RESEARCH ARTICLE

RASAL1 Attenuates Gastric Carcinogenesis in Nude Mice by Blocking RAS/ERK Signaling

Hong Chen[1]*, Ji-Yi Zhao[1], Xu-Chen Qian[1], Zheng-Yuan Cheng[2], Yang Liu[1], Zhi Wang[1]

Abstract

Recent studies have suggested that the RAS protein activator like-1 (RASAL1) functions as a tumor suppressor *in vitro* and may play an important role in the development of gastric cancer. However, whether or not RASAL1 suppresses tumor growth *in vivo* remains to be determined. In the present study, we investigated the role of RASAL1 in gastric carcinogenesis using an *in vivo* xenograft model. A lentiviral RASAL1 expression vector was constructed and utilized to transfect the human poorly differentiated gastric adenocarcinoma cell line, BGC-823. RASAL1 expression levels were verified by quantitative real-time RT-PCR and Western blotting analysis. Then, we established the nude mice xenograft model using BGC-823 cells either over-expressing RASAL1 or normal. After three weeks, the results showed that the over-expression of RASAL1 led to a significant reduction in both tumor volume and weight compared with the other two control groups. Furthermore, in xenograft tissues the increased expression of RASAL1 in BGC-823 cells caused decreased expression of *p*-ERK1/2, a downstream moleculein the RAS/RAF/MEK/ERK signal pathway. These findings demonstrated that the over-expression of RASAL1 could inhibit the growth of gastric cancer by inactivation of the RAS/RAF/MEK/ERK pathway *in vivo*. This study indicates that RASAL1 may attenuate gastric carcinogenesis.

Keywords: Gastric cancer - RASAL1 gene - BGC-823 cells - xenograft tumor

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Introduction

The RAS proteins control signaling pathways that are key regulators of several aspects of normal cell growth and malignant transformation. The aberrant activation of RAS signaling pathway due to RAS genes mutations or to alterations in upstream or downstream signaling components is common in most human cancers (Downward, 2003; Malumbres et al., 2003; Karnoub et al., 2008). Thus, it is important to understand the mechanism of activating RAS in gastric cancer.

RAS GTPase Activating Proteins (RAS GAPs) normally turn off RAS by catalyzing the hydrolysis of RAS-GTP to RAS-GDP (Cherfils et al., 2013). RAS mutations at hot spots, such as G12 and Q61, which is insensitive to RAS GAPs, result in a slower GTP-hydrolysis rate that leads to longer activated signaling pathways ultimately promoting uncontrolled cell growth and are responsible for over 25% of human tumors (Karnoub et al., 2008; Lu et al., 2011). However, in the absence of RAS mutation, RAS activity is still unusually high in gastric cancers, and the loss of RAS GAPs provides an alternative mechanism of activating RAS (Maertens et al., 2014). Mclaughlin et al. (2014) found that RASAL2, one of RAS GAPs family, is mutated or suppressed in human breast cancer, result in RAS and ERK frequently hyper-activated, and RASAL2 ablation promotes tumor growth, progression, and metastasis in mouse models. Calvisi et al. (2011) reported that in the absence of RAS mutations, down-regulation of at least one RAS GAP (RASAL1, DAB2IP, or NF1) and aberrant RAS activation was found in HCC at the same time. Reactivation of RASAL1, DAB2IP, and PITX1 inhibited proliferation and induced apoptosis, whereas their silencing increased proliferation and resistance to apoptosis. They supposed that RAS GAPs may emerge as a class of tumor suppressors and provide a novel mechanism of activating RAS signaling pathway.

The RAS protein activator like-1 (RASAL1) gene is a newly discovered member of the RAS GAPs family. Our previous study (Chen et al., 2011; Chen et al., 2013; Chen et al., 2014) found the expression of RASAL1 is down-regulated in gastric cancer tissues and various gastric cancer cell lines, which is a candidate tumor suppressor gene. Moreover, we found that RASAL1 gene could inhibit the proliferation of gastric cancer cells *In vitro* through promoting cell apoptosis, and decreasing invasion and migration by blocking RAS/ERK signaling. In this study, we investigated the effects of enforced expression of RASAL1 on nude mice xenograft model using BGC-

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823 cell line, the human poorly differentiated gastric adenocarcinoma cell line, and the associated mechanism.

