

Activator protein-1 involved in growth inhibition by RASSF1A gene in the human gastric carcinoma cell line SGC7901

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Author contributions: Deng ZH experiment design, gene transfection, tumorigenicity in nude mice and manuscript writing; Wen JF corrected the manuscript; Li JH analysed Western blot and RT-PCR; did cell culture, analysed cytobiology and EMSA; the statistical tests were carried out by Zhou JH.

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Abstract

AIM: To investigate the role of Ras association domain family protein 1 isoform A (RASSF1A) in gastric tumorigenesis.

METHODS: Through over-expression of RASSF1A gene in the SGC7901 cell line which was induced by a lipofectamine-mediated gene transfer approach. Activator protein-1 (AP-1) DNA binding activity was measured by electrophoretic mobility shift assay (EMSA).

RESULTS: Compared with the control clones, cells over-expressing RASSF1A exhibited significant inhibition of cell growth with G₁ cell cycle arrest *in vitro* and *in vivo*. The over-expression of RASSF1A significantly inhibited AP-1 activity in SGC7901 cells (0.981 ± 0.011 vs 0.354 ± 0.053 , $P < 0.001$). In addition, both Western blot analysis and immunocytochemistry demonstrated that RASSF1A down-regulated the expression of c-Fos (0.975 ± 0.02 vs 0.095 ± 0.024 , $P < 0.001$) but not c-Jun.

CONCLUSION: Over-expression of RASSF1A inhibits the growth of SGC7901 cells by negatively regulating the AP-1 activity, the latter in turn negatively signals cell proliferation.

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Key words: RASSF1A; Gastric adenocarcinoma; SGC7901; Activator protein-1

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INTRODUCTION

Gastric carcinoma is one of the most frequent tumors that seriously threaten people's health in China^[1]. Molecular genetics studies indicate that loss of 3p was observed in different types of solid tumors^[2]. Frequent loss on 3p21-23 was detected in gastric cancer^[3,4].

Ras association domain family protein 1 isoform A (RASSF1A), one transcript of RASSF1 gene, is a recently identified 3p21.3 tumor suppressor gene^[5]. Loss of expression of RASSF1A was a frequent event in primary gastric carcinoma^[6,7]. However, the exact role of RASSF1A in gastric tumorigenicity is largely unknown.

Activator protein-1 (AP-1) plays an important role in various human diseases and regulates the expression of multiple genes essential for cell proliferation, differentiation and apoptosis^[8]. AP-1 is thought to serve as a nuclear target of Ras^[9]. It is not known whether RASSF1A, as effectors of Ras signaling^[10], could or could not inhibit the activity of AP-1.

In this study, we established gastric cancer cell lines stably over expressing RASSF1A. Characterization of these cells with regard to proliferation rate and tumorigenicity *in vitro* and *in vivo* was performed. AP-1 activity was measured by electrophoretic mobility shift assay (EMSA). Our results suggest that over-expression of RASSF1A exerts inhibitory effects on the transformed phenotype of gastric cancer cells and RASSF1A inhibits AP-1 activity.

MATERIALS AND METHODS

Cell culture

The human gastric cancer cells, SGC7901 (Shanghai Cell Bank, Chinese Academy of Sciences), were maintained in RPMI 1640 medium (Life Technologies, Inc, Grand Island,

NY) supplemented with 100 mL/L fetal bovine serum plus penicillin (50 IU/mL) and streptomycin (50 µg/mL) with passage every three days. Cultures were incubated in an incubator containing 5 mL/L CO₂ at 37°C.

Gene transfection and establishment of stable cell lines

Plasmid pcDNA3.0-RASSF1A and pcDNA3.0 were gifts from Professor Michael White (Department of Cell Biology, UT Southwestern Medical Center, Dallas, TX 75390, USA). Cells were seeded in six-well plates to 70%-80% confluence. The cells were transfected with 4 µg/well plasmids using Lipofectamine 2000 (Invitrogen). After transfection for 6 h, the cells were transferred to normal medium and allowed to recover overnight. The cells were trypsinized and split 1:10, and then seeded into new six-well plates. 48 h after transfection, transfected cells were grown in RPMI containing G418 (Alexis Biochemicals) at 0.8 g/L until all of the nontransfected cells were dead (2 wk). Resistant clones were selected separately using cloning cylinders and maintained in RPMI containing 0.2 g/L G418 for further study. Meanwhile, SGC-7901 cells were transfected with the empty pcDNA3.0 vector as the control.

