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Original Article

Decreased RGS6 expression is associated with poor prognosis in pancreatic cancer patients

Nan Jiang[1], Ruihua Xue[1], Fangfang Bu[2], Xin Tong[2], Jiankun Qiang[2], Rong Liu[1]

Departments of [1]Surgical Oncology, [2]Cancer Center, Chinese People's Liberation Army (PLA) General Hospital, 28 Fuxing Road, Beijing 100853, China

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Abstract: Regulator of G-protein signaling 6 (RGS6), a member of a family of RGS proteins, has been reported to involve in multiple processes during tumor development. However, its role in pancreatic cancer has not been studied yet. In this study, we aimed to investigate the expression of RGS6 in pancreatic cancer and its role in predicting outcomes of patients with pancreatic cancer. We first measured the expression of RGS6 mRNA in 20 cases of tumor tissues and matched adjacent non-tumorous tissues by quantitative real-time PCR and examined RGS6 protein by immunohistochemistry in tissue microarrays containing 90 tumor and 90 paired adjacent non-tumor tissues. Decreased RGS6 mRNA detected in primary tumor, compared with their non-tumor counterparts. In addition, decreased RGS6 protein expression was associated with tumor differentiation (P = 0.027), pT classification (P = 0.034), smoking status (P = 0.041) and a poor survival (P = 0.007). Cox proportional hazards regression modeling analysis revealed that lymph node metastasis (P = 0.001; hazard ratio, 2.347, 95% CI, 1.387-3.972), tumor differentiation (P = 0.015; hazard ratio, 0.505, 95% CI, 0.291-0.876) and RGS6 expression (P = 0.048; hazard ratio, 0.567, 95% CI, 0.324-0.994) were three independent prognostic factors. Taken together, these date demonstrate that RGS6 decreases in tumor tissue and may serve as a novel biomarker for outcomes in pancreatic cancer patients and be a potential therapeutic target potential therapeutic target.

Keywords: RGS protein, pancreatic neoplasms, clinical pathology, prognosis

Introduction

The regulator of G protein signaling (RGS) family proteins contain a semiconserved RGS structural domain, which activate the GTPase-activating protein [1-3]. RGS proteins regulate G-protein-coupled-receptor (GPCR) signaling through accelerating guanosine triphosphate hydrolysis by association with Gα protein [4-6]. More than thirty human genes encoding proteins were found to contain RGS domain or closely related function of this domain [5, 7]. Based on sequence of RGS domain homology, RGS proteins have been distributed into eight subfamilies, including A/RZ, B/R4, C/R7, D/R12, E/RA, F/GEF, G/GRK and H/SNX [8, 9]. RGS6 proteins is a member of the R7 subfamily, which contain, besides the RGS domain, two semiconserved regions, GGL (Gγ subunit-like) domain that binding to Gβ5 subunits and function as stabilization domain for RGS6 and another is DEP domain that is assumed to interaction with R7BP protein or R9AP protein to control intracellular targeting [10-14].

Recently, RGS6 was reported to inhibit the growth of human breast cancer cells and suppress colony formation. Overexpression of RGS6 prevented breast cancer cells from G1 into S phase of the cell cycle and induced cells apoptosis by regulation of intrinsic pathway of apoptosis [15]. Loss of RGS6 expression promoted tumorigenesis and cellular transformation in vivo and in vitro [16, 17]. RGS6-mediated ROS production was reported as a mediator of cell apoptosis and growth arrest responses to doxorubicin in cytotoxic action [18]. Moreover, the expression of RGS6 is negative correlation with increasing tumor grade [15] and may reduce the risk of bladder tumor formation [19]. These finding suggest an important role of RGS6 in regulating tumorigenesis and related to progression.