Materials and Methods

Cell culture and lentiviral infection

Human poorly differentiated gastric adenocarcinoma BGC-823 cells were stored in our laboratory and were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS, Sijiqing, China), 100 μg/ml streptomycin, and 100 U/ml penicillin (Gibco). The cells were cultivated in incubator at 37˚C in atmosphere of 5% CO_2. The BGC-823 cells was infected with the concentrated viral stocks of pCDH-CMV-RASAL1-EF1-puro or pCDH-CMV-EF1-puro designed and synthesized by Moji Corporation (Nanjing, China), using Polybrene according to the manufacturer's instructions in RPMI-1640 for 6h at 37˚C. Forty-eight hours after stable transfection with RASAL1 or the control lentivirus using a concentration of 5x107 transducing units/well for 6-well plates at a multiplicity of infection (MOI) of 100. The cells were renamed BGC-823-RASAL1 (transfected with the RASAL1 lentiviral vector), BGC-823-NC (transfected with the empty lentiviral vector) and BGC-823(control untransfected). Transfected cells were selected with puromycin (Gibco) for 2 weeks, and resulting single clones were expanded to obtain stably transfected cells for subsequent experiments.

Mouse xenograft model

The BALB/c nude mice (4-6 weeks old) were purchased from Slac Laboratory Animal Corporation (Shanghai, China). All procedures were carried out according to the animal protocol approved by Southeast University Laboratory Animal Center. BGC-823 cells were trypsinized, washed in PBS, resuspended in saline solution, and $5x10^6$ cells per 0.2 ml were injected subcutaneously into 3 separate groups of nude mouse (6 or 7 mice in each experimental group). The tumor size was measured every 2 days. The tumor volume was calculated according to the formula V=L×W2×0.5, where L= the largest superficial diameter and W = the smallest superficial diameter. After 21 days, the mice were sacrificed and the tumors were weighed and photographed.

Measurement of RASAL1 mRNA using qRT-PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, USA), RNA was then reverse-transcribed using PrimeScript RT reagent Kit with cDNA Eraser (Takara, Japan), according to the manufacturer's instructions. Then cDNA was amplified using THUNDERBIRD SYBR qPCR Mix (Toyobo, Japan) by Stepone plus real-time PCR detection system (ABI). Primers used for qPCR were as follows: β-actin (upstream: 5'-CTACAATGAGCTGCGTGTGG-3'; downstream: 5'-TAGCTCTTCTCCAGGGAGGA-3', 221 bp) and RASAL1 (upstream: 5'-TGGATTTCTCTTCTTGCGATTCT-3'; downstream: 5'-TGTTGGTCCCGAAGGTCAA-3', 72 bp). Cycle parameters were: 95˚C(30 sec), followed by 40 cycles of 95˚C (5 sec),and a dissociation stage of 60˚C (30 sec) , 95˚C(15 sec), 60˚C (15 sec) and 95˚C(1 min). Fold inductions were calculated using the formula 2-(ΔΔCt), where ΔΔCt was the ΔCt(RASAL1)-ΔCt(control), ΔCt was Ct (RASAL1)-Ct (β-actin) and Ct was the cycle at which the threshold was crossed. PCR product quality was monitored by post-PCR melt curve analysis.

Western blotting detection of RASAL1, ERK1/2 and p-ERK1/2

Protein extracts were isolated from cells orxenograft tumors in RIPA (Radio-Immunoprecipitation Assay) Buffer. Protein concentration was measured by the bicinchoninic acid (BCA) method. Protein supernatants (30μg) were separated on 10% SDS-polyacrylamide gels (SDS-PAGE) (Invitrogen, USA) and transferred to nitrocellulose membranes. Expression of RASAL1, ERK1/2 and *p*-ERK1/2 at protein levels was then measured by Western blotting analysis. The goat anti-human RASAL1 polyclonal antibody (1:200, Abcam, UK), rabbit anti-human ERK1/2 polyclonal antibody(1:1000, Abcam), or rabbit anti-human phospho-ERK1/2 polyclonal antibody (1:1000, Abcam) were used as first antibodies and rabbit anti-goat-IgG (H+L) antibody conjugated with horseradish peroxidase (HRP, 1:5000, Abcam) as secondary antibody. The enhanced chemiluminescence (ECL) imaging method was used to facilitate the detection of protein bands.

