-inflammatory or inflammatory function. PLA2 family can be categorized into secreted (sPLA2), cytosolic (cPLA$_2$), and calcium-independent (iPLA2) enzymes, according to their subcellular locations and functions [7]. Cytosolic phospholipase A2a (cPLA$_2$a), also known as PLA2G4A, is a subtype of the most recognized PLA2, and it has been demonstrated to be involved in the progression and prognosis in many types of cancers including breast cancer, ovarian cancer and hepatocellular carcinoma, etc. [8–10]. Previous studies reported that cPLA$_2$a

* Corresponding author at: Department of Orthopedics, The Affiliated Hospital of Putian University, No 999 East Dongzhen Road, Licheng District, Putian, Fujian, Post code: 351100, China.
E-mail address: chenxuanhputian@163.com (X. Chen).
[1] Xumei Pang and Peng Yin contributed equally.

https://doi.org/10.1016/j.prp.2019.03.026
Received 28 January 2019; Received in revised form 11 March 2019; Accepted 26 March 2019

can promote many processes of tumor progression, such as carcinogenesis, angiogenesis and resistance to adjuvant therapies [11–13]. However, the expression, functions and clinical significance of $cPLA_2a$ in osteosarcoma have not been investigated yet.

Here in our study, the expression of $cPLA_2a$ was detected with immunohistochemistry (IHC) in 102 cases of OS, and its clinical significance was evaluated by analyzing its correlation with clinicopathological factors and prognosis. The oncogenic functions of $cPLA_2a$ in OS were investigated by detecting its role in proliferation and invasion. After confirming that $cPLA_2a$ was essential in invasion and metastasis of OS, we further detected the effects of $cPLA_2a$ on the epithelial-mesenchymal transition (EMT) by silencing $cPLA_2a$ expression or inhibiting its activity by a specific antagonist.

2. Materials and methods

2.1. Specimens and cohorts

From 2002–2016, a total of 246 patients were diagnosed as OS in Yidu Central Hospital of Weifang City and the Affiliated Hospital of Putian University, constituting the primary retrospective cohort of our study. A total of 102 patients were selected into the verification cohort following the criteria as follows: (1) specimens were enough for IHC; (2) follow-ups were available; (3) survival time was more than 5 months and no lethal operational complication. If necessary, patients underwent standard adjuvant therapy based on the multi-agent chemotherapy including high-dose methotrexate with leucovorin rescue, adriamycin, cisplatin, and ifosfamide. If neoadjuvant chemotherapy was applied before surgery, the specimens of IHC were selected in the tumors without necrosis. The patients would be excluded from the verification cohort if there was no available specimen for IHC because of neoadjuvant chemotherapy.

Our verification cohort was consisted of 80 male patients and 22 female patients, with an average follow-up time as 40.5 months. The clinical stage of OS was defined by Enneking et al. [14]. The overall survival time was calculated from the operation date to the date of death or the last follow-up. All the specimens were obtained with prior content of patients, and this study was approved by the Ethics Committee of Yidu Central Hospital of Weifang City and the Affiliated Hospital of Putian University.

2.2. Cell lines and reagents

Human OS cell lines U-2 OS, and Saos-2 were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) and 1% ampicillin/streptomycin. The antagonist of $cPLA_2a$, AACOCF3, was purchased from Selleckchem Company (Houston, TX, USA). Antibody of $cPLA_2a$ was obtained from R&D Systems (Cat. No. AF6659-SP, Minneapolis, MN, USA). EMT antibody kit was from Cell Signaling Technology (Cambrige, MA, USA).

2.3. Immunohistochemistry and evaluation

IHC with the streptavidin peroxidase complex method was applied to detect the expression of $cPLA_2a$. Briefly, the optimal antigen retrieval was realized by incubation in boiled citrate buffer (pH = 6.0) for 10 min after deparaffinization, rehydration and endogenous peroxidase inactivation. Primary antibody (1:100) of $cPLA_2a$ was applied after blocking unspecific binding with 5% bovine serum albumin. Corresponding secondary antibodies (Beyotime, Shanghai, China) were used to incubate specimens at room temperature for 2 h. Streptavidin peroxidase complex reagent and 3,3′-diaminobenzidine solution were applied orderly for antigen visualization.