Pancreatic cancer is a malignant disease with difficult to gain early diagnosis. In previous studies showed that many expression or function of proteins, including PTEN [20, 21], KRAS

Table 1. Correlation between RGS6 expression and clinicopathologic variables of pancreatic pancreatic adenocarcinoma patients

Variables	RGS6 expression		*P*-value*
	High expression	Low expression	
Age (years)			0.1065
≤65	29	28	
>65	11	22	
Gender			0.4274
Male	28	31	
Female	12	19	
Differentiation			0.0265
Well/moderate	32	29	
Poor	8	21	
PT classification			0.0339
pT1	3	1	
pT2	33	36	
pT3	3	14	
Lymph node			0.8480
pN0	24	29	
pN1	16	21	
Smoking			0.0414
Yes (> 40 pack-years)	13	27	
No	27	23	
Drink			0.2781
Yes (> 50 ml/day)	17	27	
No	23	23	
Perineural invasion			0.1117
Yes	27	41	
No	13	9	

**P*-value were two-tailed and based on the Chi-square test or fisher exact test.

[22], etc, reported in pancreatic neoplasms were of guiding significances for clinical. Nonetheless, it have not been reported that the expression of RGS6 messenger RNA (mRNA) and protein in primary pancreatic cancer. The relationship between RGS6 expression and clinical clinicopathological and prognosis in pancratic tumors are as yet unclear. Thus, in our study, the purpose is to examine the expression of RGS6 in 90 cases of pancreatic carcinoma patients and explore its correlation with clinicopathologic characteristics and survival time.

Materials and methods

Patients and tissue samples

90 primary pancreatic adenocarcinoma tissues and paired adjacent non-tumor tissues (located > 5 cm from the tumors) were collected from patients who underwent pancreatic surgical resection with informed consent at the PLA General Hospital in Beijing, China, from 2005 to 2008. Both tumor and no-tumor tissues samples were confirmed by histological proof. None of these patients received neoadjuvant or adjuvant treatment before operation. Of 90 patients, 59 were male and 31 were female. The mean age was 62 years. 52 cases were located in the pancreatic head, 13 in the body, 4 in the tail and 21 in combined locations. Tumors were classified according to UICC/AJCC tumor-node-metastasis (TNM) classification of malignant tumors (seventh edition) [23]. The follow-up data was completed on November 2013 and the range of the follow-up period was 1-87 months. Patient characteristics are shown in **Table 1**. This study was approved by the Committee on Ethics of the Chinese PLA General Hospital.

RNA extraction and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analyses

Total RNA was extracted from fresh tumorous and paired adjacent non-tumorous samples of 20 patients using Trizol Reagent (Invitrogen, USA) and was reverse-transcribed to first-strand complementary DNA (cDNA) using the Reverse Transcription System Kit (Promega, Madison, WI) according to the manufacturer's instructions. Levels of the corresponding GAPDH and RGS6 mRNA were detected by qRT-PCR using the 7500 Real-Time PCR System (Applied Biosystems, CA, USA). The PCR was run for 95°C for 3 min, followed by 40 cycles of 95°C for 3 s, 60°C for 30 s. GAPDH was used as normalization controls for RGS6 mRNA. The sequences of the qPCR primer were as follows: RGS6 forward, 5'-CCAGTTGAAGCAATACACTTGGG-3'; RGS6 reverse, 5'-TGGTGAGAACATGGTCTGAGA-3'; GADPH forward, 5'-CTTTGGTATCGTGGAAGGACTC-3; GADPH reverse, 5'-GTAGAGGCAGGGATGATGTTCT-3. Relative expression of RGS6 mRNA was cal-