Statistical analysis

Results were shown as the mean ± standard deviation (SD). Statistical analysis was performed using SPSS 18.0 software. T-test and one-way analysis of variance (ANOVA) were used. $p<0.05$ was considered to indicate a statistically significant difference.

Results

Measurement of RASAL1 mRNA and protein

After transfection with the empty lentiviral vector or the RASAL1 lentiviral vector, the expression levels of RASAL1 were evaluated in the parental gastric cancer cells. As shown in Figure 1 and Table 1, BGC-823-RASAL1 cells exhibited an obvious increase in RASAL1 mRNA levels (Figure 1B) compared with BGC-823 and BGC-823-NC cell line. Consistent with the mRNA level, there was a significant increase in the protein levels (Figure 1A, C) of RASAL1 in the BGC-823-RASAL1 cells compared to the parental BGC-823 and BGC-823-NC cell line. The result demonstrated that the cell line over-expressed RASAL1 was established successfully.

Overexpresion of RASAL1 suppresses tumor growth in vivo

To explore the effects of RASAL1 on gastric cancer *in vivo*, we established a xenograft model. As shown in Figure2, transfection of RASAL1 into BGC-823 cells led to a significant reduction in both tumor weight (Figure 2B) and volume (Figure 2A,C) compared with the other two control groups.The results indicated that RASAL1 gene inhibits xenograft tumor growth of gastric cancer in nude mice and that it might be a novel potential therapeutic target for gastric cancer therapy.

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RAS gene is a kind of proto-oncogenes which maintain several life activities in normal human cells, including cell proliferation and differentiation, apoptosis, cytoskeleton organization, cell motility, and so on. When mutation occurs in the structure or the regulatory region of RAS gene, the activity of RAS and its downstream pathway will be enhanced, resulting in aberrant excessive growth of the cells, even to form malignant tumors. Studies have found that the gene mutation was the main machanism for abnormal RAS activation in certain tumors like pancreatic cancer, colon cancer, thyroid cancer and lung cancer. However, gastric cancer is very common in China (Wei et al., 2013; Liu et al., 2014), and the frequency of RAS gene mutation was not common in gastric cancer, there was still enhanced activity of RAS gene. So it is supposed that there should be some other machanisms which responsible for the aberrant excessive activity of RAS signaling pathway, such as deregulation of RAS GTPase activating proteins (RAS GAPs) or RAS guanine nucleotide exchange factors (RAS GEFs).

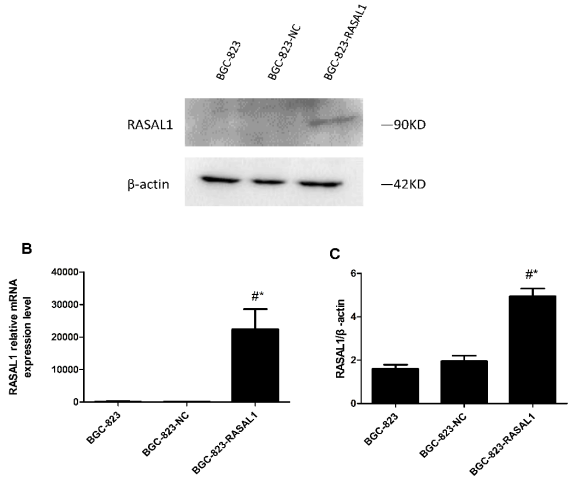

Figure 1. Expression of mRNA and Protein of RASAL1 in BGC-823 Cells. (A) RASAL1 protein levels were measured by western blotting. (B) RASAL1 relative mRNA expression levels in three groups of gastric cancer cell lines. (C) The ratio of RASAL1/β-actin in three groups of gastric cancer cell lines. **p*<0.05, BGC-823-RASAL1 cell compared to BGC-823 cell. #*p*<0.05,BGC-823-RASAL1 cell compared to BGC-823-NC cell

There are 14 predicted RAS GAP genes including RASAL1/2, DAB2IP, NF1, IQGAP1/2 and so on in the human genome. All contain a RAS GAP domain which catalyzes the hydrolysis of RAS-GTP to RAS-GDP, but share little similarity outside of this region, which suggests some RAS GAPs also have distinct RAS-independent functions. Regions flanking the RAS GAP domains are thought to promote protein–protein and protein–lipid interactions, second messenger binding and phosphorylation by protein kinases (Maertens et al., 2014). Thus, aberrant RAS GAP function will result in an important role in tumor growth. RAS GAPs are considered a newly discovered class of tumor suppressor genes.