The standard of IHC score was referred to previous studies. Results of IHC were evaluated with two independent pathologists unaware of clinical information. The IHC results were semi-quantified according to a score system comprising the score of staining intensity and the score of positive cell percentage [15]. The scores of staining intensity were set as: score 0 for negative staining; 1 for weak staining; 2 for median staining; and 3 for strong staining. The scores of positive cell percentage were defined as: score 1 for 0–25% positive cells; 2 for 25%–50% positive cells; 3 for above 50% positive cells. The final score of IHC was the multiplication product of these two scores. In the study, the cohort was divided into different groups according to the IHC score of $cPLA_2a$ by the cut-off score, which was defined as the point with the highest sum of sensitivity and specificity in receiver operating characteristic curve (ROC) according to previous studies [16].

Table 1
Basic information of patients with OS.

Characters	Number	Percentage
Sex		
Male	80	78.43%
Female	22	21.57%
Age		
< 20	19	18.63%
≥20	83	81.37%
Tumor size(cm)		
< 8	68	66.67%
≥8	35	34.31%
Site		
Femur	48	47.06%
Tibia	22	21.57%
Humerus	16	15.69%
Fibula	8	7.84%
Others	8	7.84%
Histopathology		
Osteoblastic	36	35.29%
Fibroblastic	23	22.55%
Chondroblastic	16	15.69%
Telangiectatic	12	11.76%
Others	15	14.71%
Metastasis		
No	77	75.49%
Yes	25	24.51%
Enneking stage		
I	11	10.78%
II	66	64.71%
III	25	24.51%
Response to chemotherapy		
Good	41	40.20%
Poor	61	59.80%
$cPLA_2a$		
Low	63	61.76%
High	39	38.24%

2.4. Small interfering RNA

The small interfering RNA (siRNA) of $cPLA_2a$ was purchased from Santa Cruz Biotech for $cPLA_2a$ knockdown with a scrambled RNA sequence as a control. The siRNAs were transfected with Lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the guideline.

2.5. MTT assay

Proliferation of human OS cell line U-2 OS cells was estimated with MTT assay. U-2 OS cells were seeded into a 96-well plate about 4000 per well and cultured for 48 h. At the end of incubation, 10 mg/ml MTT was added per well and incubated for 4 h at 37°C. The supernatant were discarded and the bottom crystals were dissolved in 100 μl DMSO.