Preparation of cytoplasmic and nuclear extract

Nuclear and cytoplasm extracts were prepared as described by Dignam *et al*^[11]. Confluent cells in 10 cm dishes were treated for various times with the indicated effectors. Cells were resuspended in 400 µL of buffer A [10 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 0.5 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, 1 µg/mL leupeptin, 1 µg/mL aprotinin, and 1 µg/mL pepstatin A], kept on ice for 15 min, lysed gently with 12.5 µL of 10% NP40, and centrifuged at 2000 r/min for 10 min at 4°C. The supernatant was collected and used as the cytoplasm extracts. The nuclei pellet was resuspended in 40 µL of buffer C [20 mmol/L HEPES (pH 7.9)], containing 1.5 mmol/L MgCl₂, 450 mmol/L NaCl, 25% glycerol, 0.2 mmol/L EDTA, 0.5 mmol DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, 1 µg/mL leupeptin, 1 µg/mL aprotinin, and 1 µg/mL pepstatin A] and agitated for 30 min at 4°C, and the nuclear debris was spun down at 20 000 r/min for 15 min. The supernatant (nuclear extract) was collected and stored at -80°C until ready for analysis. Proteins were measured using the BCA kit (Pierce) according to the manufacturer's protocol.

Western blot analysis

Eighty µg of cytoplasm proteins or 40 µg nuclear proteins were separated by 10% SDS-PAGE under reducing conditions, and transferred to a nitrocellulose membrane. The nitrocellulose membrane was then incubated with blocking buffer (TBST containing 5% non-fat milk) for 2 h at room temperature and with mouse monoclonal antibody against RASSF1A (Abcam, USA), c-Jun, c-Fos, CyclinD₁ (Santa Cruz Biotechnology, USA) overnight at 4°C with gentle shaking. The membrane was washed with TBST twice for 5 min, and then incubated with rabbit anti-mouse IgG conjugated horseradish peroxidase diluted at 1:2000 (Santa Cruz Biotechnology, USA) for 2 h at room temperature. After washing, RASSF1A was detected using

DAB reagents. The level of β-actin or tubulin was used as a control for equal loading of protein.

Reverse transcription-PCR analysis

Total RNA from SGC7901 cells was obtained using a RNA Mini Kit (Qiagen, Inc). Two µg of total RNA extracted from each cell line were reverse-transcribed using a RevertAid First Strand cDNA Synthesis Kit (MBI). Five ng of reverse-transcribed cDNA per sample were used to perform PCR in triplicate samples for RASSF1A and β-actin as an internal control. Reactions were carried out under the following conditions: 95°C for 5 min, followed by 35 cycles at 94°C for 30 s, and 52°C for 40 s and 72°C for 60 s. The following primers were used: RASSF1A forward, 5'-TCTGGGGCGTCGTGAGTAAA-3' reverse, 5'-CCACCACCAAGAACAGTCG-3', β-actin forward, 5'-CCTTTCCTGGGCATGGAGTCCT-3', β-actin reverse 5'-GGAGCAATGATCTTGATCTT-3'. Three independent measurements were averaged and relative gene expression levels were calculated as a ratio to β-actin expression of each.

MTT assay

Cells were cultured in 96-well microtiter plates at a density of 1×10^4 cells per well. The surviving cells were measured by MTT assay at 1 d, 2 d, 3 d, 4 d, 5 d, 6 d, 7 d after seeding. 20 µL of 5 g/L MTT [3-(4,5-dimethyl-thiazolyl-2)-2,5-diphenyltetrazolium bromide, Fluka, Buchs, Switzerland] in PBS was added to each well and the cells were incubated for another 4 h at 37°C. The supernatant was removed, and 150 µL of DMSO was added to each well. The absorbency at a wavelength of 595 nm was measured with a micro ELISA reader (BioRad, CA, USA).