Accumulating evidences have demonstrated that the changes in the expression of the RAS-GAPs are associated with tumorigenesis and development in gastric cancers. Dote et al. (2005) demonstrated that aberrant methylation of the promoter region of hDAB2IP (m2a and m2b) drives gene down-regulation, and the methylation-mediated transcriptional silencing of the hDAB2IP gene may be a critical event in tumorigenesis of gastrointestinal tumors. In a case–control study, the SNP rs2243421 of hDAB2IP gene with the minor allele C significantly revealed strong association with decreased gastric cancer susceptibility, which provided new insight into susceptibility factors of hDAB2IP gene variants in carcinogenesis of gastric cancer (Xu et al., 2013). Furthermore, the biological functions of RAS-GAPs were studied in gastric cancer. Jin et al. (2008) reported that IQGAP2 methylation is highly associated with loss of the IQGAP2 expression in the primary gastric cancer tissues as well as gastric cancer cell lines. IQGAP2 knockdown with small interfering RNA increased the invasive capacity of a gastric cancer cell line. These results suggest that silencing of IQGAP2 by promoter methylation may contribute to gastric cancer development. Interestingly, IQGAP1 promote metastasis by interaction with Rac1, Cdc42 and cadherin/â-catenin in gastric cancer (Sugimoto et al., 2001; Takemoto et al., 2001; Walch et al., 2008). These studies suggest not all RAS-GAPs function as tumor suppressors. The function of RAS GAP is highly complex and very tightly regulated.

In the context of RASAL1, there had been several findings showed RASAL1 was involved in gastric tumorigenesis. Seto et al. (2011) found RASAL1 expression was reduced in gastric cancer tissues and cell lines. Our previous study (Chen et al., 2012) also found the expression of RASAL1 was correlated with carcinoma diameter, differentiation grades, invasive depth, lymph node metastasis and TNM. The results indicated that RASAL1 may be a candidate tumor suppressor gene in gastric cancer. In this study, an *in vivo* xenograft model was established to investigate the role of RASAL1 in gastric carcinogenesis. The results suggest that alterations in RASAL1 expression levels lead to aberrant proliferation

Table 1. Expression of RASAL1 in Three Groups of Gastric Cancer Cells by q-PCR

Group	RASAL1 CT	β-actin CT	ΔCT	ΔΔCT	2-ΔΔCT
BGC-823-RASAL1	16.10±0.06	6.97±0.40	9.14±0.46	-14.41±0.38	22358.50±6221.63
BGC-823-NC	31.25±0.40	6.65±0.42	24.60±0.65	1.05±0.51	0.50±0.18
BGC-823	29.00±0.07	5.36±0.13	23.55±0.16	0.00±0.15	1.00±0.11

in gastric cancer.

Qiao et al. (2012) once reported that decreased expression of RASAL1 was not observed relationships between the RASAL1 expression level and H. pylori and Epstein-Barr virus (EBV) infection. So they speculate RASAL1 possibly contributes to gastric carcinogenesis as a tumor suppressor gene. In our previous study (Chen et al., 2012; Chen et al., 2013; Chen et al., 2014), we further explored the biological role of RASAL1 in gastric tumorigenesis *In vitro*. We found that the RASAL1 gene inhibits the proliferation of gastric cancer cells through promoting cell apoptosis, and decreasing invasion and migration by blocking RAS/ERK signaling. In the current study, we investigated the significance of the RASAL1 gene in tumor development *in vivo*. The human gastric cancer cell line, BGC-823 was transfected with a lentiviral RASAL1 expression vector to obtain a stably transfected cell line which over-expresses RASAL1. Then, we established an *in vivo* xenograft model by using it. After 3 weeks, we found that the mean size of RASAL1-overexpressing tumors was significantly smaller than that of the other two groups. The inhibitory effect of RASAL1 observed on tumor weight was highly consistent with its effect on tumor weight. It was also found that up-regulation of RASAL1 reduced *p*-ERK1/2 levels, one of downstream molecular of RAS/RAF/MEK/ERK signaling pathway. In the recent report, it was described that RASAL1 inactivation results in the activation of both the MAPK and PI3K pathways in poorly differentiated thyroid cancers (Liu et al., 2013). Thus, the relationship between RASAL1 and RAS/PI3K/AKT pathway in gastric tumorigenesis is also worthy to be further studied.

