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ORIGINAL ARTICLE

PLOD3 is Upregulated in Gastric Cancer and Correlated with Clinicopathologic Characteristics

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SUMMARY

Background: **Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3 (PLOD3) has been proven to be involved in various human cancers; however, the function of PLOD3 in gastric cancer (GC) remains unclear. In this study, the role of PLOD3 in GC was evaluated.**
Methods: **The expression of PLOD3 in GC tissues and normal tissues was predicted by The Cancer Genome Atlas (TCGA). The kmplot online tool was performed to evaluate the impact of PLOD3 expression on GC patients' survival. Real-time PCR was conducted to verify PLOD3 expression in our own clinical samples and GC cells. The Cell Counting Kit-8 and the colony formation assay were used to detect GC cell proliferation ability.**
Results: **PLOD3 was upregulated in human GC tissues (compared to adjacent normal tissues, $p < 0.001$) and GC cells. High expression of PLOD3 was significantly correlated with larger tumor size ($p = 0.007$) and poor prognosis. Inhibition of PLOD3 could suppress cell proliferation in GC.**
Conclusions: **These results revealed that PLOD3 may promote the progression of GC.**
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KEY WORDS

PLOD3, gastric cancer, clinical significance, proliferation

INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer in the world and the third leading cause of cancer-related deaths [1]. Currently, the most effective therapy for GC is surgery combined with chemotherapy, radiotherapy, and novel molecular targeted therapy. However, the 5-year survival rate of patients remains poor [2]. Thus, clarification of the molecular mechanisms underlying GC progression may lead to novel effective therapies. Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3 (PLOD3), also called lysyl hydroxylase 3 (LH3), is located on human chromosome 7q22. Its encoded pro-

tein is a multifunctional enzyme possessing three catalytic activities, namely, lysyl hydroxylase, galactosyltransferase, and glucosyltransferase activities [3]. Recently, accumulated evidence has identified that PLOD3 was involved in the regulation of human cancers. For example, Nicastri A. et al. found that PLOD3 was upregulated in colorectal cancer via a quantitative proteomic technique [4]. In hepatocellular carcinoma, knockdown of PLOD3 could inhibit tumorigenesis *in vitro* and *in vivo* by selectively modulating cell cycle and epithelial-mesenchymal transition (EMT) [5]. Furthermore, PLOD3 could induce transforming growth factor-β (TGF-β) activation and fibroblast differentiation through recruiting matrix metalloproteinase-9 (MMP-9) to the fibroblast cell surface [6]. However, the role of PLOD3 in GC remains unclear.

In the present study, we firstly predicted that the expression of PLOD3 was significantly upregulated in GC tissues by The Cancer Genome Atlas (TCGA). We verified PLOD3 expression in our own GC tissues and cells, as well as investigated the relationship with the clinicopathologic features of GC patients, including prognosis. In addition, using a series of functional assays, we detected the influences of PLOD3 on GC progression.

MATERIALS AND METHODS

Patients and tissue samples

A total of 51 paired GC tumor and adjacent normal tissues were obtained from the First Affiliated Hospital, Nanjing Medical University, China. Written informed consent was acquired from all patients without chemotherapy or radiotherapy before surgery. This study was approved by the Institutional Ethical Board of the above hospital. All tissues were transported and stored in liquid nitrogen after resection. At least two experienced pathologists confirmed all diagnoses.

GC cell culture and small interfering RNA (siRNA) transfection

Human GC cell lines (MKN45, BGC823, MGC803, and SGC7901) and human normal gastric epithelial cell line GES-1 were purchased from the Cell Center of Shanghai Institutes for Biological Sciences. All cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; WISENT, Canada) and antibiotics (1% penicillin/streptomycin, Gibco, USA) at 37°C in a humidified atmosphere under 5% CO_2. MGC803 cells were transfected with PLOD3 siRNA (Genepharma, Shanghai, China) by lipofectamine 2000 (Invitrogen, USA).