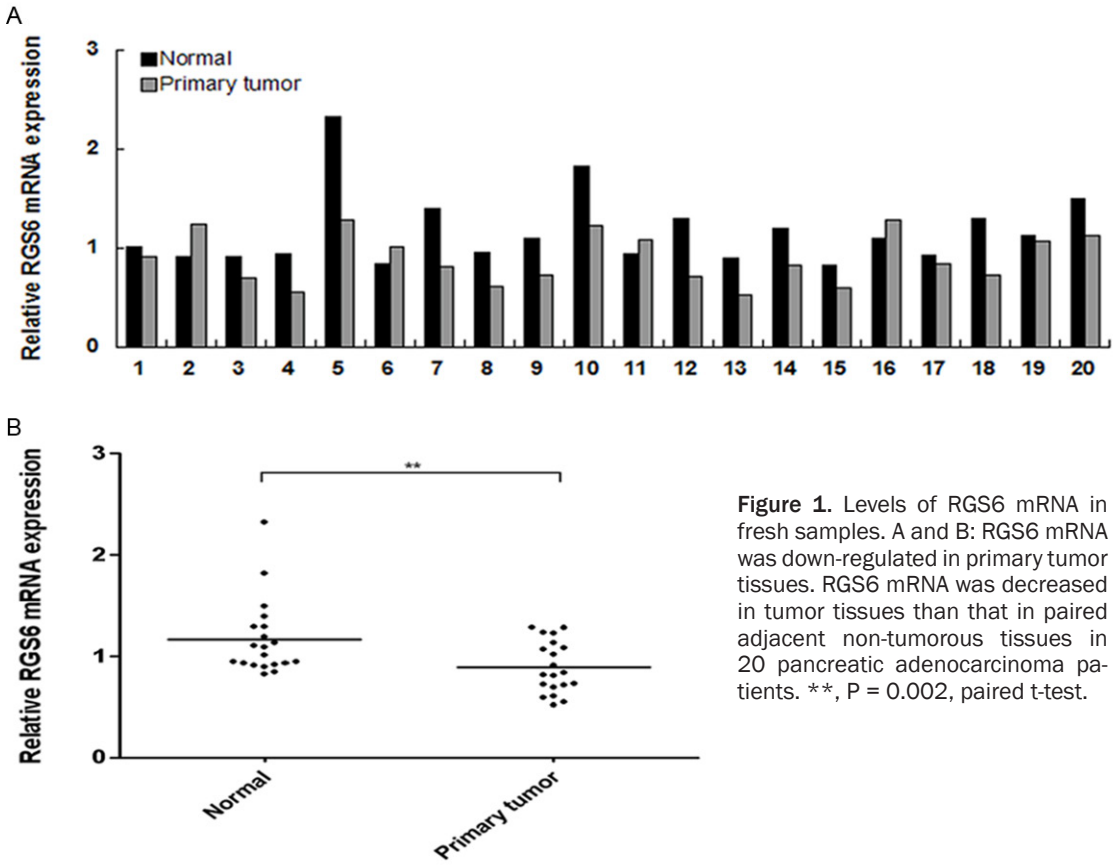

Figure 1. Levels of RGS6 mRNA in fresh samples. A and B: RGS6 mRNA was down-regulated in primary tumor tissues. RGS6 mRNA was decreased in tumor tissues than that in paired adjacent non-tumorous tissues in 20 pancreatic adenocarcinoma patients. **, P = 0.002, paired t-test.

culated and compared using the $2^{-\Delta\Delta CT}$ method as described previously [24].

Tissue microarray and immunohistochemical staining

A total of 180 formalin-fixed and paraffin-embedded tissues samples, including 90 tumor and 90 paired adjacent non-tumor tissues specimens, were constructed to tissue microarray using microarray punching instrument. The selected spots of tissues were punched out 1.5 mm in diameter (each tissue core) and were harvested into recipient blocks.