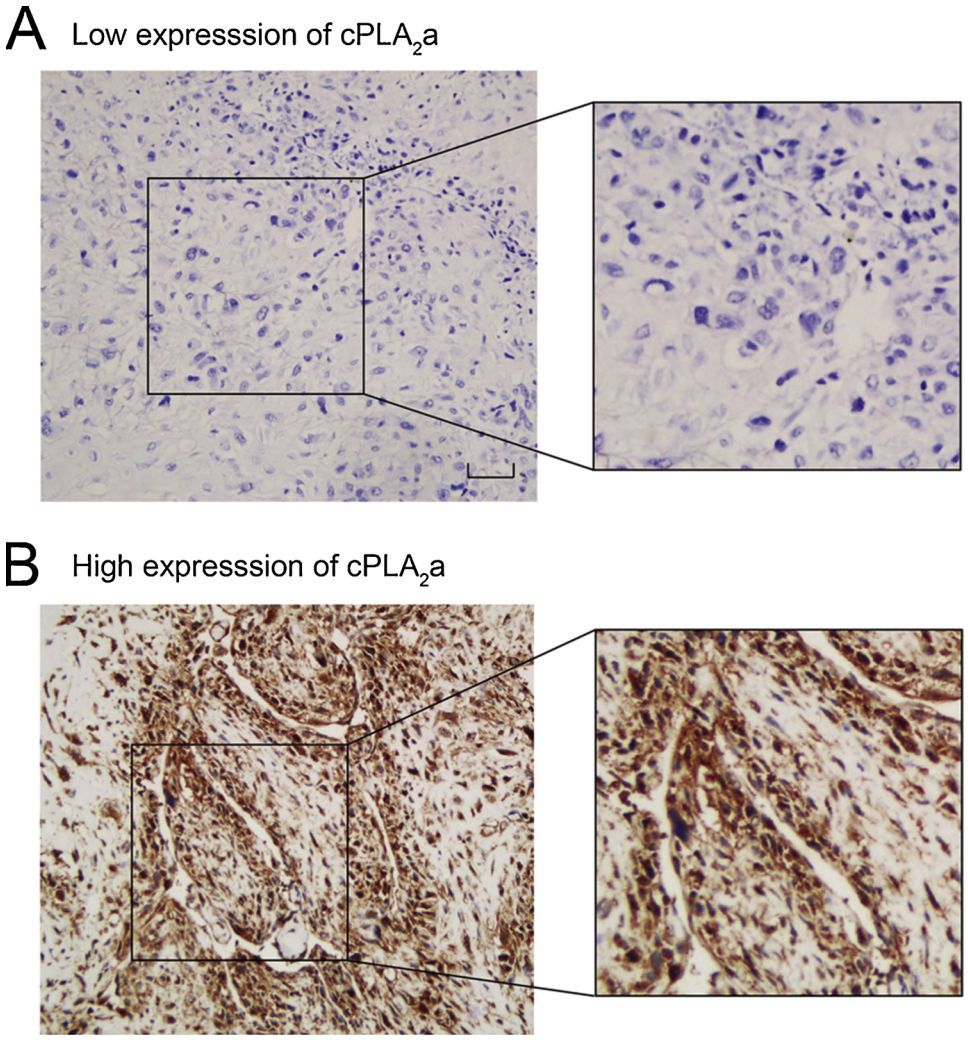

Fig. 1. Expression of cPLA$_2$a in OS tissues.
A and B. Representative IHC images of cPLA$_2$a low expression (A) and high expression (B), and their magnified images were shown. Scale bar: 50 μm.

Optimal dense of 540 nm (OD540) was read by microplate reader (SpectraMax, Molecular Devices). The OD540 of control group (without transfection) was set as baseline, used for the standardization of other groups.

2.6. Matrigel transwell assay

The cell invasion was estimated by transwell assay described in previous study [17]. In brief, cells with transfection of si-cPLA$_2$a or scrambled siRNA were seeded into the transwell chamber pre-coated with matrigel, and cultured for 12 h for cells adhesion. Fetal bovine serum was added into the bottom chamber as chemo-attractant in the presence or absence of cPLA$_2$a antagonist. After 24 h incubation, cells of upper chamber were removed and cells in the bottom chamber were fixed with paraformaldehyde and dyed with methyl violet. Invaded cells were observed and counted in at least 8 random visual fields under microscopy.

2.7. Western blotting

The expression of cPLA$_2$a and EMT markers were evaluated with western blotting. Cells were lysed with RIPA buffer (Beyotime, Shanghai, China). Equal amount of 10 μg total protein was applied for electrophoresis with SDS-PAGE, and then transferred to PVDF membrane (PALL Company, USA). After incubation in 5% bovine serum albumin for 1 h, the proteins were incubated in primary antibody (1:1000) overnight at 4 °C, and then in corresponding secondary antibody for 2 h at room temperature. Enhanced ECL (Millipore Company) was finally used for visualization of the protein bands.

2.8. Statistical analysis

SPSS17.0 software (IBM cooperation, USA) was used to analyze all the data. The correlation between cPLA2a expression and other clinicopathologic parameters was evaluated by Chi-square test, and the correlation between cPLA2a and the overall survival rate was analyzed by Kaplan-Meier method, with difference in survival curves evaluated with log-rank test. Significance of data from experiments *in vitro* was detected by student's t-test. It was considered statistically significant when $P < 0.05$.

3. Results

3.1. Basis information of patients with OS

Our validation cohort was consisted with 102 patients diagnosed with OS and underwent surgical resection, comprised of 80 male patients and 22 female patients (Table 1). The expression and subcellular locations of cPLA$_2$a in these OS tissues were investigated with IHC. The expression of cPLA$_2$a was mainly detected in cytoplasm in our study,

Table 2
The correlation between cPLA$_2$a and clinicopathological factors.