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

According to the manufacturer's instructions, total RNA was isolated from GC tissues and cells with TRIzol reagent (Invitrogen). Then we used PrimeScript RT Reagent (TaKaRa) to reverse transcribe RNA into cDNA. A 7500 Realtime PCR System (Applied Biosystems, Carlsbad, CA, USA) with SYBR Green Master Mix (Vazyme, Nanjing, China) was used to perform qRT-PCR. The primer sequences are as follows: PLOD3 forward 5'-GACCCGGTCAACCCAGAGA-3' and reverse 5'-CTCCACCAACTGTTCGAGCC-3'; GAPDH forward 5'-TCCGGGAAACTGTGGCGTGA-3' and reverse 5'-ACGGAAGGCCATGCCAGTGA-3'. Above procedures were conducted in triplicate.

Cell Counting Kit-8 (CCK-8)

According to the manufacturer's instructions, we assessed cell proliferation using CCK-8 (Dojindo, Kumamoto, Japan). We seeded GC cells into 96-well plates (2,000 cells/well), which were then cultured with RPMI 1640 (10% FBS) for 5 days. At the indicated time-point, we added CCK-8 solution (10 μL) to each well, which were incubated with cells for 2 hours at 37°C. We assessed cell proliferation by measurement of the optical density measured at 450 nm. Above procedures were conducted in triplicate.

Colony formation assay

We seeded two groups of stable GC cells in 6-well plates (400 cells/well), which were then cultured in RMPI-1640 medium for 2 weeks. Crystal violet was used to stain proliferating colonies (≥ 50 cells), then images were photographed for statistical analysis. Above procedures were conducted in triplicate.

Western blot analysis

GC tissues and cell protein extracts were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to polyvinylidene fluoride (PVDF) membrane. Next, membranes were blocked with 5% nonfat milk in tris-buffered saline for 2 hours, then incubated with primary antibodies at 4°C overnight, followed by HRP-conjugated anti-rabbit or anti-mouse IgG (1:2,000). Finally, we used enhanced chemiluminescence (ECL) to visualize protein expression levels. The primary antibodies were as follows: GAPDH (Santa Cruz Biotechnology, 1:200), PLOD3 (Abcam 1:1000). GAPDH was used as the loading control. Above procedures were conducted in triplicate.

Statistical analysis

A TCGA dataset named TCGA-STAD/Xena_Matrices/TCGA-STAD.htseq_fpkm-uq.tsv was downloaded from the website (xena.ucsc.edu) [7]. The prognosis data of GC patients was downloaded from the website (www.kmplot.com) [8]. The data were expressed as mean ± standard deviation (SD). Pearson χ^2 tests were carried out to evaluate the correlation between clinico-pathologic data and protein expression levels. Student's *t*-tests were conducted for continuous variables. $p < 0.05$ was considered to indicate statistical significance. * $p < 0.05$, ** $p < 0.01$.

Table 1. Correlation between PLOD3 expression and clinicopathological characteristics of gastric cancer patients.

Characteristics	Number	PLOD3 expression		p-value
		High group	Low group	
Age (years)				
< 60	**10**	**6**	**4**	**0.525**
≥ 60	**41**	**20**	**21**	
Gender				
Male	**34**	**19**	**15**	**0.322**
Female	**17**	**7**	**10**	
Size (cm)				
< 3	**27**	**9**	**18**	**0.007**
≥ 3	**24**	**17**	**7**	
Histology differentiation				
Well + Moderate	**16**	**5**	**11**	**0.057**
Poor + Undifferentiated	**35**	**21**	**14**	
Invasion depth				
T1 + T2	**20**	**8**	**12**	**0.208**
T3 + T4	**31**	**18**	**13**	
Lymphatic metastasis				
Yes	**25**	**16**	**9**	**0.068**
No	**26**	**10**	**16**	
Distant metastasis				
Yes	**6**	**4**	**2**	**0.413**
No	**45**	**22**	**23**	
TNM Stage				
I + II	**29**	**13**	**16**	**0.313**
III + IV	**22**	**13**	**9**	