In brief, 5 μm thick tissue sections were cut from the tissue array blocks. The serial number of the tissue sections were mounted on the slides and baked at 65°C for 90 min. For immunological histological chemistry (IHC) staining, tissue sections were deparaffinized in xylene, rehydrated in diluted alcohol series, and incubated in 3% H_2O_2 for 30 min to block endogenous peroxidase. Then slides were boiled in the steam boiler with preheating sodium citrate buffer (10 mM, pH 6.0) for 10 min to antigen retrieval. For block nonspecific binding, slides treated with 10% normal goat serum at 37°C, followed by incubating overnight with rabbit polyclonal antibody against RGS6 (1:200; Abcam company, ab155809, USA) at 4°C. For negative control, primary antibody was replaced with phosphate buffer solution (PBS) as blank control. Following day, slides were incubated with secondary antibodies that were goat anti-rabbit immunoglobulins (Santa Cruz Biotechnology, Dallas, TX, USA), stained with diaminobenzidine (DAB) and then counterstained with hematoxylin.

Evaluation of IHC staining

An immunoreactivity score was using Amend Allred scoring system as described previously [25]. The percentage of positive tumor cells was sored as follows: 0, < 5% positive tumor cells; 1, 5-25% positive tumor cells; 2, 25-50% positive tumor cells; 3, 50-75% positive tumor cells; 4, > 75% positive tumor cells. Staining intensity was scored as follows: 0, no staining;

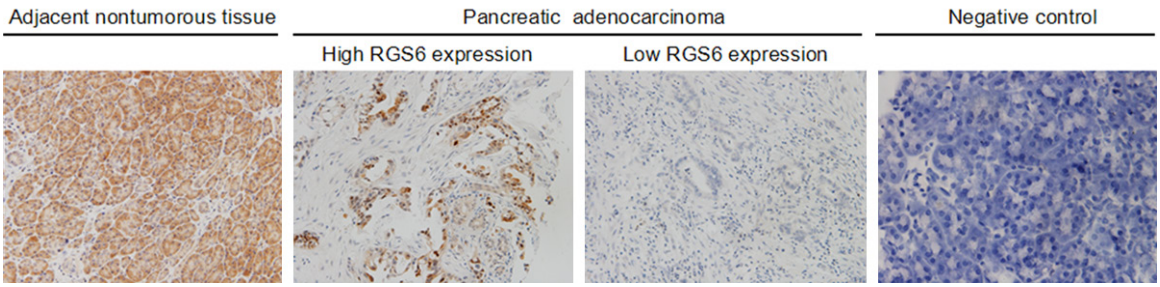

Figure 2. Representative images of immunohistochemical staining of RGS6 in adjacent non-tumorous tissue and pancreatic adenocarcinoma tumor tissues. The left image showed RGS6 staining in normal cells (original magnification × 200). The middle tow images showed different level of RGS6 in pancreatic adenocarcinoma cells (original magnification × 200). The right image was the negative control (original magnification × 400).

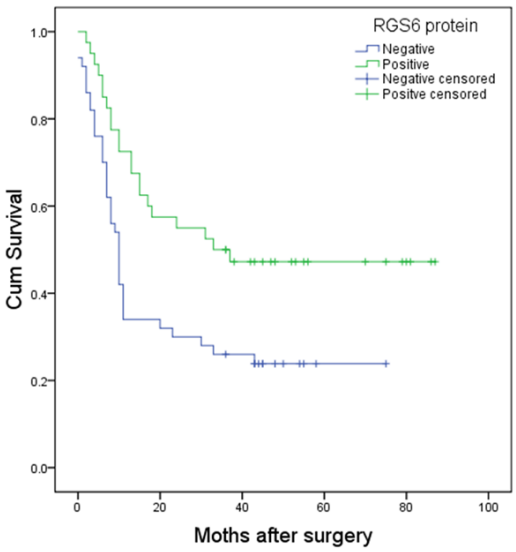

Figure 3. Kaplan-Meier analysis of the clinical outcome according to the level of RGS6 expression. Schematic representation shows that patients with low expression of RGS6 had a worse survival than those with high expression of RGS6. P = 0.007, log-rank test.

1, weak staining; 2, moderate staining; 3, intense staining. Both the percentage of positive tumor cells and staining intensity were decided independently by a double-blinded manner. The heterogeneous staining score was determined as following formula: Staining index (SI) score - intensity score × positive rate score. This present study, tissues with SI score of ≤ 4 was considered as low expression and of > 4 as high expression.

