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Clinical significance of a new oncogenic factor P5CR1 in gastric cancer

C.-X. ZHANG[1], Y. LI[2], W.-H. GONG[3], J. ZHANG[1]

[1]Department of Oncology, The First Affiliated Hospital of Dalian Medical University, Dalian, China
[2]Affiliated Hospital of Guizhou Medical University, Guiyang, China
[3]Department of Ultrasonography, Suizhou Central Hospital, Suizhou, China

Abstract. – OBJECTIVE: To explore the expression of pyrroline-5-carboxylate reductase 1 (P5CR1) and its clinical significance and function in gastric cancer (GC).

PATIENTS AND METHODS: Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) and Western blot (WB) were performed to detect the expression of P5CR1 in GC tissues and normal cells. The correlation between the expression level of P5CR1 and the clinicopathological characteristics of GC patients was analyzed by the Chi-square test. Moreover, the potential of P5CR1 in predicting the postoperative prognosis of GC was assessed by Kaplan-Meier method and Log-rank test model. Clone formation, flow cytometry, scratch wound healing, and transwell assay were performed to explore the effects of P5CR1 on cell function of GC.

RESULTS: The expression of P5CR1 significantly increased in GC tissues and cell lines. Its expression was significantly correlated with tumor differentiation and TNM stage of GC patients. Moreover, the GC patients with lower expression of P5CR1 had a better overall survival (OS). In univariate analyses and multivariate analyses, the expression of P5CR1 was an independent prognosis index of GC. Knockdown of P5CR1 significantly attenuated clone formation, migration, and invasion abilities, while the apoptotic rate of GC cells increased.

CONCLUSIONS: P5CR1 was a novel factor involved in GC progression and constituted a potential biomarker and therapeutic target of GC.

Key Words:
Pyrroline-5-carboxylate reductase 1 (P5CR1), Gastric cancer (GC), Overall survival (OS).

Introduction

As one of the most common malignant tumors of the digestive tract, gastric cancer (GC) ranks fourth in incidence rate among all types of malignant tumors in the world, and third in tumor-related mortality rate[1]. The high incidence rate and metastasis rate of GC result in a poor prognosis, posing a great threat to human health. There are nearly one million new cases of GC throughout the world every year, around 2/3 of which occur in developing countries[2]. Gastric adenocarcinoma, the most frequent subtype of GC, accounts for about 90% of all GC cases. In the current treatment of GC, surgical treatment is still the first choice, with radiotherapy and chemotherapy as adjuvant therapies[3]. However, the effect of these treatments is not satisfactory since their efficacy for metastatic tumor is low[4]. Therefore, to explore reliable predictive indexes and effective therapeutic targets are of great significance for improving the remission rate of advanced GC and reducing the mortality rate.

The progression and metastasis of GC are a multi-step, multi-stage complicated process, each stage of which is regulated by multiple genes and proteins[5,6]. Pyrroline-5-carboxylate reductase (P5CR), a widely-distributed housekeeping protein[7], comprises three isoenzymes, namely, P5CR1, P5CR2, and P5CR3[8]. P5CR1, which was first found in rabbit hepatocytes in 1956[9], locates on chromosome 17q25.3 and is present in cytoplasm and mitochondria. P5CR1 is able to reduce pyrroline-5-carboxylic acid (P5C) to proline, which is the last step in the catalytic formation of proline in almost all organisms[10,11]. During the above-mentioned reduction, NAD(P)H is oxidized to $NAD(P)^+$, and the latter one is an important buffer in cellular redox reactions[12]. Abnormally expressed P5CR1 plays an important role in many diseases[13-15]. This expression is upregulated in tumors[16,17], suggesting that it might be a potential therapeutic target. Nevertheless, evidence for the influences of P5CR1 on the occurrence and development of GC is lacking.

