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ORIGINAL ARTICLE

Expression level of PLAC1 in osteosarcoma patients and its regulatory effect on the development of osteosarcoma

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Summary

Purpose: *To detect the plasma levels of PLAC1 in osteosarcoma patients, and its regulatory effect on cell proliferation and apoptosis of osteosarcoma.*

Methods: *Plasma levels of PLAC1 in osteosarcoma patients were examined by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot. Receiver operating characteristics (ROC) and Kaplan-Meier curves were performed for assessing the diagnostic and prognostic potentials of PLAC1 in osteosarcoma, respectively. Moreover, the regulatory effects of PLAC1 on proliferative and apoptotic rates of osteosarcoma cells were determined through cell counting kit-8 (CCK-8) and flow cytometry, respectively.*

Results: *PLAC1 was highly expressed in plasma of osteosarcoma patients showing diagnostic and prognostic potentials. Overexpression of PLAC1 in U2-OS cells increased the proliferative rate but decreased the apoptotic rate, while knockdown of PLAC1 yielded the opposite results.*

Conclusions: *PLAC1 is upregulated in the plasma of osteosarcoma patients, serving as a diagnostic biomarker, and is unfavorable to the prognosis if this disease. PLAC1 promotes the development of osteosarcoma by stimulating cell proliferation and inhibiting apoptosis.*

Key words: *osteosarcoma, PLAC1, biomarker, mechanism*

Introduction

Osteosarcoma is a prevalent primary malignant bone tumor mainly affecting children and adolescents. It is hard to be cured because of high incidence of metastasis and local infiltration. About 30-40% of osteosarcoma patients develop metastases with poor prognosis although surgery, neoadjuvant chemotherapy and immune therapy have improved the survival of osteosarcoma patients [1]. The pathogenesis of osteosarcoma is complicated. Abnormally expressed genes stimulate the aggravation of osteosarcoma through mediating malignant phenotypes of cells [2,3].

With the increasing application of diagnostic technologies (e.g. body fluid biopsy and next-generation sequencing) for osteosarcoma, novel metastatic biomarkers have shown advantages in assessing the disease severity and developing therapeutic strategies. Reliable biomarkers can not only be used to diagnose osteosarcoma, but also serve as new therapeutic targets [4]. Previously, RNA sequencing has been applied in assessing the transcription variation of osteosarcoma, which is closely linked to metastasis and prognosis of this diseases [5]. Tumor-derived exosomes (TEX) are of great significance in mediating tumor cell signaling and tumor metastasis [6]. Specific proteins in plasma-derived exosomes of osteosarcoma patients can be utilized as biomarkers for predicting pulmonary metastasis. Moreover, the expression level of TEX is much higher in highly metastatic tumor patients than those of

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non-metastatic or lowly metastatic patients, further confirming its potential in predicting metastasis [7].

PLAC1 (92,641 bp) is a sex-linked gene located on human chromosome Xq26.3. It contains three exons, and the third one is the coding region that encodes a 1.7 kb mRNA with a transcription length of 1,126 bp. PLAC1 protein contains 212 amino acids, of which, the 5th to 22nd amino acids are a transmembrane spiral structure region, and the 23rd to 212th are an extracellular region [8]. Except for testis, PLAC1 is highly expressed in various types of human cancers [9]. Koslowski et al [10] reported that the expression level of PLAC1 in primary tumor samples is 37.8%, which is up to 82.3% and 54.6% in primary breast cancer tissues and cell lines, respectively. Silencing PLAC1 remarkably suppresses cell motion, proliferation and invasiveness of breast cancer. No correlation between PLAC1 and osteosarcoma has been reported yet. In the present study, we intended to detect the plasma level of PLAC1 in osteosarcoma patients, and its influence on prognosis and malignant development.

Methods

This study was approved by the Ethics Committee of The Affiliated Huaian No.1 People's Hospital of Nanjing Medical University. Signed written informed consents were obtained from all participants before the study entry.