Statistical analysis

Statistical analysis was performed using the statistical software package SPSS version 16.0 (SPSS, Chicago, IL, USA). Paired two-tailed t-test were used to evaluate the difference of RGS6 mRNA expression in primary tumorous and adjacent non-tumorous tissues. The chi-square test or Fisher's exact test were used to assess the correlation between RGS6 expression and clinicopathologic characteristics. The Kaplan-Meier method and log-rank test were used to analyze the Survival-data. Cox proportional hazards regression model were used to examine univariate and multivariate prognostic analysis. *P*-values < 0.05 were considered statistically significant.

Results

The expression of RGS6 in tumorous and adjacent non-tumorous tissues

To explore the mRNA expression level of RGS6, 20 pairs of tissues included primary tumorous and adjacent non-tumorous were detected by qRT-PCR in this study. The relative mRNA expression level of RGS6 in primary tumor was lower than most non-tumor counterparts (**Figure 1A**). qRT-PCR results show that relative RGS6 mRNA expression was significantly down-regulated in tumor tissues compared with paired non-tumor tissues (P < 0.001, t-test; **Figure 1B**). Then to further validate the results, RGS6 expression in protein level was detected in 90 primary tumor and paired adjacent non-tumor tissues specimens by IHC as described above. RGS6 predominantly located at the cytoplasm and membrane (**Figure 2**). High level expression of RGS6 was detected in 76/90 (84.4%) of adjacent non-tumor tissues, but only 40/90 (44.4%) in tumor tissues. The High level expression rate of RGS6 protein was significantly lower in tumor samples than that

Table 2. Univariate and multivariate analyses of factors associated with survival

Variables	HR (95% CI)	*P*-value*
Univariate analysis		
Age (≤ 65 vs. > 65)	0.984 (0.573-1.689)	0.952
Gender (male vs. female)	0.602 (0.338-1.070)	0.083
Differentiation (well/moderate vs. poor)	2.022 (1.201-3.405)	0.008
PT classification		
(pT2 vs. pT1)	0.844 (0.261-2.721)	0.776
(pT3 vs. pT1)	0.929 (0.257-3.351)	0.910
(pT3 vs. pT2)	1.229 (0.649-2.328)	0.527
Lymph node (pN1 vs. pN0)	2.052 (1.224-3.439)	0.006
Smoking (Yes vs. No)	1.478 (0.886-2.467)	0.135
Drink (Yes vs. No)	1.251 (0.750-2.086)	0.391
RGS6 (positive vs. negative)	0.492 (0.288-0.841)	0.009
Multivariate analysis		
Lymph node (pN1 vs. pN0)	2.347 (1.387-3.972)	0.001
Differentiation (well/moderate vs. poor)	0.505 (0.291-0.876)	0.015
RGS6 (positive vs. negative)	0.567 (0.324-0.994)	0.048

HR, hazard ratio; CI, confidence interval; *Cox's proportional hazards regression analysis (Forward stepwise).

in non-tumor samples ($P < 0.001$, χ^2 test; **Figure 2**).

Correlation between IHC expression and clinicopathologic features

The correlation between RGS6 expression and clinicopathologic features was further summarized and evaluated (**Table 1**). There was no significant correlation between the expression level of RGS6 and patient's age ($P = 0.107$), gender ($P = 0.427$), lymph node metastasis ($P = 0.848$), perineural invasion ($P = 0.112$). However, RGS6 expression was associated with tumor differentiation ($P = 0.027$), pT classification ($P = 0.034$) and smoking status ($P = 0.041$).