Flow cytometry analysis

Cells were collected and fixed in 70% of ice-cold ethanol in phosphate buffer saline (PBS) and stored at -20°C. After resuspension, 100 µL RNAase I (1 g/L) and 100 µL propidium iodide (PI, 400 g/L, Sigma, USA) were added and incubated at 37°C for 30 min. Analysis of samples was performed by flow cytometry (Coulter Epics, XL, UK). The cell cycle phase distribution was calculated from the resultant DNA histogram using Multicycle AV software (Phoenix Flow System, San Diego, CA, USA).

Plating efficiency

Plating Efficiency was prepared as described by Hu *et al*^[12]. Cells (1×10^2) were plated in 6-well plates. Colonies were scored at 14 d, fixed with 70% ethanol, stained with 5% Giemsa (Sigma), and counted under a microscope. Only those colonies containing at least 50 cells were considered to be viable survivors. The plating efficiency (PE) was calculated as follows: PE = (colonies formed/cells seeded) × 100%.

Tumorigenicity in nude mice

Single cell suspensions were trypsinized and collected. The cell viability was > 95% as determined by trypan blue staining. Cells (5×10^6) in a 0.1 mL volume of RPMI were inoculated s.c. into the right flank of 4-6 wk-old female BALB/c-nu/nu mice (Laboratory Animal Unit, Central

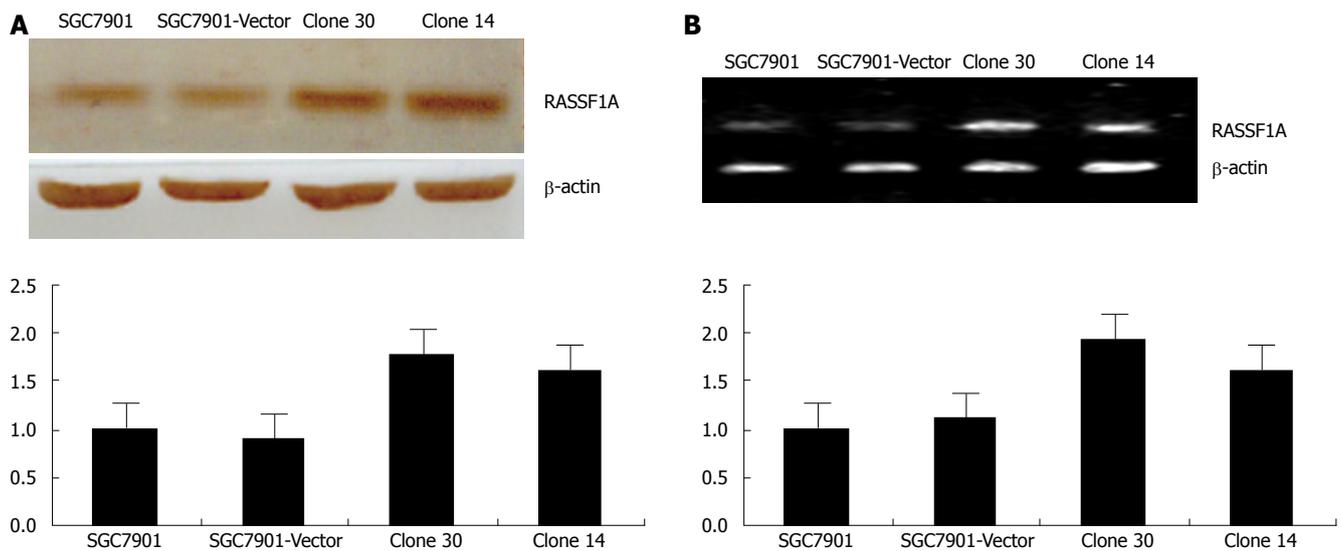


Figure 1 The over-expression of RASSF1A in gastric cancer cell line SGC7901. Cells were stably transfected using Lipofectamine 2000 with RASSF1A or empty vector and were grown in RPMI containing G418 at 0.8 g/L. Resistant clones were selected separately and were measured by Western blotting and RT-PCR, β -actin was used as an internal loading control, and clone30 was used for further study. **A:** RASSF1A expression was analyzed by Western blotting; **B:** RASSF1A expression was analyzed by RT-PCR.