RESULTS

PLOD3 was upregulated in human GC tissues and cells

Using TCGA data, we found that the expression of PLOD3 was higher in GC tissues compared to normal tissues (Figure 1A). To confirm whether PLOD3 is upregulated in our own GC tissues, we collected 51 human GC tissue samples and paired adjacent normal tissues to detect the expression of PLOD3 by qRT-PCR. The results were consistent with that of TCGA (Figure 1B). We then detected PLOD3 protein expression in six randomly selected paired GC samples and adjacent normal tissues by Western blot. Similarly, PLOD3 protein expression was higher in GC tissues (Figure 1C). We further examined PLOD3 mRNA expression in normal gastric mucosa epithelial cells (GES-1) and GC cell lines (MKN45, BGC823, MGC803, and SGC7901). As shown in Figure 1D, the GC cell lines presented higher PLOD3 expression than GES-1.

Clinical significance of PLOD3 expression in GC tissues

We analyzed the association between PLOD3 expression and the clinicopathological features of GC patients (age, gender, tumor size, histology differentiation, invasion depth, lymph node metastasis, distant metastasis, TNM Stage). According to PLOD3 expression levels, GC cases were split into two groups (high group: ≥ median PLOD3 expression; low group: < median PLOD3 expression). Table 1 indicated that the tumor size of GC patients in PLOD3 high group were more likely to be larger. Additionally, the influences of PLOD3 expression on GC patients' prognosis were evaluated by an online program. Figure 2 showed that GC subjects with high PLOD3 expression had worse overall survival (OS) and progression-free survival (PFS).

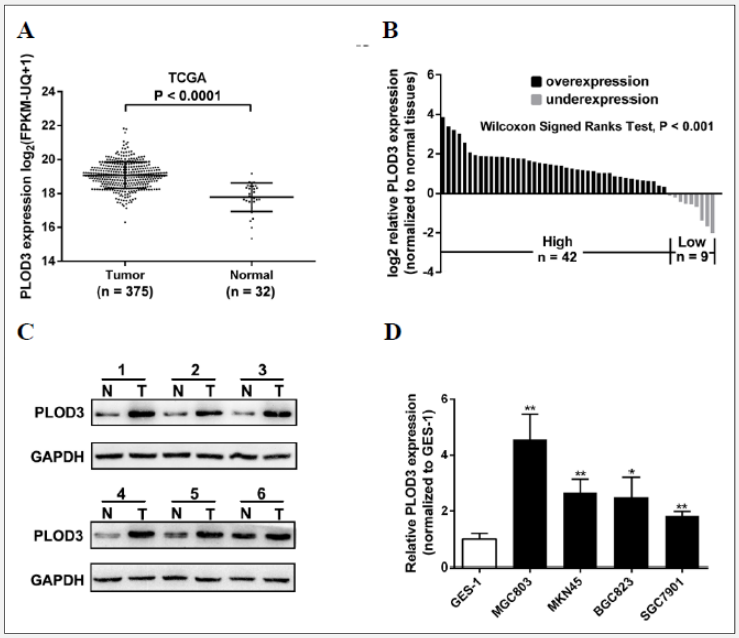

Figure 1. PLOD3 was upregulated in human GC tissues and cells.

A - The mRNA expression of PLOD3 from TCGA database in GC and normal tissues. B - PLOD3 mRNA expression in our own 51 paired GC and adjacent normal tissues. C - The protein levels of PLOD3 in six random paired GC and normal tissues. D - PLOD3 mRNA expression in GC cells and GES-1 cell. * - p < 0.05, ** - p < 0.01.