Sample collection

Sixty osteosarcoma patients and 60 traumatic fracture patients (controls) were recruited. In the next morning, 2 mL of elbow vein blood was collected from each participant who was fast overnight. Blood samples were stored in EDTA (ethylenediaminetetraacetic acid) anticoagulation tubes and centrifuged at 4°C, 1200 g for 15 min. The upper layer was collected, sub-packed in RNase-free Eppendorf (EP) tubes, and preserved at -80°C.

Cell culture

Human osteosarcoma cell line U2-OS was purchased from Cell Bank, Chinese Academy of Science (Shanghai, China). Cells were cultural in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and 1% penicillin. When cells were grown to 80% confluence, they were digested in 0.25% EDTA for 2.5 min and passaged at a ratio of 1:3. Cell passage was limited in 10 generations.

Cell transfection

Cells were seeded in 6-well plates (5×10^5 cells/mL). Transfection plasmid (50 pmol/L) was diluted in 100 μL of Opti-MEM®I, gently mixed and incubated for 5 min. Meanwhile, 2 μL of Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA) was similarly mixed in 100 μL of Opti-MEM®I. They were mixed together and incubated for 30 min and applied for 48-h transfection. Sequences of transfection plasmids were as follows: PLAC1-siRNA: GUGUUCAGCUUGUCACAGU (sense) and ACUGUGACAAGCUGAACAC (antisense); NC-siRNA: UUCUCCGAACGUGUCACGU (sense) and ACGUGACACGUUCGGAGAA (antisense).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNAs collected using TRIzol (Invitrogen, Carlsbad, CA, USA) were reversely transcribed to complementary DNAs (cDNAs) using the PrimeScript RT reagent Kit (TaKaRa, Otsu, Japan). cDNAs and the prepared PCR system were subjected to the 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) using the SYBR Primix Ex Taq II kit (TaKaRa, Otsu, Japan). Each gene was detected in triplicate. Primer sequences were as follows: PLAC1 (5'→3'): CCGGACAAAATCCAGTGACTGT (forward) and AACCCAGGCCCAAGTATAACTCA (reverse); GAPDH (5'→3'): GAAGGTGAAGGTCGGAGTC (forward) and GAAGATGGTGATGGGATTTC (reverse).

Western blot

Total protein was extracted from plasma samples using radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China). Protein samples were quantified and denaturated, and 50 μg of proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After transmembrane and incubation in tris buffered saline-tween (TBST) with 5% bovine serum albumin (BSA), membranes were washed in 0.1% phosphate buffered saline-tween (PBST) (15 min×3) and immunoblotted with primary antibodies overnight at 4°C. The other day they were immunoblotted with secondary antibodies, washed, and subjected to band exposure and gray value analysis.

Cell counting kit-8 (CCK-8)

Cells were seeded in 96-well plates (5×10^3 cells/mL), and incubated with 100 μL of CCK-8 solution (Dojindo, Kumamoto, Japan) on days 1, 2, 3 and 4. After 1-h cell culture, optical density (OD) at 450 nm was determined using a microplate reader. There were five replicates in each group.

Flow cytometry

Cells were collected and incubated in 1.5 mL of Hanks buffer. After infiltration, the mixture in the flow cytometry tube was centrifuged for 10 min. The precipitant was then gently mixed in 100 μL of binding buffer, followed by a 37°C incubation with 5 μL of Annexin V-FITC (fluorescein isothiocyanate) and 10 μL of propidium iodide (PI) in the dark for 20 min. Lastly, the mixture was incubated in 150 μL of binding buffer and subjected to flow cytometry for detecting cell apoptosis.

Follow up

Recruited osteosarcoma patients were followed up from June 2017 to July 2018 through outpatient review or telephone contacts, with an interval of one month. Overall survival was recorded as the duration from the first time of treatment to the death of any cause.