South University). The mice were maintained under sterile conditions for 30 d. At the end of the experiment, the tumors were excised and the tumor weight was measured.

Electrophoretic mobility shift assay

EMSA was prepared as described by Li *et al.*^[3]. Fifteen μ g of nuclear proteins were incubated with 1 μ g each of poly (dI-dC) in the presence of 30 fmol of digoxin (DIG)-labeled double-stranded AP-1 probe (5'-CGCTTGATG ACTCAGCCGGAA-3', BoYa Biotechnology, China) for 15 min at room temperature in a total volume of 20 μ L using DIG gel shift kit (Roche Diagnostics GmbH, Mannheim, Germany). Oligonucleotide competition experiments were performed in 50-fold excess of unlabeled oligonucleotides. DNA complexes were resolved from free probe with 4% nondenaturing polyacrylamide gels in 0.5 \times Tris-borate-EDTA (pH 8.3) and visualized by fluorography.

Immunocytochemistry

Cells were grown to 70% confluency on 22 mm \times 22 mm microscope coverslips, washed with PBS, then incubated for 1 h at room temperature with c-Jun, c-Fos monoclonal antibody (Santa Cruz Biotechnology, USA) at a final dilution of 1:200. Primary antibody was removed by repetitive washes with PBS and secondary antibody was added for 1 h at room temperature. Cells were washed in PBS and stained with DAB.

Statistical analysis

The data shown were mean values of at least three different experiments and expressed as mean \pm SD. Student's *t* test was used for comparison. $P < 0.05$ is considered statistically significant.

RESULTS

Generation of gastric cancer cells stably over-expressing RASSF1A

SGC-7901 human gastric cancer cells were transfected

with the control pcDNA3.0, pcDNA3.0-RASSF1A plasmids, respectively. Empty vector pcDNA3.0-transfected cell clones were named vector control and, together with the parental SGC-7901 cells, served as controls in this study. After G418 selection, 4 clones of pcDNA3.0-RASSF1A cells were picked, spread, and collected, including clone14, clone 19, clone 30 and clone 33. The expression of RASSF1A in clone 30 and clone 14 were further detected by Western blot analysis and RT-PCR analysis. As shown in Figure 1A, cells transfected with the control vector did not alter RASSF1A expression when compared with the SGC-7901 cells, whereas introduction of pcDNA3.0-RASSF1A resulted in marked over-expression. The protein expression of RASSF1A was increased about 77.5%, 61.2%, when compared with the parental cells. As shown in Figure 1B, RASSF1A mRNA was increased by 80.2%, 74.7%, respectively, in the corresponding cells. Clone 30, which showed the highest degree of over-expressing RASSF1A, consequently was selected for further study. Thus, the over-expression of RASSF1A was obviously increased in our established transfected cells.

Cell growth of SGC7901 cell inhibited by RASSF1A

To characterize the gastric cancer cells stably over-expressing RASSF1A protein, we first examined the possible effects on the rate of cell proliferation. Growth curves indicated that the vector control and parental cells displayed rapid growth rates, whereas the growth rate of the SGC7901-RASSF1A cells was significantly reduced (Figure 2A). The suppress rate was 28.13% at 48 h. The cell cycle distribution and apoptosis were determined by flow cytometry. As shown in Figure 2B, RASSF1A induced the cell cycle into G₁ phase. Compared with parental and vector control cells, the percentage of G₁ phase in RASSF1A transfected cells obviously increased ($P < 0.05$). And the apoptosis rate of cells expressing RASSF1A had a slight increase.