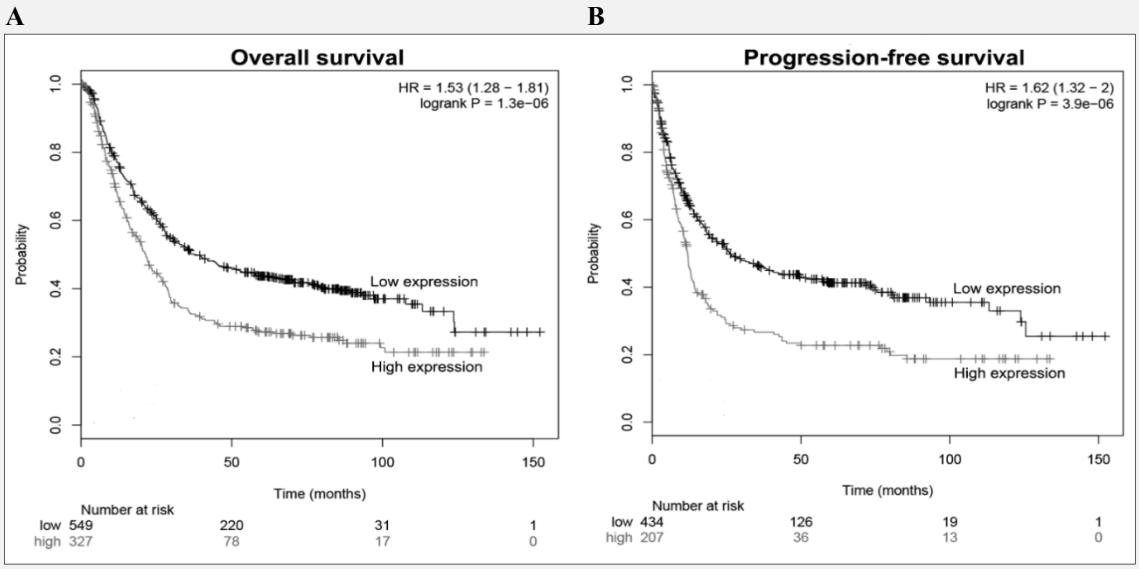

Figure 2. The impact of PLOD3 on GC patients' survival.

A - B - Kaplan-Meier survival plots demonstrated the good prognostic effect of PLOD3 upregulation correlated with a worse OS and PFS in GC patients.

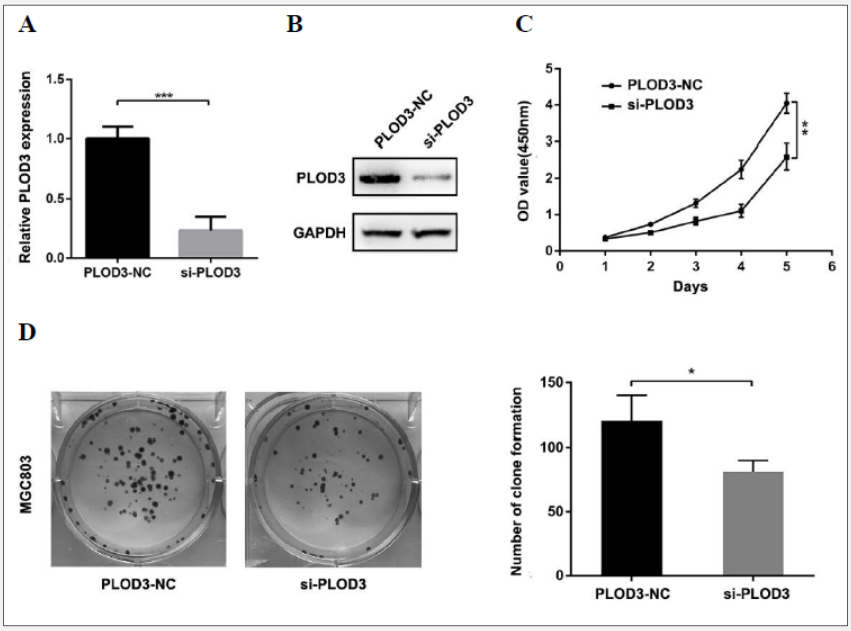

Figure 3. Inhibition of PLOD3 suppressed the proliferation of the GC cell MGC803.