Table 1. Comparison of baseline clinical data between the two groups

Variables	*Control (n=60)*	*Patients (n=60)*	t/x^2	*p*
Sex (male/female)	30/30	30/30	-	-
Age (years)	18.22±1.85	18.06±1.47	0.541	0.690
BMI* (Kg/m^2)	22.36±2.37	21.81±1.60	1.495	0.138

*Body mass index

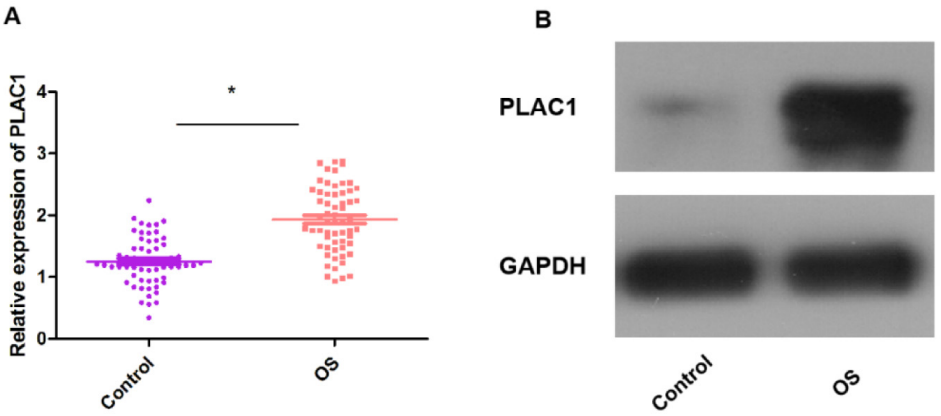

Figure 1. Upregulated PLAC1 in plasma of osteosarcoma patients. **A:** The mRNA level of PLAC1 in plasma of osteosarcoma patients and controls. **B:** The protein level of PLAC1 in plasma of osteosarcoma patients and controls (*p<0.05).

Table 2. Relationship between PLAC1 expression and clinicopathological features of osteosarcoma

Clinicopathological factors	*n*	*Low expression (n=30)*	*High expression (n=30)*	x^2	*p*
Gender					
Male	28	16	12	1.071	0.301
Female	32	14	18		
Age (years)					
<18	33	18	15	0.606	0.436
≥18	27	12	15		
Histological types					
Ordinary type	23	14	9	1.763	0.184
Special type	37	16	21		
Growth site					
Femur	23	13	10	0.635	0.426
Tibia	37	17	20		
Clinical stages					
I-II	35	20	15	1.714	0.190
III	25	10	15		
Tumor size (cm)					
≤5	18	8	10	0.317	0.573
>5	42	22	20		
Pulmonary metastasis					
Yes	20	5	15	7.500	0.013
No	40	25	15		

Statistics

SPSS 20.0 statistical package (IBM, Armonk, NY, USA) was used for statistical analyses. Data were expressed as mean ± standard deviation (SD), and compared by the Student's t-test. Diagnostic and prognostic potentials of PLAC1 in osteosarcoma were assessed by depicting receiver operating characteristics (ROC) and Kaplan-Meier curves, respectively. Significant difference was set at $p<0.05$.

Results

Baseline characteristics of participants

Sixty osteosarcoma patients and 60 traumatic fracture patients (controls) were recruited. There were 30 males and 30 females included in each group. No significant differences in the average age [osteosarcoma group: (18.06±1.47) years, control group: (18.22±1.85) years] and BMI [osteosarcoma group: (21.81±1.60) kg/m^2, control group: (22.36±2.37) kg/m^2] were detected between groups ($p>0.05$) (Table 1).

Upregulated PLAC1 in plasma of osteosarcoma patients

Compared with controls, plasma level of PLAC1 was much higher in osteosarcoma patients (Figure 1A). As expected, the protein level of PLAC1 was remarkably higher in osteosarcoma group than in control group (Figure 1B). With the cut-off value of the median plasma level of PLAC1 in 60 osteosarcoma patients, they were divided into two groups (n=30 in each group). There was a significant difference in pulmonary metastasis between two groups (Table 2). Hence, we believed that PLAC1 may influence the pulmonary metastasis of osteosarcoma.