RAS-driven cancers are among the most difficult to treat and often excluded from therapies. The RAS proteins have been termed "undruggable" based on failures from an era in which understanding of signaling transduction, feedback loops, redundancy, tumor heterogeneity, and RAS' oncogenic role was poor (Stephen et al., 2014). Progress from the studies of RAS GAPs including RASAL1 provides a novel approach of restoring GTP hydrolysis to mutant RAS proteins or not.

Taken collectively, our experiment results above confirmed that the enhanced expression of RASAL1 inhibits proliferation of gastric cancer by negatively regulating the RAS/ERK signaling mechanisms *in vivo* and provided more evidences of RASAL1 may function as a tumor suppressor gene in gastric cancer. Therefore, the therapeutic strategies targeting RASAL1 should be further investigated in gastric cancer.

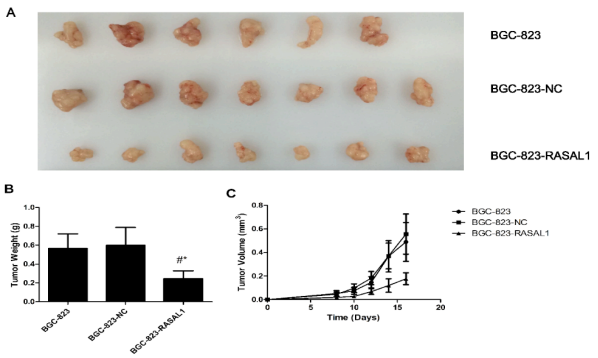

Figure 2. Suppression of Tumor Growth by RASAL1 *in vivo*. (A) RASAL1-transfected, NC-transfected and untransfected BGC-823 cells were s.c. injected into the left flank of nude mice, respectively (n=7). (B) Tumor weight was detected at the end of the study. (C) Tumor volume was monitored during the time course of 21 days. **p*<0.05, BGC-823-RASAL1 cell compared to BGC-823 group. #*p*<0.05,BGC-823-RASAL1 cell compared to BGC-823-NC group

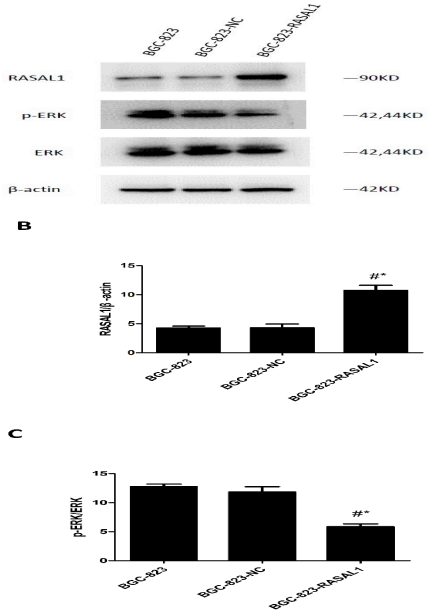

Figure 3. Expression of RASAL1, total ERK1/2 and *p*-ERK1/2 Protein in Three Groups Of Gastric Cancer Cells. (A) The protein expression was measured by western blotting. (B) The ratio of RASAL1/β-actin in three groups of gastric cancer xenograft tumor groups. (C) The ratio of (*p*-ERK1/2)/(ERK1/2) in three groups of gastric cancer xenograft tumor groups. **p*<0.05, BGC-823-RASAL1 cell compared to BGC-823 group. #*p*<0.05, BGC-823-RASAL1 cell compared to BGC-823-NC group

Discussion

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Acknowledgements

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