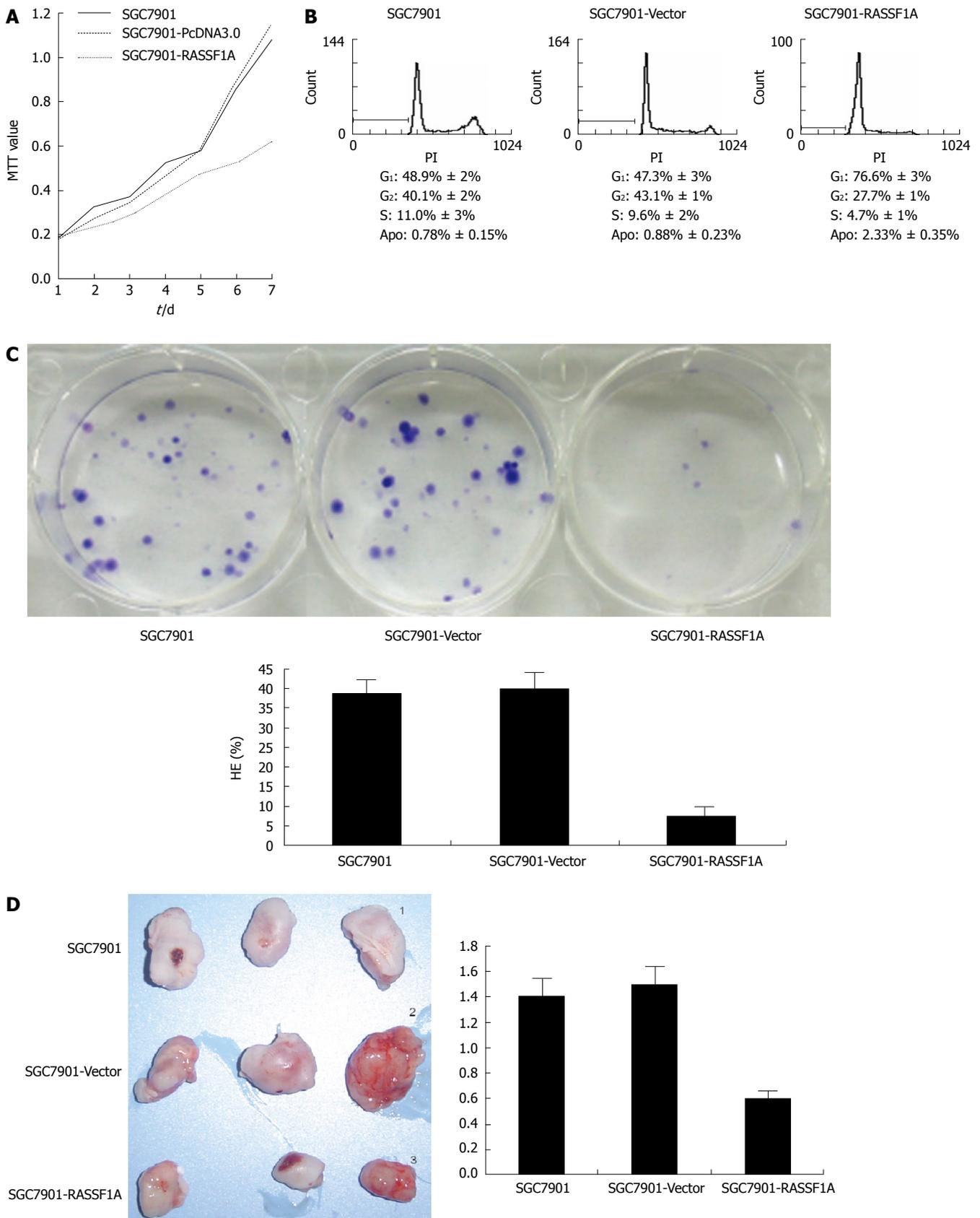


Figure 2 RASSF1A blocked gastric cancer cell line SGC7901 growth *in vivo* and *in vitro*. **A:** Cell growth curve of gastric cancer cell line SGC7901 measured by MTT assay; **B:** RASSF1A induced gastric cancer cell line SGC7901 G₁ arrest; **C:** RASSF1A inhibited the colony formation of SGC7901 cells measured by planting efficiency; **D:** RASSF1A inhibited the tumorigenicity of SGC7901 cells.