Diagnostic and prognostic potentials of PLAC1 in osteosarcoma

ROC curves were depicted to assess whether PLAC1 can be used as a biomarker for diagnosing osteosarcoma. As shown in Figure 2A, the AUC was 0.8424 (95%CI=0.7730-0.9117, $p<0.001$). The sensitivity and specificity of PLAC1 in predicting the incidence of osteosarcoma was 80% and 75%, respectively (cut-off value=1.470), suggesting a certain diagnostic potential. In addition, Kaplan-Meier curves were depicted based on follow-up data for 5 years. It was discovered that osteosarcoma patients expressing a high plasma level of PLAC1 had a shorter survival than the other groups (HR=2.866, $p=0.0013$, Figure 2B). It could be concluded that highly expressed PLAC1 was unfavorable to the prognosis of osteosarcoma.

PLAC1 promoted the development of osteosarcoma

To elucidate the involvement of PLAC1 in the development of osteosarcoma, *in vitro* experiments were performed in U2-OS cells transfected with pcDNA-PLAC1 or si-PLAC1. Their transfection efficacy was examined at both mRNA and protein levels (Figures 3A-3D). As CCK-8 curves show, overexpression of PLAC1 increased the viability of U2-OS cells, and knockdown had the opposite trend (Figure 4A,4C). In addition, the apoptotic rate was decreased in U2-OS cells overexpressing PLAC1 than in controls, which was increased by transfection of si-PLAC1 (Figure 4B,4D). Taken together, PLAC1 could stimulate osteosarcoma cells to proliferate, but inhibit their apoptosis ability.

Discussion

Osteosarcoma is a malignant osteogenic tumor derived from mesenchymal tissues. It mainly affects the metaphysis of long bones, especially in children and adolescents under 20 years of age. Owing to the high levels of metastasis and invasiveness, pulmonary metastasis of osteosarcoma occurs in the early stage. The 5-year survival of

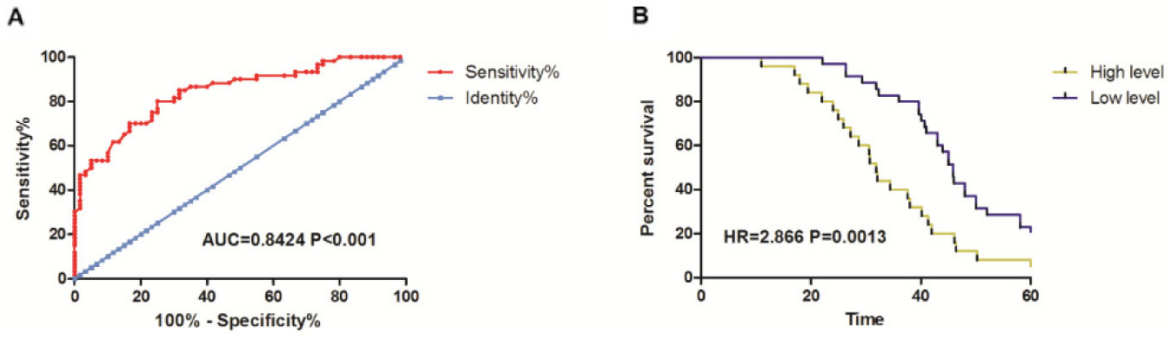

Figure 2. Diagnostic and prognostic potentials of PLAC1 in osteosarcoma. **A:** ROC curves depicted based on PLAC1 level in plasma of osteosarcoma patients; **B:** Kaplan-Meier curves depicted based on 5-year follow-up data in osteosarcoma patients expressing high or low level of PLAC1.