A - PLOD3 mRNA expression in MGC803 cells with transfected siRNA and NC. B - PLOD3 protein expression in MGC803 cells with transfected siRNA and NC. C - CCK-8 was used to conduct the proliferation of MGC803 cells transfected with siRNA and NC. D - Effects of PLOD3 alteration on the colony formation of MGC803 cells. * - $p < 0.05$, ** - $p < 0.01$.

Inhibition of PLOD3 suppressed the proliferation of GC MGC803 cells

Due to the correlation of PLOD3 expression with tumor size, we preliminarily explored the impacts of PLOD3 expression on GC cells. Based on the expression of PLOD3 in GC cells, we selected MGC803 cells for interfering PLOD3. The efficiency of interference was verified by qRT-PCR and western blot. PLOD3 was remarkably downregulated in MGC803 with PLOD3 inhibited (Figure 3A and 3B). The influence of PLOD3 on MGC803 cell proliferation was then investigated using the CCK-8 assay. Compared with the negative control (NC) group, after inhibiting PLOD3, the proliferation rate of MGC803 cell was significantly decreased (Figure 3C). Furthermore, the colony formation assay was used to assess the long-term influence of PLOD3 on cell proliferation. The results revealed that inhibiting PLOD3 expression could impair colony formation ability (Figure 3D).

DISCUSSION

PLOD genes, also known as lysyl hydroxylases, play a vital role in fibrotic processes and tissue remodeling [9]. So far, the enzyme includes three isoforms: PLOD1, PLOD2, PLOD3. Apart from the lysyl hydroxylase activity like PLOD1 and PLOD2, PLOD3 also own collagen glucosyl- and galactosyl- transferase activities [10]. For instance, Wang et al. demonstrated that deficiency of PLOD3 glucosyltransferase activities in the extracellular space could inhibit cell growth and viability, reminding us of PLOD3's potential function in cancer development [11].

Previous studies have reported that PLOD3 was upregulated and exerted critical roles in various human cancers. Overexpression of PLOD3 was observed in colorectal and pancreatic cancer [12,13]. Shen et al. revealed that inhibiting PLOD3 could induce hepatocellular carcinoma cell cycle arrest and suppress EMT, in turn, influencing tumorigenesis [5]. In gliomas, silencing PLOD3 could inhibit cell growth, migration and invasion via inducing G1 phase arrest and downregulating mesenchymal markers. In addition, PLOD3 upregulation indicated poor clinical outcomes in glioma patients [14]. Depletion of PLOD3 sensitized radioresistant human H460 lung cancer stem-like cells to radiation through increasing radiation-induced apoptosis [15]. Similarly, Cheng et al. found that PLOD3 was upregulated in GC and strongly correlated with copy number amplification [16]. However, in the present study, we not only analyzed the expression in GC tissues and cells

(including the protein level), but also the association between its expression and GC patients' clinicopathologic features, as well as prognoses. Furthermore, the CCK8 and colony formation assays were performed to evaluate the influence of PLOD3 on GC cell proliferation. Nonetheless, our study also had some limitations. Firstly, the survival data of GC patients was from the online tool (kmplot), not from our own. Secondly, according to the link of PLOD3 overexpression to larger tumor size, we only investigated the role of PLOD3 in GC cell growth, not involving migration and invasion. In the future studies, we will conduct follow-up visits and further functional experiments.

CONCLUSION

In our study, we found that PLOD3 was more upregulated in GC tissues than in paired adjacent tissues, which was accordant with TCGA data. Additionally, GC patients with high PLOD3 repression likely presented larger tumor size and worse prognosis. The initial functional assays suggested PLOD3 silencing could inhibit the proliferation of GC cells. Taken together, these results may be helpful in diagnosis or treatment of GC and predicting survival. However, more and further research is needed to verify this.

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Declaration of Interest:
The authors have no competing interests.

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