Corresponding Author: Jie Zhang, MM; e-mail: dlzhangj@163.com

Patients and Methods

Clinical Samples

GC tissues and normal tissues were harvested from 80 patients with gastric adenocarcinoma and 10 patients with benign gastric tumors undergoing radical or palliative resection in the First Affiliated Hospital of Dalian Medical University from July 2016 to December 2017. Their personal information and detailed clinical data were collected. Moreover, all patients were followed up for general conditions, clinical symptoms, and imaging by telephone and review after discharge from December 2017 to December 2018. This investigation was approved by the Ethics Committee of The First Affiliated Hospital of Dalian Medical University. Signed written informed consents were obtained from all participants before the study.

Cell Culture and Transfection

Human gastric adenocarcinoma cell line (AGS) and normal gastric mucosal epithelial cell line (RGM-1) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). All experimental cell lines were routinely cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) containing 100 Ul/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum at 37°C with 5% CO_2. The medium was changed every 2 days. After 3 to 5 days when the cells grew to 90% confluence, trypsin was used for routine digestion and passage.

Transfection

The cells in the logarithmic growth phase were digested and inoculated into a 6-well plate. When the cells grew to 60-80% confluence, the original culture medium was replaced with the serum-free one for 12 h. Transfection plasmid and lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) were dissolved into the Opti-MEM culture medium and incubated at room temperature for 5 min, separately, which were then mixed gently for reaction at room temperature for 20 min. Finally, the mixture was added to the cells. After incubation for 6 h, the cells were cultured in complete culture medium for another 48 h, and the transfection efficiency was determined and analyzed through subsequent experiments.

ORT-PCR Analysis

Total RNA was extracted by lysing cells in TRIzol (Invitrogen, Carlsbad, CA, USA), and the purity of the extracted RNA samples was determined and quantified. According to the instructions of the mRNA reverse transcription kit, 2 μg of total RNA was synthesized into complementary deoxyribonucleic acid (cDNA), and the relative expression of mRNA was detected by SYBR Green I real-time PCR, with U6 as an internal reference.

Western Blot (WB) Analysis

Proteins were extracted from the tissues and cells with radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China), and protein concentration was determined using a bicinchoninic acid (BCA) protein quantification kit (Pierce, Rockford, IL, USA). 60 μg of protein from each group were subjected to dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland) under a constant current of 200 mA. After blockage in 5% skim milk at room temperature for 1 h, membranes were incubated with primary antibodies (P5CR1 and GAPDH) in 4°C overnight. After washing with Tris-Buffered Saline with Tween-20 (TBST) for 5 min × 3 times, membranes were incubated with secondary antibodies at room temperature for another 2 h. After washing 3 times with TBST, membranes were then incubated with enhanced chemiluminescence (ECL). Grey values of exposed bands were analysis *via* Image-J software (Media Cybernetics, Silver Springs, MD, USA).

Colony Formation Assay

6×10^3 cells from different groups were cultured in 60 mm culture dishes for 14 d. Visible colonies were fixed, stained with 0.5% crystal violet for 15 min, and then washed 3 times. 10 random fields of view were observed under a light microscope. The number of colonies was counted (cell groups consisting of more than 50 cells were taken as one cell colony). The experiment was repeated three times.

Cell Apoptosis Determination

Cells were extracted, washed in pre-cooling poly butylene succinate (PBS), and suspended with 300 μL of binding buffer. The cell concentration was adjusted to 1×10^6 cells/mL. 100 μL of cell suspension was added to the flow tube. Then, 5 μL of Annexin V-fluorescein isothiocyanate 1 (FITC) and 5 μL of propidium iodide

(PI) were added, followed by incubation at room temperature for 15 min in the dark after mixing. 400 μL of PBS was added to the reaction tube. A flow cytometer was used within 1 h to detect the apoptosis of the cells.

Scratch Wound Healing Assay

The cells in the logarithmic growth phase were inoculated in a 6-well plate. When the cells grew to 80-90% confluence, scratches were made in the vertical direction using a 10 μL tip. After being rinsed twice with PBS solution, the cells were routinely cultured for 24 h. Wound healing was observed under an inverted phase-contrast microscope to assess the repair of cell scratches.