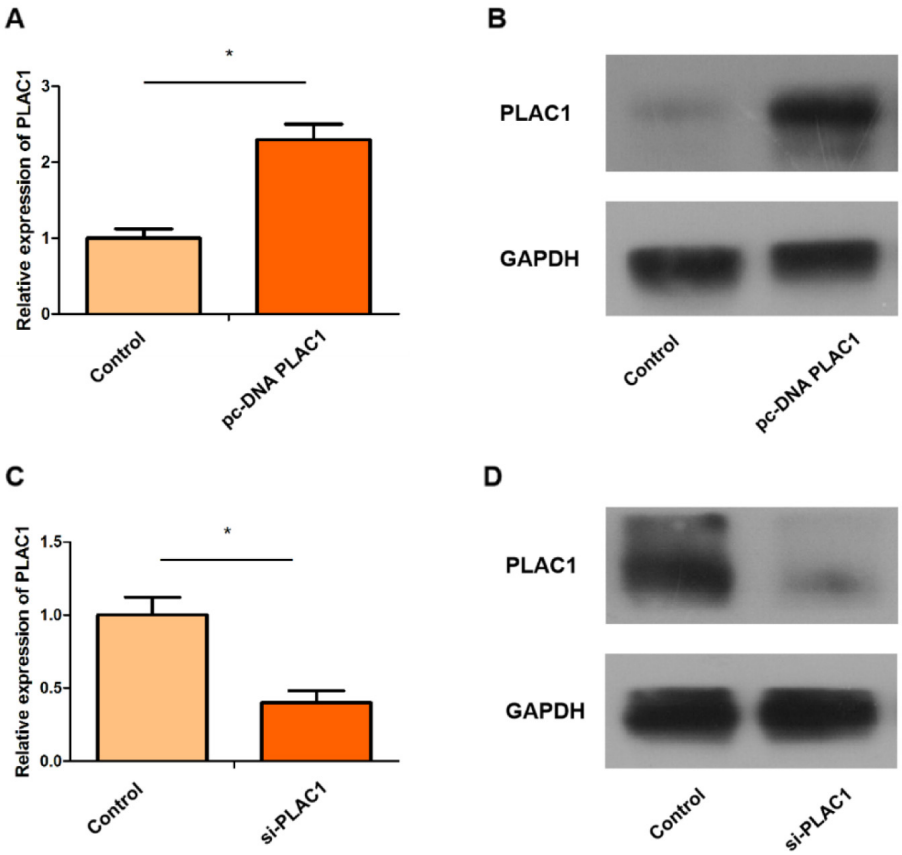

Figure 3. Intervention of PLAC1 in U2-OS cells. **A,B:** Transfection of pcDNA-PLAC1 in U2-OS cells; **C,D:** Transfection of si-PLAC1 in U2-OS cells (*p<0.05).