Colony formation and tumorigenicity inhibited by RASSF1A

We analyzed the colony forming ability of the RASSF1A

transfectants in planting because anchorage-independent growth often correlates with tumorigenicity. Plating efficiency in parent, vector control and RASSF1A

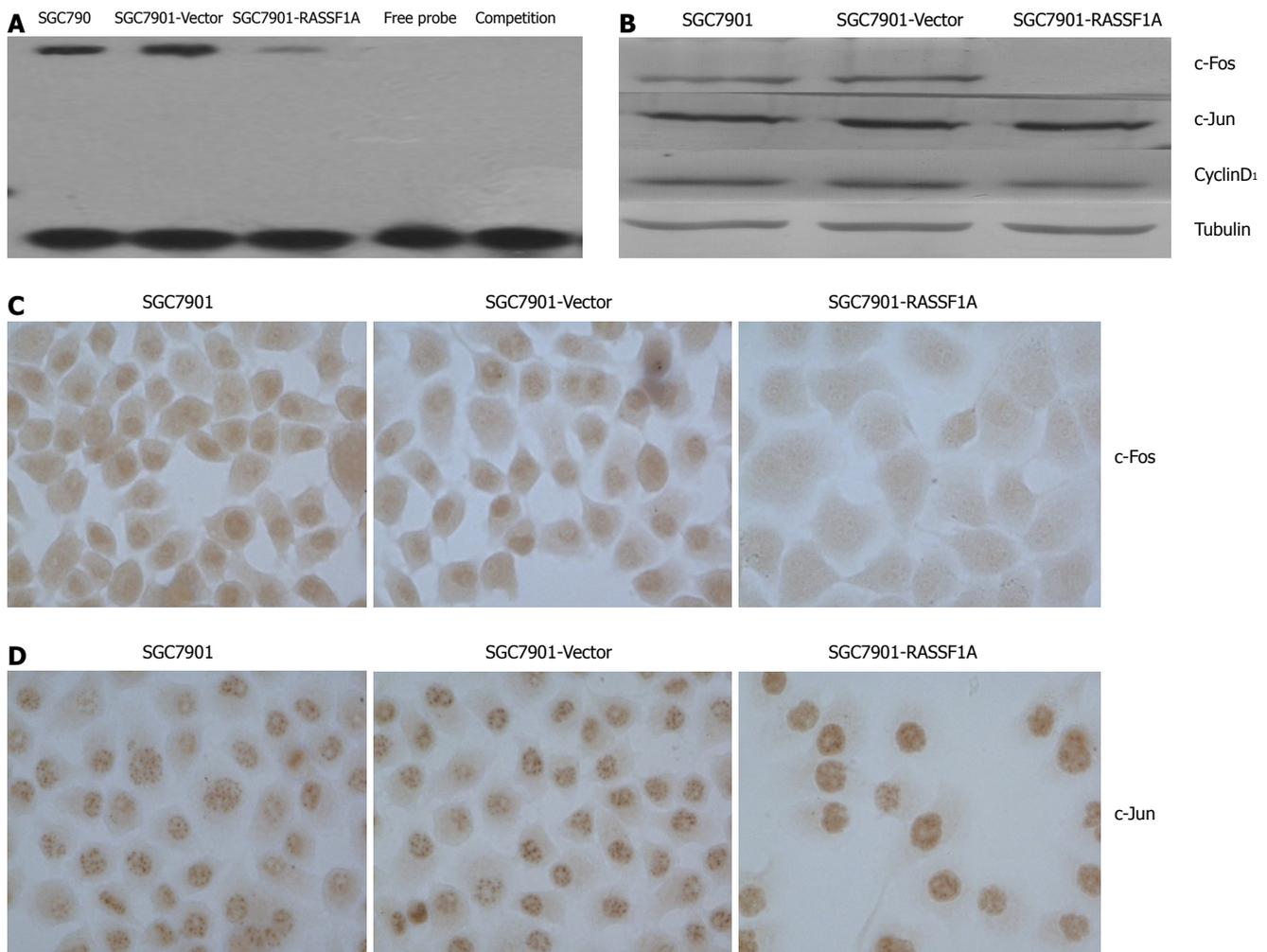


Figure 3 The over-expression of RASSF1A inhibited the AP-1 activity in gastric cancer cell line SGC7901. **A:** The over-expression of RASSF1A inhibited the AP-1 DNA binding activity measured by EMSA; **B:** The over-expression of RASSF1A inhibited the c-Fos but not c-Jun expression in nuclear extract of SGC7901 cells. (Western blotting, DAB staining); **C:** The over-expression of RASSF1A inhibited the c-Fos expression in nuclear of SGC7901 cells (immunocytochemistry, DAB staining, $\times 400$); **D:** The over-expression of RASSF1A had no influence on the c-Jun expression in nuclear of SGC7901 cells (immunocytochemistry, DAB staining, $\times 400$).

transfected cells were $38.6\% \pm 1.5\%$, $39.75\% \pm 2.1\%$ and $7.3\% \pm 0.6\%$, respectively (Figure 2C). SGC7901-RASSF1A cells displayed almost complete loss of colony-forming efficiency, and had a $> 80\%$ decrease when compared with the vector control and parental cells. In view of these results, we examined whether the RASSF1A over-expression in gastric cancer cells might affect their tumorigenicity in nude mice.

5×10^6 cells were injected s.c. into athymic nude mice and monitored for 30 d. The tumors appeared in three groups almost at the same time. At the end of the study, all of the tumors were removed and dissociated, and the weights of the tumors were measured. RASSF1A transfection revealed an obvious difference in tumor growth compared with vector control and parental cells during the observation period. The mean tumor weights in mice of parental, vector control cells and RASSF1A transfected cells were $1.4 \text{ g} \pm 0.26 \text{ g}$, $1.5 \pm 0.32 \text{ g}$ and $0.6 \pm 0.1 \text{ g}$, respectively (Figure 2D).

AP-1 activity inhibited by RASSF1A

We determined the DNA binding activity of AP-1 in SGC7901 cells by electrophoresis mobility shift assay.