Transwell Assay

Cell suspension was prepared in serum-free culture medium at 2×10^3 cells/mL. Then, the cells were inoculated in the upper chamber of the transwell inserts pre-coated with Matrigel. Complete culture medium was added to the bottom chamber, and they were cultured in an incubator at 37°C for 24 h. Later, the transwell inserts were taken out, and rinsed with PBS. Cells in the upper chamber were gently wiped with a cotton swab. After that, the transwell inserts were fixed in 90% alcohol for 30 min, stained with 0.1% crystal violet for 10 min, and rinsed with PBS for 10 min × 3 times. Finally, invasive cells were photographed and counted under the inverted microscope.

Statistical Analysis

All statistical analyses were performed with Statistical Product and Service Solutions (SPSS) 16.0 (SPSS Inc., Chicago, IL, USA) software. The relationship between P5CR1 expression and clinicopathological factors of GC patients was analyzed using the Chi-square test. Survival analysis was carried out by Kaplan-Meier method, followed by Log-rank test for comparison between curves. Other experimental results were analyzed by *t*-test and expressed as mean ± standard deviation. $p<0.05$ suggested that the differences were statistically significant.

Results

P5CR1 Expression Was Up-Regulated in GC

The expression of P5CR1 in clinical samples was detected by both PCR and Western blot. The results showed in Figure 1A indicated that the expression of P5CR1 in cancer tissues was significantly higher than that of normal tissues. Besides, P5CR1 was identically to be upregulated in GC cell lines (Figure 1B). Taken together, P5CR1 was regarded as a potential oncogene in GC.

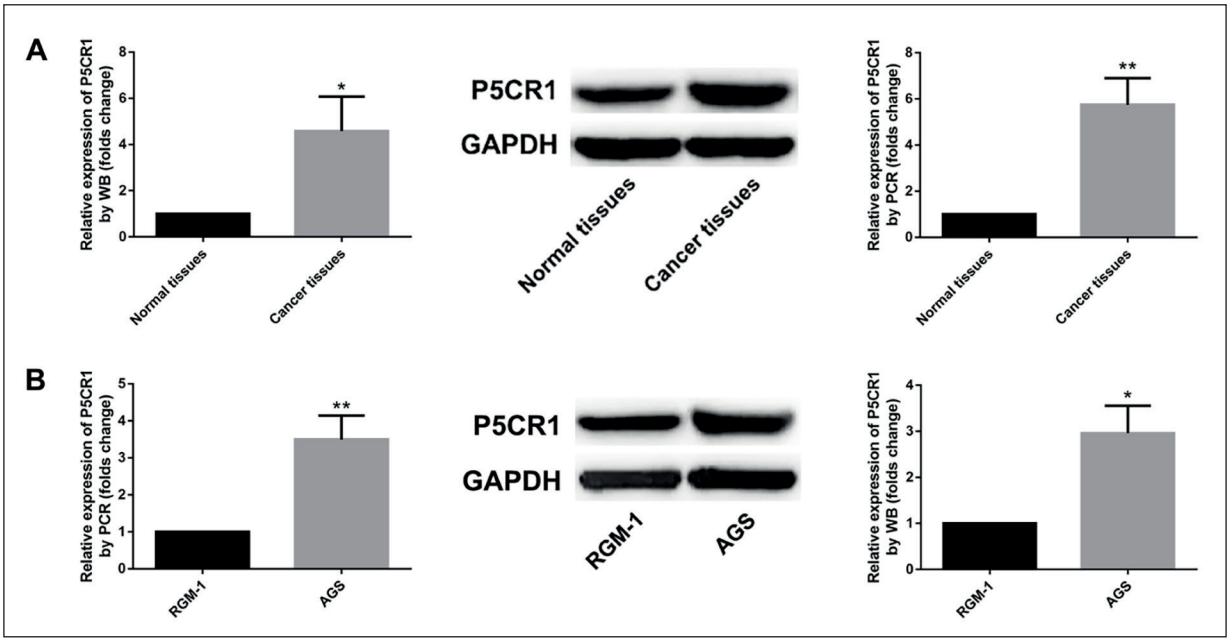

Figure 1. Different expression levels of P5CR1 at organizational level and cellular level detected by PCR and Western blot. (*$p<0.05$, **$p<0.01$).