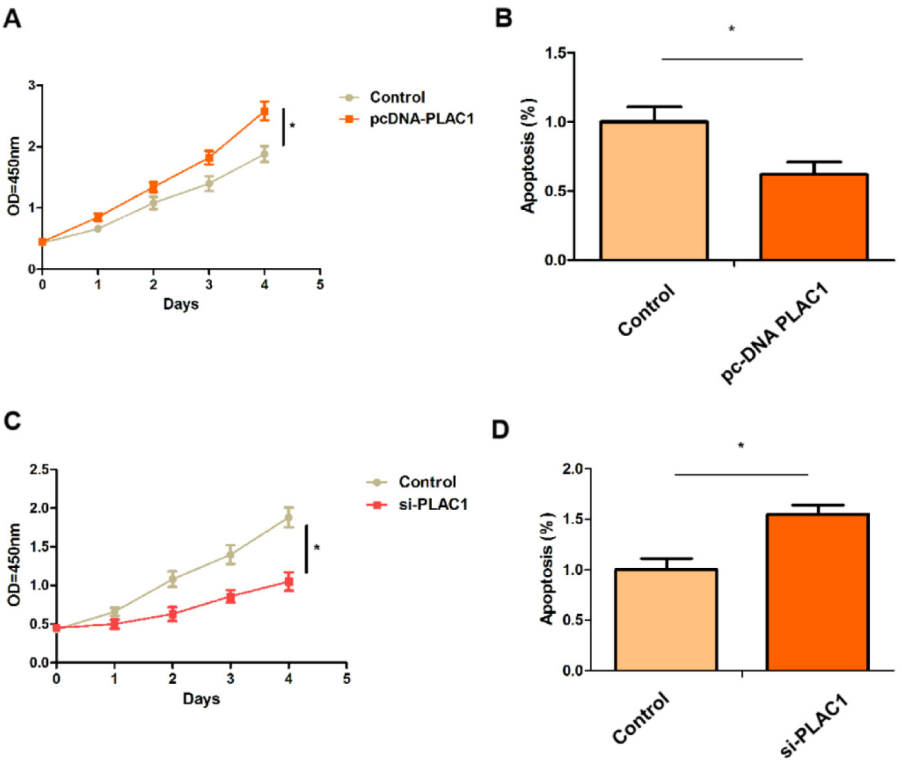

Figure 4. PLAC1 promoted the development of osteosarcoma. **A:** CCK-8 showed viability in U2-OS cells transfected with pcDNA-PLAC1. **B:** Apoptotic rate in U2-OS cells transfected with pcDNA-PLAC1. **C:** CCK-8 showed viability in U2-OS cells transfected with si-PLAC1. **D:** Apoptotic rate in U2-OS cells transfected with si-PLAC1 (*p<0.05).

metastatic osteosarcoma patients is only about 20% [11]. Many risk factors may influence the pathogenesis of osteosarcoma, including age, gender, region, race, height, birth weight, radiation and hormones. Tumor size, growth location, and metastasis are important factors that influence the prognosis of osteosarcoma [12,13].

Recent studies have shown that the expression level of PLAC1 is not limited in the placenta. Silva et al [14] first reported that PLAC1 is upregulated in multiple types of tumor cell lines (cervical cancer, colon cancer, liver cancer, prostate cancer, melanoma, multiple myeloma, choriocarcinoma, and neuroblastoma cell lines) apart from placenta tissues. Later, growing evidence has found that upregulated PLAC1 in tumor tissues is closely linked to tumor development [15-19]. PLAC1 is able to stimulate the production of many types of chemokines and inflammatory factors, thus inducing immune tolerance of tumor microenvironment and tumorigenesis [20]. The relative level of PLAC1 is identified to be linked with prognosis of gastric cancer, serving as an unfavorable biomarker for predicting the prognosis [15]. PLAC1 has tumor-specific immunogenicity, which can induce adaptive immune responses in the body and anti-tumor effect. It is expected to be a target for tumor immunotherapy [15-17,21]. Consistently, our findings showed that the plasma level of PLAC1 was remarkably upregulated in osteosarcoma patients, and correlated with pulmonary metastasis. In addition, ROC curves revealed that PLAC1 may become a biomarker for predicting the incidence of osteosarcoma. Analyzing follow-up data for 5 years, a higher level of PLAC1 predicted a shorter survival in osteosarcoma patients. It could be concluded that PLAC1 promoted the malignant development of osteosarcoma and it was unfavorable to the prognosis.

Previous studies have demonstrated the involvement of PLAC1 in tumor metastasis. Koslowski et al [10] reported that knockdown of PLAC1 decreases the proliferative rate of breast cancer cells by 80-90%, and arrests cell cycle progression in G1/S phase. Moreover, directed chemotactic metastasis of breast cancer cells is attenuated following silencing of PLAC1. The promotive effect of PLAC1 on the proliferative and invasive capacities of NSCLC has also been identified [18]. Our *in vitro* results illustrated that knockdown of PLAC1 in U2-OS cells attenuated the proliferative capacity and stimulated apoptosis while overexpression of PLAC1 yielded the opposite trends.

Collectively, PLAC1 was upregulated in the plasma of osteosarcoma patients, which was correlated with pulmonary metastasis and prognosis. It stimulated the malignant development of osteosarcoma by triggering cells to proliferate and inhibiting their apoptosis. Our findings provide theoretical references for developing PLAC1-targeted therapy of osteosarcoma.

Conclusions

PLAC1 is upregulated in the plasma of osteosarcoma patients, serving as a diagnostic biomarker, which is unfavorable to the prognosis of osteosarcoma. PLAC1 promotes the development of osteosarcoma by stimulating cell proliferation and inhibiting apoptosis.

Conflict of interests

The authors declare no conflict of interests.

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