The results showed that AP-1 DNA binding activity was significantly lower in SGC7901-RASSF1A cells than in vector control and parental cells (Figure 3A). Western blotting and immunocytochemistry data showed that c-Fos expression in the nuclear extract of SGC7901-RASSF1A cells was significantly inhibited, while, c-Jun expression in SGC7901-RASSF1A cells had no significant change. Furthermore, cyclinD₁ expression in nuclear extract was significantly lower in SGC7901-RASSF1A cells than in vector control and parental cells (Figure 3 B-D).

DISCUSSION

In this report, we have demonstrated that RASSF1A inhibited proliferation of SGC7901 cells. The cell growth was reduced by 28.13% at 48 h as determined by the MTT assay. The alteration of cell malignant phenotype was obvious as a result of loss of anchorage-independent growth ability as measured by a plating efficiency test. The tumorigenicity in nude mice was reduced significantly ($P < 0.01$). RASSF1A over-expression induced cell arrest from 48.9% to 76.6% ($P < 0.01$) in the G₁ population, and increased cell apoptosis rate from 0.78% to 2.33%

($P < 0.01$). These results are similar to the findings previously reported in other cancers, such as NSCLC^[5,10], kidney^[14], prostate^[15], nasopharyngeal carcinoma^[16], and glioma cell lines^[17]. RASSF1A differentially regulated many genes identified as having relevance to tumorigenesis including involvement in transcription, cytoskeleton, signaling, cell cycle, cell adhesion, and apoptosis^[18].

RASSF1A is predicted to encode a 39 kDa peptide that contains an N-terminal diacylglycerol (DAG)-binding domain, a Ras-association domain, a sequence PxxP and PEST sequences. The Ras-association domain is more than 50% identical and more than 70% similar to the carboxyl terminal 225 residues of mouse Nore1^[10]. RASSF1 binds Ras in a GTP-dependent manner, both *in vivo* and directly *in vitro*^[19]. It has also been shown to heterodimerize with Nore1^[20]. The presence of a Ras-association domain in both RASSF1 isoforms suggests that these proteins may function as effectors of Ras signaling (or signaling of a Ras-like molecule) in normal cells. The fact that RASSF1A can function as a tumor suppressor gene implies that RASSF1 acts in opposition to Ras-effector pathways stimulating proliferation^[10].