Characters	cPLA$_2$a		P*
	Low	High	
Sex			
Male	47	33	0.323
Female	16	6	
Age			
< 20	14	5	0.300
≥20	49	34	
Tumor size(cm)			
< 8	42	26	0.832
≥8	21	14	
Site			
Femur	29	19	0.144
Tibia	17	5	
Humerus	5	11	
Fibula	6	2	
Others	6	2	
Histopathology			
Osteoblastic	22	14	0.994
Fibroblastic	14	9	
Chondroblastic	10	6	
Telangiectatic	7	5	
Others	10	5	
Metastasis			
No	54	23	0.004
Yes	9	16	
Enneking stage			
I	9	2	0.007
II	45	21	
III	9	16	
Response to chemotherapy			
Poor	21	20	0.097
Good	42	19	

* Calculated with Chi-square test.

which was in consistent with its function as a phospholipids enzyme. With the score of IHC, we classified the validation cohort into subgroups with low expression and high expression of cPLA$_2$a (Fig. 1A and B), which accounted for 61.76% and 38.24%, respectively.

3.2. High expression of cPLA$_2$a was significantly associated with metastasis

With Chi-square test, the correlation between cPLA$_2$a and the clinicopathological parameters was evaluated to screen the possible processes influenced by cPLA$_2$a (Table 2). In our study, the expression of cPLA$_2$a was significantly correlated with tumor metastasis ($P = 0.004$). Patients with high expression of cPLA$_2$a seemed to be more vulnerable to positive metastasis, indicating that cPLA$_2$a may play an important role in OS invasion and metastasis. Moreover, high cPLA$_2$a was dramatically associated with advanced Enneking stage ($P = 0.007$), which could be a secondary consequent of the correlation between cPLA$_2$a and metastasis with metastasis as one of the determinants of Enneking stage.

3.3. cPLA$_2$a was a prognostic biomarker of OS

The prognostic significance of cPLA$_2$a was estimated with the univariate and multivariate analysis (Table 3). Univariate analysis with Kaplan-Meier method and log-rank test was first carried out to analyze correlation between survival rates and every parameter. In our test, high expression of cPLA$_2$a could indicate the unfavorable prognosis of OS (Fig. 2A). In addition, chondroblastic histopathological type had the lowest 5-year survival rate among all the histopathological types of OS, as low as 23.3% (Fig. 2B), but this result need further verification in other cohorts because the patients' number of chondroblastic OS was small (16/102). In consistent with numerous previous studies, positive metastasis, advanced Enneking stage and poor response to chemotherapywere all demonstrated to be prognostic factors of OS (Fig. 2C–E).

Table 3
Prognostic significances of cPLA2a and clinicopatholgical factors.

Characters	5-year survival rate%	P*	HR	95%CI	P#
Sex					
Male	49.3	0.726			
Female	47.3				
Age					
< 20	54.6	0.729			
≥20	49.2				
Tumor size(cm)					
< 8	58.7	0.292			
≥8	41.2				
Site					
Femur	44.8	0.118			
Tibia	80.1				
Humerus	40.8				
Fibula	36.5				
Others	0.0				
Histopathology					
Osteoblastic	45.9	0.013	1		
Fibroblastic	57.8		1.29	0.46-3.58	0.628
Chondroblastic	23.3		3.6	1.43-9.05	0.006
Telangiectatic	49.4		0.81	0.23-2.88	0.744
Others	75.4		0.5	0.13-1.90	0.306
Metastasis					
No	54.2	0.012	1		
Yes	38.2		2.61	1.10-6.22	0.03
Enneking stage					
I	75.0	0.042			
II	52.1				
III	38.2				
Response to chemotherapy					
Good	24.5	< 0.001	1		
Poor	64.3		3.19	1.52-6.68	0.002
cPLA2a					
Low	65.1	0.001	1		
High	33.1		2.72	1.32-5.63	0.007

* Calculated with log-rank test.
\# Calculated Cox-regression model.

All the prognostic factors validated with univariate analysis were selected into the Cox-regression model for multivariate analysis. Enneking stage was excluded because it was a secondary consequence of other factors such as metastasis. In our study, high cPLA$_2$a was proved to predict poor prognosis independently. Moreover, the chondroblastic histopathological type, metastasis, response to chemotherapy were all independent prognostic factors of OS.