Association Between Clinicopathological Characteristics of GC Patients and P5CR1 Expression

Enrolled GC patients were divided into P5CR1 high-expression group (n = 42) and low-expression group (n = 38) according to the mean expression level of P5CR1. Then, the association between P5CR1 expression and clinicopathological features of GC patients was analyzed (Table I). The expression level of P5CR1 was associated with tumor differentiation and TNM stage ($p<0.05$), while it was not associated with the patient's gender, age, tumor size, and tumor position ($p>0.05$).

Effect of P5CR1 on the Prognosis of Patients with GC

Kaplan-Meier survival analysis was performed to analyze the correlations between P5CR1 level and OS of GC patients. The result showed that high expression of P5CR1 in GC patients indicated a poor survival rate (Figure 2A). COX regression analysis model was used to analyze the prognostic potential of P5CR1 in GC. As shown in Table II, P5CR1 level was an independent factor for predicting the prognosis of GC.

P5CR1 Suppressed Malignant Behavior of Gastric Adenocarcinoma Cells

To further explore the effects of P5CR1 on the biological function of GC cells, AGS cells were transfected with si-P5CR1 or si-NC. Transfection of si-P5CR1 could significantly inhibit both mRNA and protein expressions of P5CR1 in AGS cells (Figure 2A, 2B). Afterwords, a series of functional experiments uncovered that knockdown of P5CR1 markedly suppressed colony number (Figure 3A), wound healing percentage (Figure 3B), and invasive cell number (Figure 3C), while apoptosis rate was elevated (Figure 3D).

Discussion

The occurrence and development of GC is a complex process that involves changes in expressions and structures of abundant tumor-related genes. It is also affected by relevant signal transduction pathways, thus forming a multi-phase, complicated network-like cascade reaction process with multiple genes involved[18,19]. A great number of genes has been identified to be related to GC development[20-23]. Differentially expressed genes between healthy population and GC patients could be promising targets of GC. These specific biomarkers attribute to improve clinical outcomes of GC patients, which are required to be urgently developed.

Phang et al[24] highlighted abnormal proline metabolism in various tumor tissues. As a non-enzy-

Table I. P5CR1 expression and clinical features of patients with gastric adenocarcinoma.

Features	No.	P5CR1		p
		High	Low	
No.	80	42	38	
Gender				0.733
Male	39	23	16	
Female	41	19	22	
Age (year)				0.121
< 60	43	19	24	
≥ 60	37	23	16	
Tumor size (cm)				0.090
< 5	32	15	17	
≥ 5	48	27	21	
Tumor position				0.068
Fundus of stomach	13	5	8	
Body of stomach	35	17	18	
Antrum of stomach	32	20	12	
Tumor differentiation				0.025
Well + moderate	46	18	28	
Poor + undifferentiated	34	24	10	
TNM stage				0.001
I + II	50	19	31	
III + IV	30	23	7	