Survival analysis

Survival curves were described by Kaplan-Meier method. Long-rank analysis showed that a low level expression of RGS6 was significantly associated with poorer survival of patients with surgically resected pancreatic adenocarcinoma (**Figure 3** and **Table 2**). Moreover, in univariate analysis, low expression level of RGS6 and poor tumor differentiation, positive lymph node metastasis were associated with decreased survival ($P < 0.05$). However, patient's age, gender, pT classification, smoking status was not significantly associated with shorter survival. Multivariate analysis using Cox proportional hazards model revealed that RGS6 expression, lymph node metastasis and tumor differentiation were three independent prognostic predictors for patient in this study.

Discussion

Recent evidences suggest that part of the RGS family protein is linked to change in growth of tumor cells. For instance, RGS16 was reported to inhibit breast cancer cell growth by regulating PI3K signaling pathways, which supports growth of many tumors, [26] and RGS17 was found to be up-regulated in human lung as well as prostate cancer and induce tumor cell proliferation through the cyclic AMP-PKA-CREB pathway [27]. RGS6, as one of the member of the RGS family protein, has been first reported to be possible antiproliferative actions in human bladder cancer [19]. Recent study found that RGS6 induced cell death and is associated with cancer progression [15-17]. Here, we found that RGS6 mRNA expression was generally lower in primary pancreatic tumor tissues than that in adjacent non-tumor through the $2^{-\Delta\Delta CT}$ method. Moreover, we detected 90 primary tumor and paired adjacent non-tumor tissues using IHC to explore the differences in protein level. The result showed that RGS6 protein expression was also significantly lower in pancreatic tumor tissues than that in adjacent non-tumor tissues. Thus, RGS6 seems to need further elucidate the mechanism in the progression of pancreatic carcinoma.

Approximately 90% of histological variant of pancreatic carcinoma is pancreatic ductal adenocarcinoma (PDAC) [28]. PDAC, characterized by easy recurrence and early metastasis, resection rate is only 20% and overall five year survival rate for postoperative patients is only 20%-29.3% [29-32]. It is known that smoking is one of risk factors for the development of pancreatic carcinoma. Interestingly, here, we found that a low expression level of RGS6 was more

frequently in patients with smoking. Meanwhile, Berman et al. has previously reported that RGS6 variant allele was associated with reduction risk in bladder cancer risk, especially in ever smokers [19]. The reason for these could be caused by mutual interference mechanism between RGS6 and smoking, which seems to need for further research.

A recent study, Maity et al [16] indicated that decreased RSG6 expression correlated with increasing human breast tumor grade. In this study, we found that RGS6 expression was associated with tumor differentiation and pT classification. These finding suggest that RGS6 may inhibit the growth of tumor in huma pancreatic cancer by inducing cell apoptosis. In previous studies indicated that RGS6 could block tumor progression by suppressing cellular proliferation and promoting apoptosis, which could be due to: (a) directly induce apoptosis via mitochondrial-dependent pathway [15]; (b) induce apoptosis with RGS6-mediated ROS generation [33]; (c) enhance pro-apoptotic genes, silenced by Dnmt1-mediated methylation, reactivation through inhibiting Dnmt1 activity [17]. Based on these data, RGS6 could be a novel target for the treatment of adenocarcinoma of the pancreas.

Furthermore, Univariate and multivariate analyses demonstrated that decreased RGS6 expression predicted a poor prognosis in patients with pancreatic cancer. Besides RGS6 expression, tumor differentiation, lymph node metastasis was risk factors of survival in patients with pancreatic cancer.

In summary, the present study, for the first time, shows that decreased RGS6 mRNA and protein expression in primary pancreatic tumor tissues. Moreover, low protein expression of RGS6 was associated with important clinicopathological parameters and predicted prognosis of patients with pancreatic cancer. Based on these finding suggested that RGS6 may provide new target for anti-cancer therapy and diagnostic for pancreatic cancer patients.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Rong Liu, Department of Surgical Oncology, Chinese People's Liberation Army (PLA) General Hospital, 28 Fuxing Road, Beijing 100853, China. Tel: (86)-10-669-39377; Fax: (86)-10-66939377; E-mail: Liurong-301@126.com

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