3.4. cPLA$_2$a promoted OS cells invasion by facilitating EMT

The correlation between high expression of cPLA$_2$a and positive metastasis was observed in clinical analyzations, so we investigated the functions of cPLA$_2$a in OS progression with experiments *in vitro*. Small interfering RNA was used to silence the expression of cPLA$_2$a in OS cell lines U2-OS and Saos-2 (Fig. 3A). After knocking down cPLA$_2$a, the proliferation and invasion were detected with MTT assay and tranwell assay respectively in both U2-OS and Saos-2 cells. It turned out that cPLA$_2$a knock down had little influence on cell proliferation but could decrease cell invasion of U2-OS and Saos-2 (Fig. 3B and C). In general, EMT is an important reason promoting cell invasion and metastasis, so

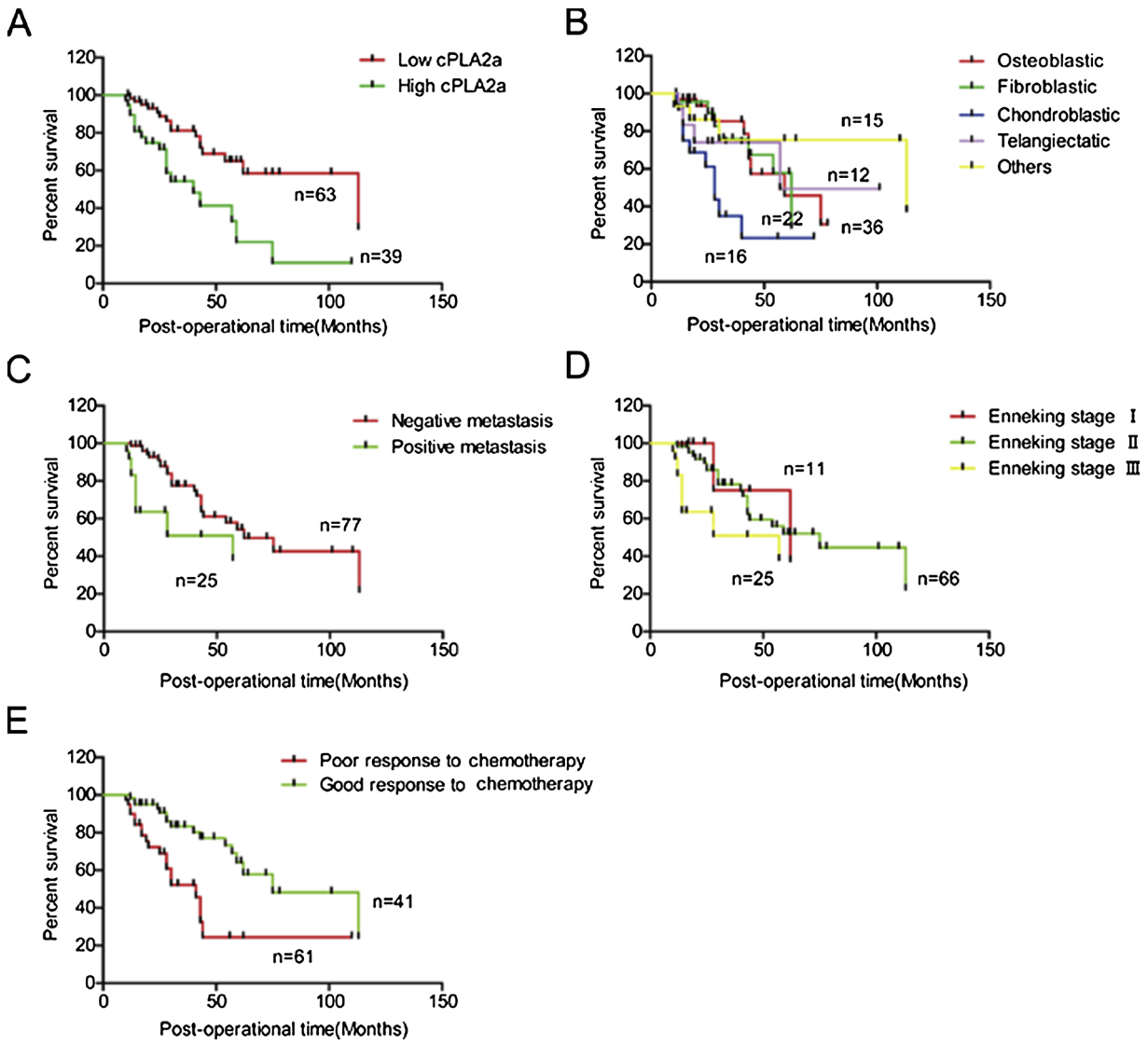

Fig. 2. The survival curves of different subgroups with OS.
The overall survival curves of OS were stratified according to cPLA$_2$a expression (A), different histopathological types (B), metastasis (C), Enneking stage (D) and response to chemotherapy (E).