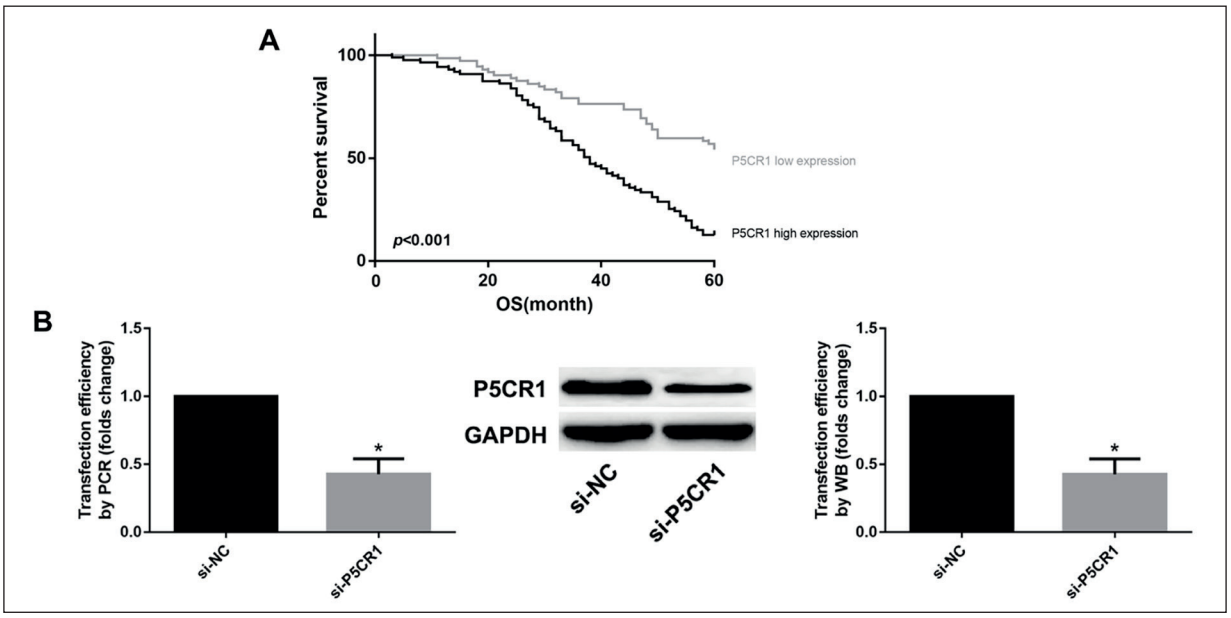

Figure 2. A, The relationship of P5CR1 expression with progression free survival and overall survival of GC patients. **B,** Transfection efficiency detected by PCR and WB. (*p<0.05).

matic antioxidant, proline could inhibit apoptosis by reducing reactive oxygen species (ROS) and retaining reduced glutathione content in cells[25]. In recent years, the biological function of P5CR1 has been recognized. As a key enzyme in the circulation pathway of proline, P5CR1 is related to the energy metabolism and functions of mitochondria, and plays a crucial role in the regulation of cell proliferation and apoptosis. Studies have demonstrated that P5CR1 is related to the occurrence and development of many tumors. Gao et al[26] found that overexpression of the oncogene Myc could promote the synthesis of glutaminase (GLS), thereby stimulating proline metabolism. In addition, Myc could upregulate P5CR1 and catalyze the synthesis of cellular proline to facilitate the growth of tumor cells. Togashi et al[27] also found that P5CR1 could increase the content of proline and reduce the production of ROS during the progression of oral tumors, significantly stimulating tumor cell survival. In prostate tumor, overexpression of P5CR1 elevates the survival rate of tumor cells[16]. All these results well proved the promoting role of P5CR1 in the occurrence and development of tumors. Silencing P5CR1 may be a novel therapeutic strategy for cancer treatment.

Our experimental results revealed the role of P5CR1 in GC for the first time. P5CR1 was highly expressed in GC. By analyzing clinical data of GC patients, the expression of P5CR1 was significantly associated with the tumor differentiation

Table II. Univariate and multivariate analyses of postoperative prognosis in patients with gastric adenocarcinoma.

	Univariate analysis		Multivariate analysis	
	Hazard ratio/CI (95%)	p	Hazard ratio/CI (95%)	p
Gender (male *vs.* female)	0.834/0.613-1.410	0.551		
Ages (< 60 *vs.* ≥ 60)	1.129/0.860-1.305	0.202		
Tumor size (< 5 cm *vs.* ≥ 5)	1.997/0.982-3.401	0.094		
Tumor position (Fundus, Body, Antrum)	1.552/0.814-2.237	0.177		
Tumor differentiation (Well *vs.* Poor)	2.878/1.905-4.371	0.012	2.106/1.659-3.308	0.041
Clinical stage (I-II *vs.* III-IV)	3.351/ 2.483-4.992	0.007	3.002/2.017-3.853	0.023
P5CR1 expression (low *vs.* high)	2.733/2.034-3.692	0.015	2.154/1.631-3.071	0.047