AP-1 is a transcription factor that consists of either a Jun-Jun homodimer or a Jun-Fos heterodimer. AP-1 regulates the expression of multiple genes essential for cell proliferation, differentiation and apoptosis^[8]. Our results showed that RASSF1A dramatically decreased basal AP-1 activity. We further detected the expression of c-Jun/c-Fos in SGC7901. Surprisingly we failed to observe any significant change of expression of c-Jun in the present study. However, expression of c-Fos had significant change. This indicated that RASSF1A could inhibit the expression of c-Fos but not c-Jun. However, in lung cancer cells, RASSF1A reduced c-Jun phosphorylation, suppresses the c-Jun-NH2-kinase pathway and inhibits cell cycle progression^[21]. We thought this difference may be due to the different histology and the role of c-Jun in RASSF1A-mediate growth inhibition in gastric carcinoma needs further investigation.

Shivakumar found that the exogenous expression of RASSF1A induced cell cycle arrest at the G₁ phase by down-regulating CyclinD₁^[22]. In agreement with this report, we also observed that the ectopic expression of RASSF1A down-regulated CyclinD₁. As a target of AP-1, CyclinD₁ plays an important role in cell proliferation^[23]. According to our results, we presumed that RASSF1A induced SGC7901 cell cycle arrest at the G₁ phase by down-regulating CyclinD₁ through inhibition of the activity of AP-1, but needs further investigation. Our current results indicate that inhibition of AP-1 activity contributes to RASSF1A mediated regulation of gastric carcinogenesis.

Thus, our data presented here clearly demonstrated that exogenous RASSF1A inhibits the growth of gastric carcinoma cells SGC7901 and RASSF1A gene may be a suppressor in gastric carcinogenesis. AP-1 may be involved in growth inhibition by the RASSF1A gene in the human gastric carcinoma cell line SGC7901.

of RASSF1 gene, is a recently identified 3p21.3 tumor suppressor gene and it is described as an effector of Ras signaling. Expression loss of RASSF1A was a frequent event in primary gastric carcinoma. However, the exact role of RASSF1A in gastric tumorigenicity is largely unknown.

Research frontiers

Gastric carcinoma is one of the most frequent tumors that seriously threaten people's health in China. Molecular genetics studies indicate that loss of 3p was observed in different types of solid tumors. Frequent loss on 3p21-23 was detected in gastric cancer. RASSF1A is predicted to encode a 39-kd peptide that contains an N-terminal diacylglycerol (DAG)-binding domain, a Ras-association domain, a sequence PxxP and PEST sequences. The presence of a Ras-association domain in both RASSF1 isoforms suggests that these proteins may function as effectors of Ras signaling (or signaling of a Ras-like molecule) in normal cells. The fact that RASSF1A can function as a tumor suppressor gene implies that RASSF1 acts in opposition to Ras-effector pathways stimulating proliferation.

Innovations and breakthroughs

Our study is believed to be the first to observe the role of RASSF1A in gastric cancer and the possible mechanisms involved.

Applications

Our research observed that RASSF1A suppressed the growth of the human gastric cancer cell line SGC7901 and RASSF1A inhibited AP-1 activity through down-regulated c-fos expression. This may have a significant clinical impact in the future.

Terminology

Electrophoretic mobility shift assay (EMSA): The EMSA technique is based on the observation that protein: DNA complexes migrate more slowly than free DNA molecules when subjected to non-denaturing polyacrylamide or agarose gel electrophoresis. Because the rate of DNA migration is shifted or retarded upon protein binding, the assay is also referred to as a gel shift or gel retardation assay.

Peer review

This is a well-written paper that suggests the role of RASSF1A in human gastric cancer. But the mechanism remains to be studied further.

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COMMENTS

Background

Ras association domain family protein 1, isoform A (RASSF1A), one transcript

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