we further detected the influence of cPLA$_2$a on EMT of OS cells. The EMT markers including E-cadherin, N-cadherin, Slug and Snail were detected with western blotting after silencing cPLA$_2$a. Knockdown of cPLA$_2$a dramatically increased E-cadherin expression and decreased expression of N-cadherin, Slug and Snail in both U2-OS and Saos-2 cells (Fig. 3D), indicating its essential role in EMT of OS cells. Moreover, the specific antagonist of cPLA$_2$a, AACOCF3, was used to inhibit cPLA$_2$a activity at different concentration. After incubation in 1 μM or 10 μM AACOCF3 for = 2 h, the invasive activity of U2-OS was robustly impaired (Fig. 3E). Moreover, AACOCF3 had similar influence on EMT markers like cPLA$_2$a knockdown, suggesting that cPLA$_2$a was required in the EMT process of OS cells.

4. Discussion

Recently, more emerging evidence indicated lipid signaling network as a promising target for cancer prevention and treatment. The cPLA$_2$ has been demonstrated to promote tumor progression and considered as a potential therapeutic target of several types of cancer, including breast cancer and prostate cancer [18]. The cPLA$_2$ was also involved in other pathological processes like tumorigenesis, angiogenesis, invasion, proliferation or chemoresistance [13]. In our study, we demonstrated that cPLA$_2$a was a prognostic biomarker of OS for the first time. Our results suggested that cPLA$_2$a detection after operation may be necessary for better stratification of patients because patients with high expression of cPLA$_2$a may have poorer prognosis. Moreover, cPLA$_2$a may be a promising drug target for OS target therapy because we proved that cPLA$_2$a could promote OS cell invasion by facilitating the EMT process.

In our study, we demonstrated that the overexpression of cPLA$_2$a was significantly associated with OS metastasis. This result was in consistent with several previous studies on other tumor types. Additionally, we for the first time proved that EMT of OS cells was promoted by cPLA$_2$a, which may be the main reason of cPLA$_2$-induced metastasis. Few previous studies reported the correlation between cPLA$_2$a and EMT. Fu et al suggested that cPLA$_2$a mediated epidermal growth factor (EGF) induced EMT through PI3K/AKT/ERK pathway in hepatocellular carcinoma cells [19]. In our study, the underlying molecular correlation between cPLA$_2$a and EMT was not elucidated but we suspected that there were cPLA$_2$-related products involved in the EMT of OS. Up-regulation of cPLA$_2$a could directly lead to increase of arachidonic acid and its product, prostaglandin (PG), mainly prostaglandin