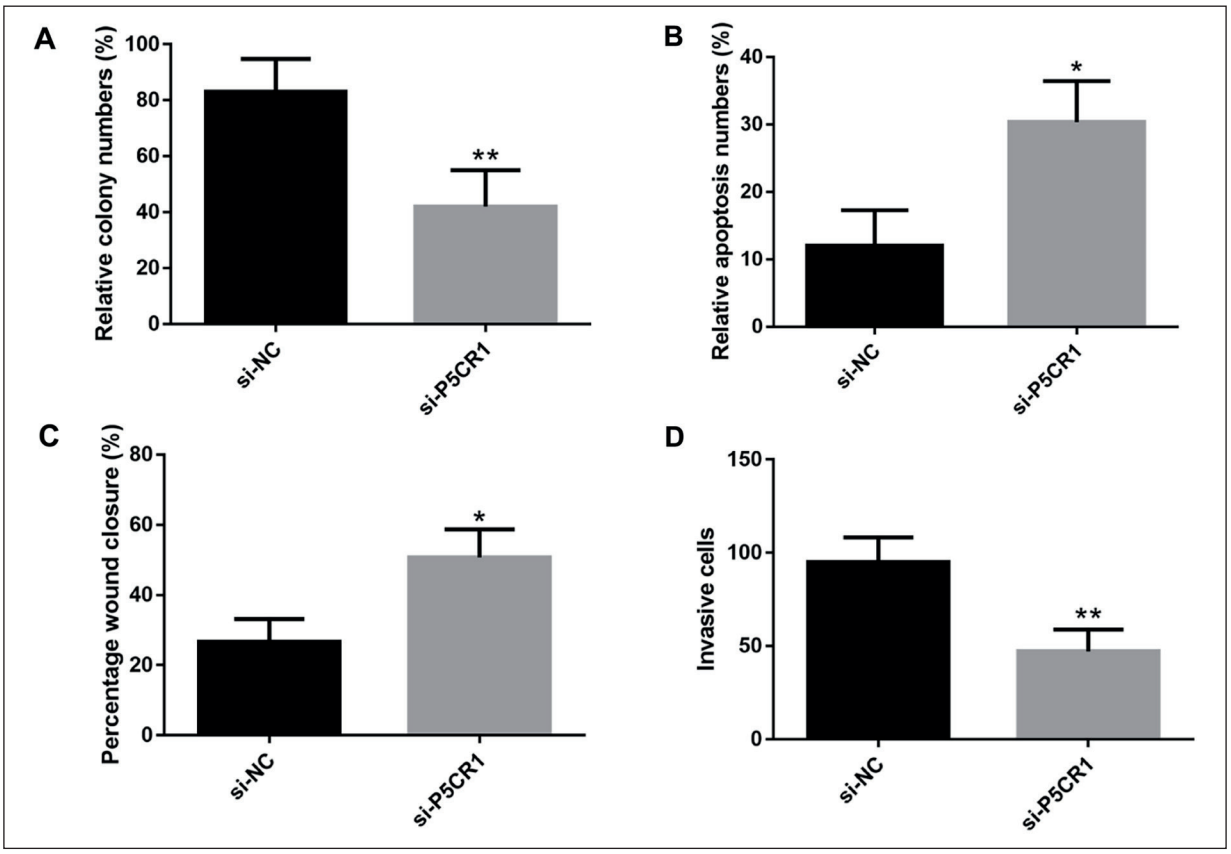

Figure 3. A, Cell clone formation. **B,** Cell apoptosis. **C,** Cell migration. **D,** Cell invasion. (*p<0.05, **p<0.01).

and TNM stage. In addition, P5CR1 was an oncogene unfavorable to the prognosis of GC. *In vitro* results indicated that down-regulation of P5CR1 could significantly inhibit cell proliferation, migration, and invasion while promoting apoptosis.

Conclusions

In brief, P5CR1 was involved in the regulation of the occurrence and development of GC, which might be a potential biomarker and therapeutic target for GC patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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