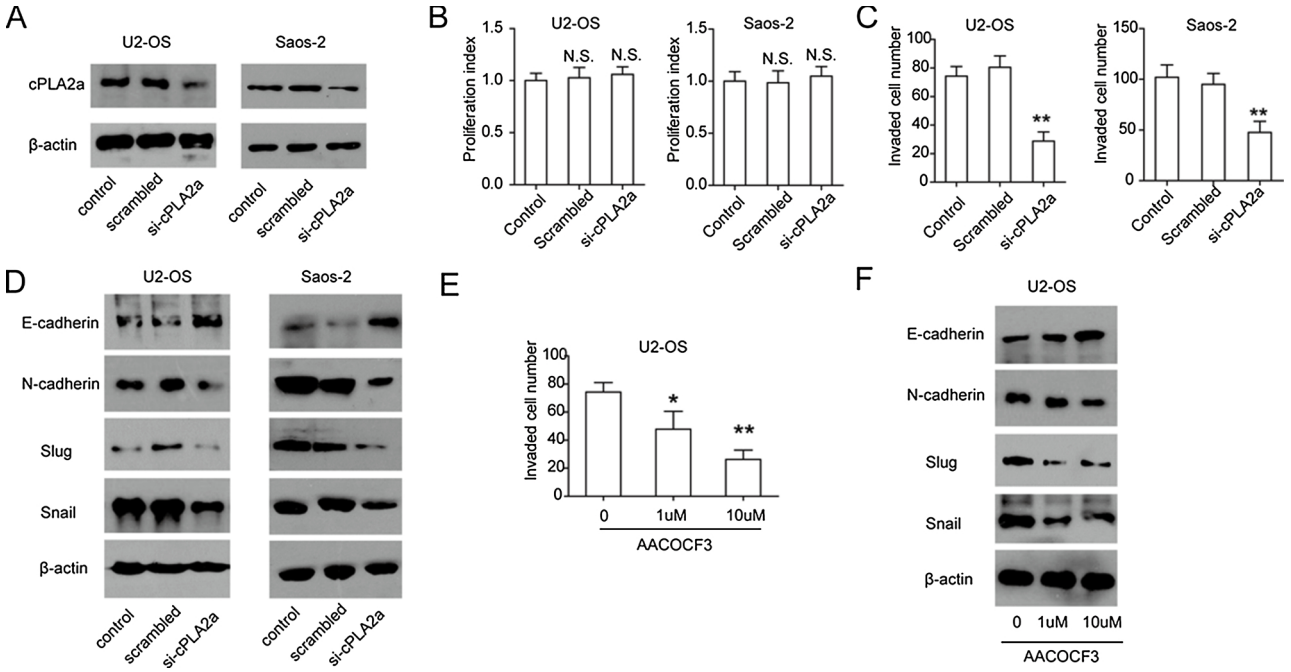

Fig. 3. cPLA$_2$a promoted cell invasion by inducing EMT process.
A. Knockdown of cPLA$_2$a in U2-OS and Saos-2 cells.
B. Effect of cPLA$_2$a knockdown on proliferation of U2-OS and Saos-2. Cells were incubated for 48 h after cPLA$_2$ knockdown and MTT assay was used. N.S. means not significant.
C. Knockdown of cPLA$_2$a decreased the invasion of U2-OS cells and Saos-2. ** means $P < 0.01$. The data of B.
D. Knockdown of cPLA$_2$a attenuated the EMT of OS cells.
E. Specific inhibitor of cPAL2a AACOCF3 impaired invasion of OS cells.* and ** means $P < 0.05$ and < 0.01, respectively. In B,C and E, data were from at least 3 independent experiments and statistical significance was calculated by student *t*-test.
F. AACOCF3 attenuated the EMT process of OS cells.

E2(PGE2). Arachidonic acid could activate mTOR signaling and thus promote breast carcinogenesis and angiogenesis [11], and PGE2 was involved in diverse cellular progresses including cell growth, therapeutic resistance and differentiation [20]. IL-6-mediated COX-2/PGE2 signaling pathway was requied in EMT of lung cancer [21], and COX-2 was also proved to be involved in the EMT process of colon cancer. In addition, cPLA$_2$/arachidonic acid/PGE2 signaling had crosstalks with other signaling pathway like mTOR or PPARD, which may influence EMT eventually.

The improvement of OS survival rate is mainly because of the application of effective adjuvant therapy, especially the chemotherapy. However, the regimen of chemotherapy remains stagnant in recent years and there is still no targeted drug applied in the treatment for OS. In general, OS is a type of highly heterogeneous cancer, increasing the difficulty of the development of OS target therapy. The application of second-generation sequencing revealed several common genetic alterations of OS such as p53 [22]. Many collaborative efforts such as the Pediatric Preclinical Testing Program are made to make more progresses of OS biomarkers exploration. Denosumab, one monoclonal antibody for the treatment of osteoporosis, was shown to have potential efficacy of OS treatment by transgenic mouse models with alterations in receptor activator of nuclear factor κB ligand [23]. Our results demonstrated that cPLA$_2$a was an independent prognostic biomarker and may be an effective drug target of OS. Anti-cPLA$_2$a therapy may be a promising approach for the future treatment of OS.

Conflicts of interest

We declare no and conflicts of interest

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